RAT CARDIAC MYOCYTE ISOLATION PROTOCOL FOR PRIMARY CULTURE

I. Preparation for cell isolation:

A. Make sure the following items are autoclaved:

1. Glassware
   a. (2) 20 ml beakers
   b. (4) 50 ml beakers
   c. (2) 100 ml beakers
   d. (3) 250 ml beakers
   e. (3) wide mouthed 1-liter bottles
   f. (2) 100 mm glass petri dishes
   g. (1) cellector

- Cover all openings with foil
- Place autoclave tape on each item
- Loosen bottle caps

2. Surgical instruments
   a. large scissors
   b. dissecting scissors
   c. (2) dissecting (needle-nosed) forceps
   d. small hemostats
   e. large forceps
   f. (2) triturating pipettes and cannula

- Place all the surgical instruments in surgical autoclave tubing
- use autoclave tape to seal the tubing

B. Prepare solutions amount will depend on how many preps are planned. Solutions are stable for 1 week:

1. Sterile ddH_20
   Filter through a .2 μm filter into an autoclaved 1L bottle

2. Add 5 ml of P/S per 500 ml of ddH_20
   Filter with the same .2 μm filter as above into another autoclaved 1L bottle

3. Ca^{2+} - free KHB- 500ml or 1 L

4. 1mM Ca^{2+} - KHB- 500 ml or 1 L

5. 2% BSA in 1 mM Ca^{2+} - KHB - 50 ml
   Filter all KHB solutions w/ the same .2 μm filter in the order 3-4-5

6. DMEM (Invitrogen #11965-092) + penicillin + streptomycin: 500 ml DMEM + 5 ml P/S

7. DMEM + P/S + 10% fetal bovine serum (FBS): 90 ml DMEM + P/S + 10 ml FBS

C. Wash out the perfusion apparatus:

1. Every month break down the apparatus and clean with 7x soap and pipe cleaners, rinse extensively with ddH_20 before setting back up.

2. At the same time, drain the water bath, wash with 70% EtOH, then refill w/ ddH_20.

3. Before each prep rinse thoroughly with the following solutions. Use approximately 250 ml per rinse.

a. 70% EtOH
b. sterile ddH_20- twice
c. sterile ddH_20 + P/S

D. Heparinize rat (by injecting 0.3 cc of 1000 U/ml heparin; 1500 U/ kg rat wt.+ 0.1 cc nembutal as a sedative) 20 min prior to starting experiment.

E. Turn on the circulating water bath.

F. Place 1mM Ca^{2+} - KHB in the left syringe and Ca^{2+} free- KHB in the right syringe. Flush system to make sure no bubbles remain and oxygenate left syringe for 10 -15 min prior to experiment.

G. Set up dissecting area:

1. Need 2 diapers down - one to wrap up rat.

2. Place the following items in a full bucket of ice.

a. 50 ml beaker filled with Ca^{2+} -KHB. This beaker is used to rinse and gently pump heart initially and then to soak surgical instruments prior to use.
b. One sterile 100 mm glass petri dish. Each half of the dish should be filled with Ca^{2+} - KHB. One half of the dish is used for dissecting away excess tissue, while the other half (cannulation dish) is used for mounting the heart onto the cannula.

3. In the cannulation dish, place cannula on 10 cc syringe containing Ca^{2+}-KHB and mounted such that the cannula is just off the floor of the dish. Pre-tie a piece of surgical silk around the cannula. Have a small pair of hemostats ready for clamping aorta to cannula.

4. One 250 ml beaker with 95 % EtOH (~50-100 ml) for sterilizing instruments.

H. Prepare collagenase solution in sterile 50 ml beaker.

12 mg hyaluronidase (Sigma H3506; Lot 44H7065)
30 mg collagenase (Worthington type II; M5E456; 331 U/mg)

20 ml KHB without Ca^{2+}. Ultimately this will be diluted up to 60 ml to obtain 0.2 mg/ml hyaluronidase and 0.5 mg/ml collagenase (165 U/ ml).

II. Heart Perfusion

A. After heparinizing rat for 15-20 minutes, anesthetize rat (0.7 - 0.8 cc of 50 U/ml nembutal; 162.5 U/kg rat wt.). Wait until rat is not responsive (check by pinching feet with forceps), then spray abdomen with EtOH (70%).

B. Put on sterile gloves (keep a second set handy) and a surgical mask.

C. Cut open thorax and remove heart above aortic arch.

D. Rinse heart in 50 ml beaker of ice cold Ca^{2+} - KHB, making sure as much blood as possible is gently squeezed from chamber. Then transfer to 100 mm petri dish and tease away excess tissue from aorta with the two needle-nosed forceps.

E. After cleaning up the heart, transfer it to the 100 mm cannulation dish and mount on cannula and hold with hemostat. Tie on once, check to see if it is firmly mounted by gently pushing KHB through heart via syringe. Then secure with second tie behind and third tie in front with surgical knot. Start slow drip on perfusion apparatus. Transfer cannula with heart to perfusion apparatus and turn on flow partially.

F. Perfuse 5 min with Ca^{2+} - KHB. Wipe off oxygenator, transfer to Ca^{2+} - free KHB after ~3 min.

G. Perfuse 5 min with Ca^{2+} - free KHB.

1. After 1 min, collect fluid for 1 min to determine flow rate (~5-6 ml/min desired).

2. After 3 min, make sure syringe has ~35 ml of Ca^{2+} - free KHB and add collagenase solution to make final volume = 80 ml (15 ml in tubing).

H. After collagenase is added, place heart in 20 ml beaker so it is immersed in collagenase solution. Tape pump tubing in beaker and begin to pump collagenase solution to the right syringe. Periodically monitor flow rate and if it is too rapid slow by partially closing off perfusion apparatus by turning the knob above the cannula. Perfuse with collagenase solution for 15 minutes.

I. At the end of the first 15 min add 3x 200 µl aliquots of 100 mM $CaCl_2$ to syringe at 30 sec intervals (final $[Ca^{2+}]$ = 1mM). Add 50 ul directly to the beaker where the heart is hanging. Continue to perfuse for an additional 15 min.

III. Cell Isolation

A. Remove heart and cannula from perfusion apparatus and transfer them to a plastic petri dish containing 20 ml of KHB + collagenase + Ca^{2+}.

1. Remove the atrium, aorta, cannula and thread from the petri dish using the small scissors and needle-nosed forceps. Mince ventricular myocardium into ~6-8 pieces with the small scissors and pour into sterile 50 ml beaker.

2. Save the enzyme solution in a fresh, sterile beaker for later digests.

B. Swirl gently for 5 minutes in water bath, then discard supernatant with pipette and resuspend ventricle in 20 ml of enzyme solution and repeat.

C. For the third digest gently triturate the minced tissue with wide bore sigma coated Pasteur pipette then swirl in water bath five minutes. Transfer supernatant into two 15 ml conical tubes; add 20 ml of enzyme solution to the remaining ventricular tissue and set aside until finished with the collected cells. Centrifuge conical tubes on setting 4 (~ 10 to 15 secs). Pour off supernatant and combine the two pellets in 5 ml of KHB + Ca^{2+} + 2%BSA. Place a drop of cell suspension on a coverslip and view on light microscope to check viability. Place cells in 37°C incubator.

D. For the fourth (or sometimes fifth if there is excess tissue after the fourth digest) and final digest repeat step C and collect the cells by passing remaining tissue and supernatant through the cellector in a 100 mm petri dish. Transfer suspension to 2 x 15 ml tubes. Centrifuge the conical tubes on setting 4 (~10 to 15 secs). Pour off supernatant and combine all of the collected pellets in a total volume of 10 ml KHB + Ca^{2+} + 2%BSA. (Based on how many cells are needed and the apparent viability can use digest 3, 4 or 5 alone or combined.)

E. Increase [Ca^{2+}] to 1.75 mM by adding 3 x 25 µl of 100mM $CaCl_2$ / 10 ml cell suspension at 5 min. intervals. After final addition and incubation, centrifuge on setting 4 (~10 – 15 secs), pour off supernatant and gently resuspend in ~ 3ml DMEM + P/S in the TC hood.

IV. Plating Cells

A. In preparation for plating cells, you will need to do the following in the hood prior to getting the cells out of the incubator:

1. Laminin coat coverslips (will need someone to do this for you): start ~ 40 min before ready to plate, place 1x18 mm^2 coverslip per well, UV (uncovered) for 10 min. Coat each coverslip with 100 µl laminin (40µg/ml), UV (uncovered) for 10 min to sterilize, cover dishes and leave in hood until plating.

2. Warm DMEM +P/S and DMEM+P/S +10% FBS at 37°C. Rinse bottles with 70% EtOH and wipe with kimwipe before placing bottles in hood.

3. Wipe down hood with lysol using paper towels and then 70% EtOH using kimwipes.

4. Place vacuum apparatus, pipette aid and tube of sterile Pasteur pipettes with holding ring in hood.

5. Dry off hemocytometer.

6. Get pen, paper and calculator.

B. Count cells:

1. Transfer a small aliquot of resuspended cells (make sure cells are resuspended completely and are not settling out) with sterile Pasteur pipette to hemocytometer.

2. Count 9 fields: live (rod-shaped) cells vs. dead (non-rod shaped) cells.

3. Calculate the percentage of rod shaped cells and the concentration of cells (concentration = # of cells in 1 field x 10^4 myocytes/ml). Multiply this concentration by the total volume of DMEM to have a total # of cells. Expect 65-75% viability (rod shaped cells) and 1-3 x 10^6 rod-shaped cells total. (Example: pellet in 3 ml of DMEM. Counted 250 rod shaped cells, 345 total cells. Viability = 250/345 = 72%. Yield = 250/9 fields = average 27.8 cells per field = 2.78 x 10^5 cells per ml. Multiply by 3 ml = 8.34 x 10^5 viable cells total.)

C. Dilute cells to 1 x 10^5 rod-shaped cells/ml and plate.

1. Add DMEM + P/S + 10% FBS to dilute the cells so that the final concentration of FBS is 5%.

2. Remove excess laminin from coverslips by aspirating.

3. Carefuly plate 200 ul cell suspension per coverslip (2 x 10^4 cells per converslip).

D. Place plate in incubator carefully so as to not disturb cells. Incubate for 2 hours.

E. After 2 hours incubation, remove serum containing media by aspirating from the edge of the coverslip. Add back 2 ml DMEM + P/S and incubate until ready to use or perform gene transfer.

IV. Troubleshooting:

A. For Low Viability

1. Check to see if there were deviations from the standard procedure, if there were note them for future reference.

2. Common errors in the setup include not turning on the water bath soon enough to properly heat the apparatus, not turning on the oxygenator and reversing the placement of the Ca2+ and Ca2+ free KHB in the two syringes.

3. Problems in the cell isolation include swirling the ventricles too vigorously during the digest and/or triturating the cells too harshly up and down during the digests. It is necessary to have a slow and steady triturating and swirling so cells are not damaged. Another problem is over digesting the cells, this can be due to too much collagenase or a highly active batch. Therefore, when digesting ventricles be aware of how much has already been digested after third digest, and divide if a full fourth digest is necessary.

4. There are also potential problems in the plating of the cells. When the cells are counted before the plating, the cells with media in the tube should be placed in the incubator so they stay in a warm environment. For each plate the cell suspension is mixed with the pipette between plating each well. This process can lead to cell damage if the mixing of the cell suspension is not done gently enough. Another possibility of poor viabilities comes from the handling of the plates when they are put in the incubator, since the media on the coverslip is easily disturbed. Finally when the media is changed after the two hours, make sure that you aspirate off the media from the edges of the coverslip. You do not want to place the pipette near the middle of the coverslip since that is where the cells are most concentrated.

B. For Contamination

1. The apparatus should be cleaned out after every third heart prep with 7x soap and then soaked over night in its own plastic bucket with filtered, deionized water.

2. After rinsing out the apparatus after each heart prep with EtOH and ddH2O immediately cover syringes and exposed tubing with parafilm to prevent airborne contamination.

3. Periodically changed the diapers (blue pad) on the table of the perfusion apparatus.

4. Wear a surgical mask and gloves when performing heart prep. The surgical mask is especially important when you are sick.

5. Wash and dry surgical equipment immediately to prevent them from rusting.

Krebs Henselait Buffer	MW	STOCK	g in 1L stock	1L Krebs	500 ml Krebs
118 mM NaCl	58.44	1 M	58.44 g	118 ml	59 ml
4.8 mM KCl	74.53	0.5 M	37.27 g	9.6 ml	4.8 ml
25 mM Hepes	260.3	add fresh		6.5 g	3.25 g
1.2 mM KH_2PO_4	136.1	0.5 M	68.05	2.4 ml	1.2 ml
1.2 mM $MgSO_4 \times 7H_2O$	246.47	100 mM	24.65 g	12 ml	6 ml
1.0 mM $CaCl_2 \times 2\ H_2O$	147	100 mM	14.70 g	10 ml	5 ml
11 mM Glucose	180.2	add fresh		1.98 g	0.99 g

*** Calcium-free Krebs-Henselait buffer: Omit $CaCl_2$
*** Oxygenate buffers for 10-15 min before using
***Adjust pH to 7.40

1 mM Ca^{2+}-Krebs + 2% BSA: 1 g BSA into 50 ml of 1 mM Ca-Krebs
2 g BSA into 100 ml of 1 mM Ca-Krebs

MW 5/98