MEF Expansion & Freezing
Using a p0 Vial Obtained from GEM

Mouse embryonic fibroblast (MEF) cells are used as a feeder layer for the culture of mouse embryonic stem (ES) cells to help maintain them in a pluripotent state. The inhibition of ES-cell differentiation provided by the MEF feeders appears to be due to their production of leukemia inhibitory factor (LIF). MEF cells are isolated from embryos, established in culture in bulk, and then frozen in convenient aliquots to be thawed for MEF feeder cell preparation.

Thaw and seed a p0 MEF vial into one 15 cm TC plate, using MEF medium (see recipes).

1-2 days Later: When the plate is confluent, split into 5 plates, 15 cm each.

a. Wash with PBS once
b. Add 1 ml of 0.25% trypsin onto the plate
c. Incubate at 37^{o}C for ~10-15 min, till single cell suspension is obtained
d. Add MEF medium and pipet up and down to fully dissociate cells
e. Divide equally to 5 plates
f. Shake plates well to achieve a homogeneous cell suspension
g. Incubate (37^{O}C, 5% CO_2 & 5%O_2 if available)

~3 Days Later: When the plates are confluent, split each plate into 5 plates each (15 cm) following the process described above. The total number of plates now is 25.

Coordinate with someone who has access to the irradiator, who will help irradiate the MEFs during the next step.

~3 Days Later:

Dissociate all 25 plates for freezing, working in batches of 7-8 plates and gather cells into 50 ml conical tubes. Spin down and re-suspend the pellets in about 40mL of MEF medium total and gather in one 50 ml tube.

After irradiation, spin down again and re-suspend the pellet in the appropriate volume.

Calculations Based on Surface Area

This method of calculations for freezing is not very accurate but works well in our hands.

This volume is determined by the following formula:

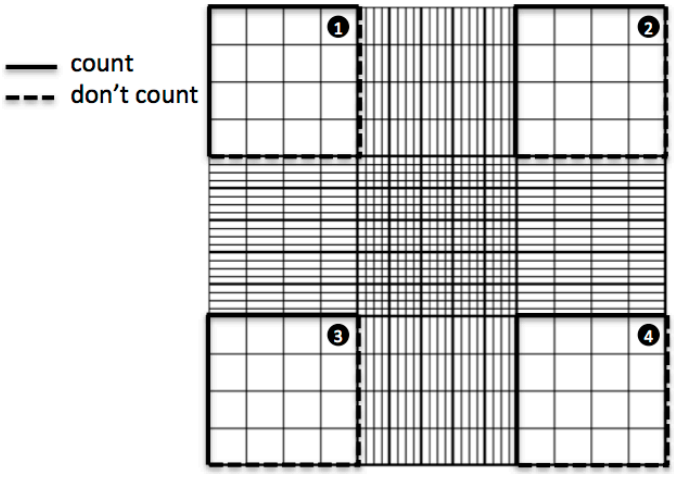
$$\frac{150 \times \text{Number of Plates} \times \text{Estimated \% Confluence (usually 0.80-1)} \times 0.8}{60}$$

150 = Roughly the area, in cm^2, of a 15 cm plate
0.8-1 = Factor for confluence of 80-100%
0.8 = Factor accounting for 20% cell death
60 = Roughly the total area, in cm^2, of a 6-well plate

This will yield the number of vials the MEFs should be frozen down into. Each vial will be good for one 6-well plate once thawed. *For 25 MEF plates, the approximate number of vials (assuming 80% confluence) is 40.*

Alternatively, for more accurate numbers, a cell count can be performed prior to freezing.

Counting Cells



- Make a 1:1 ratio of cells to trypan blue dye (will count only live cells) in and eppendorf tube – 10uL cells: 10 uL trypan blue – make sure the cell suspension is well mixed before taking 10uL
- Get the hemocytometer out and put the cover slip over the center.
- Once covered take 10uL of the 1:1 cell mixture and pipette into the slot on the hemocytometer so that the mixture spreads evenly between the surface of the hemocytometer and the cover slip (avoid bubbles)
- Under the microscope use a hand counter to count each of the 4 corners of the above image and the middle square – record the total number of cells in **all 4 corners plus the middle square. Then divide by 5 and that number is 10^4.** Multiple this number by 2 to account for the 1:1 dilution with trypan blue.
- If you want the number in 10^{x6}, then divide the number above by 100
- If you have diluted your sample then multiply by the dilution factor (e.g. if you have diluted 1:10 then multiply by 10)

Explanation: The volume of each large square (doesn't matter if it is middle or corner) is 10^{-4} cm^3

Freeze $3x10^6$/vial

MEF Freezing

First thing to know is how many vials in total you will be freezing, based on calculations above (either by area, or by number of cells). Then, resuspend your cells into the appropriate volume based on the number of vials to be frozen. Each vial will get 500 uL cell suspension and 500 ul 2x Freezing Medium. If freezing 40 vials, the total volume the cells will get resuspended in is 20 ml. The volume of Freezing Medium needed is the number of vials x 500 ul, plus a bit extra – for 40 vials, the volume needed is 20 ml, but we make 25 ml.

1) Prepare 2x Freezing Medium (50% FBS, 30% ESC Medium & 20% DMSO).
    i. 12.5 mL FBS
    ii. 7.5 mL ESC medium
    iii. 5 mL DMSO

2) Add 500uL of the MEF mixture to each cryo tube (arranged in a Styrofoam box)
3) Add 500uL of freezing medium to each cryo vial

    Important NOTE: The step of adding the Freezing Medium must be fast, as DMSO is toxic to cells. Cells must be frozen very quickly.

Place the Styrofoam box at -80oC overnight and then store the vials in a Liquid Nitrogen freezer.

Tip: After the freezing process is complete (after the overnight freezing at -80^{o}C) thaw a vial and assess whether the cells surviving cover a 6-well plate well. Sometimes, after freezing (especially for users with little experience) there is considerable cell death and more than one vial is needed to cover a 6 well plate.

Scheduling Example of Thawing & Freezing Days

The busiest day is day 8, so give yourself enough time to do everything (and remember to coordinate with the irradiation person).

Day 0 – **Thawing p0 vial:** Wednesday

Day 2 – **1st Split:** Friday

Day 5 – **2nd Split:** Monday

Day 8 – **Freezing:** Thursday