CARDIOMYOCYTES FROM ADULT RAT (150-200gr) ISOLATION KIT

Enzymes

COL G (210 U) recombinant collagenase class I + COL H (1050 U) recombinant collagenase class II + Thermolysin (1050 µg)

Stock solutions preparation

1. Dissolve COL G in 1.5 ml sterile H_2O and make 3 aliquots of 500 µl (each aliquot is the Solution A) and store at -20°C

2. Dissolve COL H in 3 ml sterile H_2O and make 3 aliquots of 1 ml (each aliquot is the Solution B) and store at -20°C

2. Dissolve the Thermolysin in 1.5 ml sterile H_2O and make 3 aliquots of 500 µl (each aliquot is the Solution C) and store at -20°C.

Buffers Preparation

1. Cardiomyocytes isolation buffer (CIB) 1 liter: Prepare a solution of NaCl 120 mM, KCl 5.4 mM, $Na_2HPO_4*7H_2O$ 0.33 mM, $MgSO_4*7H_2O$ 0.5 mM, BDM 10 mM, HEPES 25 mM, Glucose 22 mM, Taurine 30 mM. Adjust the pH (7.2-7.4) with 5 M NaOH and filter for sterility. Add 3% of antibiotic pen/strep. Add 0.05 mU of insulin per litre of cardiomyocyte isolation buffer. CIB can be stored at 4°C.

2. Perfusion Buffer (135 ml): 0.4 mM EGTA in CIB.

3. Digestion buffer (70ml): Solubilize one aliquot of Solution A, one aliquot of Solution B + $CaCl_2$ 300 µM, in CIB. Immediately before use add one aliquot Solution C

4. Stop Buffer (25ml): CIB + EDTA 2mM.

5. Culture media: MEM media, 1% Glutamine, 1% pen/strep, 1.26 mM $CaCl_2$.

Prepare all equipment

1. Put water bath, ice bath and peristaltic pump under a laminar flow fume hood, sterilize them with UV-light and set the water bath temperature at 37 ˚C.
2. Clean peristaltic pump by flushing with 70% ethanol and then remove all traces of ethanol from system by flushing with several volumes of saline serum (0.9% of NaCl in water or CIB solution).
3. Load the peristaltic pump with cold *perfusion buffer* and fill the dead space with perfusion buffer. **Ensure no air bubbles are present in the line**. Set the speed at 6 ml min^{-1}

4. Prepare a syringe with cannula needle to be used for aortic cannulation (cannula must be carefully chosen based on the aorta size, 0.6-1.3 mm in mice and 1.6-3.2 mm in rats). Attach a cannula needle to a 5 ml syringe filled with cold perfusion buffer. Remove air bubbles.

Surgical Procedure (Heart Extraction and Cannulation)

1. Administer heparin (5 IU/g body weight) to rat via intraperitoneal injection: in 20- 30 min the heparin circulate to the heart.
2. Anaesthetise the rat in an induction chamber using 5% of isoflurane (inhalant anaesthetic).
3. Immobilize the rat to the surgery platform by securing each limb. Disinfect the incision area with 70% ethanol.
4. Lift the skin with tissue forceps just below the sternum. Make a lateral incision with small scissors, cutting through the skin and abdomen.
5. Use hemostats to gently lift the ribcage via the sternum. Using small dissection scissors, extend the incision in a V-shape toward the axillae. Cut through the first rib and puncture the diaphragm on each side.
6. Continue to lift the ribcage with hemostats and use small iris scissors to completely transect the diaphragm. Take care not to puncture the heart.
7. Retract the ribcage rostrally using attached hemostats and quickly but carefully extend the V-shape incision by cutting through the ribcage toward the axillae on both sides. Retract the middle section of the ribcage fully and lay down the attached hemostats to hold in place.
8. Remove the pericardium using fine forceps.
9. While gently lifting the heart using curved forceps, transect the veins and arteries attached to the heart. Immediately place the heart in ice-cold perfusion buffer to slow muscle contraction.
10. Place the heart in a petri dish under a dissection microscope. Trim any excess non-cardiac tissue using fine iris scissors. Use caution when cutting near the aorta.
11. Transect the aorta near the brachioencephalic artery, and take care not to let bubbles or tissue debris enter the opening.
12. Fill the petri dish containing the heart with ice cold *perfusion buffer* such that the liquid level rises above the opening of the cannula. Sheath the aorta onto the cannula such that the tip is just distal to the aortic valve
13. Slide a pre-tied suture down the shaft of the cannulation needle and position just above the tip of the needle. Tighten the knot with fine forceps. Repeat this step with a second suture placed just above the first.
14. Slowly depress the syringe to eject 5 ml of perfusion buffer through the coronary vasculature.

IMPORTANT: The time from opening of the thoracic cavity to initial perfusion should be minimized to reduce hypoxic exposure and maximize the survival and health of the isolated cardiomyocytes. Ideally, this time should be less than 5 min

Perfusion and Purification

1. Put the Petri dish with the syringe, cannula and the heart inside the laminar flow fume hood to elude contamination.
2. Remove the cannula hub from the syringe and attach the needle hub to the perfusion outlet port while the pump is running at 6 ml /min.
3. Perfuse the heart until 30 ml of cold *perfusion buffer* have been aspirated by the inlet hose
4. Remove the inlet tube from the *perfusion buffer* and wash heart with 10 ml of cold *Cardiomyocyte isolation buffer, CIB* to remove traces of EGTA.
5. During the perfusion step the heart change in colour becoming light pink due to removal of blood.
6. Place the inlet tube into the warm *digestion buffer* placed in the Water bath set at 37°C
7. Perfuse the heart with warm *digestion buffer* for 4 min. The heart will begin slouching as the collagen is degraded and it loses mechanical support.

Mechanical Dissociation and Purification

1. Remove the heart from the cannula with forceps and place in a dry petri dish. Quickly trim extra-ventricular tissue. Transfer the ventricles to a fresh 6-Well plate containing 5 ml of warm *digestion buffer* with 8.4 µl of 1 M $CaCl_2$ and leave for 5 minutes. This initial solution will be then discarded.
2. Transfer heart to a new 12-Well containing 2 ml of warm (37°C) *digestion buffer* with 1.84 µl of 1 M $CaCl_2$ for 5 minutes and longitudinally divide the heart in two parts. After transferring the two parts of the heart to a new 12-Well plate containing 2 ml of warm (37°C) *digestion buffer* with 1.84 µl of 1 M $CaCl_2$. Keep monitoring state of digestion under an optical microscope and wash gently using a Pasteur pipette.
3. Repeat this step until the first cardiomyocytes with an integral structure are seen in the cell suspension (approx. 25-30 minutes). Keep repeating the previous step every **4 minutes** until all the tissue has been digested (approx. 80-90 minutes). The repeating time can be reduced to 3 min or increased to 5 min depending on the number of cells released that can be monitored with the optical microscope. Add **1.5 ml of WARM (37°C) stop buffer** immediately after transferring the heart to a new 12-Well and collect cell suspension on a 15 ml Falcon tube.
4. Centrifuge at 300G for 3 minutes and resuspend in 2 ml of warm culture media to remove enzymes and EGTA from the cell suspension. ***Note: It is advised that two operators perform this part of the procedure and the work is being carried out simultaneously in two different 12-Well plates to simplify and facilitate the cell collection and centrifugation steps.***
5. Filter cell suspensions through a 230 µm mesh and transferred to a new 50 ml Falcon tube. ***Note: if number of extracted cells is very high, collect in several 50 ml Falcon tubes to avoid the formation of big cell masses.***
6. Collect cells by centrifugation at 300 G for 3 min (acceleration and deceleration 7-7) and discard the supernatant.
7. Resuspend cells in **4 ml of WARM (37°C) culture media** and mixed by gentle inversion.

8. In order to performed a separation by Percoll gradient, on a new 50 ml Falcon tube add in this order:
    a. **5 ml of High density solution**
    b. **5 ml of Low density solution**
    c. **4 ml of cell suspension**

Centrifuge at 1000G for 5 minutes (acceleration and decelaration 3-0)Recover rings containing cells and wash traces of Percoll if needed; resuspend pellet in culture media.

9. Count cells on a haemocytometer and seed on laminin-coated (10 µg ml^{-1}) plates.

10. After centrifugation, collect the ring containing cardiomyocytes that is between the both percoll phases with care. To assess the yield of cardiomyocyte isolation, count the cells via hemacytometer under a microscope. Add pre-warmed, equilibrated *culture media* if needed and mix by gentle inversion.

Cardiomyocytes Culture

1. Pre-coat plate surfaces with laminin 221 (10 µg/ml in PBS; Biolamina) and incubate at 37° C for 2 hr prior to plating cells. Just prior to plating, aspirate the laminin solution and wash once with MEM or PBS.

2. Plate desired concentration of cells (typically 5 - 20 x 10^4 cells/cm^2).

3. Move the plates promptly to a 37 °C incubator with 5% CO_2.
4. Change media as needed to prevent acidification: every 1 - 5 days depending on cell density. Minimize handling of the culture dish during the first 3-6 days to facilitate the attachment to the cell culture surface.

***Note**: This protocol is meant to be a starting point; all isolation procedures require an individual optimization.COL G and COL H concentration, protease addition and digestion time can be experimentally adjusted.*