FACLR
FRIEDREICH'S ATAXIA CELL LINE REPOSITORY

**Napierala Lab protocol for culturing human iPSCs**

For additional details and recommendations, please refer to "Maintenance of Human Pluripotent Stem Cells in mTeSR™ Plus" Technical Manual (STEMCELL Technologies).

***Necessary Reagents***

| Reagent | Company | Catalog Number |
|---|---|---|
| Corning™ Matrigel™ hESC-Qualified Matrix | Fisher Scientific | 08-774-552 |
| mTeSR™ Plus Complete Kit | STEMCELL Technologies | 100-0276 |
| CryoStor® CS10 | STEMCELL Technologies | 07930 |
| Dispase 1U/mL, 100mL | STEMCELL Technologies | 07923 |
| Y-27632 (Dihydrochloride) | STEMCELL Technologies | 72302 |
| DMEM/F12 | Fisher Scientific | 11320-082 |

***Thawing cryopreserved iPSCs***

**NOTE:** Have all tubes, warmed medium and plates ready before starting the protocol to ensure that the thawing procedure is done as quickly as possible. See page 3 for Matrigel preparation instructions.

1.) Prepare new plate(s) by coating wells with diluted Matrigel (1 ml/well for 6-well plate), and incubate for 1 h at room temperature (in hood).

2.) Quickly thaw the iPSC cryovial in a 37°C water bath by gently shaking the cryovial continuously until **half** of frozen solution is thawed. Remove the cryovial from the water bath and wipe with 70% ethanol to disinfect.

3.) In a biosafety cabinet, using a 2 ml glass pipet, transfer the contents of the cryovial to the bottom of a 15 ml conical tube - *gently*.

4.) Add 5 ml warmed mTeSR™ Plus medium dropwise to a 15 ml conical tube, *gently* mixing while adding.

5.) Centrifuge cells at 300 x g for 5 min at room temperature (15 - 25°C).

6.) After centrifugation, aspirate supernatant, leaving the cell pellet intact. Using a 2 ml glass pipette, re-suspend the cell pellet in 2 ml of mTeSR™ Plus with 10 μM Y-27632 [final] by pipetting up and down *gently* no more than 3 times. Take care to maintain the cells as aggregates.

7.) Remove Matrigel from the coated tissue culture plate by gently tilting the plate onto one edge and allowing the excess Matrigel solution to collect. Remove the solution by aspiration. Ensure that the tip of the pipette does not scratch the coated surface. Important: Try to avoid leaving Matrigel-coated plates without any fluid – do not aspirate to complete dryness.

8.) Immediately transfer the 2 ml of cell aggregates to one well of a Matrigel-coated 6-well plate.

   a.) Unless otherwise noted, contents of 1 cryovial can be seeded into 1 well of a 6-well dish.

9.) Place the plate into the 37°C incubator and move the plate in quick side-to-side, forward-to-back motions to evenly distribute the aggregates within the wells. Culture the cells at 37°C, with 5% $CO_2$ without any motion.

10.) Check cells after 24 h and completely exchange media with 2 ml mTeSR™ Plus **without** Y-27632.

**NOTE:** Thawing is stressful for iPSCs and many cells/aggregates will not attach. There will be debris and the presence of Y-27632 will give the cells an elongated and jagged appearance. Daily medium changes might be necessary (even with mTeSR™ Plus) for the first few days after thawing to enhance cell survival and to clear debris. Colonies should regain their densely packed, smooth-edged appearance 2-3 days post-thaw.

***Passaging of iPSCs***

**Different passaging conditions are necessary depending on purpose.** The splitting ratio depends on the number of colonies before splitting/freezing, and on the nature of the cell line (e.g. growth rate, amount of differentiation, etc.). If the colonies are at an optimal density (70-80% confluence), the cells can be passaged every 4 - 7 days using 1:3 to 1:6 splits (e.g. the aggregates from 1 well of a 6-well plate can be plated into 3 wells of a 6-well plate). If the colonies are too dense or too sparse, adjust the split ratio accordingly. If there are some differentiating cells, mark them using an objective marker and remove regions of differentiation by aspiration before splitting.

1.) Prepare new plate(s) by coating wells with Matrigel (e.g. 0.3 ml/well for 24-well plate, 0.5 ml/well for 12-well plate, 1 ml/well for 6-well plate), and incubate the plate for 1 h at room temperature (in hood).

2.) Aspirate medium from the iPSC culture and rinse with DMEM/F12 (2 ml/well of 6-well plate), then aspirate.

3.) Add 1 ml/well (6-well plate) of Dispase (1 U/ml). Place at 37°C for 3-4 min.

4.) Check progress by microscope - after incubation, the colony edges will appear slightly folded back but the colonies should remain attached to the plate.

5.) Remove Dispase and gently rinse each well 2 times with 2 ml of DMEM/F12 per well, aspirating between rinses.

6.) Add 1 ml/well of mTeSR™ Plus (or DMEM/F12) per well and carefully cut colonies using a 2 ml glass pipette, then gently scrape off colonies using a cell scraper. Using a 2 ml pipet, transfer aggregates to a 15 ml conical tube. Add an additional 1 ml of mTeSR™ Plus (or DMEM/F12) to each well to rinse remaining cells from the plate and combine in the same tube.

There are different ways of splitting colonies. **A** is preferred when the ratio of splitting is small (e.g. 1:1 or 1:2); **B** is preferred if the ratio needs to be larger or centrifugation is needed (e.g. medium removal/exchange).

**A. Splitting**

1.) Pipette the suspension up and down using a 2 ml or 5 ml pipette to achieve the proper aggregate size (~3 times) and seed the cell aggregates into the wells of a new Matrigel-coated plate paying attention to equally divide between the new wells. Each well should contain a final volume of 2 ml. Optimal aggregate size is ~50–200 μm.

2.) Move the plate in several quick, short, back-and-forth and side-to-side, straight-line motions to disperse the cell aggregates across the surface of the wells.

3.) Place the plate in a 37°C incubator (5% $CO_2$). Ensure that the newly seeded aggregates are evenly dispersed across the surface of each well by again moving the plate in several quick, short, back-and-forth and side-to-side motions. Uneven distribution of cell aggregates may result in differentiation of iPSCs.

**B. Splitting or Freezing**

1.) Centrifuge cells at 300 x g for 5 min at room temperature.

a.) If passaging the cells, remove the supernatant and resuspend the pellet with a volume of fresh mTeSR™ Plus media appropriate for your desired plating. Pipette up and down using a 2 ml or 5 ml pipette to achieve the proper aggregate size (~3 times), then seed the cell aggregates into the wells of a new Matrigel-coated plate (2 ml total volume/well). Disperse the aggregates as in (**A**).

b.) If freezing the cells, carefully remove the supernatant. For each well of iPSC aggregates collected in the 15 ml tube, add 1 ml of cold (4°C) CryoStor CS10 freezing media using a 2 ml or 5 ml glass pipette. Pipette the suspension **gently** up and down several times (~2-4 times). Perform the minimum amount of pipetting necessary to achieve resuspension, trying to avoid excess pipetting. Perform this step cautiously to not disperse cell aggregates too much, but to make an evenly scattered suspension of cell clumps.

i. Transfer the cell suspension to cryovials (1 ml/vial).
ii. Freeze the vials gradually (-1°C/min) by putting them into a room temperature Mr. Frosty freezing container that is then placed at -80°C overnight. Transfer the vials to liquid nitrogen for long-term storage. Keeping vials at -80°C for longer than 1-2 days can negatively impact cell viability.

***General information and technical considerations***

Cells are cultured according to the "Maintenance of Human Pluripotent Stem Cells in mTeSR™ Plus" Technical Manual by STEMCELL Technologies with modifications depending on cell-specific characteristics.

1.) Change the mTeSR™ Plus media every day or every other day depending on the needs of your cell lines; 6-well plate, 2 ml/well; 12-well plate, 1 ml/well; 24-well plate, 0.5 ml/well.

2.) Wells should be checked daily to identify unsuitable cells and/or colonies (e.g. differentiating iPSC colonies or clumps and somatic cells that did not undergo efficient reprogramming). Mark these places using an objective marker and aspirate them while changing the medium and before splitting.
   a.) Sometimes you may have several marked places in one well. If so, do not aspirate all the media at first. Instead, aspirate some colonies/cells from one well, wash the well with media, aspirate the next area of colonies/cells, wash, and aspirate the media, etc. Be attentive and work **precisely** but also **quickly** so as to not leave the colonies without media for more than **30 sec**.
   b.) Make sure that the end of the glass Pasteur pipette is not broken before beginning aspiration steps. A pipette with a broken end does not pick up the cells, it will only aspirate medium.

3.) Always warm mTeSR™ Plus at room temperature, **do not warm at 37°C**.

4.) Complete mTeSR™ Plus is stable at +4°C for up to 2 weeks. If not used immediately, aliquot and store the complete medium at -20°C for up to 3 months. Once an aliquot is thawed, do not re-freeze. **Note**: aliquot only 40 ml into a 50 ml conical tube to allow for expansion of the media upon freezing.

5.) iPSCs are maintained using mTeSR™ Plus medium without antibiotics or ROCK inhibitor (Y-27632).

**MATRIGEL (Corning):**
Matrigel aliquots are prepared according to the manufacturer's recommended procedure and the unique product information sheet that accompanies each vial of purchased Matrigel (Corning). **Keep cold (4°C)** during the process. Thaw the vial on ice (overnight at 4°C), then keep the vial, tubes and tips cold while preparing aliquots. Store the aliquots at -80°C.

Allow a Matrigel aliquot to thaw at 4°C (usually 30 min - 1 h). When working with Matrigel, it is best to have **cold** pipettes/tips/tubes that have been kept at -20°C (at least 4°C). Transfer the thawed portion of Matrigel (kept on ice) to 25 ml of cold DMEM/F12 media using aseptic technique. **Keep it cold**. Coat a sufficient number of wells for your immediate use. Coated plates should be left at room temperature at least 1 hour prior to use. Use Parafilm to seal the plates if they need to be left for a longer period of time. **Do not allow the Matrigel to evaporate!** Diluted Matrigel can be stored at 4°C for up to 2 weeks. Be sure to label the tube with the date of dilution and be sure the lid is tightly sealed to prevent evaporation (can use Parafilm to wrap).

**mTeSR™ Plus:**
Prepare and handle complete media according to the procedure recommended by STEMCELL Technologies.

**ROCK inhibitor (STEMCELL Technologies):**
Prepare a 10 mM stock solution of Y-27632 in sterile, ultrapure $H_2O$ or PBS according to manufacturer's recommendation (STEMCELL Technologies, Cat#72302). Aliquot ~50 µl per tube/vial and store at -20°C. Once thawed, the 10 mM stock solution can be stored up to 2 weeks at 4°C; do not re-freeze. The Y-27632 molecule should be used at a final concentration of 10 µM in mTeSR™ Plus medium, freshly diluted at the time of use. The Y-27632 molecule enhances survival at thawing and when passaging iPSCs as single cell suspensions.

**Dispase solution (1 U/ml) (STEMCELL Technologies):**
Thaw bottle overnight at 4°C to prepare working aliquots (e.g. 10 ml) that can be stored at -20°C. Thaw aliquots slowly at 4°C or room temperature, and warm to room temperature shortly before using. Quick thawing at 37°C, multiple freeze/thaw cycles, or long warming of the enzyme can decrease its activity.

**DMEM/F12 medium (Life Technologies):**
Pure medium is used as a rinsing solution before and after the use of Dispase. It is also used to prepare the diluted Matrigel solution.