

## **Primary Cortical Neuron Culture--Dissociated via Trypsin**

### **Poly-D-Lysine (PDL) Substrate:**

- **1X stock:**
  - Add 333.35 mL of sterile ddH<sub>2</sub>O to 50 mg of PDL
  - Mix and filter through a 0.22 micron filter
  - Aliquot and store at -20°C
    - Use immediately or within in 1-2 weeks
    - Only freeze / thaw once
- **Coat plates:**
  - Thaw PDL in 37°C water bath
  - Filter through a 0.22 micron filter
  - Coat plates with the following volumes:

▪ 96 well plates	50-60 µL/well
▪ 4 well chamber slides	300 µL/well
▪ 24 well plates	500µL/well
▪ 6 well plates	2.5 mL/well
▪ T25 flasks	3-4 mL/well
  - Incubate coated plates at 37°C for at least 2 hours (Best if left overnight)
  - 1-2 hours before beginning the dissection, remove the Poly-D-Lysine via vacuum and wash twice with sterile ddH<sub>2</sub>O
  - Allow plates to dry completely

### **Dissection / Pre-preparation:**

- Warm the following in a 37°C H<sub>2</sub>O bath:
  - Hanks Balanced Salt Solution (HBSS)
  - 0.25% Trypsin
  - DNase
  - cFBS
  - Preparation media (for washes, trituration, etc...)
  - Plating media
- Add 7 mL of warm HBSS to three 60 mm culture dishes and 10 mL of HBSS to a 15 mL conical tube.
- Asphyxiate an E17 timed pregnant rat by CO<sub>2</sub> and sacrifice by cervical dislocation.
- Spray lower abdomen with 70% EtOH and cut through skin and muscle with a pair of scissors exposing the uterus, intestines and embryos.
- Cut embryos from amniotic sac and decapitate. Place all heads in the first dish of HBSS.
- Remove brain from skull, separate the lobes (place in dish two), remove the meninges (place in dish three) and dissect the cortices (place in 15 mL conical tube containing 10 mL of HBSS).

### **Cell Preparation:**

- Gently spin the conical tube containing the cortical tissue at 1000 rpm for 1 minute.
- In the tissue culture hood, remove the HBSS from the tissue and replace it with 5 mL of 0.25% trypsin.
- Add 75 µL of 0.1% DNase (2000 Units/mg) to the trypsin/tissue.
- Digest tissue in a 37°C water bath for 20 minutes.
- After digestion, split the tissue into two new 15 mL conical tubes (you can do this by eye). One conical tube will be prepped in 3mM NB-A and the other in 25mM NB-A
  - If you only want one glucose concentration, no split is necessary...
- Gently spin the tubes at 1000 rpm for 1 minute.
- Remove the Trypsins/DNase mix from the tissue and wash with 5 mL of cFBS (to inactivate the enzyme)

- Leave cFBS on the cells for about 2 minutes
- Gently spin the tubes at 1000 rpm for 1 minute.
- Remove the cFBS and add 5 mL of the corresponding NB-A (either 3 or 25 mM glucose)
- Gently triturate the tissue with a 10 mL plastic pipette (about 15 times).
- Triturate again with a flamed-tip, glass Pasteur pipette (about 10 times).
- Run the cell solution through a 70 micron strainer and into a 50 mL conical tube.
- Gently spin at 1500 rpm for 5 minutes to pellet cells.
- Remove NB-A media and re-suspend in 20 mL of NB-A plating media
- Dilute cells for counting by adding 20  $\mu$ L of cells to 180  $\mu$ L of plating media (10x dilution)
- Add 10 $\mu$ L of the above cell dilution to each side of a hemocytometer
  - Count cells in the 1mm center square (=25 squares, each containing 16 smaller squares) on each side. The average = # of cells x  $10^5$ /mL
  - To calculate the total # of cells/mL = (the average count per square) x (the dilution factor) x ( $10^5$ ).
    - Ex = 78 cells per square x 10 x  $10^5$  = 780 x  $10^5$ /mL = 7.8 x  $10^6$  cells/mL.
    - Therefore, the total number of cells obtained from the prep =  
7.8 x  $10^6$  cells x 20 mL = 156 x  $10^6$  cells
- Dilute the 20 mL stock to make a final concentration of 0.5 x  $10^6$  cells/mL (50% density)
- Plate cells using the following volumes:
  - For 96 well plates                      100  $\mu$ L/well
  - 4 chamber slides                      500  $\mu$ L/chamber
  - 24 well plates                      1 mL/well
  - 6 well plates                      4 mL/well
  - T25 flasks                      10 mL/flask

### Restorative Feedings:

- Feed cells every 3-4 days.
  - Always do a 50% change of media, never a 100% change.
  - Account for evaporation when keeping cells for long periods.
- The initial feeding will be on DIV3 and will be an “inhibitory” feeding with Ara-C.
  - The final concentration of Ara-C is 1  $\mu$ M.
    - Aliquots of 10 mM Ara-C in ddH<sub>2</sub>O are stored @ -20°C
    - 20  $\mu$ L of 10 mM Ara-C is added to 100 mL of feeding media = 2  $\mu$ M
    - 1/2 change of media = final of **1 $\mu$ M**.
- Check the glucose concentration remaining in the media using an AccuCheck glucometer
  - This will give you mg/dL of glucose
  - To calculate mM glucose:
    - $$\frac{[(x \text{ mg/dL} / 0.1) / (1000 * 180.57\text{g/mol})] * 1000 = \text{_____ mM}}{\text{Glucose}}$$
      - **Example:** AccuCheck reading = 42 mg/dL
        - $$[(42\text{mg/dL} / 0.1\text{dL}) / 1000 * 180.57\text{g/mol}] * 1000 = 2.33 \text{ mM}$$
    - Next, calculate the amount of glucose needed to bring the media concentration of glucose back to that at plating
    - $$[(\text{Total well volume} * \text{mM glucose at plating}) - (1/2 \text{ total well volume} * \text{current conc. of glucose})] / 1/2 \text{ total well volume} = \text{Concentration of glucose needed to bring the media back to that at plating}$$
      - **Example:**  $[(4\text{mL} * 3\text{mM}) - (2\text{mL} * 2.33\text{mM})] / 2\text{mL} = 3.67\text{mM}$
- Feed cells using NB-A media supplemented with the concentration of glucose calculated above.

- 30 minutes after feeding, check the glucose concentration of the media using the AccuCheck meter to be sure you are back at the plating concentration
  - This process must be repeated at each feeding (DIV3, DIV6, DIV8, DIV10 and DIV13) because the cells utilize increasing amounts of glucose as they age in culture.

**Solutions:**

**NB-A<sub>f</sub> Base Media** (for use in Plating and Feeding Medias)

- Defrost B27 and glutamine in 37°C H<sub>2</sub>O bath
- Remove 15 mL from 500 mL bottle of NB-A<sub>f</sub>
- Add:
  - 10 mL of B27
  - 5 mL of 200mM L-glutamine (2 mM final)
    - if necessary, add 0.5% Pen-Strep
- Filter through 0.22 micron filter system, label and store @ 4°C

**100 mM Glucose in NB-A<sub>f</sub> Base**

- Add 9.97 g of glucose to 500 mL of NB-A<sub>f</sub> Base Media (above)
- Filter through 0.22 micron filter system, label and store @ 4°C

**Product Ordering Information:**

70 micron cell strainers, Becton Dickson, 08-771-2  
Hanks Balanced Salt Solution (w/o phenol red), 114-062-101, Quality Biologics  
Ara-C, C6645, Sigma  
B27 Supplement, 17504044, Invitrogen  
DNase, D4513, Sigma  
FBS (Characterized), SH30071.03, HyClone  
L-glutamine, 25030-081, Invitrogen  
Custom Neurobasal Media w/o glucose, 05-0128DJ, Invitrogen  
6 well NUNC plates, 62407-073, VWR  
96 well NUNC plates, 25382-342, VWR  
Poly-D-Lysine, P0899, Sigma  
Trypsin (0.25%), 1505-0065, Invitrogen  
Tweezers, RS-5005, Roboz  
Forceps, 11251-35, FST  
Spring scissors, 15023-10, FST