Feeder-Dependent culture of mouse pluripotent stem cells (mPSCs) 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 while mouse induced pluripotent stem cells (miPSCs) are reprogrammed from somatic cells[1,2]. Two distinctive properties distinguish these two types of mouse pluripotent stem cells (mPSCs): their pluripotency and their capacity for self-renewal under defined conditions. Because they are pluripotent, mPSCs can be manipulated in vitro for differentiation into many cell types. In vivo, mouse ESCs contribute to a wide range of adult tissues including the germ cells and are often used as vehicles for modifying the mouse genome or for chimera studies. Media containing KnockOut™ Serum Replacement have been shown to increase the efficiency of ESC establishment[3,4], and KnockOut™ Serum Replacement – Multi-Species uses that same KnockOut™ Serum Replacement formulation.

Materials needed

- Dulbecco's Modified Eagle Medium (DMEM), High Glucose, Pyruvate (Cat. No. 11995065)
- Fetal Bovine Serum (FBS), ESC-Qualified (Cat. No. 10439024 or 16141061)
- Attachment Factor (Cat. No. S006100)
- MEM™ Non-Essential™ Amino Acids Solution, 10 mM (Cat. No. 11140050)
- KnockOut™ Serum Replacement – Multi-Species (KnockOut™ SR – Multi-Species) (Cat. No. A3181501 or A3181502)
- KnockOut™ DMEM (Cat. No. 10829018)
- Recombinant mouse LIF (leukemia inhibitory factor), 10 µg (Cat. No. PMC9484)
- Mouse Inactivated Embryonic Fibroblasts, Frozen (Cat. No. A24903)
- GlutaMAX™-I (100X) (Cat. No. 35050079)
- Dulbecco's PBS (DPBS) without Calcium and Magnesium (Cat. No. 14190144 or 14190094)
- StemPro™ Accutase™ Cell Dissociation Reagent (Cat. No. A1110501)
- TrypLE™ Express Enzyme (1X), no phenol red (Cat. No. 12604013)
- 2-mercaptoethanol, 1000X (Cat. No. 21985023)
- 37℃ water bath
- Appropriate tissue culture plates and supplies

Important guidelines for thawing and storing cells

- Upon receipt, immediately thaw cells or place into vapor-phase liquid nitrogen storage until ready to use. **Do not store the cells at –80°C**.
- Avoid short-term extreme temperature changes. When storing cells in liquid nitrogen after shipping on dry ice, allow the cells to remain in liquid nitrogen for 3-4 days before thawing.

Prepare media and materials

MEF medium (100 mL of complete medium)

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

DMEM	90 mL
FBS, ESC-Qualified	10 mL

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

Mouse pluripotent stem cell (mPSC) culture medium (100 mL of complete medium)

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

KnockOut™ DMEM	83 mL
KnockOut™ Serum Replacement – Multi-Species	15 mL
MEM™ Non-Essential™ Amino Acids Solution, 10 mM	1 mL
GlutaMAX™-I, 100X	1 mL
2-mercaptoethanol, 1000X	100 µL
LIF, 10 µg/mL	100 µL

2. Complete mPSC Culture Medium can be stored at 2–8℃ for up to 1 week.

   Note: Recombinant mouse LIF (Cat. No. PMC9484) is provided as a 10 µg/ml stock that can be added directly to the mPSC Culture Medium.

Prepare MEF dishes

AF-coated culture vessels (optional)

1. Attachment Factor (AF) is a sterile solution (1X) containing gelatin at 0.1%.

2. Cover the whole surface of each new culture vessel with Attachment Factor (AF) solution and incubate the vessels for 10–30 minutes at 37℃ or for 2 hours at room temperature.

3. Use sterile technique in a laminar flow culture hood, and completely remove the AF solution from the culture vessel by aspiration just prior to use. It is not necessary to wash the culture surface before adding cells or medium. Coated vessels may be used immediately or stored at room temperature for up to 24 hours.

   Note: Coating the vessel with AF improves attachment and spreading of MEFs but this step is dispensable.

Thaw and plate inactived MEFs

Note: Mitotically inactivated MEF (iMEF) feeder layers can be used a minimum of 16 hours after plating and must be used within 3–4 days after seeding.

1. Remove the vial of iMEFs 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℃ 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 iMEFs. This reduces osmotic shock to the cells.

8. Centrifuge the cells at 200 × *g* for 5 minutes.

9. Aspirate the supernatant. Resuspend the cell pellet in MEF Medium to a density of approximately 5×10^6 cells/mL.

10. Aspirate the AF solution from the pre-coated culture vessels.

11. Add the appropriate volume of MEF Medium into each pre-coated culture vessel (refer to Table 1).

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	5.0×10^5/well	2 mL/well
12-well plate	4 cm^2/well	2.0×10^5/well	1 mL/well
24-well plate	2 cm^2/well	1.0×10^5/well	0.5 mL/well
35-mm dish	10 cm^2	5.0×10^5	2 mL
60-mm dish	20 cm^2	1.0×10^6	5 mL
100-mm dish	60 cm^2	3.0×10^6	10 mL

12. Into each of these culture vessels, add the appropriate amount of MEF suspension (refer to Table 1).

    Note: Depending on the mouse line of origin, the required feeder density can vary. For 129 or C57 strain cells, the recommended iMEF plating density is 5×10^4 cells/cm^2.

13. Move the culture vessels in several quick figure eight motions to disperse the cells across the surface of the vessels.

14. Place iMEF culture vessels into a 37℃, 5% CO_2 incubator.

15. Maintain iMEF culture by changing medium every 2 days.

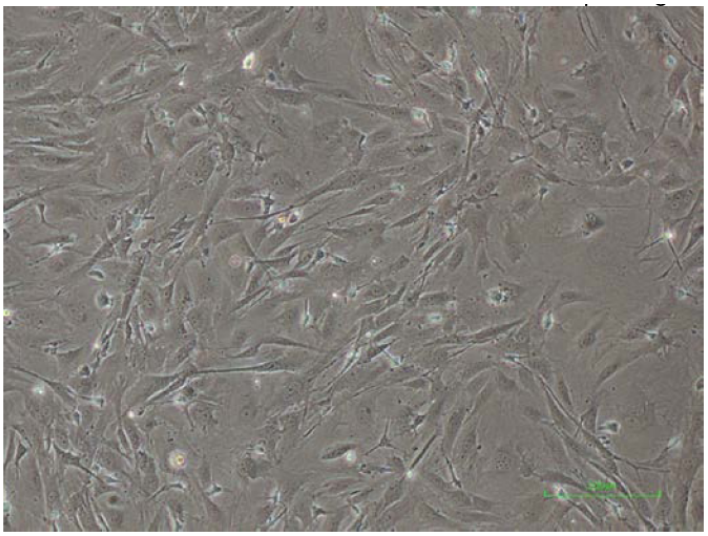

Figure 1 Mitotically inactivated mouse embryonic fibroblasts (MEFs) plated on culture vessels coated with attachment factor, shown here 24 hours after plating.

Thaw and plate mPSCs

1. Aspirate the MEF Medium from a dish containing inactivated MEFs and add pre-warmed mPSC Culture Medium to the dish. This step is performed to remove residual FBS from the iMEF culture.

2. Label the dish containing iMEF cells with the passage number from the vial, the date, and user initials.

3. Remove the vial of mPSCs from liquid nitrogen storage using metal forceps.

   Note: If the vial is going to be 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 or dry ice.

4. Immerse the vial in a 37℃ water bath without submerging the cap. Swirl the vial gently.

5. When only an ice crystal remains, remove the vial from the water bath.

6. Spray the outside of the vial with 70% ethanol and place it in hood.

7. Pipet cells gently into a sterile 15-mL conical tube using a 5-mL sterile pipette.

8. Slowly add 8 mL of mPSC Culture 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 mPSCs. This reduces osmotic shock to the cells.

9. Rinse the vial with 1 mL of mPSC Culture Medium and add to the 15-mL conical tube with cells.

10. Transfer cell suspension to a 15-mL conical tube and centrifuge the cells at 200 × *g* for 5 minutes.

11. Aspirate the supernatant and gently resuspend the cell pellet in an appropriate volume of pre-warmed mPSC Culture Medium.

12. Remove 20 µL of the cell suspension and determine the viable cell count manually using the Trypan Blue exclusion method.

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

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

Table 2 Volume of mPSC culture medium required

Culture vessel	Surface area (cm^2)	Number of mPSCs	mPSC 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

14. Aspirate the spent mPSC Culture Medium from the MEF dish and slowly add the thawed cells onto the dish at a plating density of approximately 4 × 10^4 cells/cm^2.

15. Place dish gently into the 37℃, 5% CO_2 incubator. Tilt the plate in the X and Y directions to disperse cells across the surface of the dish.

16. Incubate the cells overnight.

17. The next day, aspirate the spent medium to remove floating dead cells, and add fresh mPSC Culture Medium to the dish according to the volumes in Table 2. Return plate gently into the incubator.

18. Examine cells under the microscope and replace spent medium daily. Cells should be ready for passage in 2–3 days.

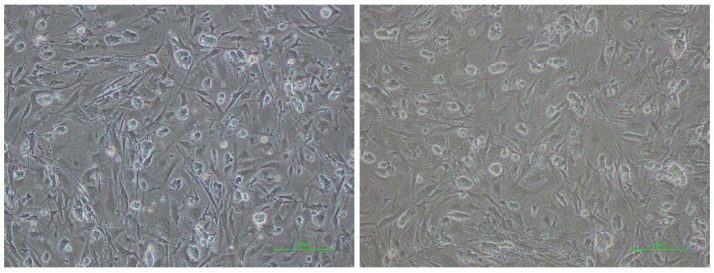

Figure 2 Mouse ESC lines cultured on mitotically inactivated MEF feeder layer in mPSC culture medium containing KnockOut™ SR – Multi-Species. The image was obtained 1 day after plating with a 10X objective. Left 129S2/SvPas line, right C57BL/6N line.

Passage mPSCs

When to split cells

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

- Culture has reached ~70-80% confluency.
- Increased differentiation occurs.

Split ratio

- The split ratio can vary, though it is generally between 1:3 and 1:5. 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 mPSC 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 2–4 days based upon appearance.

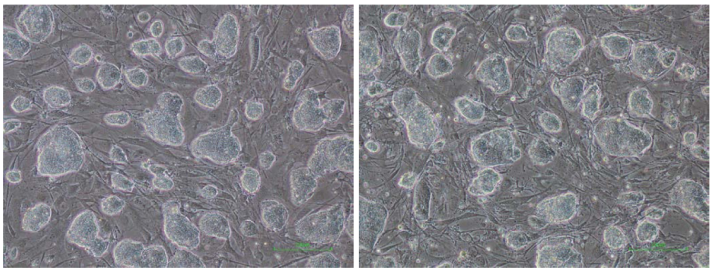

Figure 3 mESC colonies ready to be passaged. Note the large colonies and the close proximity of the colonies to each other. Left 129S2/SvPas line, right C57BL/6N line.

Enzymatically passage with StemPro™ Accutase™ solution or TrypLE™ express enzyme

1. Aspirate spent medium and feed mPSCs with pre-warmed mPSC culture medium 2–4 hours before passaging.

2. Aspirate the spent medium from the dish with a Pasteur pipette, and rinse the dish twice with Dulbecco's PBS (DPBS) without Calcium and Magnesium. Refer to Table 3 for recommended volumes.

   Table 3 Reagent volumes (mL per well or per dish)

Culture Vessel	Surface Area	DPBS	Enzyme Solution	mPSC 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

3. Aspirate DPBS and add Enzyme solution to the dish containing mPSCs. Adjust the volume of Enzyme Solution for various dish sizes (refer to Table 3).

4. Incubate the dish for 3–5 minutes in a 37℃, 5% CO_2 incubator until individual single cells start to round up.

5. Gently pipet iMEF and mPSC sheet up and down sufficiently to disperse the colonies from the feeder layers into a single-cell suspension using a p1000 pipette. Make sure to pipet gently to minimize the formation of bubbles. Add an appropriate amount of mPSC Culture Medium to the dish to stop the dissociation reaction.

6. Transfer the mPSC suspension into a 15-mL conical tube and centrifuge the tube at 200 × *g* for 5 minutes to pellet the cells.

7. Carefully aspirate the supernatant from the mPSC pellet.

8. Resuspend the pellet with an appropriate amount of mPSC Culture Medium (refer to Table 3). This is dependent on the split ratio and the number of dishes used.

9. Mix the cell suspension well with a 10-mL pipette. Be careful not to cause bubbles in the medium.

10. Add appropriate volume of cell suspension to each dish (e.g. 2–3x10^6 cells per 100-mm dish).

11. Return the dish to the incubator.

12. Move the dish in several quick figure eight motions to disperse cells across the surface of the dish.

13. Incubate cells overnight in a 37℃, 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.

Freeze mPSCs

1. Prepare cryopreservation solution on day of use by supplementing complete mPSC medium with 20% Dimethyl Sulfoxide (DMSO). Keep on ice until use.

2. Follow step 1 on page 4 through 7 in the section on Enzymatic passaging to harvest cells for cryopreservation.

3. Calculate the volume of cryopreservation solution required to give cell density of 2 × 10^6 viable cells/mL. Resuspend the pellet in half the final volume required using pre-warmed complete mPSC medium. Add an equal volume of cold complete mPSC medium + 20% DMSO in drop-wise manner to result in a final concentration of 10% DMSO. Immediately dispense suspension into cryovials (1 mL/vial).

4. Achieve cryopreservation in an automated or manual controlled rate freezing apparatus following standard procedures (1℃ decrease per minute).

5. Transfer frozen cells to liquid nitrogen; we recommend vapor phase storage at –200℃ to –150℃.

Transition FBS cultures to KnockOut™ SR – Multi-Species

- Start the transfer only after an initial bank of material has been secured.
- Frozen vials can be directly thawed into KnockOut™ SR – Multi-Species-based medium. Follow the section on Thawing and Plating mPSCs to recover vials in the medium containing KnockOut™ SR – Multi-Species.

- If difficulty is observed during recovery, recover cells in FBS-based medium first, then change to KnockOut™ SR – Multi-Species-based medium on the next day.

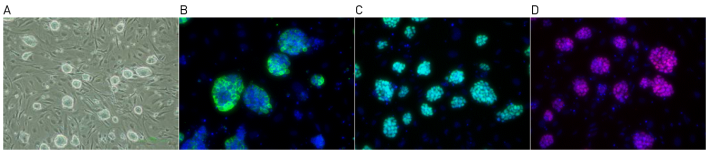

Figure 4 mESC colonies directly recovered in medium containing KnockOut™ SR – Multi-Species (A) and passaged 3 times prior to further characterization (B-D). Note the homogeneous expression of the examined pluripotent markers, SSEA1 (B), Sox2 (C) and Oct4 (D).

APPENDIX

1. A. In the mPSC Culture Medium, KnockOut™ DMEM (Cat. No. 10829-018) can be substituted with DMEM, High Glucose, Pyruvate (Cat. No. 11995-065).

2. B. In the mPSC Culture Medium, KnockOut™ SR – Multi-Species (Cat. No. A3181501 or A3181502) can be substituted with KnockOut™ Serum Replacement (Cat. No. 10828028 or 10828010).

References

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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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4. Bryja, V., Bonilla, S., Arenas, E. (2006) Derivation of mouse embryonic stem cells. Nat Protocol 1, 2082-2087.

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