**Cell Fractionation from Primary Cortical Neurons for Western Blot**

**Nucleus, Mitochondria & Cytosol**

**Preparation:**

* Turn on the benchtop centrifuge, the Sorval and the Thermo Ultra Sorval (Sign-up sheet in Emeson Lab – to begin cooling, must press vacuum button!) and cool all to 4oC
* Turn on heat block and heat to 95oC
* Have 3 ice buckets ready
* Put 1X PBS on ice to cool
* Prepare Hypotonic Buffer (HB) and Isotonic Buffer (IB) and place on ice
  + See recipe on last 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 1 x 250mL Sorval tube per condition and place on ice
* Label 2 x 50mL Sorval tube per condition and place on ice
* Label 2 x 15mL conical tubes per condition and place on ice
* Label 1 X 12mL Sorval polyalomer tube per condition and place on ice
* Label 3 microcentrifuge tubes for each condition as follows and place on ice:
  + Nuclear Pellet
  + Mitochondrial Pellet
  + Cytosol
* Label 1 x 15mL conical tube as follows:
  + Cytosolic Supernatant
    - Place on ice
* 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…):
  + Nuclear Pellet – 150uL
  + Mitochondrial Pellet – 150uL
  + Cytosolic Pellet – 150uL
  + Cytosolic Supernatant – 150uL
* For each condition, prepare a microcentrifuge tube containing 975uL of Trypan Blue (no need to place on ice)

**Nuclear Fraction Isolation:**

* Aspirate growth media and wash cells quickly with ice cold 1X PBS
* Aspirate PBS wash and add 2mL of HB per well
* Harvest cells in HB 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!
* Following spin, carefully remove the supernatant (Note: the pellet is loose, so be careful), re-suspend the cell pellet in 3mL of HB and transfer the 3mL cell suspension to the appropriately labeled, pre-cooled dounce homogenizer
* Incubate cells (in glass dounce) on ice for 30 minutes in the cold room
* Following incubation, dounce for 40 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…
* Following homogenization, transfer cells into a 15mL tube and spin at 50g in the pre-cooled benchtop centrifuge for 10 minutes.
  + This spin is meant to eliminate unbroken cells from the pellet…
* After the spin, transfer supernatant to a new 15mL conical tube and centrifuge at 800g for 10 minutes.
* Following the spin:
  + Remove the supernatant and place into a pre-cooled 50mL Sorval tube – this will be further used for mitochondrial and cytosolic preparations…
  + The resulting pellet is the nuclear pellet…
* Wash the nuclear pellet 2x with 1mL of IB – spin in mini, benchtop centrifuge in between washes – But DO NOT re-suspend the pellet…
* Re-suspend the nuclear pellet in 350uL of TNEB
  + Sonicate the nuclear pellet
  + Remove 150uL and add to WB sample tube containing 150uL Laemmli buffer
  + Boil WB nuclear samples for 10 minutes at 95oC and then store at -20oC
  + Store remaining 200uL for protein assay

**Mitochondrial Fraction Isolation:**

* Spin the supernatant from the above nuclear isolation at 13,000g for 10 minutes at 4oC
  + Following the spin:
    - Remove the supernatant and place into the second pre-cooled 50mL Sorval tube and spin again at 13,000g for 10 minutes at 4oC (this is to pull down any extra mitochondria that didn’t come down during spin 1)…
    - Re-suspend the pellet in 350uL of TNEB and keep on ice until spin two is complete
    - Following the second spin, transfer the supernatant to the pre-cooled Ultra tube – this will be further used for the cytosolic preparation…
    - Use the 350uL of TNEB / re-suspended mitochondria from above to re-suspend the additional mitochondria that have come down. (You do not want to add separate TNEB to this pellet because you don’t want to dilute your mitochondria any further.)
      * Sonicate
      * Remove 150uL and add to WB sample tube containing Laemmli buffer
      * Boil WB nuclear samples for 10 minutes at 95oC and then store at

-20oC

* + - * Store remaining 200uL for protein assay

**Cytosolic Fraction Isolation:**

* Spin the supernatant from the above mitochondrial isolation at 100,000g which is

24,200rpm when using the TH-641 rotor for 1 hour at 4oC

* + Following the spin:
    - Collect the supernatant in the appropriately labeled 15mL conical tube
    - Re-suspend the pellet in 350uL of TNEB
      * Sonicate both the supernatant and re-suspended pellet
      * Remove 150uL of each and add to WB sample tubes containing Laemmli buffer
      * Boil WB nuclear samples for 10 minutes at 95oC and then store at -20oC
      * Store remaining samples for protein assay

**Buffers:**

**Hypotonic Buffer (HB) 🡪 \*\* Add Fresh \*\***

**Calculations for preparation of 250mL of stock media:**

|  |  |  |  |
| --- | --- | --- | --- |
| **Chemical** | **[Stock]** | **Add** | **[Final]** |
| HEPES | 1M | 2.5mL | 10mM |
| MgCl2 | 4.9M | 76.5uL | 1.5mM |
| KCl |  | 0.186g | 10mM |
| EGTA | 250mM | 1mL | 1mM |
| EDTA | 0.5M | 500uL | 1mM |
| \*\* DTT \*\* |  |  | 1mM |
| \*\* Protease Inhibitor \*\* |  |  | 1:1000 |

* pH to 7.5 (this took approximately 150uL of 10M NaOH
* Bring up to 250mL with ddH20 and filter
* Store at 4oC

**Isotonic Buffer (IB) 🡪 \*\* Add Fresh \*\***

**Calculations for preparation of 250mL of stock media:**

|  |  |  |  |
| --- | --- | --- | --- |
| **Chemical** | **[Stock]** | **Add** | **[Final]** |
| HEPES | 1M | 2.5mL | 10mM |
| MgCl2 | 4.9M | 76.5uL | 1.5mM |
| KCl |  | 0.186g | 10mM |
| EDTA | 0.5M | 500uL | 1mM |
| \*\* Sucrose \*\* |  |  | 250mM |
| \*\* DTT \*\* |  |  | 1mM |
| \*\* Protease Inhibitor \*\* |  |  | 1:1000 |

* pH to 7.5 (this took approximately 150uL of 10M NaOH)
* Bring up to 250mL with ddH20 and filter
* Store at 4oC

**Notes:**