RAT Myocyte Isolation Protocol (non-Langendorff mode)

Liao Lab Protocol, modified 7/4/2014

Materials: For 1 to 3 rats → $1X10^6$ myocytes/ ml

Perfusion apparatus
4 oz specimen container. (Pcup) for second enzyme digestion
Sterile Instruments: medium size scissors
2 forceps
medium size hemostat
sterile falcon P60/p100
bulldog clamp

Filters

Millex-GV	0.22um Filter unit	Millipore (ref# SLGVR25LS)
		Corning

Solutions
All solutions should be sterile (autoclave or filter) using MilliQ-H_20

Ca^{2+} -Free Krebs buffer:
NaCl stock (68.95g/500ml H_20)
KCl stock (3.5g/500ml H_20)
$MgSO_4$ stock (1.44g/500ml H_20)
KH_2PO_4 stock (1.635g/500ml H_20)
$NaHCO_3$ stock (21.0g/500ml H_20)

Add 100ml of each to 4.32 g glucose and bring to 2000 ml.

Enzyme Solutions:
Made in 30ml Ca^{2+} free buffer with glucose
Enzyme 1: Collogenase (Worthington cat# 4177 lot#)
21560 u/200ml Ca^{2+} -Free Krebs buffer
Hyaluronidase (Sigma cat# H-2126)
??? u/200ml Ca^{2+} -Free Krebs buffer

Made in 30ml Ca^{2+} free buffer with glucose
Enzyme 2: Trypsin (sigma cat# t-0303)
0.3 mg/ml store in -20
DNAase I (Worthington cat# 2007)
0.3 mg/ml store in -20

1 M sterile $CaCl_2$ (100 cc)
70% Ethyl Alcohol (Liters)
DMEM (Gibco)
BSA (Sigma)
Penicllian-Streptomycin (Gibco cat# 15140-148)

Laminin (Invtirogen)
ACCT Media (1 g BSA, .2g L-carnitine (Sigma C-0283), .33g Creatine (C-0780), .31g Taurine (T-0625)) in DMEM + PS

Methods:

- All conditions are sterile and performed at room temperature
  1) Turn on water bath
  2) Make 2 L fresh Ca^{2+}-Free buffer (useable for 2 days)
  3) Make Enzyme 1 Solution
  4) Rinse perfusion apparatus with 70% ethyl alcohol then MilliQ H_2O
  5) Circulate Ca^{2+}-Free buffer
  6) Fill P60 with 5 cc Ca^{2+}-Free buffer –store @ 4°C
  7) Anesthetize (Pentobarbitol) rats IP
  8) Excise heart
     a. Soak chest with alcohol
     b. Open chest
     c. Remove heart by cutting just above thymus
     d. Place in Ca^{2+}-Free buffer/start perfusion pump ~ 1 ml/min
     e. Peal thymus and locate transverse/descending aorta
     f. Cut aorta at this region and cannulate tip of cannula just above pulmonary artery
     g. Use hemostat to clamp heart and tie aorta to cannula
     h. Increase pump speed ~ 7ml/min
     i. remove LA and RA
  9) Perfuse with Ca^{2+}-Free buffer for 2-3 min
  10) Stop pump/ quickly place tubing in to Enzyme 1/Perfuse with Enzyme 1 for 20 min (Note: for 3 hearts Enzyme 1 must be recirculated, but be sure NOT to let Ca^{2+}-Free buffer wash into Enzyme 1 solution)
  11) Set incubator to 37°C and set shaker at 150 rpm
  12) Thaw Trypsin and DNAase aliquots for Enzyme 2
  13) Aliquot ACCT
     a. 20 ml in 50cc tube then add 1.29 BSA
     b. 2 X 50 cc tubes for laminin 10 µg/ml
     c. 25 ml + 25 ml Ca^{2+}-Free buffer (WASH BUFFER)
  14) Add Trypsin, DNAase, 20 µl CaCl2 to 20 ml Enzyme 1 in sterile Pcup and add 5 ml of Enzyme 2 to P60
  15) Plate Laminin DMEM + PS, swirl after adding to plate
     a. P60 2ml
     b. P100 5ml
     c. 6 well plate 2ml
     d. cover slips few drops
  16) Using forceps and scissors:
     a. Cut heart down while holding tip of apex, and place in P60
     b. Cut heart 8 to 10 small pieces (save one piece a view under microscope; if rod shaped myocytes are visible, digestion went well)

17) Combine hearts with Enzyme 2 in Pcup
18) Heat and shake in incubator for 18 minutes
19) Finish platting laminin, store solutions, and clean perfusion apparatus with water and alcohol
20) Mix BSA and ACCT after platting laminin
21) Pour 15cc of wash buffer through nylon mesh filter
22) Break tip off 10cc pipett and aspirate cells up and down gently then eject cell suspension through filter Filter (*avoid running bubbles through cell suspensions)
23) Centrifuge (50 X g 3 min)
24) Aspirate supernatant with 2cc pipette (save for fibroblast isolation) to 5cc level
25) Slowly add 10cc wash media to one pellet, resuspend cells by aspirating slowly, and combine pellets.
26) Let cells settle (5-8 min), asparate supernatant, and resuspend cells in 10cc wash media
27) Again, let cells settle and asparate supernatant to about 7 cc mark
28) Using 10 cc pipette, transfer cell suspension to BSA solution by SLOWLY ejecting cells onto wall of 50cc tube. There should be two distinct layers
29) Let cells settle (6-8 minutes) [Avoid letting cells settle too long allowing a white rim to form on top of pellet]
30) Aspirate laminin solution from plates and add ACCT
    a. 1 ml/P60 or cover slips
31) Aspirate BSA/cell supernatant and resuspend cells in ACCT (500,000 cells/ml) and plate (1 ml/P60)