Guidelines for Handling RUCDR iPSC Lines

Note to the Recipient of iPSC Lines Distributed by RUCDR Infinite Biologics:

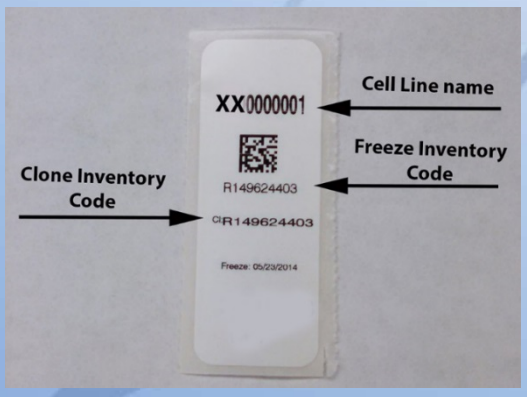
The protocol in use at RUCDR to thaw iPSC is provided along with the protocols used for routine culture and freezing.

It is very important to transfer the frozen ampoules to liquid nitrogen as soon as they are received. Failure to do so can result in serious loss of cell viability. We do not recommend storing the ampoules at -80C.

Typically we recommend thawing cells in a 6 well plate at a density of either $1x10^6$ or $2 x10^6$ cells per well. The recommended thawing density for each line can be found on the cell line Certificate of Analysis (COA), as well as the manifest you received with your shipment. We thaw and maintain all of our iPSC lines at 4% O_2 and find that this improves their survival after thawing and decreases spontaneous differentiation. If 4% O_2 is not possible, the cells will grow in normal O_2, but might need extra care such as thawing at a higher density or passaging sooner.

All of these lines are frozen as a single cell suspension in mFreSR after digestion with Accutase. The first day or so after thawing, expect the cells to look a bit odd. They will not really look much like colonies and might be a little more angular than you expect. By day 3 or so, they should be dense enough to start looking more like the colonies you expect, although at this point they will likely be interconnected colonies. Usually we passage by day 4 or 5. If there is little or no differentiation, we would passage with dispase as if they were normal colonies. If there is more differentiation than can be conveniently scraped away, we would select good colonies for manual picking and passaging. In most cases there is very little differentiation and the whole well can be passaged.

Below is an example of a label you will find on all RUCDR frozen cell line ampoules. The cell line name is a unique identifier for each subject. The clone inventory code corresponds to the different isolates we made from a single subject. Although you might want to come up with a different name or code, you should link your code to each clone inventory code as you will need to provide this clone number when you contact us for information. Since each isolate is frozen in 2 independent batches to ensure the security of the cell line, there are 2 freeze inventory codes for each clone. This number is less important for you to track, but you will need it to determine the initial plating density of the cell line after thaw.



Please don't hesitate to contact RUCDR Infinite Biologics (see www.rucdr.org for contact information) with any questions.

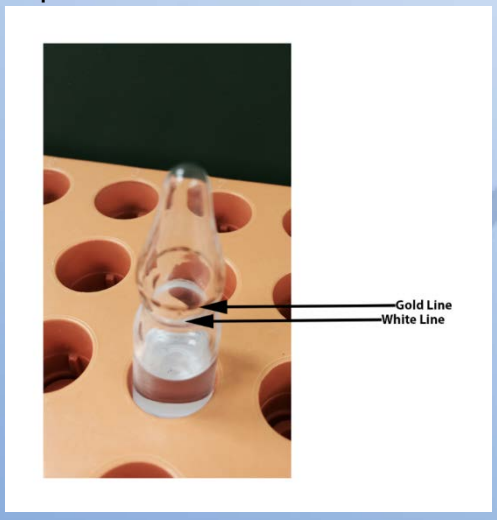
Thawing cryopreserved iPSC

Principle:
This protocol is designed to thaw cryopreserved iPSC for expansion.

Definition:
1. iPSC: Induced Pluripotent Stem Cells
2. MG: matrigel
3. DMEM/F12: Dulbecco's Modified Eagle's Medium (DMEM) and Ham's F12 with GlutaMax
4. mTeSR: Defined feeder-free maintenance medium for hESCs and hiPSC

Materials and Reagents:
1. Gloves
2. Lab Coat
3. Safety Goggles
4. Paper towels
5. Squeeze bottle with 70% ethanol solution
6. Matrigel (BD Biosciences, 354277)
7. 5mM Y27632 (Tocris, 1254)

Procedure for thawing iPS cells:
1. Prepare Matrigel (MG) coated plates according to manufacturer's instructions.
   1.1. For thawing iPSC: 1 or 2 MG well (from a 6 well plate) per cell line is needed.
   1.2. Incubate the matrigel plate at 37°C for at least 1 hour before use.
2. Remove the frozen iPSC ampule from Liquid N_2 and place it in a LN_2 vapor phase for 45 minutes.
3. Thaw samples in 37°C water bath for approximately 1 minute.
4. With 70% ethanol or an alcohol prep-pad, disinfect the neck of the glass ampule.
5. Place thawed ampule in a rack that fits 2ml cryovials (such as Wheaton 985810). Place an ampule snapper (Wheaton, 177105) on the thawed ampule and make sure the ampule snapper covers the gold line on the neck of the ampule. Using firm, steady pressure push the ampule snapper away from you and snap the neck. The break will occur at the faint white line below the gold line.



6. Transfer cellular contents to a 15ml tube containing 9mL of mTeSR.

7. Centrifuge the 15ml tube in a table top centrifuge at 1000 rpm for 5 minutes.
8. Remove liquid from the Matrigel plate and add 1mL of mTeSR.
9. Resuspend the cellular contents in mTeSR and plate in a MG coated well at the density listed on the Certificate of Assurance (COA).
10. Add 2uL of Y27632 to the well (5uM final concentration).
11. Gently shake the new plate from left to right and up to down to ensure cells are evenly distributed throughout the well.
12. Incubate in 5% CO_2/4% O_2 incubator.Hypoxic culture conditions provide the optimal environment for iPSC growth and pluripotency.
13. Exchange mTeSR every week day and once on the weekend (use 4mL of mTeSR if you skip one weekend day). Note – change the medium the day after thaw even if there are very few attached cells. Cells that do not attach within the first 24 hours will not survive.
14. Passage when colonies become ~70% confluent (approximately 3-5 days).

Dispase Passaging of iPSC

Principle:

This protocol is designed to expand iPSC by passaging colony pieces.

Definitions:

1. iPSC: Induced Pluripotent Stem Cells.
2. MG: matrigel
3. DMEM/F12: Dulbecco's Modified Eagle's Medium (DMEM) and Ham's F12 with GlutaMax
4. mTeSR: Defined feeder-free maintenance medium for hESCs and hiPSC

Materials and Reagents:

1. Gloves
2. Lab Coat
3. Safety Goggles
4. Paper towels
5. Squeeze bottle with 70% ethanol solution
6. Matrigel (BD Biosciences, 354277)
7. DMEM/F12 (Gibco, 10565-018, stored at 4°C)
8. Dispase (50 units/mL) (BD Biosciences, 354235)
9. Dispase Working solution
   - 9.1. Thaw 50 unit/mL stock dispase at room temperature
   - 9.2. Dilute 1:50 in DMEM/F12 This working solution is used for one day and works best if kept warm
   - 9.3. The 50 unit/mL stock dispase vial should be frozen and thawed a maximum of 5 times

Procedure for routine culture:

1. Prepare Matrigel coated plates according to manufacturer's instructions.
   - 1.1. For iPSC expansion: 6 to 24 MG wells (1-4 x 6 well plates) per cell line is needed.
   - 1.2. Incubate the matrigel plate at 37°C for at least 1 hour before use.
2. Remove spent medium from iPSC well.
3. Add 1ml of Dispase working solution.
4. Incubate at 37°C for 5 minutes.
5. Remove dispase working solution.
6. Rinse 2x2ml DMEM/F12.
7. Add 2ml/well of mTeSR.
8. Scrape cells with cell scraper.
9. Transfer detached cells to 15ml tube.
10. Use 1000ul micropipette to gently break the colonies into small pieces (usually only 1 or 2 triturations).
11. Plate desired ratio of iPSC colony pieces (usually 1:8-1:12) to a new matrigel coated plate.
12. Incubate at 37°C at 4% O_2/5% CO_2.
13. Exchange mTeSR every week day and once on the weekend (use 4mL of mTeSR if you skip one weekend day).
14. After 7 days the colonies should be ready to passage again.

Version 2. Last updated on 2015-01-22 by Sheldon, Michael.

Cryopreservation of iPSC

Principle:

This protocol is designed to prepare frozen stocks of iPSC lines for storage in liquid N_2.

Definitions:

1. iPSC: Induced Pluripotent Stem Cells
2. DMEM/F12: Dulbecco's Modified Eagle's medium (DMEM) and Ham's F12 with GlutaMax
3. mTeSR : Defined feeder-free maintenance medium for hESCs and hiPSC
4. mFreSR: defined serum -free cryopreservation medium for hESCs and hiPSC
5. LN_2: Liquid Nitrogen
6. DPBS : Dulbecco's phosphate buffered saline

Materials and Reagents:

1. Gloves
2. Lab Coat
3. Safety Goggles
4. Paper towels
5. Squeeze bottle with 70% ethanol solution
6. DMEM/F12 (Gibco, 10565-018, stored at 4°C)
7. Accutase (Stem Cell Technologies, 07920)
8. DPBS (Fisher Scientific, SH3002802)

Procedure for freezing iPSC:

1. Examine all wells of the iPSC line that needs to be frozen under a microscope to locate the differentiated cells.
2. Manually remove differentiated cells with a sterile drawn-out glass pipette.
3. Aspirate media from wells.
4. Wash wells 2x2ml of PBS.
5. Add 1ml of Accutase to each well.
6. Incubate at 37^{o}C for 5 minutes.
7. Ensure that the cells are loosened from the plate.
8. Using 1000ul pipet tip, take the cell suspension and rinse wells twice before collecting cells.
9. Transfer cell suspension to 50ml tube.
10. Add an equal amount of PBS to the 50ml tube.
11. Remove 10ul of cell suspension for counting on a Hemacytometer.
12. Centrifuge 50ml tube at 1000rpm for 5 minutes.
13. Calculate volume of mFreSR necessary to freeze cells at 2e6 cells/vial.
    Volume of mFreSR (ml) = total cells counted/2e6
14. Aspirate supernatant and resuspend the pellet in the amount of mFreSR calculated in step 13.
15. Dispense 1ml of mFreSR/cell mixture into ampules and place on ice.
16. Transfer to LN_2 for long term storage.

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