

## GTR1 (a.k.a., $\alpha$ MPCH 2-10-1) ES Cell Lines

General information on culturing ES cells.

All reagents used for the culturing of ES cells should be “tissue culture grade” or “tissue culture tested”. All procedures are to be done under sterile conditions in a laminar flow hood using sterile instruments and detergent-free glassware. Dulbecco’s modified Eagle’s media (DMEM) (Gibco #12100-046) is prepared for ES cell medium as described below. The plates must be specially treated before seeding the ES cells. This is performed either by coating the plates with 0.1-0.2% gelatin (Sigma #G-1890) or preparing a layer of mitotically inactivated embryonic fibroblasts for the ES cells to grow on. ES cells should be fed every day and split every second day (approximately 70-80% confluency, colonies almost touching each other) using a 1:5 split ratio (this might need to be adjusted depending on the cell line). It is generally not recommended to grow ES cells for long periods of time in LIF alone (i.e., on gelatin-coated plates) without a feeder layer unless you have commercially purchased LIF with known activity units (Chemicon). If the cells grow to over-confluency it can result in their differentiation and loss of germline competence.

### Media Preparation

#### Embryonic Stem Cell Medium (ES)

Dulbecco’s modified Eagle’s Medium (DMEM) with high glucose (Gibco #11960-044)

To the 500ml bottle add:

- 6mls of GlutaMAX, (GlutaMAX-1, Gibco 35050-061)
  - 6ml of diluted  $\beta$ -mercaptoethanol, 100 $\mu$ M final\*\*
- \*\*Note: Diluted 2-mercaptoethanol is made by adding 70ul of 2-mercaptoethanol (Sigma, M7522) to 100ml of sterile PBS or water.
- 6mls of Sodium Pyruvate, 1mM final (Gibco, 11360-070)
  - 6mls of Non-essential amino acids, 100  $\mu$ M final (Gibco, 11140-050)
  - 1000U/ml of LIF (Leukaemia inhibitory factor, Chemicon ESG1107)
  - penicillin/streptomycin (Gibco #15140-148, final concentration 50ug/ml each)

Plus the required amount of FBS for the different media types  
-normal ES media has 15% which = 95ml of FBS added

#### Embryoid Body Medium

Made the same as the ES media except that the LIF is omitted and 15% FBS is used. It is sometimes best to test other lots of serum for differentiation of the ES cells.

Thawing Cells:

- Thaw the vial quickly in 37°C water
- Wipe the vial with 70% ethanol
- Transfer the contents of the vial to a tube with at least 5mls of media in it
- Spin at 1000 RMP for 5 min
- Aspirate off the media

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- Resuspend the pellet in 4ml of media
- Transfer contents to a 6cm tissue culture treated dish which has feeder cells on it
- Change the media the next day
- ES cells should be passaged every other day with a 1:5 split
- One 6cm dish can be passaged onto two 10cm dishes

Passage of ES cells on 10cm tissue culture plates:

- Remove the media from the plate.
- Rinse the plate with PBS (Gibco cat# 14190-250), aspirate off the PBS
- Add 2mls of trypsin (Sigma T4049) to the plate.
- Incubate at 37°C for 5 minutes.
- After 5 minutes, add 3ml of media to the plate and resuspend the cells well.
- Dispense 1ml of cell suspension to each new plate distributing the cells well.
- Return the plates to the incubator.
- Feed the plates the next day.

Freezing Cells:

- ES cells can be frozen down using 50% complete DMEM, 40% FBS and 10% DMSO. Four 1ml vials of cells can be frozen from each 10cm plate of cells (thaw back onto a 6cm plate).

## Embryoid Body (EB) Assay for Cardiomyocyte Selection

### Day 0 (Outline of Dispase Treatment)

Before EBs can be made, the ES cells must be passaged onto gelatinised plates to remove the majority of feeder cells. Cells must be grown on gelatinised plates to about 80% confluency. Over confluent layers do not make nice EBs.

We use speciality plates from Costar (Costar - 96 well #3474, 24 well #3473, and 6 well #3471) since the normal non-tissue culture treated plates still allow a large percentage of the EBs to attach, preventing proper EB formation. You can also prepare plates using pluronic acid (Sigma) as a coating on the plate. Protocols are available in EB papers.

Prepare ultra low cluster plates by adding enough EB medium (15% FBS, **NO LIF**) to cover the bottom of the well and placing them at 37°C for at least 15 minutes. It is a good idea to use more than one well per clone since then you can choose the best culture based on density and appearance of the EBs. It is best to err on the side of low densities of EBs to limit aggregation.

The amount of **dispase** (Collaborative Research Labs #40235 or Roche #295825) working solution required is 500ul/6cm plate, 2ml/10cm plate or just enough to cover the well in which the cells are growing. Dilute the dispase 1:3 with cold PBS (this amount may have to be changed depending on the activity level of the dispase) and filter using a 0.45um filter. If the dispase/PBS solution is not filtered a large amount of precipitant will be present in the wells the next day interfering with the EB formation and appearance. Place the dispase on ice until ready to use. Note: if the dispase is old, it will need to be diluted less for the same amount of activity.

- Remove the ES medium from the plate of ES cells.
- Wash with PBS

- Aspirate off the PBS
- Add the correct amount of cold dispase
- Let sit at room temperature for about 2 minutes, until you see the colonies lifting off with gentle tapping of the plate. The time may be less if dispase is very active.
- Add EB media and resuspend the colonies by pipeting up and down 5-6 times. It is possible to break the colonies apart too much, so don't resuspend excessively.
- Transfer the colonies into the ultra low cluster plates using a 1:2 or 1:3 dilution of the colonies (ie. One 6cm dish of ES colonies can be split into 3 or 4 wells of an ultra low cluster 6 well plate).

### Day 1, 2 and 3

- Feed the dispase plates by removing about half of the medium, being careful not to suck up the EBs which will be lying on the bottom of the plate but not attached to the plastic.
- Refill the well with fresh EB medium.

### Day 3 or 4: Transfer EBs to Tissue Culture Plates

- Prepare the desired number of normal tissue culture 24 or 6 well plates by adding EB media to each well. Two or three wells per cell line should be set up. **Prepare a separate plate for each time point of interest, multiple cell lines can be done in the same plate for each time point.** The EBs can be transferred on day 3 or 4. If your culture is really healthy then day 4 is ideal. If you notice that there are dead cells within the EB then the transfer should be done on day 3. If the EBs are very large you will see more dead cells occurring in the EB.
- Pipette the EBs up and down to mix them well and transfer about 1:6<sup>th</sup> of the well to the prepared TC plates. Make sure that you mix well before each transfer since the EBs will settle very quickly. It is good to have 10 to 20 EBs per well of a six well plate, 5-10 EBs for a well in a 24 well plate. Too many EBs per well results in the EBs not developing to their full potential and early cell death.
- The EBs should attach and spread out within 24 hours

### Every Second Day

- Feed the EB plates

By day 7 or 8, you should be able to see the cardiomyocytes beating within the wells.

### Day 8

On day 8 of EB formation, start selection with 2ug/ml of puromycin in the EB media.

### Day 10

Carefully passage cells 1:1 (use cell dissociation buffer or collagenase to disassociate the cells, centrifuge, and replate in the half the volume and half the number of wells originally used). Because only the cardiomyocytes are resistant to puromycin, most of the EB cells will die. If the cells are not passaged, cardiomyocyte foci will begin floating in the wells since they are

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surrounded by, and attached to the dish, by many other cell types. If you notice that the cardiomyocytes are already floating, then remove the floating cells and treat those cells in a tube, centrifuging down the cells and disassociate within the tube as well.

### **Day 12 and afterwards**

If cells are still dying or cardiomyocytes are floating, then passage cells again (be careful of losing beating foci that are not attached to plastic). Cardiomyocytes can be expanded by passaging 1:2 as needed.

### **Embryonic Stem Cell Media**

DMEM (Gibco cat# 11960-044, 11965-084, 10829-018 or Specialty Media cat# SLM-220B)

To each 500mls of media add:

- penicillin/streptomycin, final concentration 50ug/ml each (Gibco cat# 15140-122)
- 6mls of L-glutamine (200mM, Sigma #G7513) or Glutamax from Gibco (200mM, Sigma #G7513)
- 6mls of B-mercaptoethanol stock solution (Sigma M7522, stock solution prepared by diluting 70ul of B-mercapto into 100ml of PBS, only 6mls of this is added to the 500mls of media)
- 6mls of Sodium Pyruvate (Gibco, 11360-070)
- 6mls of Non-essential amino acids (Gibco, 11140-050)
- Normal ES media has 15% FBS added which = 95ml of FBS
- \*\*Leukemia inhibitory factor (LIF- Gibco cat# 13275-029, Chemicon) is added (1000 U/ml).

### **Embroid Body Media**

\*\*Prepared the same as above with the exception that leukemia inhibitory factor is not added.