

# Neonatal Heart Cell Isolation Procedure

Adapted from  
Worthington Biochemical Corporation



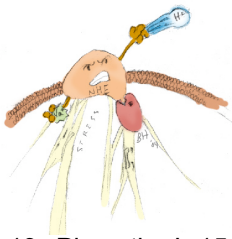
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## Day 1:

1. Take Reagent 1, which is the Calcium- and Magnesium-free Hank's Balanced Salt Solution (CMF HBSS), and Vial 2, which is trypsin, from the refrigerator.
2. Under the sterile hood, pipet 30 ml of CMF HBSS into two sterile 50-ml plastic centrifuge tubes (30 ml in each) and place these tubes on ice.
3. Combine 2 ml of CMF HBSS into the vial of trypsin, using a sterile pipette tip in the hood, mixing thoroughly, and place the vial on ice.
4. Put a sterile 10 cm petri dish on ice in the hood using a small glass dish.
5. Cover the Styrofoam "operating table" with a new lab cover and stick pins in it for later use. Put a small, brown, plastic bag in one of the containers for the rat pup bodies. Also add lab cover to the second container for the rat pups and retrieve the rat pups from the animal room.
6. Carefully remove the heads from the rats and pin down three appendages. Sterilize the abdomen of each rat with 70% ethanol solution, and surgically remove the beating heart. Immediately place the heart in the centrifuge tube on ice.
7. After all rat hearts have been extracted, cap the centrifuge tube tightly and swirl it to rinse the hearts. Pour off most of the liquid and add 10 ml of CMF HBSS to the tube. Swirl and pour off the liquid as before.
8. Transfer the hearts to the petri dish and mince the tissue with a sterile scalpel to approximately less than 1 mm<sup>3</sup> pieces, keeping the dish holding the tissue on ice.
9. If the rats are 3 days old or younger, add 9 ml of CMF HBSS to the tissue and 1 ml trypsin solution. If the rats are 4 days old or older, add 8 ml of CMF HBSS to the tissue and 2 ml of the trypsin solution.
10. Mix the tissue and trypsin completely by swirling and place in refrigerator overnight. Do not leave the tissue in the refrigerator for more than 20 hours.

## Day 2:

11. Start Hybridization Oven (37.0°C) and Shaking Water Bath.
12. Obtain  
From the refrigerator:
  - Hank's Balanced Salts Solution with antibiotic and antimycotic (**HBSS**)
  - Leibovitz L-15 **dissociation media**
  - Leibovitz L-15 **culture media**
  - Leibovitz L-15 **culture media with 0.1 mM BrdU**
  - Vial 3 (trypsin inhibitor)
  - Vial 4 (collagenase)
  - sterile plastic strainer  
From Other Areas:
  - 500 ml beaker of ice
  - 300 – 500 ml beaker for waste liquids
  - Coverslips which have been autoclaved



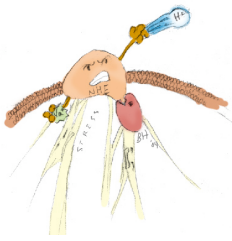
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13. Place the L-15 **culture media** and L-15 **culture media with 0.1 mM BrdU** in the water bath to warm it up.
14. Sterilely pipette 31 ml of **HBSS** into a 50-ml centrifuge tube and place this on ice.
15. Sterilely pipette 30 ml of L-15 **dissociation media** into each of two 50-ml centrifuge tubes. Leave these two tubes under the hood at room temperature.
16. Pipette 1 ml of **HBSS** from the centrifuge tube into **Vial 3** (trypsin inhibitor) and mix thoroughly. Leave this vial under the hood at room temperature.
17. Pipette 5 ml of **dissociation media** from one of the centrifuge tubes into **Vial 4** (collagenase) and mix thoroughly. Leave this vial under the hood at room temperature.
18. Remove petri dish containing heart tissue from the refrigerator bring this to the hood.
19. Using a 25 ml wide mouth pipette, **quickly** transfer tissue and solution in the petri dish to the centrifuge tube containing **HBSS**.
20. Add the 1 ml of solution from **Vial 3** (trypsin inhibitor) to the centrifuge tube containing heart tissue and HBSS. Cap the tube tightly and mix by inverting several times.
21. Warm the centrifuge tube containing the heart tissue and solution for 30 minutes in the 37°C shaking water bath.
22. Bring the centrifuge tube back to the hood and slowly add the entire contents of **Vial 4** (collagenase) to the 50 ml tube containing the tissue. Cap the tube tightly and mix.
23. Wrap the tube with 3 paper towels that have been folded in half twice. Place the centrifuge tube in the hybridization oven. Incubate the rotating cells for 30 minutes.
24. Remove the tube from the hybridization oven and return it to the hood.
25. With a 10-ml pipet tip, triturate about 15–20 times to break up the cells. (Trituration is cell dispersion through mild pumping action.) **Do this gently and slowly so that the cells remain intact.** Allow the tissue residue to settle 3 – 4 minutes.
26. Using a new 50 ml centrifuge tube, rinse the plastic cell strainer with 2 ml of the **dissociation media**.
27. Filter the supernatant (cloudy media with cells) from step 14 through the cell strainer into the new 50-ml centrifuge tube containing 2 ml of **dissociation media**.
28. If undissociated tissue remains:
  - Add 5 ml additional **dissociation media** to tissue residue
  - Repeat the trituration step.
  - Allow tissue residue to settle as before (3 – 4 minutes).
  - Filter the cells through the same cell strainer, adding to the media that was strained before.
29. After all the supernatant has been filtered, rinse the strainer with 2 ml **dissociation media**.
30. Allow the filtered cells to remain undisturbed for 20 minutes at room temperature. The cells can be held up to 1 hour at this point.



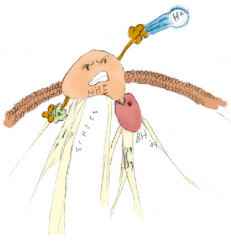
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31. Place coverslips into 35 mm petri dishes:
  - General rule is one petri dish for every 5 rat pups for pH experiments.
  - Place 3 or 4 dry, sterile coverslips into each petri dish.
  - Add 2 ml of **culture media** to each dish.
  - Incubate the coverslips at 37°C in the incubator for a minimum of 30 minutes. The coverslips can be incubated until the cells are through the preplating phase of the procedure.
32. Swirl the cells from step 21 gently and add all of the media and cells to four 15-ml centrifuge tubes.
33. Centrifuge the four tubes in a clinical centrifuge at speed 2 for 5 minutes to form a pellet of cells at the bottom of the tubes.
34. Bring the tubes back to the hood and pour off the supernatant.
35. Add 10 ml dissociation media to each tube and gently pipet up and down to break up the pellet.
36. After the pellet is broken up, cap the tube tightly and centrifuge as before.
37. Bring the tubes back to the hood and pour off the supernatant.
38. Preplate the cells to remove fibroblasts.
  - Disperse the cells in all four tubes in a total of 16 ml **culture media**.
  - Add the 16 ml of **culture media** to a T75 flask.
  - Incubate at 37°C in the incubator for 30 minutes.
39. Following the incubation remove the 16 ml media from the T75 flask with a minimum of shaking or disruption.
40. Add 8 ml of media to each of two 15 ml centrifuge tubes.
41. Centrifuge for 3 minutes at setting 2 in a clinical centrifuge.
42. Bring the tubes back to the hood and pour off the supernatant.
43. Add **culture media with 0.1 mM BrdU** to the cells and disperse the pellet.
  - Add 2 ml **culture media with 0.1 mM BrdU** for each petri dish of cells which you wish to plate.
  - Combine all the cell suspension and mix prior to adding cells to culture dishes.
44. Obtain the petri dishes with the coverslips from the incubator.
45. Remove **culture media** from the dishes.
46. Add 2 ml of cell suspension in **culture media with 0.1 mM BrdU** to each of the dishes.
47. Do not touch or disturb the cells for at least 24 hours.
48. The following day, rinse the cells with PBS and add 2 ml of fresh **culture media with 0.1 mM BrdU** to each dish.



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## Directions for making L-15 Dissociation and Culture Media

1. Sterilely add 2.5 ml (2 ½ eppendorf tubes) of antibiotic/antimycotic solution from the freezer to the 500 ml of CMF HBBS media. There is nothing more to add to this.
2. Take the pouch containing Leibovitz L-15 Media powder from the Worthington Kit. Cut open the top of the envelope and pour contents into a beaker containing 900 ml of cell culture grade water (water from the brown box in lab). Rinse pouch 2-3 times with the remaining 100 ml of water to get a final volume of 1 L in the beaker.
3. Add 5 ml (5 eppendorf tubes) of antibiotic/antimycotic solution from freezer to media.
4. Split media into two beakers with 500 ml of media in each beaker.
5. Filter one of the beakers of this media (500 ml) into a 1L bottle and label as **Leibovitz L-15 Dissociation Media**.
6. Take the other 500 ml of media and add 23 ml of calf serum (in -80° freezer) and 1.05g of sodium bicarbonate (NaHCO<sub>3</sub>). Mix thoroughly and filter into a 1 L bottle. Fill 2 sterile 50 ml Sigma bottles with this media. Label as **Leibovitz L-15 Culture Media**.