Protocol for Neonatal Rat Primary Cardiomyocytes Assays

Introduction

Reagents for NRPCM isolation

a) Fibronectin: Sigma; Catalog # F1141
b) Phosphate-Buffered Saline (PBS) Ca/Mg free: HyClone; Catalog # SH30028.02
c) Hanks' Balanced Salt Solution (HBSS) Ca/Mg free: HyClone; Catalog #SH30588.02
d) Trypsin: Gibco; Catalog # 27250-018
e) Collagenase Type II: Gibco; Catalog # 17101-015
f) DMEM: Hyclone; Catalog # SH30022.01
g) Fetal Bovine Serum (FBS): Gibco; Catalog # 16000-044

Media and solutions

a) NRPCM culture medium
- DMEM: 500 ml
- FBS: 55 ml (10%)

b) Digestion solution
- Trypsin: 0.07g (0.07%)
- Collagenase Type II: 0.05g (0.05%)
- HBSS: 100 ml

Day 0

A: Coat the E-Plate CardioECR 48 well with Fibronectin

Time: About 3.5 hours-16 hours

1. Dilute the fibronectin (FN) stock 1:100 with PBS to 10 µg/mL.
2. Transfer 50 µl of diluted FN to each well of the E-Plate® CardioECR 48.
3. Incubate the coated E-Plate CardioECR 48 at 37 °C for 3 hours or at 4 °C overnight.

Day 1

A: Isolation of neonatal rat primary cardiomyocytes

Time: About 3 hours

The experiment is performed in accordance with the Guide for the Care and Use of Laboratory Animals published by the US National Institutes of Health (NIH Publication No.85-23, revised 1996).

Neonatal rat primary cardiomyocytes are prepared from 1- to 2-day-old Sprague-Dawley rats.

1. Harvest ventricular tissue

1. Decapitate each rat pup after they are decontaminated with 70% EtOH.
2. Cut the thoracic wall open and surgically remove the beating heart with scissors.
3. Remove the atria from the isolated heart and immediately transfer the ventricles to the cell culture dish containing ice-cold DMEM. Squeeze hearts gently with forceps to expel the blood from the lumen. Transfer hearts to the

2nd and 3rd cell culture dishes containing ice-cold DMEM to further clean/wash the hearts. Repeat for the remaining pups.

4. After washing, collect the ventricles in a 5 ml sterile bottle containing 2 ml of ice cold HBSS.
5. Mince the ventricles into small pieces (1-3 mm^3) with fine scissors.

2. Digest tissue fragments with digestion solution

Incubate the tissue fragments in digestion solution (0.07% Trypsin with 0.05% Collagenase Type II in HBSS) as described in the following.

1. Digest tissue fragments in 1.5 ml of digestion solution for 8 min in the incubator. Gently shake the tube every 4 min. Discard the supernatant with a 10 ml pipette after the 8 min digestion.
2. Repeat step 1 eleven more times until there is a very small amount of tissue fragments left in the bottle.
3. Discard the supernatant with a 10 ml pipette, then add 3 ml of DMEM supplemented with 10% FBS. Gently triturate the remaining tissue fragments with a 10 ml plastic serological pipette about 10-20 times to release the cells.
4. Rinse a 70 µm cell strainer with 1 ml of DMEM plus 10% FBS. Allow tissue residue to settle for 2-3 minutes, then filter the supernatant through the cell strainer into a fresh 50 ml centrifuge tube.
5. Centrifuge the collected cell suspension for 5 min at 1000 rpm and re-suspend the cell pellet in 5 ml of culture medium.

3. Enrich and purify cardiomyocytes

1. Pre-plate collected cell suspension on 1 X T-25 or T-75 flask (depending on the number of isolated cells) 2 times, 45 min each to remove fibroblasts and other types of cells.
2. Transfer the suspension cells (cardiomyocytes) from the T-25 or T-75 flask to a new 15 ml tube for cell counting.

B: Measure the background

Time: About 5-10 minutes

1. Replace the coating buffer in the E-plate CardioECR 48 with 50 µl of pre-warmed NRPCM culture medium.
2. Engage the E-plate CardioECR 48 on the CardioECR station inside the CO2 incubator.
3. Open the RTCA CardioECR aquistion software and click on the Layout tab. Enable the sample wells by highlighting the area in the well map corresponding to the sample wells. Enter requested information about the cells, including cell number.
4. Click on the Schedule tab, then add Step_1 for the background measurement

   ⚠ Do not change the preset parameters which are important for the background calculation.
5. Click on the Start/Continue icon to obtain the background reading. The background reading step will take 1 min.

C: Count and seed NRPCM on the E-plate CardioECR 48

Time: About 30 minutes

1. Pipette 10 µl of the cell suspension and mix with 40 µl of PBS plus 50 µl of trypan blue in an eppendorf tube. In-

cubate for 5 minutes at 37 °C in the incubator. Count the viable cells, which are not stained in blue, using a hemocytometer and adjust the volume in order to obtain 240,000 viable cells/mL.

⚠ It is always helpful to count cells twice and to use the average value to calculate the actual number of viable cells.

2. Use a repeat pipettor or multichannel pipette to transfer 100 µl of the cell suspension to the wells of the E-plate CardioECR 48 (24,000 viable cells/well).
3. Gently tap each side of the E-plate CardioECR 48 to ensure even distribution of cells on the electrode sensors in the bottom of the E-plate CardioECR 48.
4. **Keep the E-plate CardioECR 48 in the laminar flow hood for 30 min at room temperature to further ensure even cell distribution in the wells.**
5. Transfer E-Plate CardioECR 48 seeded with cells to the incubator. **Allow 15 min of temperature equilibration before engaging the plate into the station.**

⚠ Temperature equilibration is recommended for preventing condensation on the bottom of the E-plate CardioECR 48, which will interfere with accurate assessment of the field potential signal.

6. Click on the Schedule tab and add Step_2 to monitor cell attachment and growth (optional).

   Recommended schedule step for cell status QC measurement:

   1) 100 sweeps of 20-second block duration (extra sweeps can be aborted)
   2) 6 hr interval between each sweep
   3) Sampling rate:

      Impedance: 2 or 12.8 ms

      ECR: 0.1 ms (10 KHz)
7. Click on Start/Continue icon to start Step_2.

Day 2-4

A: Change culture medium every day

Time: About 10 minutes

1. Warm up NRPCM culture medium (10% FBS DMEM) in a 37 °C water bath.

   ⚠ It is important to use warm medium for medium change.
2. Pause the RTCA Cardio System monitoring by clicking on the Pause button in the RTCA CardioECR aquistion software. Disengage the E-plate CardioECR 48 from the CardioECR station and transfer the plate to the laminar flow hood. Slightly tilt the plate and use a multichannel pipette to gently remove the media from the wells.
3. Cautiously add 150 µl of the pre-warmed media on top of the cells by tilting the E-plate CardioECR 48 at an angle and adding the media to the side of the well.

   ⚠ Do not disturb the cell monolayer during the medium change.
4. Bring the E-Plate CardioECR 48 back to the incubator. After 15 min temperature equilibration, engage the E-plate CardioECR 48 into the CardioECR Station and resume measurements by clicking on the Start/Continue icon.

Tip: Based on experience, the NRPCM beating signal should be detectable within 20-28 hours after cell seeding.

Overview

Day 5

A: Change culture medium at least 4 hours prior to compound treatment

Time: About 10 minutes

1. Change medium at least **4** hours prior to compound treatment with **90** µl of fresh and pre- warmed culture medium and continue monitoring the cells.
2. Check the beating activity of cells and make sure the cells are beating at regular intervals without any intermittent gaps.

B: Measure baseline of beating and electrical activities of cells before compound treatment

Click schedule tab to add next Step for the baseline measurement of beating and electrical activity.

Recommended schedule step for baseline measurement:

1) 7 sweeps of 60-second block duration (extra sweeps can be aborted)
2) 5 min interval between each sweep
3) Sampling rate:

   Impedance: 2 or 12.8 ms

   ECR: 0.1 ms (10 KHz)

C: Compound preparation and dilution

Time: About 1 hour

1. Determine the effective concentration ranges for the test compounds.
2. Prepare stock solutions by dissolving the compounds in an appropriate solvent. If DMSO is used as the solvent, dissolve the compound in a high stock concentration if possible (ideally 1000-fold of the highest tested concentration) and store at -20˚C.
3. Prepare a set of 100 X concentrated solutions from the compound stock solution in culture medium, which correspond to the tested concentrations of the compound in a 96-well V bottom plate.
4. Transfer 10 µl of 100X stock solutions using a multichannel pipette from the V bottom microtiter plate to the E-plate CardioECR 48.

⚠ It is very important to include wells treated with solvent only at the same final concentration as it is in compound treated wells for the negative control.

D: Add compound and monitor for short-term (<1 hr) or long-term (24 hr)

Abort Step_3 for baseline measurement, by clicking on the Pause icon first. A pop-up window will appear asking you to confirm termination of the step. Click yes.

1. Set up Steps for compound testing in the Schedule section, and make sure the Auto reading box is checked.

Recommended schedule step for short-term monitoring (<1 hr)

Step 1:

1) 3 sweeps of 60-second block duration
2) 5 minutes interval

Step 2:

1) 30 sweeps of 60-second block duration
2) 2 minutes interval

Sampling rate:

Impedance: 2 or 12.8 ms

ECR: 0.1 ms (10 KHz)

Recommended schedule step for long-term monitoring (<24 hr)

1) 24 sweeps of 60-second block duration
2) 1 hr interval between each sweep

Sampling rate:

Impedance: 2 or 12.8 ms

ECR: 0.1 ms (10 KHz)

⚠ Make sure to enter compound names and tested concentrations in the layout page.

2. Before removing cells from the incubator for compound treatment, disengage the E-Plate CardioECR 48 from the RTCA CardioECR Station. Transfer the plate with cells onto the stabilization core of the Temperature Tool to minimize temperature fluctuation.
3. Carefully, but quickly, add the compound to the cells.
   Using a multichannel pipette, transfer 10 µL of compounds diluted in culture medium from the V bottom microtiter plate to the E-Plate CardioECR 48.

⚠ It is very important to include wells treated with the solvent only at the same final concentration as compound treated wells for the negative control.

⚠ It is important to have a multichannel pipette, tips and tip waste box ready. This will ensure the minimum time for adding compounds and exposing cells to the ambient temperature and oxygen levels outside of the tissue culture incubator.

4. Return the E-Plate CardioECR 48 to the CardioECR Station. Initiate the next step immediately.

⚠ In order to capture all the information on electrical and beating activity changes after compound addition, the process of compound addition should not exceed 3-5 minutes. Ensure that all the recording schedules are pre-programmed to help minimize this time.

Day 6

A: Termination of experiment

1. The experiment can be terminated 24 hours after compound treatment.
2. Alternatively, the experiment can continue to obtain additional recording of beating activity, or the compound may be washed out and replaced with fresh medium to restore beating activity.

B: Analyze data

Published by:

ACEA Biosciences, Inc., 6779 Mesa Ridge Road Ste. 100, San Diego, CA 92121, U.S.A.