**Mitochondrial Isolation from Primary Cortical Neurons for Western Blot**

**Preparation:**

* Turn on Sorval and cool to 4oC
* Turn on heat block and heat to 95oC
* Have 3 ice buckets ready
* Prepare Mitochondrial Isolation Buffer (MIB) and place on ice
  + + BSA
  + – BSA
    - See recipe on next page
* If volume to be harvested is over 50mL:
  + Label one 250mL bottle and one 50mL tube for each condition and place on ice
* If volume to be harvested is under 50mL:
  + Label one 50mL tube per condition and place on ice
* Label one, 7mL glass dounce homogenizer fitted with a “tight” pestal for each condition and place on ice
* Label 4 microcentrifuge tubes for each condition as follows and place on ice:
  + Pellet
  + Spin 2
  + Spin 3
  + Mitochondria
* Label an additional 4 microcentrifuge tubes for each condition the same as above and add the following amounts of Laemmli Buffer + BME to each (no need for these to be on ice…):
  + Pellet – 500uL
  + Spin 2 – 500uL
  + Spin 3 – 500uL
  + Mitochondria – 75uL
* Prepare a microcentrifuge tube containing 975uL of Trypan Blue (no need to place on ice)

**Mitochondrial Isolation:**

* Harvest cells in their growth media via cell scraper
* Collect cell suspension from all similar wells and place into the pre-cooled 50mL tube or 250mL bottle (depending on volume)
* Spin bottles at 3000g in the pre-cooled 4oC Sorval for 15 minutes
  + Rotor = SLA-1500 (Stored in cold room)
  + Note: 3000g is the same as 3000rcf – but 3000rpm is MUCH slower so make sure you choose the appropriate setting!
* If your cells are already in the 50mL vessel, you can skip the following (in red):
  + Following spin, remove all but 20mL of growth media from the 250mL bottle
    - Be careful… The pellet is fairly lose, not compact!
  + Using the remaining 20mL of growth media, re-suspend the pellet and transfer to the appropriately labeled, pre-cooled 50mL tube
  + Spin the 50mL tubes at 3000g in the pre-cooled 4oC Sorval for 5 minutes
    - Rotor = SS-34 (Stored in cold room)
* Following spin, remove the supernatant and wash/re-suspend the cell pellet in 3mL of MIB
* Spin to pellet at 3000g in the pre-cooled 4oC Sorval for 5 minutes
* Following spin, estimate the pellet size and add 2X volume of MIB
* In the cold room, transfer the re-suspended pellet into the pre-cooled homogenizer and break apart cells by douncing for 20 strokes
  + Add 25uL of the homogenized cell suspension to the Trypan Blue tube
  + Incubate at room temp for three minutes
  + Add 10uL of cell / Trypan Blue mixture to hemocytometer and be sure you have at least broken apart 80% of your cells…
    - If less than 80% of cells are sheared, dounce for 20 more strokes in the glass homogenizer and Trypan Blue again…
  + When cells are appropriately sheared, collect suspension from glass homogenizer and place into the microcentrifuge tube labeled “pellet”
  + Spin “pellet” tube in the centrifuge located in the cold room at 600g for 10 minutes
  + Following the spin:
    - Transfer the supernatant to the tube labeled “Mitochondria” and keep on ice
    - Re-suspend the nuclear pellet in 500uL of MIB to release any trapped mitochondria
      * Spin the “pellet” tube again in the centrifuge located in the cold room at 600g for 10 minutes
  + Following the spin:
    - Transfer the supernatant to the tube labeled “mitochondria”
      * At this point, the “mitochondria” tube should have the supernatants from two individual spins combined…
    - Re-suspend the nuclear “pellet” in 1mL of MIB w/o BSA using a P1000 tip with the end cut off and place on ice until later
  + Spin the “mitochondria” tube in the centrifuge located in the cold room at 8000g for 15 minutes (This spin will result in a mitochondrial pellet!)
  + Following the spin:
    - Remove the supernatant via pipette and place into tube labeled “Spin 2” – keep tube on ice until later
    - Add 1mL of MIB
    - Wash and re-suspend the mitochondrial pellet using a P1000 tip with the end cut off
  + Spin the “mitochondria” tube in the centrifuge located in the cold room at 8000g for 15 minutes (This spin will result in a hopefully pure mitochondrial pellet!)
  + Following this spin:
    - Remove the supernatant via pipette and place into tube labeled “Spin 3” – keep tube on ice until later
    - Re-suspend the mitochondrial pellet in 90uL of TNEB lysis buffer and place on ice
  + Sonicate the nuclear pellet, the mitochondrial pellet, Spin 2 and Spin 3 for 5 seconds each at 5 watts (setting 1-2 on our sonicator)
  + Following sonication, remove the following volumes from each tube and add to the tubes already containing Laemmli Buffer + BME:
    - Pellet – 500uL
    - Spin 2 – 500uL
    - Spin 3 – 500uL
    - Mitochondria – 75uL
      * Note: this is a 1:1 dilution in Laemmli Buffer
  + Save the remaining samples without Laemmli Buffer for protein assay at -20oC
  + Boil samples with Laemmli Buffer for 10 minutes at 95oC and then store at -20oC

**Buffers:**

**Mitochondrial Isolation Buffer (MIB) – 250mL**

|  |  |  |  |
| --- | --- | --- | --- |
| **Chemical** | **[Stock]** | **Add** | **[Final]** |
| Mannitol |  |  | 110mM |
| Sucrose |  | 10.02g | 34mM |
| KCl |  | 5.82g | 40mM |
| EGTA | 0.5mM | 6.67mL (or 1.49g) | 0.25mM |
| MgAc2 | 250mM | 0.5mL | 1mM |
| HEPES (pH 7.4) | 1M | 0.5mL | 5mM |

* Bring up to 250mL with ddH20 and filter
* Store at 4oC
* On day of experiment, calculate volume of MIB with or without BSA needed and add the following components fresh:
  + Fatty Acid Free BSA – at a final of 2mg/mL
  + Protease Inhibitor Cocktail – 1:1000

**Notes:**

* In the end, the tubes consist of:
  + Pellet = Nuclei and cellular debris
  + Spin 2 = Cytosolic fraction and small organelles
  + Spin 3 = Cytosolic fraction and small organelles – but more dilute due to the wash step
  + Mitochondria = Purified mitochondrial fraction
* **The flowing dilutions work well for being in range for protein assays following isolation:**
  + Pellet 🡪 Usually about 2.5mg/mL of protein in final sample
    - 1:25
    - 1:50
    - 1:100
  + Spin 2 🡪 Usually about 1.75mg/mL of protein in final sample
    - No dilution
    - 1:2
  + Spin 3 🡪 Usually about 1.25mg/mL of protein in final sample
    - No Dilution
    - 1:2
  + Mitochondria 🡪 Usually about 1mg/mL of protein in final sample
    - No dilution