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Basic Maintenance Protocol for Human Induced Pluripotent Stem Cells (hiPSCs) Introduction V.3

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Protocol status: Working
We use this protocol and it's working

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Abstract

This protocol details the maintenance of human induced pluripotent stem cells (hiPSCs), covering thawing, passaging, media changes, single-cell dissociation, and cryopreservation. It begins with thawing hiPSCs, followed by plating on an extracellular matrix (Matrigel) and regular media changes every 1-2 days. Cells are passaged upon reaching 60-70% confluency. Finally, the protocol includes guidelines for freezing hiPSCs for long-term storage. This streamlined approach ensures effective culture and management of hiPSCs.

Materials

ROCKi **STEMCELL Technologies Inc. Catalog #**72304 -20 °C

Note

- iPSCs like being in clusters. Single cell iPSCs tend to die. ROCKi prevents that from happening
- Need a 10mM stock: dilute 5mg of the powder in 1.56 ml nuclease free water. Aliquot into smaller quantities. Store in -20C.
- RevitaCell is a good alternative, either can be used

ReLeSR **STEMCELL Technologies Inc. Catalog #**05872 Room temperature

TrypLE Select (1X) no phenyl red **Thermo Fisher Scientific Catalog #**12563-011 Room temperature

Matrigel Matrix hESC qualified **Corning Catalog #**354277 20 °C

DMEM/F12 (1:1) **Thermofisher Catalog #**11320033 4 °C

CryoStor CS10-100ml **STEMCELL Technologies Inc. Catalog #**07930 4 °C

DBPS -/- (without calcium / magnesium) **Thermo Fisher Scientific Catalog #**14190144 Room temperature

Antibiotic/Antimycotic (100X) **Thermo Fisher Scientific Catalog #**15240062 -20 °C

Thawing iPSCs

9m

1 Quickly thaw the frozen vial of iPSCs in a water bath at **37°C**. Gently agitate the vial in the water bath until the cells are just thawed (usually takes about 1-2 minutes).

> Note
>
> Swirl the tube as the ice melts until only a tiny amount of ice is in the vial, and then remove it from the water bath.

2 Using a **10 mL serological pipette**, add **9 mL** of media into a **15 mL conical tube**.

2m

3 Using a **5 mL serological pipette**, transfer the contents of the thawed cryovial into the **15 mL conical tube** containing the media.

4 Spin down the contents of the conical tube at **200 x g** for **5 minutes**. While the cells are centrifuging, add **2 mL of mTeSR media+ ROCKi** to **one well of a 6-well plate**

5 After centrifugation, carefully aspirate the supernatant without disturbing the cell pellet.

6 Resuspend the cell pellet in **1 mL of mTeSR media** containing ROCKi (Y-27632):

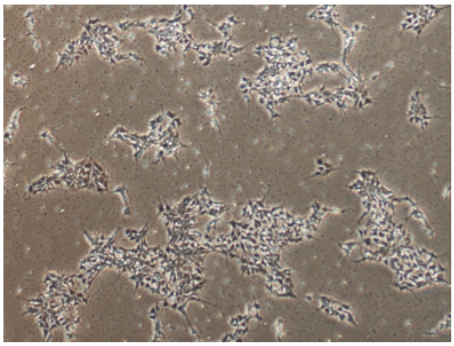
- **ROCKi:** Use Y-27632 (STEMCELL Technologies Inc., Catalog #72304) at a final concentration of **1 µL per 1 mL of media**

7 Transfer the resuspended cells to the well containing **2 mL of mTeSR media** in the **6-well plate**.

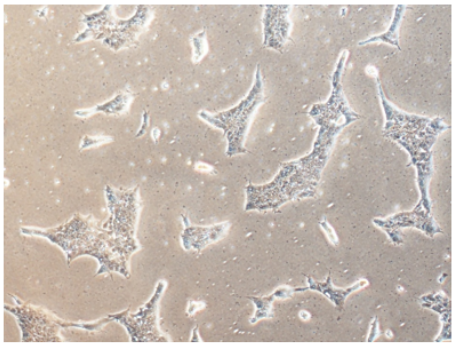
Perform the transfer with **minimal trituration** to avoid excessive cell clumping or damage

Cells immediately after thawing; high density due to some cell death.

- 24 hours post thaw before removing rocki



- 26 hours post thaw (2 hours after removing rocki)

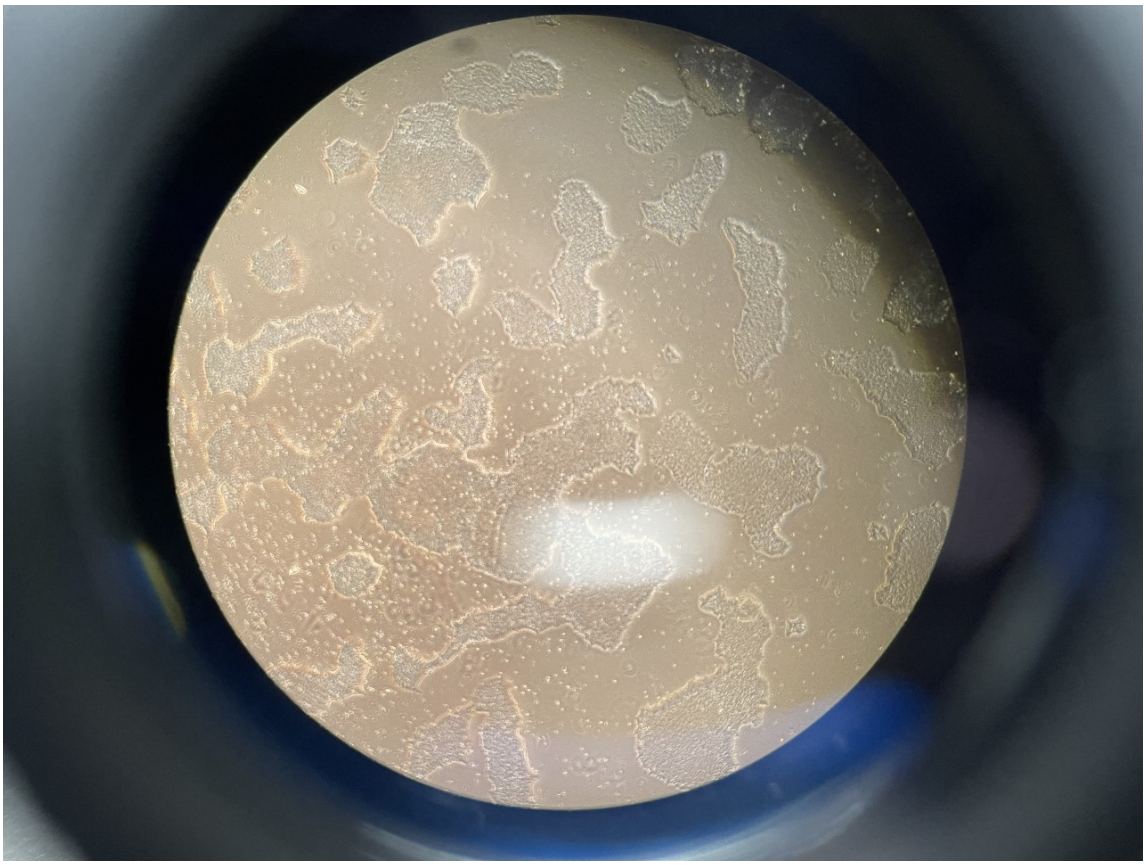


Passaging IPSCs

6m

8 Ideal confluency for passaging iPSCs is approximately **60%**, which corresponds to about **1 million cells per well** in a **6-well plate**.

Do not allow cells to reach 90% confluence. Pass cells more frequently if needed, such as on Monday and Friday. Some cells may be cultured at different concentrations to ensure availability on various days. Passage sooner if separate colonies begin to merge.



Example image of ideal confluency for passaging at ~ 60%. This would be about 1 million cells in a 6-well plate.

9 Aspirate the media from each well or flask.

10 Rinse each well or flask with **DPBS -/-** to remove residual media and non-adherent cells:
- **6-well plate:** Add **1 mL of DPBS -/-** per well.
- **T-25 flask:** Add **3 mL of DPBS -/-**.

11 Aspirate the DPBS -/- from each well or flask.

12 Add **1 mL of ReLeSR** to each well of the **6-well plate** or **3 mL of ReLeSR** to the **T-25 flask**. Start your timer and allow ReLeSR to act on the cells for **1 minute**.

Note

*ReLeSR is an enzyme-free reagent for the dissociation of cells as aggregates **without** manual selection of differentiated areas or scraping to remove cell aggregates*

13 Fully aspirate ReLeSR and leave the plate in the incubator for 00:05:00 5m

Note

If cells were recently treated in ROCKi, leave cells for 5-10 more minutes). After the incubation period, you may observe gap formations in the colonies.

14 Remove the plate/flask from the incubator.

Use a **5 mL serological pipette** to add **1 mL of mTeSR plus media** to each well of the **6-well plate** or **3 mL of mTeSR plus media** to the **T-25 flask**.

Note: This step inactivates the ReLeSR

15 Use the same serological pipette to rinse the well **once,** if needed, to collect the aggregates. Avoid being aggressive to prevent breaking down the clusters.

Do not use a P-1000 tip for aspiration, as its fine bore can reduce aggregate size.

16 Transfer the cell suspension from the wells/flask to a **15 mL conical tube**.

Gently triturate the cell suspension once with a P-1000 pipette, taking care to avoid bubbles. **One trituration only!**

17 **Transfer the desired amount of cells** (typically a 1:10 split) into a new **Matrigel-coated well**. **Ensure that the Matrigel is removed from the new well** and that the well is pre-warmed with **2 ml of mTeSR media** before adding the cells.

Plate the resuspended cells at a 1:10 dilution in each well of a **6-well plate**.

- For example, if you are plating into a 6-well plate and have a total of **1 mL of cell suspension**, add **100 µL of the cell suspension per well**.

Use the distributed technique: Aspirate **100 µL with a P-1000 pipette**, go to a corner of the plate, and put the tip just under the surface. Watch the liquid level and dispense about **⅕ of the**

pipette tip's volume, count to 3, then keep the tip submerged, move to the other corners, and finally to the center of the plate, repeating the process.

Lid the plate and immediately gently tap on two sides in a **left/right motion** and a **front/back motion** to distribute the cells (avoid circular motion as it leads to poor distribution).

What the transferred cells look like immediately after plating (this is low density, see first image for a higher density version and add more cells)

Performing a Media Change for iPSCs

18 Ideally, you want to bring media to RT before use, however if you are under time constraints, using cold media is okay unless you are dealing with a problematic cell line.

Do NOT warm media in a water bath at 37 C. To bring to RT, simply take out of the fridge for 30min - 1 hr.

Daily Media Change: Aspirate old media from wells or flasks and add 2mL of fresh mTeSR per well.

Freezing iPSCs

7m

19 Aspirate the media and rinse with 1 ml DPBS-/- per well. Add 1mL of ReLeSR to each well. Aspirate ReLeSR after 1-minute incubation

20 Add 1mL of ReLeSR to each well. Aspirate ReLeSR after 1-minute incubation

21 Allow the vessel to sit for 5-7 minutes in the incubator. Checking every minute to ensure gaps are forming in the clusters.

22 Aspirate 1mL of Cryostor CS10 (keep Cryostor on ice) for each well using a 5mL serological pipette.

23 Dispense 1mL of Cryostor CS10 into each well. Each well is rinsed 2-3 times, then the resuspension is transferred to a vial (keep Cryostor on ice) using the 5mL serological bore to scrape/press on the surface of each well