

## Protocols for Hematopoietic Differentiation of Murine ES Cells (in 6 or 24 well plates)

### Media Preparation

#### A) 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, Gibco #13275-029)
  - 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

-gradual reduction of FBS for low serum plates 10% and 5% EB media

10% = 60ml

5% = 30ml

\*The activin assay requires only 0.2% FBS = 1.4ml

#### B) Embryoid Body Medium

Made the same as the ES media except that the LIF is omitted and 15% FBS is used.

#### C) OP9 Medium

Minimum Essential Medium- Alpha ( $\alpha$ -MEM) powdered media is used (Gibco #12000-022)

The powder is added to 900ml of sterile distilled water while stirring. The package is rinsed out at least twice.

Once the powder has dissolved add the following:

- 2.2g of Sodium Bicarbonate (Tissue culture tested)
- 6ml of L-glutamine (200mM stock, Sigma #G7513)
- 6ml of diluted  $\beta$ -mercaptoethanol\*\*

\*\*Note: Diluted 2-mercaptoethanol is made by adding 70ul of 2-mercaptoethanol (Sigma, M7522) to 100ml of sterile PBS or water.

- 5ml of penicillin/streptomycin solution (Gibco #15140-148, 50ug/ml final conc.)

Top up to 1L with sterile dH<sub>2</sub>O

Filter-sterilise using a 0.22 $\mu$ m bottle top filter.

\*Before use add 95ml of FBS to the 500ml bottle.

#### D) Collagen IV Medium

Prepared the same as the OP9 medium with the exception that only 10% FBS is added.

### **\*\*Fetal Bovine Serum**

We purchase our FBS from Hyclone and the lot# we use is AHH8832 but this lot expires at the end of 2002. You can ask Hyclone to cross-match for a new lot that is close to AHH8832 but you still must test it for growing ES cells since some lots of FBS will cause differentiation of the ES cells.

## **General Culturing Information**

### **Culturing OP9 Cells**

Thaw the OP9 cells at 37C and then transfer the contents of the vial to a tube containing 5mls of OP9 media. Spin down the cells at 1000 rpms for 5min. Resuspend the cell pellet in 3mls of OP9 media and transfer to a 10cm plate containing 10ml of OP9 media. Once a confluent plate is achieved, usually 2-3 days of growth, the OP9 cells can be easily maintained by passing them every Monday, Wednesday, and Friday. The media does not need to be changed in between passages.

When freezing down OP9 cells you will need to freeze down one confluent plate into 2 vials. We freeze in 50% FBS, 10% DMSO and 40% OP9 media.

If large vacuole-containing cells start to form in the dish then a new vial must be thawed since they will not go away with passaging and will only get worse. As long as they don't go more than three days between passages and don't get over confluent then they should stay healthy for a long time

After the cells have been growing for a few weeks you might notice that upon trypsinization the cell layer comes off whole and is very hard to resuspend, even after five minutes in trypsin. If this is the case then you might have to split them every so often using a 1:4 ratio to decrease the number of dividing cells.

### **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) is prepared for ES cell medium as described above.

The plates must be specially treated before seeding the ES cells. This is performed either by coating the plates with 0.1% gelatin (BioRad #170-6537, Sigma and BDH also carry TC tested gelatin) 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). Use 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. Also, if the cells grow to over-confluency it can result in their differentiation and loss of germline competence.

Thawing ES Cells:

- Thaw the vial quickly in 37°C water
  - Transfer the contents of the vial to a tube with at least 5mls of media in it to remove the DMSO
  - Spin at 1000 RMP for 5 min
  - Aspirate off the media
  - 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
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- ES cells should be passaged every other day with a 1:5 split
  - OP9 cells should be passaged every other day with a 1:3 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 (Gibco cat#25300-047) 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.

Note: For electroporations: ES cells should be ready for electroporation 2 days after they were passaged 1:5.

#### 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 (to be thawed back onto a 6cm plate).

## Preparation and Protocols for Differentiation of Murine ES Cells

**Preparation** for the Collagen IV and OP9 assays set up in 6 or 24 well plates. To be done at least one day in advance of setting up the assays.

### Collagen IV

For the Collagen IV assay the alpha MEM medium is used with the exception that only 10% FBS is added. Two plates per set of clones are to be done.

- Coat the desired number of plates with collagen type IV
  - Use a 250µg/ml solution (Sigma C5533). Dilute the collagen in PBS.
  - For 24 well plates use 200ul/well
  - For 6 well plates use 0.5ml/well
- Let the collagen adhere to the wells for about 10 minutes (can be over night).
- Aspirate off the collagen and let plates dry.
- One the same day as setting up the assays, add alpha-MEM media (with only 10% FBS) to each well
  - 24 well - add 1ml of media/well
  - 6 well - add 2.5ml of media/well

### OP9

For the OP9 assay, alpha MEM media is used containing 15% FBS. Since two sets are required for this assay two plates of OP9 cells are needed for each cell line to be tested. One 10cm plate of a two day culture will make three 6 well plates or four 24 well plates.

- Use a two-day old 10cm plate of OP9 cells.
- Aspirate off the media
- Rinse the plate with 5ml of PBS
- Aspirate off the PBS
- Add 2ml of trypsin
- Incubate at 37°C for 5 minutes
  - For 24 well plates, add 8.2ml of media and resuspend the cells well.
    - Dispense 50ul/well of OP9 cell suspension
  - For 6 well plates, add 7.2ml of media and resuspend the cells well.
    - Dispense 250ul/well of OP9 cell suspension
- Plates should be used within 24 hours, checking to make sure that a complete layer has formed.
- Add fresh media to each well before using in the OP9 assay

## Setting up of the OP9 and Collagen IV Assays

For the Collagen IV and OP9 assays, the same plate of ES cells (grown on feeders) will be used to set up all of the assays. Make sure that the cells are not overcrowded (<80% confluency). Before starting this section make sure that you have done the preparation for each of the assays required.

Preparation of the ES cells (on a 10cm feeder plate) for the Collagen IV, OP9 and neurosphere assays.

- Aspirate off the ES media
- Wash the plate with PBS
- Aspirate off the PBS
- Add 2ml of trypsin.
- Incubate at 37°C for 5 minutes
- Add 4ml of Collagen IV media (alpha-MEM with 10% FBS) and resuspend the cells well.
- Determine the number of cells/ml.
- The ES clones are now ready to be transferred to the Collagen IV and OP9 plates.

**\*\*Note: The same cells will be used for the OP9, Collagen IV and Neurosphere assay. After the OP9 and Collagen assays are set up the cells must be washed with neurosphere media to remove any FBS present before being used in the neurosphere assay.**

### A) Collagen IV -Endothelial Assay

For this assay you don't want the cells to be over crowded in the dish. It is usually a good idea to do three replications per cell line. Since two sets are required for each cell line the same plate can be used for multiple cell lines as long as you keep all of the set #1s together on the same plate since the whole plate has to be X-Gal stained on day 4. After the ES cells have been resuspended, transfer the appropriate number of cells according to which size of plate you are using. **(Day 0)**

- 24 well plate, use 1500-2000 cells/well
- 6 well plate, use ~3000 cells/well
- Incubate at 37°C.
- Feed the plates on day 2 by aspirating off the media and replacing it with fresh media
  - 24 well - add 1ml of media/well
  - 6 well - add 2.5ml of media/well
- **On day 4**, X-gal stain set #1 (angioblasts and ECs) and feed the 2<sup>nd</sup> set with fresh medium.
- **On day 7**, X-gal stain set #2 (differentiated ECs)

## B) OP9 Differentiation -Mesoderm and Hematopoietic

- Two sets of plates are required for each cell line tested.
- After the ES clones have been prepared as above and the number of cells determined, transfer the appropriate number of cells for the type of plate being used. **(Day 0)** It is a good idea to have 2 or 3 different wells for each cell line in order to pick the well with the best differentiated colonies in it for transfer on the fifth day.
  - 24 well plate, ~500 cells/well.
  - 6 well plate, ~3000-4000 cells/well.
- **On day 3**, feed the plates.
- **On day 3** set up a new OP9 plate (as described above) for the transfer of set #2 plates. These plates will be used for the transfer of set #2 on day 5 and it is a good idea to have three wells per cell line to analyze.
- **On day 5**, X-gal stain set #1 for mesoderm and endothelial cells.
- **Also on day 5**, transfer set #2 of each clone onto a new OP9 layer.
  - Aspirate the media off the ES clones.
  - Wash the cells with PBS.
  - Add trypsin to each well (200ul for 24 well plate, 0.5ml for 6 well plate)
  - Incubate at 37°C for 5 minutes
  - Add OP9 media (500ul for 24 well, 2ml for 6 well) and resuspend well.
  - Transfer either 1:15<sup>th</sup> or 1:20<sup>th</sup> of the well onto the new OP9 layers
- **On days 7 and 9**, feed the plates with OP9 media by replacing 2/3rds with fresh media.
- **On day 10**, X-Gal stain the OP9 set#2 plates.

## C) Embroid Body Assay

### Day 0

To be done when cells are <80% confluent in a gelatinized plate. Over confluent layers do not make good EBs

Prepare an ultra low cluster plate (24 well plates seem to work the best for EB formation) by adding 500 µl/well of EB medium (15% FBS, **NO LIF**) to the desired number of wells (Costar) and place them at 37°C for at least 15 minutes. Usually three to four wells per cell line is enough. These plates can be prepared the day before or 15 minutes prior to the dispase treatment.

The amount of dispase (Dispase II Roche #10038100) required is 500ul/6cm plate or 2ml/10cm. Dilute the dispase 1:3 with sterile PBS before use. If precipitation occurs after the dilution filter through a 0.45 µm syringe filter before use.

- Remove the ES medium from the plate of ES cells.
- Wash with PBS
- Aspirate off the PBS
- Add the correct amount of dispase

- Incubate for a maximum of 2-3 minutes, just until you see the colonies start to lift off the plate.
- Add EB media (6cm = 3mls, 10cm=5mls) and resuspend gently (2-3 times)
- Transfer the correct amount to an ultra low cluster plate (Costar). A number of wells can be prepared and then the wells containing the best EBs can be selected for attachment. Do not over resuspend since you don't want to break apart the colonies.
  - When transferring from a 6cm dish to 24 well use 200-400ul
  - When transferring from a 10cm dish to 24 well use 100-200ulDetermining the number of EBs transferred is important for good differentiation to occur. If there are too many then the EBs will stick together and create long chains which will not make good EBs.

### **Day 1 and 2**

- Feed the plates by removing about half of the medium. Be very 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 500-800µl of fresh EB medium.

### **Day 3 (or day 4) Transfer of EBs**

- Prepare the desired number of normal tissue culture 24 well plates by adding 500µl of 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.**
- Pipette the EBs up and down only once or twice between transfers since they will break apart easily. Transfer about 1:6<sup>th</sup> of the well to the prepared normal TC plates..
- The EBs should attach and spread out within 24 hours

### **Every Second Day**

- Feed the EB plates

**X-Gal stain the plates at the desired time points.**

## Methylcellulose Culturing of Differentiated ES Cells.

(Used to count myeloid, erythroid, GM and GEMM colony development)

For methylcellulose (MEC) cultures we use MEC from Stem Cell Technologies type M3434. When diluting cells we use IMDM media in a final volume 10% of the total amount of MEC being used. **The amount of MEC used per well is 300ul for 24 well plates and 1.8ml for 6 well plates.**

**For the EB cultures** the differentiated cells are transferred to MEC on day 10 of differentiation. The EBs are trypsonized into single cell suspensions (you can also treat the EBs in several mls of 0.2% collagenase in 1XPBS at 37C for 30min and resuspend well, using a needle if necessary).

Once the cells are resuspended in IMDM we **plate them** out at **10<sup>5</sup> cells/well** of a six well plate. Make sure to keep a few wells empty to use for hydration of the cultures. **Count** different types of colonies **on day 8-10**. The wells can be stained with benzidine to help with identifying heme-positive colonies \*\*.

**For the OP9 cultures** the differentiated cells are **transferred to MEC on day 8-10** of culturing on the OP9 layer. Two cultures can be set up for MEC analysis from the OP9 differentiation assay.

**First step:** **Without removing** the media, pipette up and down onto the layer being careful not to dislodge the OP9 layer. This will isolate any loose or floating hematopoietic cells. Remove the media and spin down the cells at 1000rpm for five minutes. Resuspend the cells **in IMDM using a volume 10-12%** of the final MEC volume that will be used. Gently add the correct volume of MEC, resuspending very gently so as to prevent bubbles. Transfer the MEC and cells to one well. Culture for 8-10 days and count colonies. **Make sure to fill at least one quarter of the wells with just sterile distilled water to help keep the MEC hydrated** for the 8-10 days of culturing. Regular incubator humidity is not enough.

**Second step:** Trypsinize the remaining OP9 layer for 5 minutes at 37°C, making sure that the layer is coming apart before you stop the reaction since it is very important to have single cell suspensions. Resuspend well and plate out at 10<sup>4</sup> cells/well for a 24 well plate and 10<sup>5</sup> cells/well for a 6-well plate. **Make sure to fill at least one quarter of the wells with just sterile distilled water to keep the MEC hydrated** for the 8-10 days of culturing. Stain with benzidine and count colonies.

When looking for **CFU-E** clusters you have to look at the cultures on **day 2** and benzidine staining is usually required in order to identify them so you might have to set up a separate plate if these are of interest to you.

I've included the outline for MEC colony assays from bone marrow at the end of this in case they are of interest to you.

\*\*Sometimes, when there is a deficiency in hematopoiesis, proper erythroid colonies will not form but heme-positive cells can be present within larger multicell-type colonies. Therefore, the benzidine staining is very helpful to identify this occurrence.

## Direct Benzidine Staining

### Stock Benzidine Base

Benzidine Base (Sigma cat# B3503, p,p'-Diaminobiphenyl)

Make a 3% solution in a 90% glacial acetic acid and 10% d water solution. (make about 10mls)

Store in the dark at 4C since it is light sensitive. The solution will appear dark brown. Sometimes a precipitant forms. If this occurs just take the solution from the top, avoiding the precipitant. This stock solution should last at least 6 months, we've used on that was a year old and it worked fine.

### Working solution

Make this solution fresh, immediately prior to use.

1 part Benzidine base stock

1 part H<sub>2</sub>O<sub>2</sub> (hydrogen peroxide)

5 parts sterile d water

Add 0.2ml of the working solution per 2ml of TC adherent cells or methylcellulose culture. The supernatant will turn bluish/brown. The positive cells will turn blue after a few minutes. The staining will fade so only add the solution immediately prior to counting the colonies. If desired, rinse the wells with one times PBS before photography if methylcellulose isn't being used.

This stain is not very stable. Photograph within one hour of adding the cells. The cells will not remain consistently stained after overnight storage at 4C in the dark.

## Methylcellulose Colony Forming Assays From Bone Marrow

### Bone Marrow

Flush marrow from femurs and tibias in IMDM (with 10% FBS)  
Wash in IMDM  
Resuspend in 1 ml IMDM  
Count (dilute 1:10)

### For CFU-E

Aliquot  $10^4$  cells in 30  $\mu$ l IMDM (with 10% FBS) into 3-14ml tubes  
Add 270  $\mu$ l M3434 Stem Cell Technologies Complete Methylcellulose  
Mix without generating bubbles  
Plate into a well of a 24-well plate (1 well/tube)  
Add water to some wells for humidity  
Count day 2 by staining with benzidine

### For BFU-E/ CFU-C

Aliquot  $2 \times 10^4$  cells in 150  $\mu$ l IMDM (with 10% FBS) into 3-50ml tubes  
Add 1.2 ml M3434 Stem Cell Technologies Complete Methylcellulose  
Mix without generating bubbles  
Plate into a well of a 6-well plate (1 well/tube)  
Add water to some wells for humidity  
Count day 7-10