Culture of LT2e-H9CAGGFP on Feeder Cells

1.MATERIALS

1.1. Reagents

1. DMEM (1X), liquid (high glucose) (Life#10569-010)
2. Hyg resistant inactivated MEF (Millipore# PMEF-H)
3. DMEM/F12 with GlutaMAX™-I (Life#10565-018)
4. Fetal Bovine Serum, ES Cell-Qualified (Life # 10439-024)
5. Non-essential amino acids solution (NEAA, Life#11140-050)
6. Attachment factor (Life# S-006-100) or sterile 0.1% gelatin solution
7. Distilled water (Life#15230-162)
8. KnockOut™ Serum Replacement (KSR, Life#10828-028)
9. β-Mercaptoethanol (55 mM, Life#21985-023)
10. Basic fibroblast growth factor (bFGF or FGF2, Life#PHG0264)
11. PBS without $CaCl_2$ and $MgCl_2$ (Life#14190-250)
12. Dimethyl sulfoxide (DMSO, Sigma-Aldrich#D2650)
13. Trypan blue (Life#15250-061)
14. Hygromycin B (Life#10687-010)
15. Zeocin™ (Life#17104)
16. Collagenase IV (Life# 17104019)

1.2. Solution and medium preparation

MEF medium (For 500 mL)

DMEM	450 mL
FBS	50 mL

Medium lasts for up to 7 days at 4°C.

Basic FGF Solution (10 µg/mL, For 1 mL)

Basic FGF	10 µg
PBS	996 µL
KSR	4 µL

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

Human hESC Medium (For 100 mL)

DMEM-F12	79 mL
KSR	20 mL
NEAA	1 mL
β-Mercaptoethanol	182 µl
Basic FGF solution	40 µl

Medium lasts for up to 7 days at 4°C.

Note: Maintain the LT2e-H9CAGGFP cell line under drug selection during culture, except for the day cells are passaged, and for the first 2-3 days after thaw. To maintain selection, supplement hESC medium with Hygromycin B at a concentration of 10 µg/mL and with Zeocin at a concentration of 1 µg/mL.

10X (10 mg/mL) Collagenase IV Solution

Collagenase IV	500 mg
DMEM/F-12	50 mL

Filter sterilize solution with 0.2 um PES filter unit. Aliquot 1 mL into 15 mL conical tubes and store at -20°C for up to 6 months. At time of use, add 9 mL of DMEM/F-12 to a tube to make 1X (1mg/mL) solution. Discard any unused 1X solution.

2X hESC cryo-preservation medium A

hESC medium (50%) + KSR (50%)

2X hESC cryo-preservation medium B

hESC medium (80%) + DMSO (20%)

Note: Make fresh cryo-preservation medium A and B, and store at 4^{o}C for up to 3 days.

2. METHODS

2.1 Preparing Feeder dishes

2.1.1 Coating culture vessels with attachment factor

Add attachment factor solution (or 0.1% gelatin solution) into each well of the culture vessels according to the following table. Incubate for 1 hour at room temperature.

4-chamber (1.8 cm^2)	6-well plate (9.6 cm^2)	35mm dish (11.78cm^2)	60mm dish (19.5 cm^2)	100mm dish (58.95cm^2)	T25 flask (25 cm^2)	T75 flask (75 cm^2)	T175 flask (175 cm^2)
0.3 mL	1 mL	1 mL	2 mL	4-5 mL	3 mL	4-5 mL	6-7 mL

2.1.2 Preparing MEF culture vessels

1. When attachment factor coated culture vessels are ready, warm MEF medium in a 37^{o}C water bath.

2. Wear eye protection as cryo-vials stored in the liquid phase of liquid nitrogen may accidentally explode when warmed.

3. Wear ultra-low temperature cryo-gloves. Remove cryo-vials of inactivated MEF from the liquid nitrogen storage tank using metal forceps.

4. Roll vials between hands for about 10 – 15 seconds to remove frost.

5. Immerse vials in a 37°C water bath. Swirl gently. **Note**: Do not submerge the cap.

6. When only an ice crystal remains, remove vial(s) from water bath.

7. Spray outside of vials with 70% ethanol and place in hood.

8. Pipette cells into a 15 mL conical tube with a 1mL pipette.

9. Add 10 mL MEF medium to cells drop-wise. While adding the medium, gently move the tube back and forth to mix the cells. This reduces osmotic shock to the cells. Pipette up and down to mix gently.

10. Centrifuge at 200 x g for 5 minutes and aspirate supernatant.

11. Reconstitute the pellet in 5 mL MEF Medium.

12. Centrifuge at 200 x g for 5 minutes and aspirate supernatant.

13. To estimate cell concentration, reconstitute the small pellet with **appropriate amount** of MEF Medium. *(Note: Cell concentration needs to be more than 2.5x10^6 cells /mL after reconstitution).*

14. Add 90 µl trypan blue into an eppendorf tube. Use a P10 pipette to remove 10 µl of the cell suspension, add cells into the eppendorf tube and mix well. Load 10 µl of the cell mixture to the hemacytometer chamber and count the total cell number in 4 squares to determine cell concentration. *(Note: Carefully touch the edge of the coverslip with the pipette tip and allow the chamber to fill by capillary action. Do not overfill or under fill the chamber).*

15. Reconstitute MEF in MEF medium to a concentration of 2.5x10^6 cells /mL.

16. Aspirate attachment factor solution (or gelatin solution) from coated culture vessels.

17. Add pre-warmed MEF medium into each well of culture vessels according to the following table.

4chamber (1.8 cm^2)	6-well (9.6 cm^2)	35 mm dish (11.78 cm^2)	60 mm dish (19.5 cm^2)	100 mm dish (58.95 cm^2)	T25 flask (25 cm^2)	T75 flask (75 cm^2)	T175 flask (175 cm^2)
0.5 mL	2.5 mL	2.5 mL	5 mL	10 mL	5 mL	15 mL	35 mL

18. Add appropriate amount of cell suspension into culture vessels according to the table below to plate MEFs at the density of **2.5x10^4** cells/cm^2

4-well (1.8 cm^2)	6-well (9.6 cm^2)	35 mm dish (11.78 cm^2)	60 mm dish (19.5 cm^2)	100 mm dish (58.95 cm^2)	T25 flask (25 cm^2)	T75 flask (75 cm^2)	T175 flask (175 cm^2)
18 µL	96 µL	117.8 µL	195 µL	589.5 µL	250 µL	750 µL	1,750 µL

19. Move the culture vessels in several quick back-and-forth and side-to-side motions to disperse cells across the surface of the culture vessels. Return the culture vessels to the incubator after plating the MEFs. *(Note: MEF culture vessels can be used on the next day and should be used within 3-4 days after MEF plating.)*

2.2 Thawing and plating human hESCs

1. Warm up human hESC medium in a 37°C water bath. Aspirate the MEF medium from a 60 mm dish containing inactivated MEFs and add 2.5 mL pre-warmed human hESC medium to the dish 3-4 hours before plating human hESCs.

2. Wear eye protection as cryo-vials stored in the liquid phase of liquid nitrogen may accidentally explode when warmed.

3. Wear ultra low temperature cryo-gloves. Remove a cryo-vial of hESCs from the liquid nitrogen storage tank using metal forceps.

4. Roll the vial between your gloved hands until the outside is free of frost. This should take between 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. Quickly remove the sticker or copy the information written on the vial in your notebook. (The writing may come off the vial after spraying outside of vial with 70% ethanol).

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

9. Transfer cells gently into a sterile 15 mL conical tube using a 1 mL pipette.

10. Add 10 mL hESC medium to the 15 mL conical tube drop-wise. While adding the medium, gently move the tube back and forth to mix the human hESCs. This reduces osmotic shock to the human hESCs.

11. Centrifuge the cells at 200 x g for 5 minutes. Aspirate the supernatant.

12. Re-suspend the cell pellet in 5 mL hESC medium.

13. Centrifuge hESCs at 200 x g for 5 minutes and aspirate the supernatant. (Rinse twice to eliminate DMSO).

14. Re-suspend the cell pellet in 5 mL hESC medium.

15. Aspirate medium from the 60 mm dish containing inactivated MEF cells and label it with the passage number of hESC (increase the passage number on the vial by one), the date and your initials.

16. Slowly add hESC suspension into the dish.

17. Move the dish in several quick back-and-forth motions to disperse cells across the surface of the dish. Place the dish gently into the incubator.

18. Replace spent medium daily. If feeding more than one dish, use a different pipette for each dish to reduce risk of contamination. Cells may be overfed once a week to allow skipping one feed (skip feeding one day over the weekend).

19. Observe hESCs every day and passage cells whenever the colonies are too big or crowded. The ratio of splitting depends on the total number of hESCs in culture vessels. (Approximately 1:3 for hESCs at the first time of recovery).

2.3 Passaging hESCs

In general, split cells when the first of the followings occurs: (a) MEF feeder layer is one week old; (b) hESC colonies are becoming too dense or too large; (c) Increased differentiation occurs.

1. Prepare the new MEF plates and dishes **one or two days** before hESC splitting.
2. Warm up human hESC medium in a 37°C water bath. Aspirate the MEF medium from each MEF plate to be used, and add 2.5 mL pre-warmed human hESC medium to the dish 3-4 hours before plating human hESCs.
3. Warm the appropriate amount of Collagenase IV and hESC medium to 37 °C in a water bath.
4. Add 2 mL of 1 X Collagenase IV solution to a 60-mm dish containing ESC. Adjust the volume of Collagenase IV for various dish sizes (e.g., 35-mm dishes require 1 mL of Collagenase IV).
5. Incubate 30–45 minutes in a 37°C, 5% CO_2 incubator. ***Note:*** Incubation times may vary among different batches of collagenase, therefore, you need to determine the appropriate incubation time by examining the colonies.
6. Stop the incubation when the edges of the colonies are starting to pull away from the plate.
7. After incubation, add 2 mL of hESC medium and gently dislodge the colonies with a 5-mL pipette. Gently pipette colonies up and down across the surface of the plate 5 to 8 times to break up the colonies into smaller clumps.
8. Transfer the cell solution to a 15-mL conical tube. Add 2 mL of pre-warmed ESC media to the original dish and pipette across the surface of the dish to dislodge any remaining colonies. Transfer this 2 mL suspension to the 15- mL conical tube and pipette up and down another 2-3 times to resuspend colonies. (Be sure to not introduce bubbles or shear colonies too much. You may want to break up the colonies into small clusters (50–500 cells) for normal passaging.
9. Centrifuge the cells at 200 x g for 2 minutes at room temperature. Alternatively, you can let the colonies settle to the bottom of the tube via gravity by allowing the tube to stand at room temperature for 5-10 minutes. Gravity separation will allow any MEFs, differentiated cells, dead cells, single cells, and undesirably small colony fragments from settlings and thus can be removed from the desired colonies to be re-plated.
10. Aspirate and discard the supernatant, and then gently tap the tube to loosen the cell pellet from the bottom of the tube.
11. Add 2-5 mL of ESC media and resuspend the colonies by gently pipetting up and down. ***Note:*** Avoid making single cell suspensions.
12. Seed the cells onto the MEF plate(s) prepared in step 2. Generally ESCs are passaged at a 1:3 to 1:6 dilution. The final volume of medium depends on the plates used (see Table 2).

13. Place the plates in a 37°C incubator with 5% CO_2. Shake the plates gently to evenly spread out the cell clusters.

2.4 Cryopreserving human hESCs

1. Prepare the appropriate amount of Cryopreservation Medium A and B (0.5 mL of each for each vial).

2. Warm the appropriate amount of Collagenase IV, hESC medium, and Cryopreservation Medium A in a 37°C in a water bath.

3. Aspirate the medium from culture vessels containing confluent hESC. Add the Collagenase IV into each well of the culture vessels according to **Table 4**. Incubate for 30-45 minutes at 37°C.

4. Stop the incubation when the edges of the colonies start to pull away from the plate.

5. After incubation, add 2mL of hESC medium and gently dislodge the colonies with a 5-mL pipette by gently pipetting colonies up and down, across the surface of the plate 5 to 8 times. This will also help break up the colonies into smaller clumps.

6. Transfer the cell solution to a 15-mL conical tube. Add 2 mL of pre-warmed hESC media to the original dish and pipette across the surface of the dish to dislodge any remaining colonies. Transfer this 2 mL suspension to the 15- mL conical tube and pipette up and down another 2-3 times to re-suspend colonies. (Be sure to not introduce bubbles or shear colonies too much. You may want to break up the colonies into small clusters (50–500 cells) for freezing).

7. Centrifuge the cells at 200 × g for 2 minutes at room temperature. Alternatively, you can let the colonies settle to the bottom of the tube via gravity by allowing the tube to stand at room temperature for 5 minutes. Gravity separation will allow any MEFS, differentiated cells, dead cells, single cells, and undesirably small colony fragments from settlings and thus can be removed from the desired colonies to be re-plated.

8. Aspirate and discard the supernatant, and then gently tap the tube to loosen the cell pellet from the bottom of the tube.

9. Gently resuspend the cell pellet in 0.5 volumes (0.5 mL ver vial) of Cryopreservation Medium A. ***Note:*** Avoid making single cell suspensions.

10. Slowly add 0.5 volumes (0.5 mL per vial) of cold Cryopreservation Medium B to the cells while gently swirling the tube to mix.

11. Aliquot 1 mL of cells into each vial. Place vials into Mr. Frosty at -80°C overnight, then transfer to liquid nitrogen freezer for long term storage.