Protocols

iPS cell culturing

Media

Standard hES Media (500 ml)

400 ml DMEM/F12

100 ml (20%) KOSR

2mM L-Glutamine

100 units/ml penicillin/streptomycin

0.1 mM MEM-NEAA

0.1mM 2-Mercapetoethanol

10 ng/ml bFGF

MEFs Medium

500 ml DMEM

15% ml FBS

100 units/ml penicillin/streptomycin

2mM L-Glutamine

0.1mM MEM-NEAA

0.1 mM 2-Mercapetoethanol

2X Freezing Media (10 ml)

8 ml defined FBS

2 ml DMSO

Plating MEFs

The iPS cells are typically maintained on 0.1% gelatin coated plates with MEFs. It is recommended to plate 1 million MEFs per 10 cm plate (~170,000 per well of a 6-well plate). Optimization of the MEF density might depend on the level of differentiation of your iPS cells. Allow MEFs to settle overnight (or at least 8 hours) before adding iPS cells. MEFs should ideally be used within 4 days.

Thawing human iPS cells

These cells should be plated as large clumps to increase survival efficiency, so pipetting should be minimized during thawing.

1. Set up 2X 15 ml conical tubes. In tube 1, add 1 ml of pre-warmed hES media. In tube 2, add 9 ml of pre-warmed hES media.
2. Partially thaw the frozen vial of iPS cells at 37ºC, until a small piece of ice remains. Spray the vial with 70% ethanol to sterilize.
3. Taking 1 ml of media at a time from tube 2, slowly (drop by drop) add the pre-warmed media to the vial and transfer the liquid content with cells into tube 1. Repeat until all 9 ml have been used.
4. Spin at 1000 RM for 2 min.
5. Meanwhile, wash with PBS one well of a 6 well plate containing with MEFs on gelatin coating. Add 2 ml hES media. **Although not required, it is highly recommended that you add 10 uM ROCK inhibitor Y-27632 (both to 9 ml thawing media in tube 2 and to final 3 ml of plating media) to improve efficiency. Do not add this ROCK inhibitor to any subsequent feeds.**

   The ROCK inhibitor Y-27632 enhances the survival rate of human embryonic stem cells following cryopreservation.

6. Aspirate the media from the spun down tube 1, and gently resuspend the pellet with 1 ml of hES media. Pipet slowly 1 or 2x maximum, trying to avoid disrupting the chunks of cells, and transfer to one well of a 6-well plate.
7. Change the medium after 36 to 48 hours.
8. Feed cells daily with 2 ml medium. Colonies should start to form between5 to 10 days after plating.
9. The first split should be mechanical (ratio depending on cell density observed).

NOTE: it is highly recommended that you perform a mycoplasma test upon successful thawing of these cells. It is also recommended that you check the karyotype of the line after every 10 passages.

Passaging human iPS cells

1. Before splitting, remove differentiated colonies under a microscope in sterile conditions (i.e. via slow-vacuum aspiration or pipet scraping). Be careful not to leave the plate out of the incubator for too long and make sure cells do not dry out if using vacuum method).
2. Wash cells with either warm hES medium (without bFGF) or PBS.
3. Add 1 ml of Collagenase IV (1 mg/ml) per well of a 6-well plate and incubate at 37ºC for 5-10 minutes (expect to see visible curling of thickening of colonies around the edges).
4. Remove the enzyme by aspiration and add 1 ml of hES medium. Using a cell lifter, scrape the entire well to lift the colonies.
5. Transfer the solution into a conical tube; wash the well with an additional 1 ml hES medium and transfer this to the tube as well.
6. Centrifuge at 1000 RPM (200xg) for 2 min.
7. Remove the medium and resuspend pellet in 1 ml mediuma per well that you wish to plate (ratio depends on cell density just prior to splitting).
8. Triturate to get medium-small fragments (~50-200 cells per fragment). Avoid over-triturating since that will lead to cell death, especially when colonies are broken down to single cell suspension.
9. Plate 1 ml of cell suspension into a well of a 6-well plate containing MEFs. MEFs should be pre-washed with PBS and should contain 1 ml of hES media.

Freezing cells

Note: For cells grown on Materigel, the cells should be frozen in same manner, except using 500 ul of mFreSR per 6-well.

As with thawing, it is very important to minimize the amount of pipetting to ensure cell survival later on.

1. Prepare the cells as described in steps 1-6 of “Passaging hiPS cells”.
2. Aspirate the media and carefully add 250 ul of hES media + Rock inhibitor for every vial you intend to freeze (freeze 1 vial per well of a 6-well plate, or 5 vials per 10 cm dish).
3. Add 250 ul of 2X freezing media (keeping cells in as large of chunks as possible; generally pipetting 2X should be enough).
4. Quickly transfer 500 ul per cryo-vial, and place inside isopropanol-containing freezing container.
5. Store 24-48 hrs at -80ºC and then transfer to liquid nitrogen. (once DMSO in contact with cells, work quickly and ideally get the cells at -80 within 3 min. of contact).

Vendor list:

DMEM/F12: Invitrogen 31330-095

KOSR: Invitrogen cat# 10828-028

L-Glutamine cat# 25030-24

Penicillin/streptomycin: Invitrogen cat# 15140-155

MEM-NEAA: Invitrogen cat# 11140-035

2-Mercaptoethanol: Sigma cat# M-7522

bFGF: Peprothec cat# 100-18B

DEMEM: Invitrogen cat# 11965-118

FBS: Invitrogen cat# 16000-044

Defined FBS: Hyclone cat# SH30070.01

DMSO: sigma cat# D-2650

*Irradiated MEFs: GlobalStem cat# 6001G

* available by iPS facility

Collagenase Type IV, Powder: Gibco cat# 17104-019

Rock inhibitor Y27632, Calbiocam: cat# 688000-10MG

0.1% gelatin: Sigma G-2500-100

cell lifter: Corning #3008

Metaphase chromosome spreading of human ES/iPS cells

Day 1

- Prepare single cell suspension of 2 confluent wells of a 6-well plate using TrypLE Express (1x) Gibco, cat. #12604-013
- Seed single cells on 3-wells of materigel coated wells of a 6-well plate in presence of 10µM of Rock inhibitor.

Day 2

- Pre-warm hypotonic sloution of 0.075 M KCL to 37ºC.
- Deattach cells from plate using TrypLE Express, resuspend cells in 6ml of mTeSR1 medium in a tube.
- Add 0.1 ml colcemid (10 µg/ml) to a tube & incubate at 37C in waterbath for 30 min.
- Centrifuge tubes for 10 min at 1100 rpm.
- Add 6 ml of pre-warmed 0.075 M KCL per tube & resuspend cells gently using a plastic pasteur pipet.
- Incubate at 37ºC waterbath for 30 min.

In between prepare fresh Carny's fixative sloution by mixing 3:1 ratio methanol: Acetic Acid)

- Incubate at RT for another 10 min.
- To allow cells to adapt to the fixative, add about 0.5-1 ml fixative to cells en mix gently using a plastic Pasteur pipet.
- Centrifuge tubes for 10 min at 1100 rpm.
- Aspirate supernatant using vaccum pomp.
- Add 6 ml of fixative to cells & resuspend gently.
- Centrifuge tubes for 10 min at 1100 rpm.
- Add 6 ml of fixative to cells & resuspend gently.
- Centrifuge tubes for 10 min at 1100 rpm.
- Prepare fresh fixative
- Add 6 ml of fixative to cells & resuspend gently.
- Centrifuge tubes for 10 min at 1100 rpm.
- After the last round, leave about 200 µl medium on the cell pellet.
- It is possible to store cells at this step at -20ºC or directly continue making a slide.

Making a slide

- Clean the microscope slides with 70% EtOH
- Take 10 – 15µl cell suspension and make a streak on the slide. Dry the slide.
- Mount the slide with Vectashield Mounting with Dapi, Vector Laboratories, cat. #H-1200.