

iPSC Maintenance – Thawing, Passaging, and Cryopreservation

Revision Number: 05

Effective Date: 12March2025

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1. PURPOSE

To describe the procedure for thawing, passaging, and cryopreserving induced pluripotent stem cell (iPSC) lines.

2. SUPPLIES

GFR or hESC Matrigel coated flasks (Refer to GFR Matrigel SOP)

Feeding Medium Options:

 mTeSR Plus (100-0276, StemCell Technologies)

 mTeSR1 (85850, StemCell Technologies)

 Essential 8 Complete Medium (E8) (A1517001, Life Technologies)

ROCKi, 10mM stock (Ri) Y-27632 (S1049, Selleck Chemicals)

DPBS (-/-) (14190250, Thermo Fisher)

Accutase (07920, Stem Cell Technologies)

ReLeSR (05872, Stem Cell Technologies)

Trypan Blue

Cryovials

Controlled rate freezing container (such as Mr. Frosty or Freeze Cube)

CryoStor (07930, Stem Cell Technologies)

3. PROCEDURE

Note: All work must be performed in a sterile environment such as a biosafety cabinet.

Note: mTeSR Plus or E8 may be substituted in place of mTeSR1 for some cell lines.

hESC Matrigel may be substituted in place of GFR Matrigel for some cell lines.

Table 1.

Vessel Format	Volume of Feeding Media	Surface area (cm ² /well)
12-well plate	1mL/well (12mL/plate)	3.8
6-well plate	2mL/well (12mL/plate)	9.6
10cm dish	10mL	56.7
T25 flask	4mL	25
T75 flask	12mL	75

3.1. Thawing iPSCs

3.1.1. Prepare GFR Matrigel coated flasks by placing them at 37°C for at least one hour.

3.1.2. Label flasks with cell line, passage number (increase passage number from vial label), date, and operator initials.

- 3.1.3. Remove vial of frozen cells from the liquid nitrogen tank and place on dry ice.
- 3.1.4. Prepare an appropriate volume of mTeSR1+Ri (1:1000).
- 3.1.5. Thaw vial in a 37°C water bath until only a small ice pellet can be seen. Quickly transfer the cell suspension to a 15mL conical.
 - 3.1.5.1. **Optional:** Rinse the vial with 1mL mTeSR1+Ri and add to the conical containing cells.
- 3.1.6. Add 8mL mTeSR1+Ri to the cell suspension. Invert the conical 2-3 times to mix.
- 3.1.7. Centrifuge for 3-4 minutes at 200-300xg (RCF).
- 3.1.8. Carefully aspirate the supernatant and resuspend in an appropriate volume mTeSR1+Ri according to Table 1.
- 3.1.9. Aspirate excess Matrigel from flasks and add the cell suspension to the pre-coated flasks. Add dropwise across wells in multi-well plates for an even distribution.
- 3.1.10. Place in a 37°C incubator, gently shaking flasks left/right and up/down to ensure even seeding.
- 3.1.11. Replace media daily with mTeSR1 (without Ri) until flasks reach 70-80% confluence.

3.2. Single-Cell Passaging iPSCs using Accutase

- 3.2.1. Prepare GFR Matrigel coated flasks by placing them at 37°C for at least one hour.
- 3.2.2. Label flasks with cell line, passage number (increase passage number by one), date, and operator initials.
- 3.2.3. Remove 70-80% confluent flasks from incubator and aspirate spent media.
- 3.2.4. Wash flask with an appropriate volume of DPBS (-/-) based on flask size.
- 3.2.5. Aspirate DPBS and add an appropriate volume of Accutase.
- 3.2.6. Incubate at 37°C for 5 minutes. Check cells for detachment by gently tapping the side of the flask. If < 90% detachment is observed, incubate for up to an additional 5 minutes.
- 3.2.7. Pipette the cell suspension up and down 3-5 times to singularize before transferring to an appropriately sized conical. Add an equal volume DPBS or mTeSR1 to the conical.
- 3.2.8. Centrifuge at 200-300xg (RCF) for 3-4 minutes.
- 3.2.9. Aspirate supernatant and resuspend in an appropriate volume mTeSR1+Ri (1:1000).
- 3.2.10. If splitting by ratio:
 - 3.2.10.1. Aspirate excess Matrigel from flasks and pre-fill with an appropriate volume mTeSR1+Ri according to Table 1 above.
 - 3.2.10.2. Add cell suspension volume based on the chosen ratio using the following calculation:
$$V_{cell\ suspension} \div [Ratio\ (i.e.\ 1:4 = 4)] = V_{cells/flask}$$
- 3.2.11. If splitting by cell count:
 - 3.2.11.1. In a 1.5mL microcentrifuge tube, mix 10µL trypan blue with 10µL cell suspension.

- 3.2.11.2.** Pipette 10µL from the stained cell suspension into the chamber of a Countess cell counting slide.
- 3.2.11.3.** Insert the slide into the Countess and wait for the instrument to adjust the light intensity and choose the most in focus image.
- 3.2.11.4.** Click on the “Capture” button and wait for cells to be counted.
- 3.2.11.5.** Determine the volume of cell suspension to seed using the following calculation:
- $$Total\ Cells\ per\ flask \div C_{live\ cells/mL} = V_{cells/flask}(mL)$$
- Note:** If the volume to seed is less than 200µL, dilute the cell suspension and recalculate the volume to reduce the variability between flasks.
- 3.2.12.** Aspirate excess Matrigel from flasks and pre-fill with an appropriate volume mTeSR1+Ri according to Table 1 minus the volume calculated above.
- 3.2.13.** Add the volume of cell suspension calculated above.
- 3.2.14.** Place in a 37°C incubator, shaking flasks left/right and up/down to ensure even seeding.
- 3.2.15.** Replace media daily with mTeSR1 (without Ri) until flasks reach 70-80% confluence.

3.3. Clump Passaging iPSCs using ReLeSR

- 3.3.1.** Prepare GFR Matrigel coated flasks by placing them at 37°C for at least one hour.
- 3.3.2.** Label flasks with cell line, passage number (increase passage number by one), date, and operator initials.
- 3.3.3.** Remove 70-80% confluent flasks from incubator and aspirate spent media.
- 3.3.4.** Wash flask with an appropriate volume of DPBS (-/-) based on flask size.
- 3.3.5.** Aspirate DPBS and add an appropriate volume of ReLeSR to completely cover the bottom of the flask.
- 3.3.6.** Aspirate ReLeSR within 1 minute to leave a thin layer behind.
- 3.3.7.** Incubate flasks according to the table below. Check cells for detachment by observing under the microscope. Colonies should appear to be detaching, with cells starting to ball up. Differentiated cells should not appear detached.

Table 2.

mTeSR Cultures	37°C for 5-7 minutes
E8 Cultures	15-25°C (Room Temp) for 7-9 minutes
Troubleshooting:	Aggregate size too large Colonies remain attached • Increase incubation time by 1-2 minutes • For E8 cultures, increase incubation temperature to 37°C Aggregate size too small Differentiated cells are also detached • Decrease incubation time by 1-2 minutes • For mTeSR cultures, decrease incubation temperature to 15-25°C (room temp)

3.3.8. Add an appropriate volume of mTeSR1 to the flask and firmly tap the side of the plate for 30-60 seconds to fully detach colonies. Visually assess colony size, and manually break up colonies using a serological pipette if necessary.

3.3.9. Aspirate excess Matrigel from flasks and pre-fill with an appropriate volume mTeSR1 according to Table 1 above.

3.3.10. Add cell suspension volume based on the chosen ratio using the following calculation:

$$V_{cell\ suspension} \div [Ratio\ (i.e.\ 1:4 = 4)] = V_{cells/flask}$$

3.3.11. Place in a 37°C incubator, shaking flasks left/right and up/down to ensure even seeding.

3.3.12. Replace media daily with mTeSR1 until flasks reach 70-80% confluence.

3.4. Freezing iPSCs

3.4.1. Prepare cryovials by labeling with cell line, passage number, number of cells per vial, date, and operator initials.

3.4.2. Remove 70-80% confluent flasks from incubator and aspirate spent media.

3.4.3. Wash flask with an appropriate volume of DPBS (-/-) based on flask size.

3.4.4. Aspirate DPBS and add an appropriate volume of Accutase.

3.4.5. Incubate at 37°C for 5 minutes. Check cells for detachment by gently tapping the side of the flask. If < 90% detachment is observed, incubate for up to an additional 5 minutes.

3.4.6. Pipette the cell suspension up and down 3-5 times to singularize before transferring to an appropriately sized conical. Add an equal volume DPBS or mTeSR1 to the conical.

3.4.7. In a 1.5mL microcentrifuge tube, mix 10µL trypan blue with 10µL cell suspension.

3.4.8. Pipette 10µL from the stained cell suspension into the chamber of a Countess cell counting slide.

3.4.9. Insert the slide into the Countess and wait for the instrument to adjust the light intensity and choose the most in focus image.

3.4.10. Click on the "Capture" button and wait for cells to be counted.

3.4.11. Centrifuge cell suspension at 200-300xg (RCF) for 3-4 minutes.

3.4.12. Aspirate supernatant and resuspend in the volume of CryoStor calculated below:

$$Total\ Cells\ (V_{Total} \times C_{live\ cells/ml}) \div Cells/vial = V_{CryoStor}(mL)$$

3.4.13. Dispense 1mL cell suspension into pre-labeled cryovials and place in a controlled rate freezing container. If there is a large number of vials to be filled, place cell suspension on ice while dispensing.

3.4.14. Store the controlled rate freezing container at -80°C overnight and transfer to the liquid nitrogen tank the following day. Keep vials on dry ice while transporting to the liquid nitrogen and transfer vials as quickly as possible without warming.