Culturing of C57BL/6 Mouse Embryonic Stem (ES) Cell Line

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[Abstract] Using ATCC ES C57BL/6 as an example, this protocol shows how to culture mouse embryonic stem (ES) cell line. The clonal embryonic stem cell line #693 ES C57BL/6 was derived from a strain C57BL/6J (B6) mouse blastocyst (PubMed: 11730008). The ES cells were shown to populate the germ line of two host blastocyst donors, FVB/NJ (FVB) and the co-isogenic strain C57BL/6-Tyrc-2J (c2J). Coat-color chimera production was high using c2J blastocysts while FVB blastocysts produced a low number of chimeras (PubMed: 11730008).

Materials and Reagents

A. Cells and cell line:
1. C57BL/6J (B6) mouse blastocyst (PubMed: 11730008)
2. #693 C57BL/6 mouse ES cell line (ATCC, catalog number: SCRC-1002™)
3. CF1 mouse embryonic fibroblast (MEF) feeder cells (self-made from day 12.5 CF1 strain mouse embryos) or other mouse feeder cells such as DR4 (Applied StemCell, catalog number: 1013)

B. Medium and growth factors:
1. DMEM (Life Technologies, Invitrogen™, catalog number: 11995-073)
2. Fetal bovine serum (FBS), Qualified (US) (Life Technologies, Invitrogen™, catalog number: 26140-079)
3. 100x MEM non-essential amino acids (NEAA) (Life Technologies, Invitrogen™, catalog number: 11140-050)
4. 1,000x 2-Mercaptoethanol, liquid (Life Technologies, Invitrogen™, catalog number: 21985-023)
5. 100x L-Glutamine (20 mM), liquid (Life Technologies, Invitrogen™, catalog number: 25030-081)
6. Penicillin/streptomycin (Pen/Strep), liquid (Life Technologies, Invitrogen™, catalog number: 15140-122)
7. 1,000 U/ml ESGRO® mouse leukemia inhibitory factor (LIF) (Merck KGaA, catalog number: ESG1107)

8. TrypLE™ express stable trypsin-like enzyme with phenol red (Life Technologies, Invitrogen™, catalog number: 12605-028)
9. Gelatin from porcine skin-BioReagent, Type A, powder (Sigma-Aldrich, catalog number: G1890-100G)
10. 1x PBS (pH 7.4), liquid (Life Technologies, Invitrogen™, catalog number: 10010-049)
11. Ethanol
12. 0.25% gelatin (see Recipes)
13. Mouse ES cell medium (see Recipes)

Equipment

1. Incubator: 5% CO_2 in humidified air, 37 °C (Thermo Fisher Scientific)
2. Centrifuges and rotor (Thermo IEC)
3. BD Primaria* Tissue Culture Dishes, 100 x 20 mm, 08-772-4F (Thermo Fisher Scientific, catalog number: 353803)
4. 10 cm tissue culture dish
5. Water bath
6. 0.22 μm filter

Procedure

A. Day 0

1. Coat the 10 cm tissue culture dish with 10 ml 0.25% gelatin in a laminar flow hood at room temperature (RT) for 20-30 min.
2. Remove gelatin, do not wash, plate 2 x 10^6 mitotically arrested MEF (CF-1 or DR4) as a feeder layer in 10 ml MEF medium (everything is the same as MES cell medium with the exception of LIF). MEF can stay for 4 days before plating mESC.

B. Day 1

1. One hour before thawing the vial of ES cells, perform PBS wash twice, then a 100% medium change using 9 ml mESC medium.
2. Thaw the vial of C57BL/6 mouse ES cell line by gentle agitation in a 37 °C water bath. To reduce the possibility of contamination, keep the O-ring and cap out of the water. Thawing should be rapid (approximately 90 sec).
3. Remove the vial from the water bath as soon as the contents are thawed, and decontaminate by dipping in or spraying with 70% ethanol. All of the operations from this point on should be carried out under strict aseptic conditions.

4. Transfer the cells from the vial to a 50 ml centrifuge tube. Use an additional 1 ml of media to rinse the vial and transfer the liquid to the tube. Add 8 ml of mESC medium dropwise to bring the total volume to 10 ml.
5. Spin the cells at 270 *x g* for 3 min. Aspirate the supernatant and resuspend the pellet in 1 ml of mESC medium.
6. Add the 1 ml of cell suspension to the previously prepared 10 cm dish containing feeder cells. Shake to move the cells to distribute evenly.
7. Incubate the culture at 37 °C in a humidified 5% CO_2/95% air incubator.

C. Perform medium change every day, and passage cells every 1 to 3 days. Also, the subcultivation ratio is 1:4 to 1:7 as recommended.
   1. Subculturing:
      a. Aspirate the medium from the 10 cm culture dish containing the C57BL/6 mouse ES cells and rinse with 10 ml of PBS twice.
      b. Aspirate the PBS and add pre-warmed 5 ml of TrypLE™ Express, place the dish in a incubator for 5 min or until the ES cells are dissociated.
      c. Add 5 ml of ES cell medium and gently neutralize the contents of the dish.
      d. Transfer the cell suspension to the 50 ml centrifuge tube, spin the cells at 270 *x g* for 3 min. Aspirate the supernatant and resuspend the pellet in 4 ml or 7 ml of mESC medium depending on the subcultivation ratio.
      e. Add the 1 ml of cell suspension to the previous prepared 10 cm dish containing irradiated feeder cells. Shake to move the cells to distribute evenly.
      f. Place the dish in the 37 °C incubator overnight.

D. When freeze the cells, use the following freezing medium:
   1. 10% DMSO.
   2. 90% FBS.

Notes

1. Place vials in liquid nitrogen immediately upon receipt until it is convenient to proceed to culture.
2. To insure the highest level of viability, be sure to warm media to 37 °C before using it on the cells.
3. C57BL/6 mouse ES cells grow as small, tight colonies with phase bright borders. It's optimal to passage them timely before they grow to over confluence.

Recipes

1. 0.25% gelatin (800 ml)
   1.75 g gelatin
   Add Mill Q H_2O to final volume 800 ml, autoclave before use.
2. Mouse ES cell medium (500 ml)

DMEM	409 ml
FBS	75 ml
NEAA (100x)	5 ml
Pen/Strep (100x)	5 ml
L-Glutamine (100x)	5 ml
2-mercaptoethanol (1,000x)	1 ml
1,000 U/ml LIF	50 µl

   Filtered by 0.22 µm filter unit, kept at 4 °C

References

1. Brook, F. A. and Gardner, R. L. (1997). The origin and efficient derivation of embryonic stem cells in the mouse. *Proc Natl Acad Sci U S A* 94(11): 5709-5712.
2. Brook, F. A., Evans, E. P., Lord, C. J., Lyons, P. A., Rainbow, D. B., Howlett, S. K., Wicker, L. S., Todd, J. A. and Gardner, R. L. (2003). The derivation of highly germline-competent embryonic stem cells containing NOD-derived genome. *Diabetes* 52(1): 205-208.
3. C57BL/6 mouse ES cell line product datasheet (ATCC).
4. Evans, M. J. and Kaufman, M. H. (1981). Establishment in culture of pluripotential cells from mouse embryos. *Nature* 292(5819): 154-156.

