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Feeder-Dependent Culture and Passaging Mouse Embryonic Stem Cells (mESCs) in KnockOut™ Serum Replacement with Leukemia Inhibitory Factor (LIF)

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Introduction

Mouse embryonic stem cells (mESCs) are pluripotent stem cells derived from the inner cell mass of the blastocyst, an early- stage embryo[1,2]. Two distinctive properties distinguish embryonic stem cells, their pluripotency and their capacity for self-renewal under defined conditions. They are pluripotent, capable of differentiating into all derivatives of the primary germ layers, including ectoderm, endoderm, and mesoderm, thus generating every cell type in the body. Mouse ESCs also contribute to a wide range of adult tissues in chimeras with intact embryos, including the germ cells.

Recombinant Leukemia Inhibitory Factor (LIF) is a bioactive protein that induces macrophage differentiation in cell culture applications.

Materials Needed

- Dulbecco's Modified Eagle Medium (DMEM), High Glucose, Pyruvate (Cat. no. 11995-065)
- Fetal Bovine Serum (FBS), ESC-Qualified (Cat. no. 10439-024)
- Attachment Factor (Cat. no. S006100)
- MEM Non-Essential Amino Acids Solution, 10 mM (Cat. no. 11140-050)
- KnockOut™ Serum Replacement (KSR) (Cat. no. 10828-028)
- KnockOut™ DMEM (Cat. no. 10829-018)
- Recombinant human LIF (leukemia inhibitory factor), 10 µg (Cat. no. PHC9464)
- Gibco® Mouse Embryonic Fibroblasts (Irradiated), Frozen (Cat. no. S1520-100) or Mitomycin C-treated MEFs
- GlutaMAX™-I (100X) (Cat. no. 35050-079)
- Gibco® Mouse Embryonic Stem Cells (mESCs), ~1 × 10^6 cells/vial (Cat. no. S1503-100)
- Dulbecco's PBS (DPBS) without Calcium and Magnesium (Cat. no. 14190-144)
- StemPro® Accutase® Cell Dissociation Reagent (Cat. no. A11105-01)
- β-mercaptoethanol, 1000X (Cat. no. 21985-023)
- 37°C water bath
- Appropriate tissue culture plates and supplies

Preparing Media and Materials

10 µg/mL Recombinant Human Leukemia Inhibitory Factor (LIF) (1000 µL)

1. Briefly centrifuge LIF vial prior to opening to bring the contents to the bottom.
2. To prepare 1 mL of 10 µg/mL LIF solution, aseptically mix the following components:

LIF	10 µg
DPBS without Calcium and Magnesium	989 µL
0.1% KnockOut™ Serum Replacement (KSR)	1 µL

3. Aliquot and store at –20°C for up to 6 months.

MEF Medium (100 mL of complete medium)

1. To prepare 100 mL of complete MEF Medium, aseptically mix the following components:

DMEM	89 mL
FBS, ESC-Qualified	10 mL
MEM Non-Essential Amino Acids Solution, 10 mM	1 mL
β-mercaptoethanol, 1000X	100 µL

2. Complete MEF Medium can be stored at 2–8°C for up to 1 week.

Mouse Embryonic Stem Cell (mESC) Culture Medium (100 mL of complete medium)

1. To prepare 100 mL of complete mESC Culture Medium, aseptically mix the following components:

KnockOut™ DMEM	83 mL
KnockOut™ Serum Replacement (KSR)	15 mL
MEM Non-Essential Amino Acids Solution, 10 mM	1 mL
GlutaMAX™-I	1 mL

2. Complete mESC Culture Medium can be stored at 2–8°C for up to 1 week.
3. Before using the medium, add the components below and mix:

LIF (10 µg/mL stock solution)	100 µL (final concentration = 10 ng/mL)
β-mercaptoethanol, 1000X	100 µL

Preparing MEF Dishes

AF-coated culture vessels

1. Cover the whole surface of each new culture vessel with Attachment Factor (AF) solution and incubate the vessels for 30 minutes at 37°C or for 2 hours at room temperature.
2. Using sterile technique in a laminar flow culture hood, completely remove the AF solution from the culture vessel by aspiration just prior to use. Coated vessels may be used immediately or stored at room temperature for up to 24 hours.

Note: It is not necessary to wash the culture surface before adding cells or medium.

Thawing and Plating MEFs

Note: Mitotically inactivated MEFs must be plated 3–4 days before initiating or passaging mESC culture.

1. Remove the vial of mitotically inactivated MEFs from liquid nitrogen storage using metal forceps.
2. Roll the vial between your gloved hands until the outside is free of frost. This should take ~10–15 seconds.
3. Immerse the vial in a 37°C water bath without submerging the cap. Swirl the vial gently.
4. When only an ice crystal remains, remove the vial from the water bath.
5. Spray the outside of the vial with 70% ethanol and place it in hood.
6. Pipet the thawed cells gently into a sterile 15-mL conical tube.
7. Slowly add 4 mL of pre-warmed MEF Medium drop-wise to cells in the 15-mL conical tube. While adding the medium, gently move the tube back and forth to mix the MEFs. This reduces osmotic shock to the cells.
8. Rinse the vial with 1 mL of pre-warmed MEF Medium and add to the 15-mL conical tube with cells.
9. Centrifuge the cells at 200 × g for 5 minutes.
10. Aspirate the supernatant. Resuspend the cell pellet in MEF Medium to a density of approximately 2.5×10^6 cells/mL.
11. Aspirate the AF solution from the pre-coated culture vessels.
12. Add the appropriate volume of MEF Medium into each pre-coated culture vessel (refer to Table 1).
13. Into each of these culture vessels, add the appropriate amount of MEF suspension (refer to Table 1).

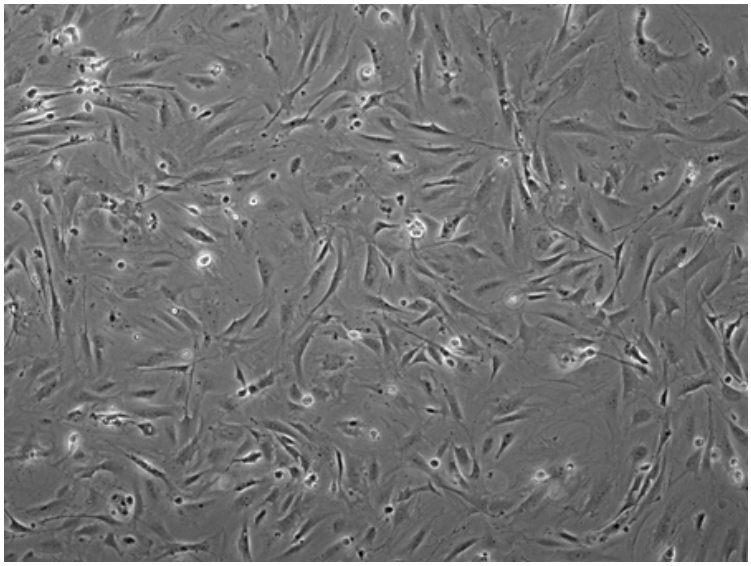
Note: The recommended plating density for mitotically inactivated MEFs is 8×10^4 cells/cm^2.

14. Move the culture vessels in several quick figure eight motions to disperse the cells across the surface of the vessels.
15. Place MEF culture vessels into a 37°C, 5% CO_2 incubator.
16. Use MEF culture vessels within 3–4 days after plating.

Table 1 Amount of Inactivated MEFs Needed

Culture Vessel	Surface Area (cm^2)	Number of MEFs	Optimal Volume (mL)
6-well plate	10 cm^2/well	8.0×10^5/well	2 mL/well
12-well plate	4 cm^2/well	3.2×10^5/well	1 mL/well
24-well plate	2 cm^2/well	1.6×10^5/well	0.5 mL/well
35-mm dish	10 cm^2	8.0×10^5	2 mL
60-mm dish	20 cm^2	1.6×10^6	4 mL
100-mm dish	60 cm^2	4.8×10^6	12 mL

Figure 1 Mitotically inactivated Mouse Embryonic Fibroblasts (MEFs) plated on culture vessels coated with Attachment Factor, shown here 4 days after plating.



Thawing and Plating mESCs

1. Aspirate the MEF Medium from a dish containing inactivated MEFs and add pre-warmed mESC Culture Medium to the dish, 3–4 hours before plating mESCs.
2. Label the dish containing inactivated MEF cells with the passage number from the vial, the date, and user initials.
3. Remove the vial of mESCs from liquid nitrogen storage using metal forceps.

Note: If the vial is exposed to ambient temperatures for more than 15 seconds between removal and thawing, transfer the vial into a container containing a small amount of liquid nitrogen.

4. Roll the vial between your gloved hands until the outside is free of frost. This should take ~10–15 seconds.
5. Immerse the vial in a 37°C water bath without submerging the cap. Swirl the vial gently.
6. When only an ice crystal remains, remove the vial from the water bath.
7. Spray the outside of the vial with 70% ethanol and place it in hood.
8. Pipet cells gently into a sterile 50-mL conical tube using a 5-mL sterile pipette.
9. Slowly add 10 mL of mESC Culture Medium drop-wise to cells in the 50-mL conical tube. While adding the medium, gently move the tube back and forth to mix the mESCs. This reduces osmotic shock to the cells.
10. Rinse the vial with 1 mL of mESC Culture Medium and add to the 50-mL conical tube with cells.
11. Transfer cell suspension to a 15-mL conical tube and centrifuge the cells at 200 × *g* for 5 minutes.
12. Aspirate the supernatant and resuspend the cell pellet in an appropriate volume of pre-warmed mESC Culture Medium.
13. Remove 20 µL of the cell suspension and determine the viable cell count manually using the Trypan Blue exclusion method.

Note: Frozen vials of mESC may also contain MEFs if the mESCs were cultured on MEF feeder layers before cryopreservation. We recommend determining the viable cell count manually, because automatic cell counters cannot distinguish between mESCs and MEFs. The mESCs will be smaller and rounder than the feeder cells, and more uniform in shape.

14. Resuspend the cell pellet in sufficient volume of mESC Culture Medium according to Table 2 by gently pipetting the cells up and down in the tube a few times.

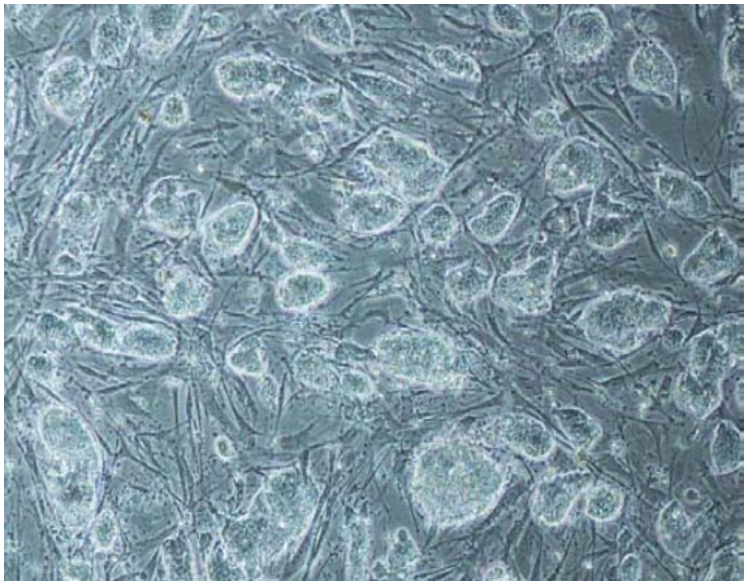
15. Aspirate the spent mESC Culture Medium from the MEF dish and slowly add the thawed colonies onto the dish at a plating density of approximately 4×10^4 cells/cm^2.
16. Place dish gently into the 37°C, 5% CO_2 incubator. Move the dish in several quick figure eight motions to disperse cells across the surface of the dishes.
17. Incubate the cells overnight.
18. The next day, remove the spent medium with debris using a sterile serological pipet and transfer it into a prepared MEF dish. You can use this dish as a backup in case there is a problem with the main dish.
19. Add fresh mESC Culture Medium to each dish according to the volumes in Table 2. Place both plates gently into a 37°C, 5% CO_2 incubator overnight.

Table 2 Volume of mESC Culture Medium Required

Culture Vessel	Surface Area (cm^2)	Number of mESCs	mESC Culture Medium
6-well plate	10 cm^2/well	4.0×10^5/well	2 mL/well
12-well plate	4 cm^2/well	1.6×10^5/well	1 mL/well
24-well plate	2 cm^2/well	8×10^4/well	0.5 mL/well
35-mm dish	10 cm^2	4.0×10^5	2 mL
60-mm dish	20 cm^2	8.0×10^5	4 mL
100-mm dish	60 cm^2	2.4×10^6	12 mL

20. Examine cells under the microscope and replace spent medium daily from both plates. If feeding more than one plate, use a different pipette for each well to reduce risk of contamination. Colonies may not be visible for up to a week.

Figure 2 Mouse (C57BL/6) ESCs at passage 21 cultured on mitotically inactivated MEF feeder layer in mESC Culture Medium containing KSR. The image was obtained 2 days after plating with a 10X objective.



Passaging mESCs

When to Split Cells

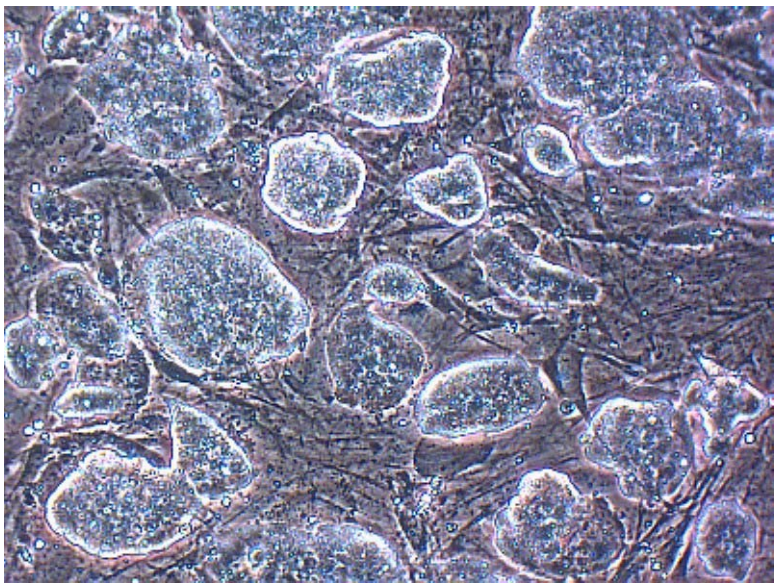
In general, split cells when one of the following occurs:

- mESC colonies are becoming too dense or too large.
- Increased differentiation occurs.

Split Ratio

- The split ratio can vary, though it is generally between 1:3 and 1:8. Occasionally, cells will grow at a different rate and the split ratio will need to be adjusted. A general rule is to observe the last split ratio and adjust the ratio according to the appearance of the mESC colonies.
- If the cells look healthy and colonies have enough space, split using the same ratio. If they are overly dense and crowding, increase the ratio. If the cells are sparse, decrease the ratio. Cells will need to be split every 4–10 days based upon appearance.
- mESCs do well in MEF plates that have been conditioned with mESC Culture Medium. It is common practice to condition new feeder plates before passaging mESCs into them.

Figure 3 mESC colonies ready to be passaged. Note the large colony and the close proximity of the colonies to each other.



Enzymatic Passaging Using StemPro® Accutase® Solution

1. Aspirate the MEF Medium from a dish containing inactivated MEFs and add pre-warmed mESC Culture Medium to the dish, 3–4 hours before plating mESCs.
2. Label the new MEF dish with the cell line name, the new passage number, the date, the split ratio, and user initials. Return the dish to the incubator.
3. Under a dissecting microscope, remove any differentiated colonies from the mESC dish to be passaged.
4. Aspirate the spent medium from the dish with a Pasteur pipette, and rinse the dish once with Dulbecco's PBS (DPBS) without Calcium and Magnesium. Refer to Table 3 for recommended volumes.
5. Aspirate DPBS and add StemPro® Accutase® Solution to the dish containing mESCs. Adjust the volume of StemPro® Accutase® Solution for various dish sizes (refer to Table 3).

Table 3 Reagent Volumes (in mL per well or per dish)

Culture Vessel	Surface Area	DPBS	StemPro® Accutase® Solution	mESC Culture Medium
6-well plate	10 cm^2/well	2 mL/well	1 mL/well	2 mL/well
12-well plate	4 cm^2/well	1 mL/well	0.5 mL/well	1 mL/well
24-well plate	2 cm^2/well	0.5 mL/well	0.3 mL/well	0.5 mL/well
35-mm dish	10 cm^2	2 mL	1 mL	2 mL
60-mm dish	20 cm^2	4 mL	2 mL	4 mL
100-mm dish	60 cm^2	12 mL	5 mL	12 mL

6. Incubate the dish(es) for 1–2 minutes in a 37°C, 5% CO_2 incubator until individual single cells start to round up. Gently tap the sides of the culture vessels to detach the majority of the cells from the surface of the culture vessels.
7. Add an appropriate amount of mESC Culture Medium to each dish to stop the dissociation reaction. Gently pipet the dissociated mESCs up and down sufficiently to disperse the colonies into a single-cell suspension. Make sure to pipet gently to minimize the formation of bubbles.
8. Transfer the mESC suspension into a 15-mL conical tube and centrifuge the tube at 250 × *g* for 5 minutes to pellet the cells.
9. Carefully aspirate the supernatant from the mESC pellet.
10. Resuspend the pellet with an appropriate amount of mESC Culture Medium (refer to Table 3). This is dependent on the split ratio and the number of dishes used.
11. Mix the cell suspension well with a 10-mL pipette. Be careful not to cause bubbles in the medium.
12. Add appropriate volume of cell suspension to each dish. Return the dish to the incubator.
13. Move the dish in several quick figure eight motions to disperse cells across the surface of the dishes.
14. Incubate cells overnight in a 37°C, 5% CO_2 incubator. Replace spent medium daily.

Note: While cells are attaching, be careful when opening and closing the incubator doors to avoid disturbing the even distribution of cells on the surface of the wells.

APPENDIX

A. In the mESC Culture Medium, KnockOut™ DMEM (Cat. no. 10829-018) can be substituted with the following alternative**:**

i. KnockOut™ DMEM/F-12 (Cat. no. 12660-012)

To prepare 100 mL of complete mESC Culture Medium using KnockOut™ DMEM/F-12, aseptically combine the components listed in the table below.

Component	Stock Concentration	Final Concentration	Volume
KnockOut™ DMEM/F-12 (Cat. no. 12660-012)	—	1X	83 mL
KnockOut™ Serum Replacement (KSR) (Cat. no. 10828-028)	—	15%	15 mL
GlutaMAX™-I (Cat. no. 35050-061)	200 mM	2 mM	1 mL
MEM Non-Essential Amino Acids Solution (Cat. no. 11140-050)	10 mM	0.1 mM	1 mL
LIF (Cat. no. PHC9464)	10 µg/mL	10 ng/mL	100 µL
β-mercaptoethanol (Cat. no. 21985-023)	1000X	1X	100 µL

B. Dissociation Enzymes/Tools for Harvesting mESC

Dissociation Enzyme /Tools	Application	Suggested concentration
TrypLE™ Express (Cat. no. 12604-021)	Dissociation to single cells	1X ready to use

References

1. Evans, M., Kaufman, M. (1981) Establishment in culture of pluripotent cells from mouse embryos. Nature *292*, 154–156.

2. Martin, G. (1981) Isolation of a pluripotent cell line from early mouse embryos cultured in medium conditioned by teratocarcinoma stem cells. Proc Natl Acad Sci USA *78*, 7634–7638.

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