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BIOMEDICUM

Stem Cell Center

FEEDER CULTURES

The function of feeder cultures is to support the undifferentiated growth of hPSC. Typically primary fibroblasts are used for this purpose. We prepare our mouse feeder cells from ICR mice. We recommend using ICR mice for feeder fibroblast cells for routine expansion of our own cell lines to ensure optimal growth of hPSC without differentiation

Stock fibroblasts are isolated from E12.5-13.5 mouse embryos and are used as feeders between passages three and five. Prior to passage 3, fibroblast cultures may be able to support hECS growth, but also may still contain other cell types from the mouse foetus. After passage five (in some other strains at passage seven) fibroblast lose their ability to maintain hPSC in an undifferentiated state, and the fibroblasts may begin to senesce. The MEF have to inactivate before using in stem cell culture. In our lab we use Mitomycin C for inactivation. It is extremely toxic to both cells and humans. Medium containing mitomycin as well as the first wash of cells that have been exposed to mitomycin-containing medium must be disposed of as a hazardous waste. Embryo cells are very sensitive to even amounts of mitomycin (as well as many chemicals, such as antibiotics). For best results, trypsinize fibroblast and wash several times by centrifugation after mitomycin treatment. Feeder cell density is crucial for hPSC culture.

Thawing MEFs (Mouse Embryonic Fibroblasts (MEFs)

Materials and media needed:

- +37°C water bath
- ICR p1 cells
- 70% ethanol
- 50ml centrifuge tube
- MEF media (see SOP ‘Reagents and media preparation/MEF media’)
- centrifuge
- T150 cell culture flask
- +37°C incubator (+37 ^{0}C, 5 % CO_2)

Procedure:

1. Remove ICR p1 vial from -150 ^{0}C or liquid nitrogen.
2. Thaw vial quickly in +37°C water bath.
3. When just a small crystal of ice remains, treat the outside of the vial with 70% ethanol. Wipe ethanol away before opening the vial.
4. Transfer cell suspension (1ml) into a 50 ml centrifuge tube and add 10 ml of MEF media into the tube.
5. Centrifuge cell suspension 1100 rpm for 5min.
6. Remove supernatant.
7. Resuspend cell pellet in 20 ml (T150) of culture medium (MEF) and add to a cell culture flask.
8. Place flask into an incubator set at +37 ^{0}C with 5 % CO_2 in air.
9. Change the media next day. From now on change the media every third day.

10. Passage ICRs 3 to 4 days after thawing (1:3 - 1:4), when they become ~90% confluent.
11. ICRs are passaged four times (up to passage 5) after which they are Mitomycin C treated and freeze for later use.

PASSAGING FIBROBLAST

When feeder cells cover more than 90% of the surface of the flask, the cells are confluent and should passaged.

Material and media needed:

- 50ml centrifuge tube
- MEF media (see SOP 'Reagents and media preparation/MEF media')
- centrifuge
- Trypsin-EDTA/TrypLe (Life Technologies # 12563-029)
- T150 or triple cell culture flask
- +37°C incubator (+37 ^{0}C, 5 % CO_2)

Precedure:

1. Aspirate media from the flask
2. Rinse cells on flask with 10ml PBS and aspirate
3. Add 5ml Trypsin/TrypLe to flask and gently swirl to ensure cells are covered with the solution.
4. Incubate for 5 minutes at into an incubator set at +37 ^{0}C with 5 % CO_2 in air.
5. Tap side of flask to dislodge cells and check under microscope to ensure that cells are in single-cell suspension.
6. Neutralize Trypsin by adding 10 ml MEF media to flask, and transfer the flask contents to a 50ml centrifuge tube (one tube per flask)
7. Centrifuge 5 min 1100rpm
8. Aspirate supernatant and resuspend cells with 10 ml MEF media
9. Plate suspension in three T150 flasks containing MEF medium to final volume 25ml per flask or in one triple flask containing MEF media to final volume 100 ml.
10. Incubate until growth in confluent

NOTE: There are variations from lab to lab. What is important is to culture feeders carefully so that they never overgrowth and the medium is not depleted of nutrients or too acidic

MITOMYCIN C TREATMENT AND CRYOPRESERVATION OF ICRS

NOTE: Fibroblasts that have not yet been mitomycin treated are considered stock fibroblasts. Fibroblast may be frozen for stock within low passage number (1-2) without mitomycin treatment. When ICR p5 flasks are ~90% confluent, inactivate the cell proliferation by treating the flasks with Mitomycin C treat and freeze mouse embryonic fibroblasts (MEFs) in cryovials for later use.

Materials and media needed:

- cryovials
- 1000μg/ml Mitomycin C solution (see SOP 'Reagents and media preparation/Mitomycin C')
- 1μg/ml Mitomycin C solution in MEF media
- MEF media (see SOP 'Reagents and media preparation/MEF media')
- +37°C incubator (+37 ^{0}C, 5 % CO_2)
- 1xPBS (w/o Ca^{2+}/Mg^{2+})
- 1x trypsin/EDTA solution
- 50ml centrifuge tubes
- haemocytometer chamber
- centrifuge
- freezing media for HFFs and MEFs (see SOP 'Reagents and media preparation/ Freezing media for HFFs and MEFs')
- –70°C
- liquid nitrogen

Procedure:

1. Pre-label cryovials.
2. Aspirate media from the flask(s) and add 10ml /T150, 30 ml/Triple flask MEF media containing Mitomycin C such that the final concentration is 2μg/ml.
3. Incubate 2.5-3h into an incubator set at +37 ^{0}C with 5 % CO_2 in air.
4. Aspirate/puor medium from the flask(s) to **a waste bottle**.
5. Wash 3 times with 1xPBS (w/o Ca^{2+}/Mg^{2+}).
6. Add 1x trypsin/EDTA to cells 6ml/ T150, 15 ml/Triple flask. Incubate approximately 5min into an incubator set at +37 ^{0}C with 5 % CO_2 in air, until cells start to detach. Inactivate trypsin/EDTA with MEF media (10ml /T150, 20 ml/Triple flask).
7. Centrifuge cell suspension 1100rpm for 5 min.
8. Remove supernatant and resuspend cell pellet to 10 ml MEF media and pool to a one tube.
9. Count the cells (freeze 2x10^{6} cell/vial).
10. Centrifuge cell suspension 1100rpm for 5 min.

11. Remove supernatant and resuspend cell pellet to X ml freezing media (1 ml per cryovial).
12. Place 1ml of cell mixture into each vial and transfer vials immediately into freezing box and place it in –70 °C. Next day transfer vials to -150 ^{0}C or liquid nitrogen.

THAWING AND PLATING MITOMYCIN C TREATED MEFS ON CULTURE DISHES

Materials and media needed:

- 0.1% gelatine (see SOP 'Reagents and media preparation/0.1 % gelatine')
- Mitomycin C treated MEFs
- 50 ml centrifuge tube
- cell culture dishes
- MEF media (see SOP 'Reagents and media preparation/MEF media')
- +37°C incubator (+37 ^{0}C, 5 % CO_2)

Procedure:

1. Coat cell culture dishes with 0.1% gelatine solution. Incubate at RT ~30min. Remove gelatine solution and let the dishes dry before plating the feeders.
2. Thaw Mitomycin C treated MEFs as describe in section **Thawing MEFs** .
3. Resuspend the cell pellet in an appropriate volume of MEF media and plate on gelatinized dishes (at a density of 10 000-20 000 MEFs per cm^2).

MEF –dishes	
3,5 cm ∅	0,2x10^6 cells
6 cm ∅	0,5x10^6 cells
10 cm ∅	1,4x10^6 cells

MEF –plates	
6-well	0,2x10^6 cells/well
12-well	0,08-0,1x10^6 cells/well
24-well	0,05x10^6 cells/well

Chamberslide 8 wells 0,2x10^6 cells

4. Move carefully the plated dishes into an incubator set at +37 ^{0}C with 5 % CO_2 in air and let the MEFs to settle o/n.

THAWING AND MAINTAINING HFFS
(Human Foreskin Fibroblast Feeders (HFFs) from American type Culture Collection (CRL-2429, htpp://www.atcc.org)

Materials and media needed:

- +37°C water bath
- 70% ethanol
- conical centrifuge tube
- MEF media (see SOP 'Reagents and media preparation/MEF media')
- centrifuge
- T150 cell culture flask
- +37°C incubator (+37 °C, 5 % CO_2)

Procedure:

1. Remove vial from -150°C or liquid nitrogen.
2. Thaw vial quickly in +37°C water bath.
3. When just a small crystal of ice remains, treat the outside of the vial with 70% ethanol. Wipe ethanol away before opening the vial.
4. Transfer cell suspension (1ml) into a 50ml conical centrifuge tube and add 10ml of MEF media into the tube.
5. Centrifuge cell suspension 1100rpm for 5min.
6. Remove supernatant.
7. Resuspend cell pellet in 20ml of culture medium (MEF) and add to a cell culture flask (T150).
8. Place flask in +37°C incubator.
9. Change the media next day. From now on change the media every third day.
10. Passage HFFs 3 to 4 days after thawing (1:3 - 1:4), when they become ~90% confluent.
11. HFFs are used up to passage 15.

MITOMYCIN C TREATMENT AND CRYOPRESERVATION OF HFFS

When HFF flasks are confluent, inactivate the cell proliferation by treating the flasks with Mitomycin C. Freeze HFFs in cryovials in liquid nitrogen for later use.

Materials and media needed:

- cryovials
- 1000μg/ml Mitomycin C solution (see SOP 'Reagents and media preparation/ Mitomycin C')
- 5μg/ml Mitomycin C solution in MEF media
- MEF media (see 'Media preparation/MEF media')
- 1xPBS (w/o Ca^{2+}/Mg^{2+})
- 1x trypsin/EDTA solution
- +37°C incubator (+37 ^{0}C, 5 % CO_2)
- haemocytometer chamber
- centrifuge
- freezing media for HFFs and MEFs (see SOP 'Reagents and media preparation/ Freezing media for HFFs and MEFs')
- –70°C
- liquid nitrogen

Procedure:

1. Pre-label cryovials.
2. Aspirate medium from the flask(s) and add 10ml (T150) MEF media containing Mitomycin C such that the final concentration is 1μg/ml.
3. Incubate 2.5-3h into an incubator set at +37 ^{0}C with 5 % CO_2 in air.
4. Aspirate medium from the flask(s) to **a waste bottle**.
5. Wash 3 times with 1xPBS (w/o Ca^{2+}/Mg^{2+}).
6. Add 1x trypsin/EDTA to cells (6ml per T150). Incubate approximately 5min into an incubator set at +37 ^{0}C with 5 % CO_2 in air, until cells start to detach. Inactivate typsin/EDTA with 10 ml MEF media.
7. Centrifuge cell suspension 1100rpm for 5 min.
8. Remove supernatant and resuspend cell pellet to 10 ml MEF media and pool to a one tube.
9. Count the cells (freeze 2x10^6 cell/vial).
10. Centrifuge cell suspension 1100rpm for 5 min.
11. Remove supernatant and resuspend cell pellet to X ml freezing media (1 ml per cryovial.
12. Place 1ml of cell mixture into each vial and transfer vials immediately into freezing box and place it in –70 °C. Next day transfer vials to -150 ^{0}C or liquid nitrogen.

THAWING AND PLATING MITOMYCIN C TREATED HFFS ON CULTURE DISHES

Materials and media needed:

- 0.1% gelatine (see SOP 'Reagents and media preparation/0.1 % gelatine')
- Mitomycin C treated HFFs
- 50 ml centrifuge tube
- cell culture dishes
- MEF media (see SOP 'Reagents and media preparation/MEF media')
- +37°C incubator (+37 ^{0}C, 5 % CO_2)

Procedure:

1. Coat cell culture dishes with 0.1% gelatine solution. Incubate at RT ~30min. Remove gelatine solution and let the dishes dry before plating the feeders.
2. Thaw Mitomycin C treated HFFs as describe in section **Thawing HFFs** .
3. Resuspend the cell pellet in an appropriate volume of MEF media and plate on gelatinized dishes (at a density of 45 000-50 000 HFFs per cm^2), for 3,5 cm ∅ plate $0{,}3x10^6$ cells per plate
4. Move carefully the plated dishes into an incubator set at +37 ^{0}C with 5 % CO_2 in air and let the HFFs to settle o/n.