

Embryonic Stem Cells – General Notes

If you are embarking in growing ES cells, be prepared to refeed them DAILY. All procedures should be carried out using sterile techniques. The growth and maintenance media for ES cells is M-15: DMEM (no pyruvate, high glucose) 15% FBS, 1X GPS, 1XBME. Handling ES cells: growth, maintenance, passing, freezing and thawing is conducted in a manner to protect and maintain the quality of the cells and keep them in a pluripotential state. Serum quality is critical for successful growth of ES cells and especially true for blastocysts. The quality of the feeders is very instrumental. Remember also that in passing, freezing, and electroporating ES cells; it is best that the cells are still at exponential growth (80% confluence) for optimal results.

THAWING (QUICK THAW)

1. Remove cells from the freezer and quickly thaw in a 37°C waterbath.
2. Transfer the cell suspension to a sterile 15 ml tube. Add 10 - 12 mls of M15 media to 1 ml of cell suspension.
3. Gently mix and pellet the cells by centrifuging at 1000 rpm for 7 minutes.
4. Aspirate off supernatant and resuspend cells into 6 mls of M15, and plate out cells in a 6-cm feeder plate.
5. Refeed cells daily with fresh M15. Upon 80-85% confluence, cells need to be passaged or frozen.

PASSAGING ES CELLS

ES cells typically should be passaged every 2-4 days (apart from colonies under selection). If passaging is neglected the cells will differentiate and you will select for variants that might have lost totipotency. Cells must be fed when media begins to turn orange. Yellow media (acid pH) is very bad for ES cells and should be avoided at all costs. If you are planning to passage and believe that the cells might turn yellow overnight feed last thing in the evening and again the next morning before passaging. DO NOT PASSAGE CELLS WHEN MEDIA IS YELLOW.

1. Check cells under the microscope for 80-85% confluence.
2. Refeed cells 3 - 4 hours before passing them. (VERY IMPORTANT)
3. Aspirate media off. Wash one time with PBS. Add trypsin (see table below)
4. Incubate at 37 °C for 15 minutes.

5. Inactivate the trypsin by adding at least an equal volume of media (the serum inactivates the trypsin).
6. Pipet up and down several times to separate the cells and break any colonies.
7. Add fresh media to fresh feeder (see table below). Split ratios for ES cells can vary from 1:1 to 1:10; it is not recommended to dilute them more than 1:10.

The area relationships for the various dishes are as follows:

Dish	Media	Trypsin	Area (cm ²)	Diameter cm (actual)
96 well plate	200 µl/well	30-50 µl	0.3	0.6
24 well plate	1 ml/well	200 µl	1.8	1.5
6-well plate	3-4 ml	400 µl	9.6	3.5
6-cm dish	6 ml	0.6 ml	21.2	5.2
10-cm dish	12 ml	1.5 ml	60	8.7
15-cm dish	30 ml	3 ml	154	14

Some typical passaging ratios:

- 1:6 = 1 x 60 mm to 2 x 90 mm
- 1:6 = 1 x 30 mm to 1 x 90 mm
- 1:4 = 1 x 30 mm to 2 x 60 mm
- 1:5 = 1 x 24 well to 1 x 30 mm (6-well plate)
- 1:6 = 1 x 96 well to 1 x 24 well

8. Aliquot the cell suspension into plates in the volume specified for each plate. Always check the feeders before using them. They should be confluent, no gaps, not contaminated and not dividing. Use feeders that are older, (1-2 weeks old), the advantages are many: any contamination is assessed, also any dividing run-away cells can be detected, and the passage will be earlier. Also, older feeders have settled nicely and flattened.
9. Mix carefully, moving the plate in a cross (up and back, then sideways) to distribute the cells evenly. Swirling them is not a good idea!

FREEZING ES CELLS (SLOW FREEZE)

1. Check cells under the microscope : 80-85% confluence is best.
2. Refeed cells 2 - 4 hours before freezing them.
3. Aspirate media off. Wash one time with PBS. Add an appropriate amount of trypsin.

4. Incubate at 37°C for 15 minutes.
5. Inactivate the trypsin by adding at least an equal volume of media (the serum inactivates the trypsin).
6. Pipet up and down several times to separate the cells and break any colonies. Collect cell suspension in a centrifuge tube and note the volume.
7. Count a 200 µl aliquot and calculate the total cell number. From this, calculate the volume of media required to give a final density of 3.0×10^7 cells/ml.
8. Pellet cells by centrifuging @ 1000 rpm for 7 minutes.
9. Aspirate off supernatant and resuspend the pellet in half the volume calculated in Step 7 of M15.
10. Add half the volume with 2X Freezing Media (60% DMEM, 20% FBS, 20% DMSO, freshly prepared); the cell suspension is diluted as a result: 10% DMSO is the final conc. Add the freezing media dropwise, mixing well after each addition.
11. Aliquot the suspension into sterile freezing vials, pre-labeled with the cell type (AB2.2, AB1, etc.), clone number, passage number and date. A typical aliquot would have 0.3 ml - 0.4 ml of ES cells. This is about 9×10^6 - 12×10^6 cells total/vial.
12. Place vials into a freezing container. Optimally, the temperature should drop about 1°C per minute, and the isopropanol freezing containers are best for this application. Freeze cells overnight at -80°C.
13. Next day, transfer cells to the Liquid Nitrogen freezer.