

## Primary Hypothalamic Neuron Culture--Dissociated via Papain

- **Papain Kit Storage:**
  - Store kit at 4°C
    - Keep Papain and DNase vials in a dessicator
    - Reconstitute ovomucoid inhibitor in 37 mL of EBSS
      - Make five, 7.5 mL aliquots and store in dessicator
- **Poly-D-Lysine (PDL) coating:**
  - **To prepare 1X stock:**
    - 1) Add 333.35 mL of sterile ddH<sub>2</sub>O to 50 mg of PDL.
    - 2) Mix and filter thru 0.22 µm filter.
    - 3) Aliquot and store @ -20°C.  
Use within 1-2 weeks  
Only freeze / thaw once!
  - **To coat plates:**
    - 1) Thaw PDL in 37°C water bath
    - 2) Filter thru 0.22 µm filter
    - 3) Coat plates as follows:

<b>Plating volumes:</b>	
96 well plates	50-60 µL/well.
4 well slides	300 µL/well
24 well plates	500 µL/well
6 well plates	2 mL/well
T25 flasks	4 mL/flask
    - 4) Incubate at 37°C for *at least* 2 hr  
Best if left on overnight
    - 5) 1-2 hours prior to the prep remove plates from incubator and wash twice (with sterile ddH<sub>2</sub>O)
    - 6) Allow to dry completely in hood.
- **Dissection/Enzyme Preparation:**
  - 1) Warm dissection media (HBSS) and place in appropriate dishes.
  - 2) Warm plating media in 37°C H<sub>2</sub>O bath.
  - 3) Asphyxiate the mother (optimum is E16 timed pregnant) and then sacrifice by cervical dislocation.
  - 4) Spray lower abdomen with 70% EtOH and cut through skin and muscle with a pair of scissors exposing the uterus, intestines and embryos.
  - 5) Cut embryos from amniotic sac.
  - 6) Remove brain from skull.
  - 7) Dissect hypothalamus from head.
  - 8) Prepare papain when prep is 50% complete
    - Reconstitute vial of Papain in 5 mL of Earl's Balanced Salt Solution (EBSS)
    - Place in incubator until dissection is complete
  - 10) While Papain is equilibrating, finish the dissection and place all hypothalamic tissue into a 15 mL conical tube filled with HBSS.
- **Plating Procedure:**
  - 1) Reconstitute DNase in 500 µL of EBSS.
  - 2) Add 250 µL of DNase to the papain solution.

- 3) Remove EBSS media from hypothalamic tissue (as much as possible via glass pipette) and  
add the papain/DNase solution to the tissue.
- 4) Place the tissue/enzyme in a 37°C H<sub>2</sub>O bath and allow to incubate as follows:  
1 rat (~15 pups) = 18 minutes
- 5) Invert the tissue/enzyme tube 3 times every four minutes.
- 6) After the incubation is complete, triturate tissue with a 10 mL pipette (13 times) followed by a  
flamed tip glass pipette (13 times).
- 7) Place tube in rack and allow any remaining chunks of tissue to settle to the bottom of the  
tube.
- 8) Once tissue has settled, remove the cloudy supernatant (via glass pipette) and place into a  
new 15 mL conical tube.
- 9) Centrifuge the supernatant at 2000 rpm for 5 minutes.
- 10) While centrifuging, prepare re-suspension media as follows:
  - Place 2.7 mL of EBSS in a 15 mL conical tube
  - Add 300 µL of albumin-ovomuroid inhibitor to tube
  - Add 150 µL of DNase to tube
- 11) After the 5 minute spin is complete, remove supernatant and re-suspend the cell pellet with  
the above re-suspension media using a *flamed tip* glass pipette (Triturate ~ 7 times).
- 12) Filter the re-suspended cells through a 70 µm cell strainer to remove any left-over tissue  
debris.
- 13) Repeat step 12 with a 40 µm cell strainer.
- 14) Prepare a continuous density gradient as follows:
  - Add 5 mL of albumin inhibitor solution to a new 15 mL conical tube.
  - **Carefully** layer the cell suspension from step 13 unto the top of the 5 mL of inhibitor solution (Add the cell suspension very slowly!)
    - You should begin to see three layers appear:
      - Cloudy pink on top
      - Clear, lighter pink in the middle
      - Yellow on the bottom
- 14) Centrifuge the gradient solution at 1000 rpm for 5 minutes.
- 15) After the 5 minute spin, remove the supernatant and re-suspend the pellet as follows via a  
flamed tip glass pipette:
  - 1 rat (15 pups) = 5 mL
  - 2 rat (30 pups) = 10 mL
- 16) Count cells on hemacytometer by diluting 20vµL of cell suspension in 180 µL  
of plating  
media (10x dilution)
- 17). Dilute cells to 0.5 x 10<sup>6</sup> cells/mL
- 18) Plate cells using the following volumes:
 

96 well plates	100 µL/well
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4 chamber slides	500 $\mu$ L/chamber
24 well plates	1 mL/well
12 well plates	2 mL/well
6 well plates	4 mL/well
T25 flasks	10 mL/flask

▪ **Feeding/Inhibition:**

- Feed cells every 2-3 days. Remove 50% of the media and replace with fresh feeding media. Never do a 100% change
- Account for evaporation when keeping cells for long periods.
  - ie... for a 96 well plate, remove 40  $\mu$ L and add back 60  $\mu$ L
- The initial feeding will be on DIV3 and will be the 'inhibiting' 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**.

**Solutions:**

**NB/B27 Plating and Feeding Media**

- 1). Defrost B27 and Glutamine in 37°C H<sub>2</sub>O bath.
- 2). From 500 mL Neurobasal bottle, remove 15 mL
- 3). Add:
  - 10 mL of B27 (2% final)
  - 5 mL of 200 mM Glutamine (2 mM final)
  - \*\*\* if necessary, may add 0.5-1.0% pen/strep.
- 4). Filter through 0.22  $\mu$ m filter system, label and store @ 4°C

**NB-A Base Media**

- 1). Defrost B27 and Glutamine in 37°C H<sub>2</sub>O bath.
- 2). From 500 mL Neurobasal-A bottle, remove 15 mL.
- 3). Add:
  - 10 mL of B27 (2% final)
  - 5 mL of 200 mM Glutamine (2 mM final)
  - \*\*\* if necessary, may add 0.5-1.0% pen/strep.
- 4). Filter through 0.22  $\mu$ m filter system, label and store @ 4°C
- 5). Add necessary amount of 100 mM glucose in NB-A base to get to the desired glucose concentration...

**100mM Glucose in NB-A Base**

- 1). Defrost B27 and Glutamine in 37°C H<sub>2</sub>O bath.
- 2). From 500 mL Neurobasal-A bottle, remove 15 mL.
- 3). Add:
  - 10 mL of B27 (2% final)
  - 5 mL of 200 mM Glutamine (2mM final)
  - \*\*\* if necessary, may add 0.5-1.0% pen/strep.
- 5). Remove 50 mL of NB-A base and replace with 50 mL of 1 M glucose made up in NB-A base.
- 4). Filter through 0.22  $\mu$ m filter system, label and store @ 4°C

**Notes:**

- We are plating the cells at 65% density =  $0.65 \times 10^6$  cells/mL

- We are using restorative feedings only!

**Product Ordering Information:**

Hanks Balanced Salt Solution (w/o phenol red), 14170-112, Invitrogen

Papain Kit, LK003153, Worthington

Ara-C, C6645, Sigma

B27 Supplement, 17504044, Invitrogen

40 micron cell strainer, 352340, Becton Dickson

70 micron cell strainer, 08-771-2, Becton Dickson via Fisher

L-glutamine, 25030-081, Invitrogen

Neurobasal Media, 21103-049, Invitrogen

Custom Neurobasal w/o glucose, 05-0128DJ, Invitrogen

NUNC 6 well plates, 140675, Fisher

Costar 24 well plates, 3526, Fisher

Falcon 12 well plates, 524101, Fisher

Poly-D-Lysine, P0899, Sigma