

CULTURE MEDIA /COMPONENTS AND SUPPLEMENTATION

DMEM: Dulbecco's Modified Eagle's Medium-Gibco # 11965-084
purchased with L-glutamine and glucose (4.5g/L already added), no
pyruvate
FBS: Fetal Bovine Serum (Use serum certified for ES cells)
BME: 1000X stock = 0.1M or 100mM
DMSO: Dimethylsulfoxide-Sigma (hybri-max)# D 2650
NEAA: Non-essential Amino Acids Gibco # 1114-050¹
Trypsin: (0.25%) Gibco # 25200-056

CMT ES Cell Medium

75 mls ES screened FBS(15% FBS)
5 mls of 100x NEAA
5 ml 100X Nucleosides (from Specialty Media)
0.5 ml of 1000X BME*
QS to 500 mls with high glucose DMEM.

Sterile Filter
Then add 50 µl of 10⁷/ml ESGRO

2X Freezing medium

This should be freshly prepared for each use. Wear gloves when
working with DMSO. Filter before use.

2.1 ml (60% DMEM)
0.7 ml(20% FBS)
0.7 ml(20% DMSO)

EAP1 EMBRYONIC STEM CELLS

EAP1 cells are derived from C57BL/6NCr1 mice. The EAP1 ES cells must be fed daily. All procedures should be carried out using sterile techniques. 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 of ES cells (quick thaw)

1. Remove cells from liquid nitrogen and immediately vent the vial.
2. After venting the vials, vigorously shake the vials in a 37°C water bath until all ice is dissolved.
4. Transfer the cell suspension to a sterile 15 ml tube. Add 4 mls of ES media to 1 ml of cell suspension.
5. Pellet the cells by centrifuging @ 1000 rpm for 4 minutes.
6. Aspirate the supernatant and resuspend cells into 10 mls of ES media, and plate the cells in a 10-cm plate (plate has mouse embryonic fibroblasts that were plated at least one day prior) such that the density is $2 \times 10^6/10\text{cm}$ dish.
7. Feed cells daily with fresh media. Upon 80-85% confluence, cells need to be passed or frozen.

Passage of ES cells

The EAP1 cells take longer than most ES cells to recover and should not be passed until day 3 after thawing. After the first passage, they can be passed every second day. If passing is neglected the cells will differentiate and you will select for variants that might have lost pluripotency. The cells must be fed when the media begins to turn orange.

1. Check cells under the microscope for 80-85% confluence.
2. Refeed cells 2-3 hours before passing them. (VERY IMPORTANT)
3. Aspirate media off. Add 2.0 ml of trypsin to a 10-cm plate.
4. Incubate @ 37 °C for 4 minutes.

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5. Add 3.0 ml of ES media to inactivate the trypsin.
 6. Pipet up and down several times to separate the cells and break-up any colonies. Count the stem cells. Pass at 2×10^6 cells/10cm dish.
 9. Return plates to the 37 °C incubator.

Freezing ES cells (slow freezing)

1. Check cells under the microscope for 80-85% confluence.
2. Refeed cells 2-3 hours before passing them.
3. Aspirate media off. Add 500 µl of trypsin to a well of a 6-well plate, or 2.0 ml of trypsin to one 10-cm plate.
4. Incubate @ 37 °C for 4 minutes.
5. Add ES media (about 1.5 ml to a well of a 6-well plate, or 3.0 ml to a 10-cm dish) to inactivate the trypsin.
6. Pipet up and down several times to separate the cells and break-up any colonies. Count the ES cells.
7. Pellet cells by centrifuging @ 1000 rpm for 4 minutes.
8. Aspirate the supernatant off and resuspend the pellet in media to ½ the desired freeze volume. Add (½ the volume) 2X Freezing Media.
9. Place vials in a freezing container. It is critical that the freezing rate is not faster than 1 °C/minute. Freeze cells overnight at -80 °C, (24 hours).
10. Next day, transfer cells to a liquid nitrogen freezer (or -135 °C freezer). Freezing and thawing is counted as one passage.