Mouse Embryonic Stem Cell Maintenance for Differentiation

Hogune Im*

Molecular and Cellular Pharmacology Program, Department of Pharmacology, University of Wisconsin Medical School, Madison, USA
*For correspondence: hoguneim@stanford.edu

[Abstract] Embryonic stem cells are derived from inner cell mass of an embryo that can differentiate into every cell type in the body. Clinically, cultured red blood cell supply is of great interest. However, some of the hurdles need to be overcome. This protocol describes a protocol to maintain mouse stem cells that will be used for hematopoietic lineage differentiation assay in the lab.

Materials and Reagents

1. Mouse embryonic stem cells (ES cells)
2. STO cells
3. Fetal calf serum (FCS) Gemini (pre-selected) (catalog number: 100-500)
4. ATLAS biologicals, Summit (Summit Biotechnology, catalog number: FP-200-05)
5. DMSO (Sigma-Aldrich)
6. Phosphate buffered saline (PBS) (Life Technologies, Gibco®, catalog number: 14190-144)
7. Gelatin (STEMCELL Technologies, catalog number: 07903)
8. Monothiolglycerol (MTG): (Sigma-Aldrich, catalog number: M-1753 25 ml)
9. IMDM media (Life Technologies, Gibco®, catalog number: 12440-053)
10. CCE cells (ATCC, catalog number: SCRC-1023™)
11. Mitomycine C (Sigma-Aldrich, catalog number: M0503)
12. Penicillin/Streptomycin (Pen/Strep) (10,000 U) (Life Technologies, Gibco®, catalog number: 15140-122)
13. $NaHCO_3$ (Sigma-Aldrich, catalog number: S5761)
14. Trypsin /EDTA (Lonza Group, catalog number: 17-161E)
15. HEPES buffer (Life Technologies, Gibco®)
16. MTG
17. BME
18. Eosin
19. Iscove's modified dulbecco medium (IMDM) (10% FCS) media
20. β-Mercaptoethanol (BME) (see Recipes)

21. ES-DMEM media (see Recipes)
22. STO media (see Recipes)
23. ES freezing media (see Recipes)
24. Leukemia inhibitory factor (LIF) (screened) (see Recipes)
25. Dulbecco's modified eagle medium (DMEM) (Life Technologies, Gibco®, catalog number: 12100-046, 1 L/pack) (Liquid, catalog number: 11965) (see Recipes)

Equipment

1. 1 L with volumetric flask
2. 0.45 micron filter
3. 0.22 micron filter
4. T25 flask
5. T75 or T175 flask
6. Water bath

Procedure

A. Notes before beginning:

1. ES cells are routinely maintained on feeder cells. STO-neo (neo gene) cells are used as feeder. STO cells should not be used if grown over more than one month.
2. ES cells divide every 8 h, so you need to split ES cells more often than most other cell lines. Typically, split ES cells every 2 day at a density of 8 x 10^5 cells per T25 flask. This number of cells will reach confluency in 2 day.
3. It is not good to keep ES cells in culture for a long time after the cells are thawed. It could affect the differentiation efficiency of the ES cells (it's recommended to thaw new ones after 5-6 passages). I count the number of passages after thawing regardless of number of passage at time cells were frozen. However, if possible thaw and use cells with lower passage numbers. Generally, cells are thawed in T25 and transferred to T75 or T175 to get enough cells for experiment and maintenance.
4. When splitting ES cells, it is important to count ES cells only! STO cells are easily recognized, as they are granular and somewhat bigger in cell size. ES cells are generally smaller more transparent and clear. You have to use 400x magnifications to distinguish ES from STO. Dissociating the cells to single cell makes it easy.
5. Media is prepared in small quantity. Prepare more as you scale up your experiments. Typically ~200 ml will last 1-2 wk. Do not heat media. Use right away from the fridge and return it after finishing.

B. Prepare feeder

Day 1:

1. Add appropriate amount (*i.e.* 3 ml per T25 or 7 ml per T75, just to cover the flask/dish) of 0.1% gelatin in PBS and let it sit at room temperature (RT) for minimum 20 min.
2. Aspirate gelatin right before you add the media. Flask/dishes with 0.1% gelatin in PBS can be stored at 4 °C as well.
3. Split STO cells at a density of 5 x 10^4/cm^2 (*i.e.* you need 1.25 x 10^6 cells per T25 flask; look at table) in 2-3 ml of STO media.

STO cell No. seeding for next day Mito C treatment			
T25	1.25 × 10^6	6 well	4.8 × 10^5
T75	3.75 × 10^6	24 well	1 × 10^5
P100	2.84 × 10^6	96 well	1.92 × 10^4
P60	1 × 10^6	T175	1.25 × 10^7

Day 2: (mitomycine C treatment)

4. Remove media from the flask by aspiration.
5. Add 4 ml of STO media + mitomycine C (1 x fianl). Incubate for 2 h in 37 °C.
6. Aspirate media from the flask and add 2-3 ml of STO media. Let it incubate in 37 °C O/N. At this point your feeders are ready for use the next day.
7. If ES cells are not ready to split, mitomycine C treated STO cells could go for one more day. If grown for more than 2 days, STO cells will start dividing again.

C. Split ES cells

Day 3: (subculture of ES cells)

1. Remove media and add ES cells (8 x 10^5 cells per T25 flask) with ES-DMEM media.
2. Aspirate medium from the flask.
3. Add 1 ml of 1% trypsin /EDTA to ES cells, swirl around, and quickly remove.
4. Add 1 ml of 1% trypsin/EDTA and wait until cells start to detach. It usually takes about 1-2 min. Do not over-trypsinize cells.
5. Deactivate the trypsin by adding 1 ml FCS (maintenance serum) and 4ml ES-DMEM and pipette up and down to make single cell suspensions. It is important not to have cell clumps.
6. Using eosin (0.2% eosin in 1x PBS) count live ES cells only under 40x objective lenz. Dead cells will stain red.
7. Remove STO media from feeder cells and add 2-3 ml of ES-DMEM. Add desired number of ES cells.

D. Thawing and freezing

1. Prepare mitomycine treated feeder. Remove STO media and add 3 ml of ES-DMEM.
2. Quickly thaw cells in 37 °C water bath and transfer everything to mitomycine treated feeder.
3. You will not see any ES cells at this point. Change media daily and inspect under microscope. It will take 2-3 days for ES cells to form visible colony.
4. Change media daily. Do not let the ES cells to over grow. Overgrown ES cell colonies spontaneously differentiate, indicated by vacuole like structure in the middle of colony.
5. ES cells are frozen with the STOs at density of 1~2 x 10^6 cells (ES only)/ml. Add 1 ml of cells to each freezing vial and freeze in -80 °C for >1 day. For short-term stock (<6 month), it could be kept in the -80 °C and for long-term stock (>6 month) transfer to liquid nitrogen. When thawing ES cells for the first time, passage the ES cells on STO cells for twice to ensure the health of ES cells. Once thawed, you need at least 2 days to see ES cells.

Recipes

1. β-Mercaptoethanol (BME)
   BME is added at 5 x 10^{-5} M final, add 0.5 ml of 100x stock per 100 ml media.
2. DMEM (1 L)
   a. from powder
      1 packages DMEM powder
      10 ml Pen/Strep
      25 ml 1 M HEPES buffer
      3.024g $NaHCO_3$
      Bring up to 1 L with volumetric flask w/ autoclaved H_2O filter sterilize (0.22 µm filter)
   b. from liquid
      5.26 ml of 10,000 U Pen/Strep
      21.19 ml of 1 M HEPES
3. ES-DMEM media
   a. 15% FCS (pre-screened ES FCS)
   b. 1.5% LIF
   c. MTG (12.4 µl per 100 ml of media).
   d. MTG is pre-diluted 1:10 (*i.e.* take 0.1 ml of MTG and 0.9 ml of DMEM, mix, and take 12.4 µl for 100 ml of media; use only for one day).
   e. BME is alternatively used at 1 x 10^{-4} M.

f. (Make 100x stock solution by adding 72 µl of 14 M BME to 100 ml of 1x PBS, and add 1 ml per 100 ml of ES-media).

g. Make sure that MTG or BME is made fresh every time you make media. Used for STO+ES cell culture.

4. STO media

a. 10% non-heat inactivated FCS (regular serum) in DMEM.

1) MTG: add 6.2 µl per 100ml of media of MTG that is pre-diluted 1:10 in DMEM.

2) BME: BME is added at 5 x 10^{-5} M final, add 0.5 ml of 100× stock per 100 ml media.

b. Add MTG or BME. Do not use MTG older than 1 month. Always close cap right after use to prevent oxidation.

5. ES freezing media

90% FCS with 10% DMSO

6. LIF (screened)

a. Grow LIF producing cell line in IMDM (10% FCS) media, change media when it reaches 70~80% confluency to DMEM (4% FSC) and collect the sup in 3-4 days.

b. Spin and filter through 0.45 micron filter, aliquot and keep them in -80 °C or -20 °C.

c. Using CCE cells, test for optimum LIF concentration by generating series of titration (0.5, 1, 2, 4%).

d. ES cell colony should look round, tightly packed, and clear. When you see spreading or vacuole like structures on the colony this indicates the LIF concentration is too low.

7. Mitomycine C stock

a. 1 mg/ml in PBS, this is 100x store in dark 4 °C.

b. Best to use the feeder cells 1 day after mitomycine C treatment.

8. FCS

a. You have to screen the serum for differentiation and maintenance purpose.

b. Use CCE cells (these cells do not need feeder) for maintenance serum screen and look for cell morphology.

c. For differentiation serum, perform differentiation in small scale and compare the efficiency of differentiation from previous batch to that of the new serum.

d. One checks for flic-1 expression level by FACS in day 4 embryoid bodies to screen differentiation serum. Usually ~30% of the cells express flic-1 on a good day.

e. Alternatively, ask the representative for prescreened differentiation serum. These batches of serum have been tested in other stem cell labs. However, some variability exits. So it's better to test it on your own.

f. Some source of FCS companies.

g. ATLAS biologicals, Summit for Differentiation.

h. Gemini for ES culture.

References

1. Park, C., Ma, Y. D. and Choi, K. (2005). Evidence for the hemangioblast. *Exp Hematol* 33(9): 965-970.
2. Zhang, W. J., Chung, Y. S., Eades, B. and Choi, K. (2003). Gene targeting strategies for the isolation of hematopoietic and endothelial precursors from differentiated ES cells. *Methods Enzymol* 365: 186-202.

