GLADSTONE INSTITUTES | STEM CELL CORE
GLADSTONE CORE SERVICES

iPSC Maintenance – Thawing, Passaging, and Cryopreservation	SOP Number:
	Effective Date: 19 February 2019
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Table of Contents:

Version Number	Summary of Changes	Revised by/Date
01	Original Document	Wendy Runyon 9Oct17
02	Updated incorrect formula in cryo section, decreased T25 feeding volume, added instructions for clump passaging	Wendy Runyon 5Apr18
03	Added StemFit+50µg FGF as an option for feeding medium, added vendor and catalog information to supplies	Wendy Runyon 12Sep18
04	Streamlined version picking one culture medium, ECM, and indicate when single cell passing is required. (For ENCODE.)	Po-Lin So 8Feb19
05	Added option to use E8 back into SOP. (For ENCODE.)	Wendy Runyon 19Feb19

1. PURPOSE

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

2. SUPPLIES

hESC Matrigel coated flasks/plates (hESC Matrigel stock: 354277, Corning)
mTeSR (85850, Stem Cell Technologies)
or 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) or ReLeSR (05872, Stem Cell Technologies)
Trypan Blue
Cryovials
Mr. Frosty freezing container
CryoStor (07930, Stem Cell Technologies)

3. SCOPE

This procedure applies to iPSC lines cultured in mTeSR medium and single-cell passaged using Accutase and clump passaged using ReLeSR.

Note: E8 medium may be substituted for mTeSR in some cell lines.

4. PROCEDURE

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

Table 1.

Vessel Format	Volume of 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

4.1. Thawing iPSCs

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

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

4.1.3. Remove vial of frozen cells from the liquid nitrogen tank and place on dry ice.

4.1.4. Prepare a 15mL conical with 8mL mTeSR+Ri (1:1000).

4.1.5. Thaw vial in a 37°C water bath until only a small ice pellet can be seen. Quickly transfer the cell suspension to the prepared 15mL conical.

4.1.6. Rinse the vial with 1mL mTeSR+Ri and add to the conical containing cells. Invert the conical 2-3 times to mix.

4.1.7. Centrifuge for 3-4 minutes at 800 rpm.

4.1.8. Carefully aspirate the supernatant and resuspend in an appropriate volume mTeSR+Ri according to Table 1.

4.1.9. Aspirate excess Matrigel from flasks and add the cell suspension to the pre-filled flasks. Add dropwise to multi-well plates for an even distribution.

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

4.1.11. Replace media daily with mTeSR (without Ri) until flasks reach 70-80% confluence.

4.2. Single-Cell Passaging iPSCs using Accutase, for plating known numbers of iPSCs (i.e. for cell lineage differentiations, transfection, transduction). For general passaging, use ReLeSR (see 4.3) for clump passaging.

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

4.2.2. Label flasks with cell line, passage number (increase passage number by one), date, and operator initials.

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

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

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

4.2.6. 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.

4.2.7. Pipette the cell suspension up and down before transferring to an appropriately sized conical. Add an equal volume DPBS to the conical to stop the dissociation.

4.2.8. Centrifuge at 800 rpm for 3 minutes.

4.2.9. Aspirate supernatant and resuspend in an appropriate volume mTeSR+Ri (1:1000).

4.2.10. Count cells using the Countess cell counter:

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

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

4.2.10.3. Insert the slide into the Countess and press the "zoom in" button. Focus the cells using the knob on the right of the machine.

4.2.10.4. Count cells, obtaining the number of live cells. Click on the "details" button to make sure < 5 cells are excluded. Excluded cells are marked by black rings. If there are many cells excluded, further dissociate the original cell suspension by pipetting up and down and prepare a new sample for re-counting.

4.2.10.5. 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.

4.2.10.6. Aspirate excess Matrigel from flasks and pre-fill with an appropriate volume mTeSR+Ri according to Table 1 minus the volume calculated above.

4.2.10.7. Add the volume of cell suspension calculated above.

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

4.2.12. Replace media daily with mTeSR (without Ri) until flasks reach 70-80% confluence.

4.3. Clump Passaging iPSCs using ReLeSR

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

4.3.2. Label flasks with cell line, passage number (increase passage number by one), date, and operator initials.

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

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

4.3.5. Aspirate DPBS and add an appropriate volume of ReLeSR to completely cover the bottom of the flask.

4.3.6. Aspirate ReLeSR within 1 minute to leave a thin layer behind.

4.3.7. 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 temperature to 37°C Aggregate size too small Differentiated cells are also detached • Decrease incubation time by 1-2 minutes • Decrease incubation temperature to 15-25°C (room temp)

4.3.8. Add an appropriate volume of mTeSR+Ri 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.

4.3.9. Aspirate excess Matrigel from flasks and pre-fill with an appropriate volume mTeSR+Ri according to Table 1 above.

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

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

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

4.3.12. Replace media daily with mTeSR until flasks reach 70-80% confluence.

4.4. Freezing iPSCs

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

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

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

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

4.4.5. 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.

4.4.6. Pipette the cell suspension up and down before transferring to an appropriately sized conical. Add an equal volume DPBS to the conical to stop the dissociation.

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

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

4.4.9. Insert the slide into the Countess and press the "zoom in" button. Focus the cells using the knob on the right of the machine.

4.4.10. Count cells, obtaining the number of live cells. Click on the "details" button to make sure less than 5 cells are excluded. Excluded cells are marked by black rings. If there are many cells excluded, further dissociate the original cell suspension by pipetting up and down and prepare a new sample for re-counting.

4.4.11. Centrifuge cell suspension at 800 rpm for 3 minutes.

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

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

4.4.13. Dispense 1mL cell suspension into pre-labeled cryovials and place in a Mr. Frosty. If there is a large number of vials to be filled, place cell suspension on ice while dispensing.

4.4.14. Store the Mr. Frosty 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.