Protocol:

Human induced pluripotent stem cell (iPSC) culture and maintenance

Written by Faria Zafar

Purpose: Described in this protocol is the daily culture of iPSC maintenance and passaging to expand.

Reagents and media formulations:

1. StemFlex media kit (Thermo Fisher Scientific, A3349401)
2. KnockOut DMEM (Thermo Fisher Scientific, 10829018)
3. Matrigel GFR Membrane Matrix (Thermo Fisher Scientific, CB-40230)
4. *rhLaminin-521 (Thermo Fisher Scientific, A29249)
5. Thiazovivin (THZ) (ReproCell, 04-0017)
6. Bambanker (Fisher Scientific, NC9582225)
7. DPBS with Ca 2+ and Mg 2+ (Fisher Scientific, 14-040-133)
8. PBS without Ca 2+ and Mg 2+
9. ReLeSR (StemCell Technologies, 05872)

*iPSCs can be cultured in Matrigel or rhLaminin

Consumables

10. 500ml Filter unit (Fisher Scientific, 0974105)
11. BD 21G, 1" Luer lock syringe (Fisher Scientific, 1482123)
12. Falcon 6 well plate (Fisher Scientific, 087721B)
13. 15 ml Conical Tubes, Nunc (Fisher Scientific, 12565268)
14. 2ml cryovial, Corning (Fisher Scientific, 0337421)
15. Serological pipettes
16. Pasteur pipettes
17. 1000ul Pipet tips
18. 20ul Pipet tips

Equipment:

Centrifuge

Media preparation:

StemFlex media (add supplement directly to the media bottle)
https://www.thermofisher.com/order/catalog/product/A3349401
Note: Thaw the supplement in refrigerator temperature 2ºC to 8ºC overnight

Ingredients	Stock concentration	Final concentration	In 500ml
StemFlex basal media			450ml
StemFlex supplement	10X	1X	50ml

- StemFlex supplement can be aliquoted and stored at -20C for later use (Please avoid freeze-thaw cycle)
- Once prepared, complete StemFlex media can be stored at 2ºC to 8ºC for two weeks
- Warm complete media as needed as daily basis at room temperature or 37ºC water bath
- StemFlex supplement is aliquoted in 50ml conical tubes as 5 ml

Preparation of Matrigel (MG) plates: (24 hours in advance) - passaging
Note: Thaw 5ml of MG matrix on ice at 2ºC to 8ºC overnight to make working aliquots (e.g. 500ul/aliquot in 50ml conical tubes, make sure all the consumables are cold, store at -20C)

1. Thaw MG aliquot on ice for one hour or at 2ºC to 8ºC overnight until fully liquefied
2. For 1/90 dilution, add 44.5ml cold (4ºC) KnockOut DMEM into 500ul MG
3. Mix thoroughly with a cold 50ml pipette. Do not introduce air bubbles. MG has a tendency to clump if not mixed smoothly.
4. Add 1-1.5ml of diluted MG solution to cover the entire growth surface area of a well ($9.6cm^2$) for 6-well plate on ice.
5. Seal plate(s) with parafilm and incubate overnight (or at least an hour) at 37ºC
    a. Coated plates can be stored up to two weeks at 2ºC to 8ºC or one week at 37ºC ready to use

Preparation of rhLaminin (LN521) plates: (24 hours in advance) - thawing
1. Thaw 1ml of LN521 (1mg/ml) on ice at 2ºC to 8ºC overnight. Once thaw, the vial can be kept at 4ºC for three months.
2. Dilute the stock LN521 to 5-10ug/ml (1:100 to 1:200) in cold DPBS with Ca^{2+} and MG^{2+} on ice.
3. Mix thoroughly with a cold pipette. Do not introduce air bubbles.
4. Add 1ml of diluted LN521 solution to cover the entire growth surface area of a well ($9.6cm^2$) for 6-well plate
5. Seal plate(s) with parafilm and incubate overnight at 4ºC.
    a. Coated plates can be stored up to four weeks at 2ºC to 8ºC
6. On the day of passaging, warm up the plate in 37ºC for at least an hour.

Working Steps:

i) iPSCs Manually Passaged For Maintenance: use MG coated plates

A. Maintaining iPSC
1. Change StemFlex media to feed cells every other day
2. Monitor cells every day. Cells should be round with clear borders
3. Manually remove any differentiation from colonies
    a. To manually clean cell, use a 20ul pipet tip to scrape off differentiated areas or colonies under a microscope in laminar flow hood
    b. Change media after removal of differentiated cells

B. Manual passaging of iPSC
1. Colonies are confluent typically 7-10 days after passaging. Best to passage iPSCs as clumps.
2. Before passaging, incubate a new MG-coated plate at 37ºC for one hour.
3. Aspirate MG from the plate and add fresh 1.5ml StemFlex media to equilibrate at 37ºC before cells are seeded.
4. Add 2ul of 1mM THZ to the new well to have a final concentration of 1uM. Shake the plate to mix THZ to media, incubate plate at 37ºC.
5. Inspect the plate under the microscope and remove any differentiated cells/colonies before passaging by scraping off with a P20 pipette tip.
6. Change StemFlex media before passaging the cells.
7. 4-5 fully-grown colonies are recommended to passage in one well of a 6-well plate.

8. Using a 21G needle with syringe, make grids on the colonies under a microscope under laminar hood (see also YouTube video for passaging: https://www.youtube.com/watch?v=dKTmQuIFHcs)
9. Using a P200 pipette aspirate the pieces of iPSC colonies from old plate and gently transfer them to new plate.

(Note: monitor time while cleaning and transferring cells. iPSCs **should not** be kept at room temperature for more than 15 minutes)

10. Incubate at 37ºC and 5% CO_2 overnight.
11. Next day, change StemFlex media to remove THZ.

ii) **Enzyme-free iPSCs Passaging:** use MG-coated plates

A. Enzyme-free passaging of iPSC

1. Colonies are confluent typically 4-6 days after single cell passaging.
2. Before passaging, incubate a new LN521 or MG coated plate at 37ºC for one hour.
3. While the plate is incubating, aspirate spent StemFlex media from the well with cells.
4. Wash cells with 1ml PBS without Ca 2^+ and Mg 2^+.
5. Add 1ml of room temperature ReleSR in each well of 6-well plate.
6. Incubate the plate at room temperature for 5– 7 minutes until the cells look like a white sheet of cells on the bottom when you tilt the plate.
7. Remove ReleSR carefully with a 1000ul pipettes tip without disturbing the cells.
8. Add 1ml of warm StemFlex media to the well and collect the cells by pipetting up and down to wash the cells from the bottom.
9. Transfer the cells gently with 2ml of Stem Flex media with 1uM THZ in a 15ml conical and count.
10. Take out the coated plate from incubator and aspirate LN521/MG.
11. Add 3-5x10^5 or 1:20 split of iPSCs suspension onto the plate. Typically one 100% confluence well of a 6-well plate generates 5 – 8 million cells.
12. Incubate at 37ºC and 5% CO_2 overnight.
13. Next day, change media to remove THZ.
14. Passage cells whenever they reach 100% confluence.
    Note: use LN521 only for difficult cell lines that grow slowly. Reagent cost is very high.

B. EDTA passaging of iPSCs

1. Aspirate the spent medium from the well to be split and wash once with 2 mL PBS.
2. Aspirate PBS and add 2ml 0.5 mM EDTA/PBS then put in incubator for 5 min.
3. In a 15mL falcon: Prepare the required amount of E8 media then add rock inhibitor (1:1000), e.g. 3 ml per cell line (1ml splitting, 1 ml resuspension, 2 ml for well/feeding)
4. Add 1 ml of E8 medium with rock inhibitor per well and collect everything in a 15mL falcon
5. Centrifuge at 200xg for 3min then aspirate the medium with EDTA and resuspend the pellet in 1 ml of E8 medium with rock inhibitor
6. Aspirate matrigel and add 2 ml of E8 medium with rock inhibitor then plate cells at 1:10 in the new well (~100 ul, discard remaining cells.
7. Change medium after 24 hrs (no rock inhibitor required).

iii) Cryopreservation of iPSCs

1. Aspirate the spent media.
2. Wash cells with 1ml PBS without Ca 2+ and Mg 2+.

3. Add 1ml of room temperature ReleSR in each well of 6-well plate.
4. Incubate the plate at room temperature for 5– 7 minutes until the cells look like a white sheet of cells on the bottom when you tilt the plate.
5. Remove ReleSR carefully with a 1000ul pipettes tip without disturbing the cells.
6. Add 1ml of PBS to the well and collect the cells by pipetting up and down to wash the cells from the bottom.
7. Transfer the cells gently in a 15ml conical.
8. Wash the wells with 1ml PBS to collect any residual cells, and add to 15ml conical.
9. Count if needed.
10. Centrifuge at 1,000 rpm for 3 minutes at room temperature.
11. Aspirate supernatant.
12. Re-suspend cells in 1 ml Bambanker/ 1-1.5x10^6 cells/cryovial.
13. Transfer cells into cryovial.
14. Store vials in a freezing container (e.g. Mr. Frosty) for 24 hours at -80ºC
    (Note: Mr. Frosty needs to be equilibrate at room temperature before cell storage and then directly place Mr. Frosty at -80ºC)
15. Transfer and store frozen vials in liquid nitrogen tank for long-term storage

iv) Thawing iPSCs

1. Prepare one well of a 6-well plate with MG or 10ug/ml LN521. Incubate at 37ºC and 5% CO2 before use (as described above).
2. Add fresh 1ml of Stemflex media + 3ul of 1mM THZ to the well to have final concentration of 1.5uM. Shake the plate to distribute THZ to media. Keep plate in 37ºC incubator.
3. Prepare a 15ml conical tube with 3 ml pre-warmed Stemflex media.
4. Obtain vial of frozen iPSCs from liquid nitrogen tank (bring the vial to tissue culture room on dry ice) and place it in a 37ºC water bath until only a thin piece of ice remains.
5. Using a P1000 pipette, add 700-800ul of pre-warmed Stemflex media to the vial dropwise and gently stir to mix, avoid introducing bubbles or spill.
6. Collect cell suspension in the 15ml conical tube containing Stemflex media.
7. Centrifuge at 1,000 rpm for 3 minutes at room temperature.
8. Aspirate supernatant.
9. Re-suspend cells gently with 1ml of StemFlex media.
10. Add iPSC suspension onto the MG or LN521 coated plate and incubate overnight at 37ºC.
11. Change media daily and observe the well for iPSC colonies to emerge.

Notes:

Gentle passaging and pipetting. Keep small colony aggregates. Do not dissociate into single cells, as this often leads to poor survival and abnormal karyotypes. When passaging, aim to plate cell aggregates of ~20 cells each.