

Protocols for Differentiation of Gene Trapped Clones in 96 Well Plates

Media Preparation

A) Embryonic Stem Cell Medium (ES)

Dulbecco's modified Eagle's Medium (DMEM) with high glucose (Gibco #12100-046) Prepare according to directions on package, start with only 900ml of sterile water and make sure to rinse out the packet at least twice.

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 β -mercaptoethanol, 100 μ 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 μ 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

-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 (α -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)
- 6mls of GlutaMAX, (GlutaMAX-1, Gibco 35050-061)
- 6ml of diluted β -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 dH₂O
Filter-sterilise using a 0.22um 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 North-Bio and is the Gemini brand. Lots must be tested to verify that it sustain the ES cells in an undifferentiated state.

E) Neurosphere Media

Stock Solutions for Neurosphere Assay

- all solutions are to be made using sterile distilled water

30% glucose (Sigma G-5000)

weigh 30g of glucose and add slowly to 80mls of sterile distilled water while stirring. Once all of the powder has dissolved, bring the total volume up to 100ml. Transfer to the hood and filter sterilize through a 0.2um filter and aliquot into sterile 15ml centrifuge tubes, approximately 10mls per tube. Store at 4C.

7.5% NaHCO₃ (Sigma S5761)

weigh 7.5g of glucose and add slowly to 80mls of sterile distilled water while stirring. Once all of the powder has dissolved, bring the total volume up to 100ml. Transfer to the hood and filter sterilize through a 0.2um filter and aliquot into sterile 15ml centrifuge tubes, approximately 10mls per tube. Store at 4C.

1M Hepes (Sigma #H0887)

Already comes as a stock solution. Store at 4C

5X DMEM/F12, (Gibco 430-2100EB/430-1700EB)

Measure 200mls of cool sterile distilled water into a 400ml beaker containing a stir bar. Gradually add the contents of the DMEM to the stirring water, rinsing out the empty package using a 10ml pipette and media solution. Once all of the DMEM is in solution, slowly add the package of F12, rinsing the packet with media as above. Filter sterilize in the hood with a 0.2um filter.

Hormone mixture for Neurosphere media

(10X concentration, total amount made per batch = 200mls)

To prepare the hormone mixture, begin by making up the following media to which you will add the various additions.

Base media

20mls of 5X DMEM/F12 media

4mls of 30% Glucose

3mls of 7.5% NaHCO₃

1ml of 1M Hepes

150mls of sterile distilled water

(No glutamine is added at this step as it precipitates upon freezing)

To the above media add: (be sure to follow the instructions for dissolving the different additives)

200mg of Transferrin (Sigma T-2252)

- empty contents of the bottles carefully into the above media. Once all of the powder has dissolved, rinse the bottles once with media.

50mg Insulin (Sigma I-5500)

- carefully add 2mls of acidified water (filtered 0.1N HCl or water acidified with acetic acid, instructions for dissolving the insulin can be found under neurosphere section in my protocol binder) to the 50mg vial of insulin. Cap and vortex the vial until all of the powder has dissolved. Add the 2mls to the above media, rinsing the vial 2X.

19.23mg Putrescine (Sigma P-7505)

- dissolve 19.32mg of putrescine in 20mls of sterile distilled water. Add to the media mixture, the final concentration is 6×10^{-3} M.

20ul of 3×10^{-3} M Selenium (Sigma S-9133)

- add 1.93mls of sterile distilled water to 1mg vial of selenium. Mix and transfer to a small sterile tube. Once made, this can be stored and -20 and reused. Add 20ul to the media mixture

20ul 2×10^{-3} M Progesterone (Sigma P-6149)

- add 1.59mls of 95% ETOH to the 1mg vial, mix and transfer to a small sterile tube. Store at -20 C. Can be reused. Add 20ul to the media mixture.

Once all of the 5 ingredients have been added and mixed, filter sterilize using a 0.2um filter and aliquot into 5ml aliquots in 15ml sterile centrifuge tubes. Freeze at -20 C. Do not refreeze.

Complete Neurosphere Serum-Free Media

	50ml	100ml	200ml
Sterile water	37.5	75.0	150.0
5X DMEM/F12	5.0	10.0	20.0
30% Glucose	1.0	2.0	4.0
7.5% NaHCO ₃	0.75	1.5	3.0
1M HEPES	0.25	0.5	1.0
L-glutamine	0.5	1.0	2.0
Hormone mix	5.0	10.0	20.0
LIF	4.15ul	8.3ul	16.6ul

*For the differentiation on ECM gel 1% FBS is added and the LIF is omitted.
Once the complete media is made it should be used within 2 days.

General Culturing information

Culturing OP9 Cells

Thaw the OP9 cells at 37 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% gelatine (BioRad #170-6537, Sigma and BDH also carry TC tested gelatine) 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 gelatine-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 the Differentiated Assays

Preparation for the Collagen IV, OP9 and Neurosphere assays set up in 96 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 40ul/well of collagen type IV (250µg/ml) (Sigma C5533)
- Leave to adhere to the wells for at least 10 minutes.
- Aspirate off the collagen.
- Leave empty until the day of use at which time you add 200ul of alpha-MEM media (10% FBS) to each well

OP9 Layers

For the OP9 assay, alpha MEM media is used containing 15% FBS. Two plates of OP9 cells are needed for each set of clones picked. One 10cm plate of a two day culture can make a maximum of eight 96 well plates. These plates can be prepared one or two days in advance.

- 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
- If preparing the OP9 layers **one day in advance** use a 1:4 dilution of a two-day-old OP9 culture (10cm dish). Therefore, after trypsinization, add 6ml of OP9 media and resuspend the cells well. For each 96 well plate required, dilute 2ml of resuspended OP9 cells into 8.2ml of media and dispense 100ul/well into the plate.
- If preparing two days in advance use a 1:8 dilution of the two-day-old OP9 culture (10cm dish). Therefore add 6ml of media and resuspend the cells well. For each 96 well plate required, dilute 1ml of resuspended OP9 cells into 9.2 ml of media and dispense 100ul/well into the plate.
- Let grow for 24-48 hours or until the OP9 layer covers the bottom of the well. If the layer is not complete, good differentiation of the ES cells will not occur.
- On the day of use, gamma irradiate the plates at 500 RAD (4.0 minutes for the irradiator in the mouse colony). This will slow down the growth of the OP9 layers and prevent the layers from coming off during the assay.
- Add fresh media to each well before use in the OP9 assay.

Neurosphere assay

For the neurosphere assay a special serum free media is used. Directions for making this media can be found in the separate media section. Once a stock of 1X completed media is prepared, lacking the hormone mix and LIF, it can be stored at 4°C for up to six weeks. If stored longer than six weeks, additional L-glutamine must be added.

*****One the day** of setting up the assay, hormone mix and LIF must be added to the serum free media before use.

Prepare one 96 well plate for each set of clones by adding 200ul of completed neurosphere media to each well.

Setting up of the OP9, Collagen IV and Neurosphere Assays

For the Collagen IV, OP9 and neurosphere assays, the same 96 well plate of ES clones (grown on feeders) will be used. In order to maximize the number of cells analyzed the clones should be grown as close to confluency as possible without jeopardizing the health of the cells.

Before starting this section make sure that you have done the preparation for each of the assays required.

Preparation of the ES clones (on the feeder plate) for the Collagen IV, OP9 and neurosphere assays.

- Aspirate off the ES media
- Wash each well with 100ul of PBS
- Aspirate off the PBS
- Add 50µl/well of trypsin
- Incubate at 37°C for 5 minutes
- Add 150µl of Collagen IV media (completed alpha-MEM with only 10% FBS added) to each well
- Resuspend each row of clones, changing the tips in between each row
- The ES clones are now ready to be transferred to the Collagen IV and OP9 plates.

****Note: Once the OP9 and Collagen IV are set up DO NOT throw out the plate. It will also be used for setting up the Neurosphere assay.**

A) Collagen IV -Endothelial Assay

- After the ES clones have been prepared as above and all of the rows have been resuspended, transfer 2-3µl/well onto the prepared Collagen IV plates. (**Day 0**)
- Incubate at 37°C until day 2.
- Feed the plates **on day 2** by aspirating off the media and replacing it with 200µl of fresh Collagen IV media

- **On day 4**, X-gal stain set #1 (angioblasts and ECs) and feed the 2nd set with fresh medium.
- Feed on Day 6 if media is turning yellow
- **On day 7**, X-gal stain set #2 (differentiated ECs)

B) OP9 Differentiation -Mesoderm and Hematopoietic

- After the ES clones have been prepared as above and all of the rows have been resuspended, transfer 0.5µl/well onto the prepared OP9 layers. (**Day 0**)
- **On day 3**, feed the plates.
- **On day 3** set up 1 new OP9 plate (as described above) for each set of ES clones. These plates will be used for the transfer of set #2 on day 5.
- **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 100µl/well of PBS.
 - Add 50µl of trypsin to each well
 - Incubate at 37°C for 5 minutes
 - Add 200µl of OP9 media to each well and resuspend well.
 - Transfer 5-7µl (depending on how many mesodermal colonies resulted from the first five days of differentiation) from each well onto the new OP9 layers\

X-Gal stain the OP-9 plates whenever there are good hematopoietic colonies present. This should be done before day 7 or else the hematopoietic cells will float off during staining.

C) Neurosphere Assay -Neural stem cells and differentiated progeny

***Once the Collagen IV and the OP9 assays are set up, spin down the plate containing the ES cells at 1000 rpms for 5 min. The cells are then resuspended in neurosphere media (without the hormone mix or LIF) and the neurosphere assay is set up as follows:

- Transfer 50µl from each well of the ES clones, which have been resuspended in the serum free media, into a prepared plate containing 200µl/well of serum free neurosphere media (containing hormone mix and LIF).
- Leave the plate at 37°C for 7 days **without changing the media**. If the media turns yellow before the 5th day then a portion of the media can be changed (<50% to be replaced, care must also be taken not to aspirate off any of the neurospheres since they can float off of the bottom of the plate).
- **On day 6**, prepare one 96 well plate for each plate of ES clones by coating the wells with ECM gel. The ECM gel is prepared by using an 8mg/ml stock and diluting it 1:25 in neurosphere media. Add 50µl/well of the diluted ECM gel and incubate at 37°C for at least 10 minutes. Leaving the ECM overnight at 37 works well too. Before use on day 7, aspirate off the ECM gel and add 100µl of neurosphere media containing 1% FBS and hormone mix **but NO LIF**

- **On Day 7**, transfer the neurospheres by resuspending them well and transferring 50ul (depending on the number of neurospheres present) over to the newly prepared ECM plate.
- Media does not need to be changed for the rest of the culture period.
- **On day 10**, remove ~50% of the media and replace with fresh neurosphere media (with 1% FBS, FBS, no LIF)
- **On day 13-14**, X-gal stain the differentiated neurospheres.

D) RNA isolation from undifferentiated ES clones

- aspirate the media from the confluent plate of ES clones (on gelatin)
- Wash the wells with 200µl of PBS
- Aspirate off the PBS
- Add 175µl/well of RNA lysis buffer (Qiagen) (**10µl of β-mercaptoethanol, per 1ml of RNA lysis buffer, must be added to the lysis buffer before use**).
- Cover the plate with a foil Costar cover, label the foil clearly with the plate number and date lysed/frozen. Freeze right away at -86

E) Embryoid 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 the ultra low cluster 96 well plates by adding 100 µl/well of EB medium (15% FBS, **NO LIF**) to the desired number of ultra low cluster plates (Costar 3474) and place them at 37°C for at least 15 minutes. These plates can be prepared ahead of time. **Two-four 96 well plates are needed for each plate of ES clones picked, depending on the number of assays to be preformed. From each plate of suspension EBs 3-4 attached EB plates can be made.**

The amount of dispase (Dispase II Roche #10038100) required is 50ul/well. 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 wells.
- Wash with at least 50 ul of 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. One minute can be long enough.
- Add 150ul-200ul of EB media and resuspend gently only 2-3 times
- One well of dispased ES colonies can be transferred up to 2-3 wells.
- Incubate at 37C

Day 1 and 2

- 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 100-150 μ l of fresh EB medium.

Day 4 Transfer of EBs (This shouldn't be done before day 4)

- Prepare the desired number of normal tissue culture 96 well plates, which dependent on the number of assays to be preformed by adding 175 μ l of EB media to each well. **For the first set of plates that will be used for Retinoic acid, Activin and low serum control, use EB medium containing only 10% FBS (3 plates in total).**
- Label the plates with **EBs**, up in the left hand corner, **the date and plate #** as well as one of the following treatments:
 - Set #1 Retinoic Acid
 - Set #1 Activin
 - Set #1 Low serum control
 - Set #1 Control
 - Set #1 γ -irradiation
 - Set #1 Hypoxia
- Repeat these labels for Set #2 plates as well.
- From each of the dispase plates 6 EB plates can be prepared. Pipette the EBs up and down to mix them and transfer 30 μ l-60 μ l to each of the prepared TC 96 well plates. (The amount transferred depends on the number of EBs which have formed in the well. Ideally you want 3-6 EBs per well). Make sure that you mix before each transfer since the EBs will settle very quickly.
- The EBs should attach and spread out within 24 hours

Day 5

- Feed all of the EB plates. **For the set#1 plates that will be used for Retinoic acid, Activin, and low serum control, change the media to EB medium containing only 5% FBS.**

Day 6

- Set up the Retinoic acid and Activin assays as described below.
- Feed the rest of the EB plates

Retinoic Acid Assay

- The media that is used in the retinoic acid assay is EB media with only 5% FBS added to it. The stock of retinoic acid is at a 1000000X concentration. Therefore, for each plate of EB clones you will need to prepare 20ml of media containing retinoic acid. Add 1 μ l of retinoic acid for every 100mls of media.
- Aspirate off the EB media.
- Wash the wells once with EB media containing only 5% FBS
- Add 200 μ l of EB media (5% FBS) containing retinoic acid to each well.
- Incubate for 2 days at 37°C
- X-Gal stain 2 days after treatment.

TGFB Factor Assay

- For each plate of EBs you will need 10mls of media containing the TGFB factor on the first day and 10ml for the second day. It is better to prepare the media containing the factor on the day of use. The dilution of the factor depends on which one is being used. Activin is in a 10 000X stock and must be made in a low serum media (0.2%). Prepare the media by adding 2 μ l of activin for every 20mls of media needed in EB media which contains only 0.2% FBS. The BMP2 factor is only a 1500 dilution. Therefore you will need 10ul of BMP2 for every 15ml of media required. This factor also required low serum media of only 0.2% FBS.
- Remove the EB media from the plate of EBs. Wash the wells with 100 μ l of serum free media to remove extra FBS.
- Add 100 μ l/well of EB media (0.2% FBS) containing the factor.
- Incubate at 37°C overnight. On the next day add 100 μ l more of the factor media
- Incubate over night at 37°C.
- X-Gal-stain the plates the following day (2 days after treatment began).

Day 7

- Place an EB plate from set #1 in hypoxic conditions
- Gamma-irradiate an EB plate from set #1. Irradiate at 400 RADs.
- Make sure you add the other 100ul/well of media containing the TGFB factor
- Feed the rest of the EB plates (except for the low serum control, activin or retinoic acid set #1 plates).

Day 8

- X-gal stain all set #1 EB plates
- **For the set of plates that will be used for Retinoic acid, Activin, and low serum control, use EB medium containing only 10% FBS (3 plates).**

Day 9

- Feed set #2 EB plates. **For the set of plates that will be used for Retinoic acid, TGF-B, and low serum control, use EB medium containing only 5% FBS (3 plates).**

Day 10

- Set up the Retinoic acid and Activin assays as described above.

Day 11

- Place an EB plate from set one in hypoxic conditions
- Gamma-irradiate an EB plate from set one. Irradiate at 400 RADs.

Day 12

- X-gal stain set #2 EB plates