

## Protocols for Mouse Embryonic Fibroblast, Human Embryonic Stem Cell, and Embryoid Body Culture

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### Aliquoting Fetal Bovine Serum (FBS)

Thaw the 500 mL bottle of FBS from -20° C in water bath for a few hours. Shake well. Using a 50 mL pipette aliquot in 50 mL tubes. Save a few mL additional serum in a 15 mL tube. Store at -20° C.

### Aliquoting Cell Freezing Medium

Thaw the 50 mL bottle from -20° C. Aliquot 1.5 mL in each vial. Store at -20° C.

### Trypsin solution (0.25%v/v)

1. Prepare trypsin solution by adding 100ml (0.5%) trypsin to 100ml PBSA or DPBS (pipette trypsin, there might be about 105 ml trypsin in the bottle)
2. Sterilize with a 0.22 µm low protein-binding filter (250ml size)
3. Aliquot 8ml increments and freeze at -20°C until needed

### Murine embryonic fibroblast (MEF) medium

1. Fetal bovine serum (FBS) 10% - 50 mL (from -20° C, thaw in water bath before use)
2. Dulbecco's Modified Eagle medium (DMEM) 90% - 450 mL
3. Filter the solution using a 0.22 µm filter bottle (500ml size).
4. Procedure: For filtering, pour the Normal DMEM from its bottle directly in vacuum filter system which is attached on top of a wide sterile bowl. Empty out the aliquoted 50ml FBS on that. Attach the air vacuum tube to it so the medium is suctioned down (Bacteria is 1µm (1 micron) and cannot pass the 0.22 µm filter. Remove the filter, and carefully remove the sterile cap from plastic wrap and tighten on the bowl.
5. Label both the top and the side of the bottle (MEF, initials, date).
6. Store at 4° C in TC room.

### Seeding MEF from cryopreservation

1. Place MEF media in 37°C water bath from 4° C refrigerator
2. Remove the cells from the liquid nitrogen tank or from -80° C freezer (cells can be kept for up to 6 months at -80° C).
3. Leave the cryogenic vial in a 37° C water bath until the contents begin to melt (keep it in water bath for less than 2 minutes, one drop ice must be still there when take it out from bath, fast thaw is the key).
4. Transfer to a sterile hood (This step must be done very fast b/c MEF in freezing medium dies very quickly).
5. Put the cells in a 15 ml centrifuge tube (use a separate 5ml pipette for each vial).
6. In a drop by drop manner add 5 ml of MEF medium to the tube (This way the cells will not burst from osmotic pressure).
7. Centrifuge the tube at 1200 rpm for 3 min
8. Meanwhile, add 15 ml of MEF medium to a 75 cm<sup>2</sup> tissue culture flask (if it is 1 to 2 split, then 2 T75 flasks are needed)
9. Aspirate medium from the tube and resuspend cells in 2 ml of medium
10. Seed the cells in two 75-flasks (1 mL per flask) and place them in the CO<sub>2</sub> incubator (1:2 split). Write the same passage number on flask as is written on vial.
11. MEF must reach to at least 80% confluency before it can be inactivated.

### Passage of MEF cells

1. Remove the MEF medium (Turn around the flask so the bottom is to back, tilt the flask with one hand, to aspirate the residual medium b/c medium will interfere with trypsin).
2. Add 10 mL of PBS to wash the cell culture (After adding PBS lay down the flask for a minute or less, can repeat for 3 times, also can use one 50ml pipet to aliquot into flasks).
3. Remove the PBS and add 2 mL of trypsin
4. Incubate the tissue culture flask in the CO<sub>2</sub> incubator for 3 minutes (meanwhile get the cryovials and label them).
5. After ca. 3 min remove the flask and hit the flask on sides so the cells get detached. Add 5 mL of MEF medium to neutralize the trypsin,. Pipet up and down with 10 ml pipet to resuspend well
6. Transfer the cell suspension to a falcon tube (15 mL)
7. Centrifuge the suspension at 1200 rpm, 3 min (for hES cells 800 rpm for 3 min)
8. Aspirate the medium and resuspend the cells in 4 mL of MEF medium
9. Add 15 mL of MEF medium to 4 flasks and then 1 mL of cell suspension to each one
10. Place the tissue culture flasks in the CO<sub>2</sub> incubator
11. If you want to **freeze**, 1 tube goes for three freezing vials for 1 to 3 split. Label 3 vials, write the passage number of the cells for one more than the flask, write 1→3.
12. Take one vial containing 1.5 mL of freezing medium from -80 ° C. Thaw it in water bath for a few minutes.
13. Resuspend pellet in 0.5 mL MEF medium per vial by pipetting up and down very gently (This is one third the final volume required for freezing).
14. Dropwise, add 1 mL of Freezing/Cryopreservative Medium and mix by pipetting up and down very gently.
15. Place 1 ml of cells in each labelled vial ( pre-labelled for cells, passage number, initials, date, split)..
16. Rapidly transfer the cells to a freezing container and place at -80°C overnight (cells do not like to be in DMSO at room temperature for long periods of time).
17. Transfer cells to liquid nitrogen the next day for long-term storage.

**Mitomycin C solution (8 µg/ml in DMEM)**

*Care should be taken when handling the powdered form of mitomycin C.*

1. Use a syringe to add 4 ml of DMEM (cat.#11965, Invitrogen) to the closed bottle of mitomycin by puncturing the top. Once resuspended in the bottle, use the syringe to draw the DMEM and mitomycin out of the bottle, and then dilute it to 8 µg/ml in DMEM by adding 246 ml DMEM to the 4ml of mitomycin (pipette 4 ml warm medium into a 15 ml tube. First inject 2.5ml of it to mitomycin tube, then 1.5ml to dissolve the powder. Remove the rubber cap and pipette the entire solution into the filter containing 246ml DMEM.
2. Sterilize with a 0.22 µm low protein-binding filter (250ml size filter)
3. Aliquot 7ml increments and freeze at -20° C until needed

**Gelatin solution (0.1 % w/v)**

1. Wash empty 500 ml glass bottle 3 times with Milli Q deionized water
2. Pour into the bottle a 500 ml bottle of autoclaved (sterile) Milli Q water
3. Add 0.5 g of gelatine (Type A for hES)
4. Wrap the cap of the bottle with aluminium foil and autoclave tape, loosen the cap
5. Autoclave the bottle for 45 min
6. Wait 30 min. before using the gelatin solution in the petri dishes
7. The gelatine solution is colourless and slightly more viscous than water. Keep it at room temperature in the shelf.

**Inactivation of MEFs**

1. Aspirate media, and wash with 10 ml DPBS (for T75 flask) for 1X
2. Add 7 ml of the mitomycin C solution
3. Leave the flask in the incubator for exactly 2 hrs
4. While MEFs are incubating with mitomycin, put 10 ml of 0.1% gelatin type A in the bottom of four 10-cm Petri dishes. Spread the gelatin to cover the entire surface and incubate at 37°C until needed (at least 2 hrs).
5. After 2 h, aspirate the mitomycin C
6. Add 10ml DPBS then aspirate – repeat 3X
7. Add **2 ml** of trypsin solution to each flask
8. Put the flask in the incubator until the cells are free-floating (ca. 3 min.)
9. Take the flask from the incubator and add **5 ml** of MEF medium to stop the trypsin
10. Pipette up and down to break cell clumps and then move the contents to a 15 ml centrifuge tube (make one tube from each T75 flask).
11. Spin down at **1200 rpm** for **3 min**.
12. While centrifuging, aspirate uncoated gelatin from petri dishes and add 10 ml of MEF media to each dish
13. Resuspend the cells (there is no specific volume, depends on the final concentration that you want- 4 mL), and divide equally among the pre-treated dishes, quickly and evenly: usually 1 flask into 4, 10-cm dishes
14. If you want to **freeze**, 1 tube goes for two freezing vials for 1 to 2 split. Take one vial of freezing medium from -80 ° C. Thaw it in water bath for a few minutes. Meanwhile, label 2 vials, write the passage number of the cells for one more than the plate, write 1→2.
15. Resuspend pellet in 0.5 ml MEF medium per vial by pipetting up and down very gently (This is one half the final volume required for freezing).
16. Dropwise, add an equivalent volume (0.5 ml per vial) of Freezing/Cryopreservative Medium and mix by pipetting up and down very gently. Your DMSO concentration is now 5%.
17. Place 1 ml of cells in each labelled vial ( pre-labelled for cells, passage number, initials,

date, split)..

18. Rapidly transfer the cells to a freezing container and place at  $-80^{\circ}\text{C}$  overnight (cells do not like to be in DMSO at room temperature for long periods of time).

### **New iMEF**

1. At least 2 hours before melting the frozen vials of iMEF, put 10 ml of 0.1% gelatin in the bottom of four 10-cm Petri dishes (2 dishes per vial for 1→2 split). Spread the gelatin to cover the entire surface and incubate at  $37^{\circ}\text{C}$  until needed.
2. Place iMEF media in  $37^{\circ}\text{C}$  water bath from  $4^{\circ}\text{C}$  refrigerator
3. Remove the cells from  $-80^{\circ}\text{C}$  freezer (iMEF bank can be kept for up to 6 months at  $-80^{\circ}\text{C}$ ).
4. Leave the vial in a  $37^{\circ}\text{C}$  water bath until the contents begin to melt (keep it in water bath for less than 2 minutes, one drop ice must be still there when take it out from bath, fast thaw is the key).
5. Transfer to a sterile hood (This step must be done very fast b/c iMEF in freezing medium dies very quickly).
6. Put the cells in a 15 ml centrifuge tube (use a separate 5ml pipette for each vial).
7. In a drop by drop manner add 5 ml of MEF medium to the tube (This way the cells will not burst from pressure).
8. Centrifuge the tube at 1200 rpm for 3 min
9. While centrifuging, aspirate unsolidified gelatin from petri dishes and add 10 ml of MEF media to each dish
10. Aspirate medium from centrifuged tube and resuspend the cells by adding 2 ml of medium (1mL per dish for 1→2 split)
11. Seed the cells in Petri dishes (1 mL per dish) and place them in the  $\text{CO}_2$  incubator.
12. Label the top of Petri dishes with the same passage number as one written on vial.
13. iMEF does not replicate
14. iMEF has inactivated DNA. It does not replicate. It only needs to attach to the dish, which takes at least 4 to 5 hours. It must be at least 50% confluence for seeding hES. It takes two days for iMEF to start secreting the inhibiting factors for differentiation and to be capable for seeding hES.

### **Viable Cell Counts Using Trypan Blue**

*Trypan Blue is a vital dye. The reactivity of trypan blue is based on the fact that the chromophore is negatively charged and does not interact with the cell unless the membrane is damaged. Therefore, all the cells which exclude the dye are viable.*

1. Place 0.5 ml of a suitable cell suspension (dilute cells in complete medium without serum to an approximate concentration of  $1 \times 10^5$  to  $2 \times 10^5$  cells per ml) in a screw cap test tube.
2. Add 0.1 ml of 0.4% Trypan Blue Stain. Mix thoroughly
3. Allow to stand 5 min at 15 to 30 C (room temperature)
4. Fill a hemocytometer as for cell counting
5. Under a microscope, observe if non-viable are stained and viable cells excluded the stain

### **L-glutamine**

1. Aliquot L-glutamine (200mM), that comes in 100ml bottle, into 15 ml tubes, 2.5ml in each.
2. Label and store at -20° C freezer.

### **Aliquoting Knockout Serum Replacement**

Thaw the 500 mL bottle of Knockout Serum Replacement from -20° C in water bath for a few hours. Shake well. Using a 50 mL pipette aliquot in 50 mL tubes. Save a few mL additional serum in a 15 mL tube. Store at -20° C.

### **bFGF solution (10ug/ml in 0.1% BSA in PBSA)**

1. Aliquot (50 mg/ml) BSA in 100 ul increments (freeze all but one).
2. Prepare 0.1 % bovine serum albumin (BSA) by adding 100ul (50mg/ml) BSA to 4.9 ml DPBS (Dulbecco's phosphate buffered saline lacking Ca<sup>2+</sup> and Mg<sup>2+</sup>) or PBSA
3. Dissolve 10 µg bFGF in 1 ml of 0.1 % BSA (bFGF comes in 1.5ml tube in powdered form. Transfer 1 ml of 0.1% BSA to it). Throw away the rest 4ml.
4. Immediately after resuspension, prepare 250 µL aliquots of bFGF and store at -20° C

### **hES cell medium**

1. Knockout (KO) Serum Replacement, 20 % - 100 mL (Thaw from -20° C)
2. KO DMEM, 78.3 % - 400 mL, kept at 4° C
3. Non essential amino acid solution, 1 % - 5 mL ( kept at 4° C)
4. 2-Mercaptoethanol, 0.2% (55 mM in DPBS) – 1 mL ( kept at 4° C)
5. L-glutamine, 0.5 % (200 mM in 0.85 % NaCl) – 2.5 mL ( kept at -20° C)
6. bFGF from a stock of 5 ng / ml – 0.250 mL (250 ul), aliquoted and kept at -20° C (bFGF helps to keep hES in undifferentiated state)
7. Filter the solution using a 0.22 µm filter bottle (500 mL size)

### **Seeding hES from cryopreservation**

1. Immediately after removal from frozen storage the cell containing vials are placed in a 37°C water bath until they are almost completely defrosted (1-3 min)
2. Transfer the cell suspension to a sterile 15 mL tube, add 5 mL of pre-warmed media drop-wise to avoid osmotic shock to cells, pipette up and down gently to mix.
3. Spin at 800 rpm for 3 min, at 4° C (don't leave cells long; they die of cold and of DMSO in freezing medium).
4. Meanwhile, take out iMEF Petri dishes out of incubator. Aspirate MEF medium. Replace it with 10 mL hES medium in each plate.
5. Aspirate the supernatant from centrifuged tube.
6. Add 4 mL of hES medium to the tube (1 mL/dish), and gently pipette up and down to dissociate the cells.
7. Place the cell suspension onto a Petri dish (1 mL/dish), and place in the CO<sub>2</sub> incubator at 37 C.
8. When the cells reach approximately 70% confluence (several days) they are ready to passage.

### Passage of hES cells

*Collagenase IV is the preferred enzyme for passaging hES cells since it selectively removes the ES cell aggregates from the co-cultures, without disturbing the MEF monolayer. Thus, it is possible to enrich for hES cells during the passaging process.*

1. Aspirate the medium, rinse with 10 mL DPBS, aspirate (use same pipette to distribute DPBS)
2. Add 4 ml of 200 unit/ml collagenase solution to each dish (pre-warm from 4° C)
3. Leave the dish in the incubator for 45 min
4. Take out the dishes from incubator, put away the lid, scrape the dish with 5mL-pipette tip holding at 45 degree relative to the surface of the dish. Scrape 20 times back and forth at two different angles with 90 degrees difference, pipette up and down
5. Transfer the ES colonies to a 15 ml centrifuge tube.
6. Wash the plate a second time with 4 ml ES medium to collect any ES colonies which were not taken the first time, and transfer to 15 mL tube.
7. Spin down at 800 rpm for about 3 min.
8. During centrifugation, take plates prepared with mitomycin inactivated MEF from the CO<sub>2</sub> incubator, remove the MEF medium and add 10mL of hES medium to each plate (use 50 mL pipette to distribute).
9. Take the tubes out of centrifuge. Aspirate the supernatant, add 4 mL new medium slowly (1 ml per dish), and pipette up and down gently to resuspend the colonies (firmly not harshly).
10. Add the resuspended colonies to the mitomycin treated MEF plates, quickly and evenly. Split in the range from 1:3 to 1:4 (1 mL per dish).
11. Label the dishes with cells, passage #, initials, and date. Move the plates cross-ward so the cells do not accumulate in the middle. Put the dishes in the incubator.
12. Change the medium daily.
13. If you want to **freeze**, 1 tube goes for two freezing vials for 1 to 2 split. Take one vial of freezing medium from -80° C. Thaw it in water bath for a few minutes. Meanwhile, label 2 vials, write the passage number of the cells for one more than the plate, write 1→2.
14. Resuspend the cells in remaining medium by flipping on the tube or pipetting up and down very gently.
15. Transfer freezing medium drop-wise, very slowly.
16. Pipette up and down very gently to resuspend the cells in the medium. Divide it into two, labelled vials (cells, passage number, initials, date, split). Put in the freezing box at the -80° C freezer.

### Collagenase type IV solution (200units/ml)

Calculate 200 units collagenase per ml of Knockout DMEM and filter through a 0.22 µm filter, 50 mL size for 50 mL of total volume.

$$\frac{200\text{units}}{\text{mlDMEM}} = \frac{\text{number.of.units.on.bottle}}{\text{mg}} \times \frac{\text{Xmg}}{50\text{ml}}$$

**Solution can be used for up to 2 weeks stored at 4°C.**

### Preparation of Embryoid Bodies (EBs)

*EBs can be formed by a number of methods including suspending cells in gels that restrict the migration of the cells, placing cells within non-adhesive dishes, or seeding cells within hanging drops that induce aggregate formation of the cells.*

1. Aspirate the medium, rinse with 10 mL DPBS, aspirate (use same pipette to distribute DPBS)
2. Add 4 ml of 200 unit/ml collagenase solution to each dish (pre-warm from 4° C)
3. Leave the dish in the incubator for 45 min
4. Take out the dishes from incubator, put away the lid, pipette up and down the collagenase to collect the detached colonies
5. Transfer the ES colonies to a 15 ml centrifuge tube.
6. Wash the plate a second time with 4 ml EB medium to collect any ES colonies which were not taken the first time, and transfer to 15 mL tube.
7. Spin down at 800 rpm for about 3 min.
8. Meanwhile, add 2 mL of EB medium in each well of a 6-well ultra low attachment polystyrene plate.
9. Resuspend the colonies in each tube in 1mL EB medium and add to each well
10. Usually cells are seeded so that each plate goes for one well
11. Put the dishes in incubator
12. After 2 days, change media. Then change media every other day. The cells typically form clusters that range in size from 50 to 1000 µm.

### Changing EB Media

1. Pipette all the cells and medium from one well, and transfer it to a 15 mL tube. Make 6 tubes from a 6-well plate.
2. Centrifuge the tubes at 200 rpm for 1 min. (If the EBs look cystic or hollow, it is better to leave them in the tubes for 15 min until they sediment: usually after 4 days EBs become cystic).
3. Aspirate the supernatant so that about 1 mL is left in the tube, in the conical part.
4. Resuspend EBs in 3 mL EB medium and add to each well.
5. Keep them in CO2 incubator.

### Aliquoting hES Fetal Bovine Serum (FBS)

Thaw the 500 mL bottle of ES Cell Qualified FBS from -20° C in water bath for a few hours. Shake well. Using a 50 mL pipette aliquot in 50 mL tubes. Save a few mL additional serum in a 15 mL tube. Store at -20° C.

### EB Medium Formulation

*EB medium is the same as hES but it does not have bFGF and instead of KO Serum Replacement, ES cell qualified FBS is used.*

1. ES cell qualified FBS, 20 % - 100 mL (Thaw from -20° C)
2. KO DMEM, 78.3 % - 400 mL, kept at 4° C
3. Non essential amino acid solution, 1 % - 5 mL ( kept at 4° C)
4. 2-Mercaptoethanol, 0.2% (55 mM in DPBS) – 1 mL ( kept at 4° C)
5. L-glutamine, 0.5 % (200 mM in 0.85 % NaCl) – 2.5 mL ( kept at -20° C)
6. Filter the solution using a 0.22 µm filter bottle (500 mL size)