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EDDU Protocols

iPSC Culture

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1 Introduction

1.1 Objectives

This protocol describes how to:

- Culture human induced pluripotent stem cells (iPSCs) in mTeSR1 or Essential 8 media
- Freeze cryovials of iPSCs

1.2 Protocol overview

Human iPSCs generated from adult somatic cells can be cultured in mTeSR1 or Essential 8 media to maintain pluripotency. iPSC cultures may be guided to populations of lineage-specific progenitor cells (e.g. neural progenitor cells) which can be differentiated into enriched populations of functionally mature cells (e.g. neurons).

Culturing of human iPSCs in mTeSR1 or Essential 8 media may require different techniques than culturing in other media formulations. The procedures described in this protocol are general and may require optimization for use with different cell lines.

1.3 Technical and safety considerations

The following information should be read before starting:

- iPSCs must be handled within a Class II biosafety laminar flow hood to protect the worker from possible adventitious agents. McGill University Environmental Health and Safety (EHS) office regulations must be followed.
- iPSCs may be recovered from a frozen stock or reprogrammed from peripheral blood mononuclear cells (PBMCs).
- iPSC cultures consist of cell aggregates. Use a 5-mL glass pipet during resuspension and transfer of iPSC cultures to minimize breakage of cell aggregates.
- The choice between mTeSR1 and Essential 8 media depends on the cell line. If the type of media is unknown, compare culture in mTeSR1 and Essential 8 media to determine the optimal media.

- Maintain a stable culture environment for cells during incubation:
  - Culture vessels should be placed toward the back of the cell culture incubator shelf to maintain stable temperature and CO_2 levels when the door of the incubator is opened and closed.
  - When dissociating cells, use a different 37°C incubator than the cell culture incubator to minimize opening and closing the door of the cell culture incubator. Note that the dissociation incubator should be sterile but does not require a CO_2 supply.

2 Materials

The quality of materials used in this protocol is critical to its success. The suppliers and catalogue numbers listed in this section allowed for successful culture of iPSCs that expressed pluripotency markers. There is significant lot-to-lot variability in the quality of materials which can negatively impact neuronal cultures. Note that specific lots may no longer be available.

Refer to the product datasheet from the supplier for further details on storage and preparation instructions.

2.1 Labware

Item	Supplier	Catalogue #
Cell scraper	Corning	3010
Conical tube, 15-mL	ThermoFisher	352097
Conical tubes 50-mL	Sarstedt	72.379
Cryo-box	Biocision	CoolCell LX
Culture dish, 10-cm	BD Falcon	354277
Culture dish, 35-mm	Eppendorf	0030701112
Culture dish, 60-mm	Eppendorf	0030701119
Culture flask, T25	ThermoFisher	12-556-009
Culture plate, 6-well	ThermoFisher	087721B
Glass pipet, 5-mL	ThermoFisher	13-678-27E
Low protein-binding filter, 0.2-µm	VWR	28145-501
Parafilm	ThermoFisher	13-374-12
Plastic serological pipet, 10-mL	Sarstedt	86.1254.001
Plastic serological pipet, 5-mL	Sarstedt	86.1253.001
Glass serological pipet, borosilicate disposable	Fisherbrand	13-678-27E

2.2 Culture reagents

Working aliquots of culture reagents that require storage at 4°C can be stored for up to 2 weeks unless otherwise stated.

Item	Supplier	Catalogue #	Stock conc.	Working conc.	Storage temp.
Antibiotic-Antimycotic	Gibco	15240062	100x	1x	Stock: −20°C Working: 4°C
DMEM/F12	Gibco	10565018	1x	1x	4°C
DMSO	Fisher	BP231-1	100%	20%	Room temperature
Essential 8 50x supplement	Gibco	A1517001 (component A1517101)	50x	1x	Stock: −20°C Working: 4°C
Essential 8 basal media	Gibco	A1517001 (component A1516901)	1x	1x	4°C
FBS	Gibco	12484028	1x	1x	Stock: −80°C Working: Room temperature
Gentle Cell Dissociation Reagent	STEMCELL Technologies	07174	1x	1x	Room temperature
Matrigel Matrix hESC-qualified	Corning Millipore	354277	100x	1x	Stock: −80°C Working: 4°C*
mTeSR1 5X supplement	STEMCELL Technologies	05850 (component #05852)	5x	1x	Stock: −20°C Working: 4°C
mTeSR1 basal media	STEMCELL Technologies	05850 (component #05851)	1x	1x	4°C
Y27632 (ROCK inhibitor)	Selleckchem	S1049	10 mM	10 µM	Stock: −80°C Working: 4°C

*Matrigel working solution must be used immediately or stored at –20°C for later use.

2.3 Equipment

Item	Supplier	Catalogue #
Cell culture incubator	ThermoScientific	Steri-Cycle Model 370 Ref#20
Cell culture water bath	FisherScientific	IsoTemp GPD20
Centrifuge	Eppendorf 5702	022626001
Light microscope	Motic	AE2000
LUNA-II Automated cell counter	Logos Biosystems	L40002
Cell counting slide	Logos Biosystems	05181401
EVOS XL Core cell imaging system	ThermoScientific	EVOS XL Core

3 Protocol

3.1 Coating culture vessels

Materials:

- Culture vessels (flasks or plates; if using coverslips, add to plate wells)
- Parafilm
- Matrigel (150-µL aliquot)
- DMEM/F12
- Antibiotic-Antimycotic
- 37°C/5% CO_2 cell culture incubator

Procedure:

1. Prepare Matrigel working solution on ice by adding 1 aliquot of Matrigel and 150 µL of Antibiotic-Antimycotic to 15 mL of cold DMEM/F12. Mix solution well.

    - **IMPORTANT:** Thaw Matrigel on ice. Keep DMEM/F12 with Antibiotic-Antimycotic on ice.
    - Recommended volumes of Matrigel working solution needed depending on the type of culture vessel are listed in Table 1.
    - Use immediately after preparation. Matrigel working solution can be stored at –20°C for up to 1 week. Thaw the Matrigel working solution on ice and use immediately. Do not re-freeze.

2. Immediately apply recommended volume of Matrigel solution to culture vessel (Table 1) and swirl to spread across surfaces.

    - **IMPORTANT:** Ensure culture surfaces are coated completely and evenly. Do not let surfaces dry. Uneven coating or evaporation of coating may affect cell distribution.

3. Incubate culture vessel at 37°C for at least 1 hour.

4. If plating cells on culture vessel immediately, gently tilt culture vessel to one side to allow the excess Matrigel solution to collect in a corner. Remove the Matrigel solution with a serological pipet or by aspiration. Ensure coated surface is not scratched. Immediately add an appropriate volume of culture media to the culture vessel and then plate cells. If not plating cells on culture vessel immediately, do not aspirate Matrigel solution, seal with Parafilm, and store at 4°C for up to 7 days.

    - **IMPORTANT:** If using Matrigel-coated culture vessels that have been stored (i.e. if not using immediately after coating procedure), allow coated culture vessels to come to room temperature for 30 minutes and check quality of coating before plating cells. Uneven coating or evaporation of coating may affect cell distribution.

Table 1. Recommended volumes of Matrigel working solutions based on type of culture vessel.

Culture vessel	Volume of Matrigel solution
T25 flask	3 mL/flask
6-well plate	1 mL/well
35-mm dish	1 mL/dish
60-mm dish	2 mL/dish
100-mm dish	5 mL/dish

3.2 Culturing iPSCs

Materials:

- Frozen cryovial of iPSCs
- Matrigel-coated 10-cm dish
- 5-mL glass pipet
- 15-mL conical tubes
- 50-mL conical tubes
- Cell scraper
- DMEM/F12 containing 1x Antibiotic-Antimycotic
- Media (use mTesR1 or Essential 8):

Media	Preparation instructions	Storage
mTeSR1	• Thaw mTeSR1 5x supplement at 4°C overnight. • Add 100 mL of mTeSR1 5x supplement to 400 mL mTeSR1 basal media to obtain 500 mL of complete media. Mix well. • Complete media is ready for use and does not require filtering. However, the complete media can be filtered using a 0.2-µm low protein-binding filter. • Warm complete media at room temperature (do not warm in at 37°C).	• Once supplement is thawed, use immediately or store at 4°C for up to 2 days. • Complete media is stable when stored at 4°C for up to 2 weeks or at –20°C for up to 6 months. Prepare 40-mL aliquots in 50-mL conical tubes, seal with Parafilm, and store at –20°C. Thaw aliquots overnight at 4°C (do not warm in a 37°C water bath). Do not refreeze aliquots after thawing.
Essential 8	• Thaw Essential 8 50x supplement for about 1 hour at room temperature or overnight at 4°C (do not thaw at 37°C). Mix gently. • Add entire bottle (10 mL) of Essential 8 50x supplement to 500 mL of Essential 8 basal media. • Complete media is ready for use and does not require filtering. • Warm complete media at room temperature (do not warm in at 37°C).	• See instructions for mTeSR1 media.

- Y-27632 (ROCK inhibitor)
- Gentle Cell Dissociation Reagent
- 37°C cell culture water bath
- 37°C/5% CO_2 cell culture incubator
- Centrifuge
- Light microscope
- EVOS XL Core cell imaging system

Procedure:

1. Thaw frozen cryovial of iPSCs in 37°C water bath.

    - Prepare all materials before thawing the cryovial to ensure that the cells can be plated as quickly as possible.
    - Transfer frozen cryovial from liquid nitrogen tank in small liquid nitrogen transfer vessel or dry ice.
    - Do not thaw frozen cryovial in your hand as you will not be able to maintain a constant temperature.
    - Thaw cryovial quickly by gently shaking the cryovial continuously until only a small, frozen cell pellet remains.
    - After cells have thawed, sterilize the outside of the cryovial with 70% ethanol.

2. Transfer cells to a 15-mL conical tube containing 4 mL DMEM/F12 containing 1x Antibiotic-Antimycotic and mix gently. Pellet cells by centrifuging tube at 1200 rpm (200 g) for 3 minutes.

    - Use a 5-mL glass pipet to minimize breakage of cell aggregates.

3. Remove supernatant. Gently resuspend cell pellet in 8 mL of complete media with a glass pipet.

    - Take care to avoid breaking up cell aggregates.

4. Add ROCK inhibitor to cell suspension at a final concentration of 10 µM. Mix the cells by pipetting gently up and down two times. Transfer entire cell suspension to a Matrigel-coated 10-cm dish. Incubate cells at 37°C and do not disturb for 24 hours.

5. After 24 hours, change media daily (do not add ROCK inhibitor). Monitor culture for the presence of undifferentiated cultures that are ready to be passaged. Increase the volume of media in the dish from 8 mL (days 2 and 3) to 10 mL (days 4 and 5) and then to 12 mL (days 6 and 7).

   - Undifferentiated colonies are typically ready to be passaged about 5 to 7 days after thaw/recovery. Examples of iPSC cultures at days 1 to 6 after thaw/recovery are shown in mTeSR1 in Figure 1 and Figure 2 and for Essential 8 in Figure 3 and Figure 4.
   - If regions of differentiation are observed, mark area with fine-point marker on bottom of dish. Remove regions of differentiation by aspiration with a 200-µL tip while frequently turning the dish to avoid exposing the cells to air. Aspirate media, add 8 mL of fresh media, and incubate cells at 37°C. Regions of differentiation should not exceed 10% of the area of the dish. Examples of regions of differentiation in iPSC cultures are shown in Figure 5.

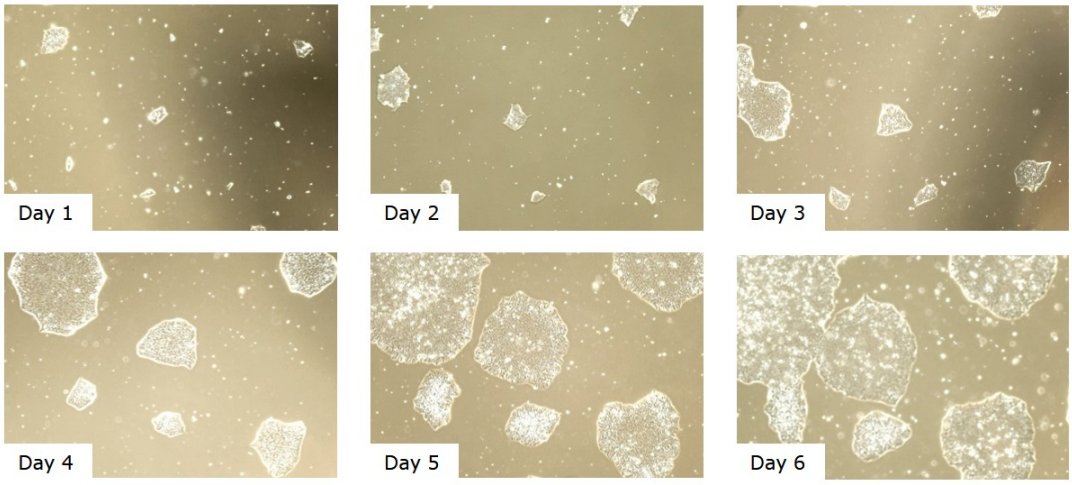

Figure 1. Bright-field phase contrast of NCRM1 cells in mTeSR1 media on days 1 to 6 after thaw/recovery (4X objective).

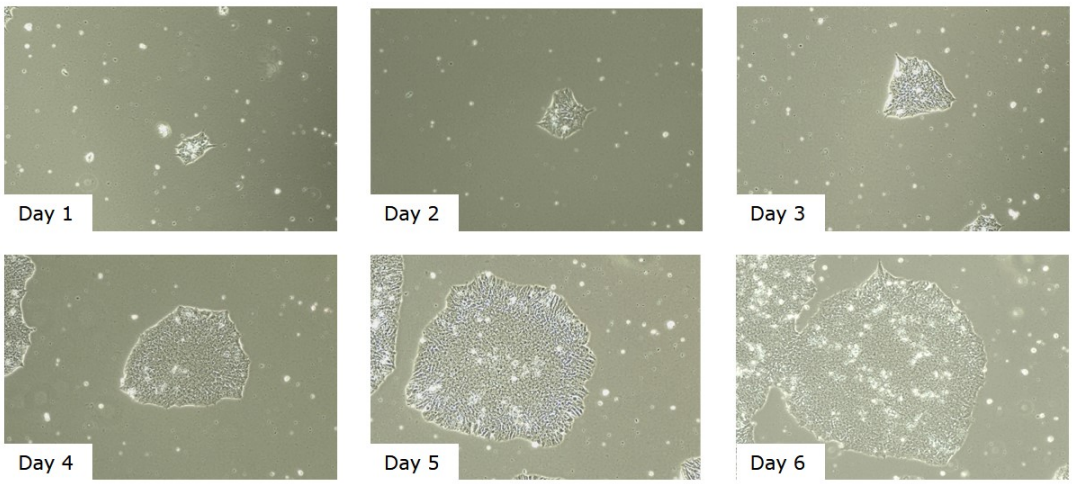

Figure 2. Bright-field phase contrast of NCRM1 cells in mTeSR1 media on days 1 to 6 after thaw/recovery (10X objective).

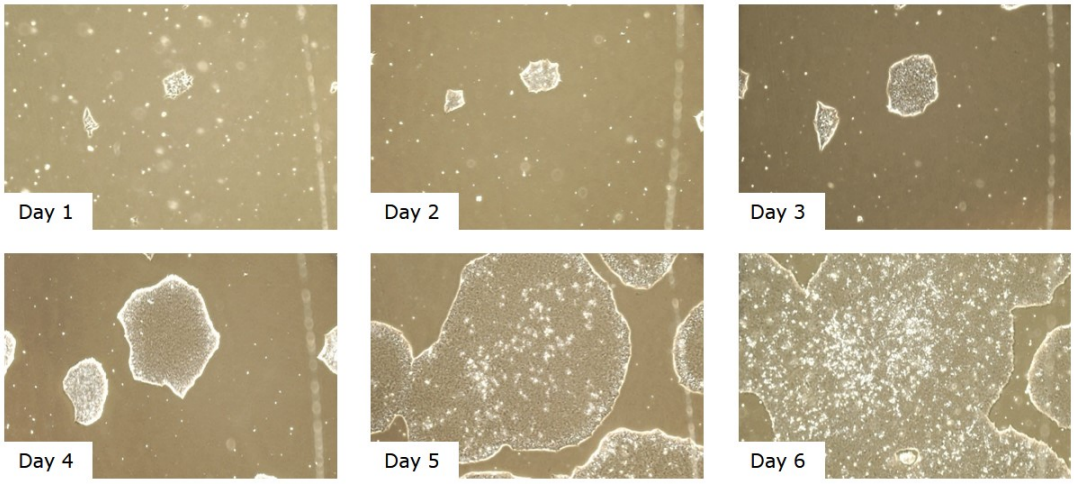

Figure 3. Bright-field phase contrast of NCRM1 cells in Essential 8 media on days 1 to 6 after thaw/recovery (4X objective).

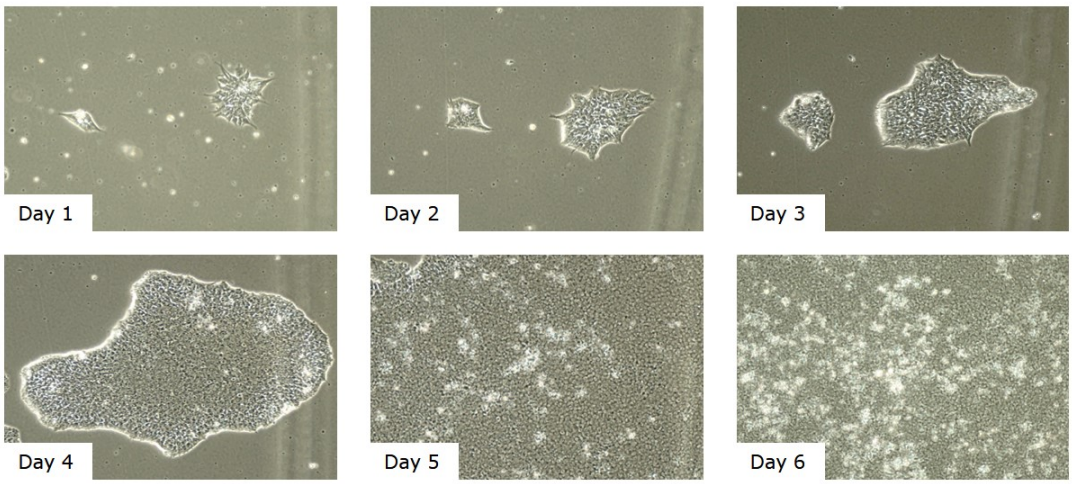

Figure 4. Bright-field phase contrast of NCRM1 cells in Essential 8 media on days 1 to 6 after thaw/recovery (10X objective).

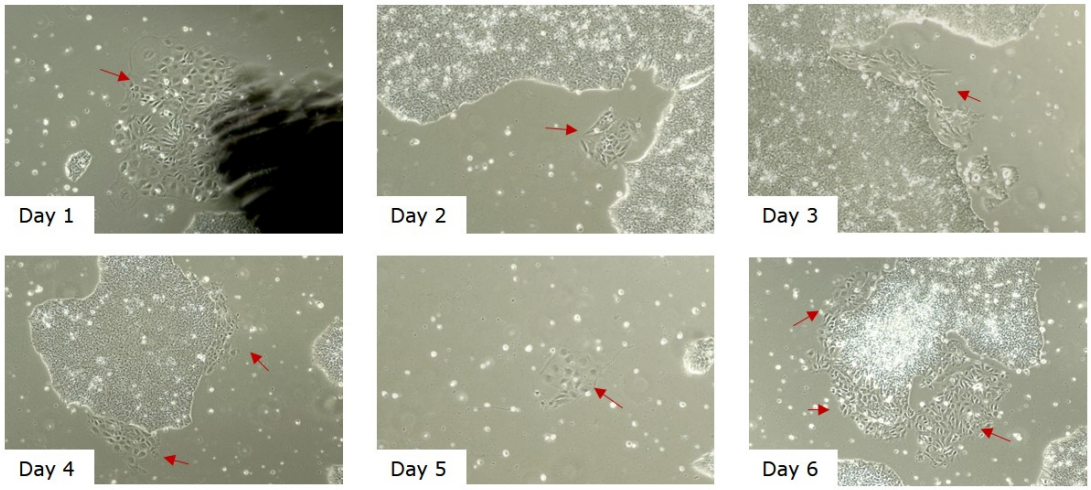

Figure 5. Bright-field phase contrast of NRCM1 cells in mTeSR1 media (10X objective). Red arrows indicate regions of differentiation.

6. When undifferentiated colonies are ready to passage, assess confluency and follow recommendations outlined in Table 2.

Table 2. Recommendations depending on confluency of 10-cm dish when ready to passage.

Confluency	Recommendations
60%–70% (ideal)	Proceed to step 7.
70%–80%	Proceed to step 7.
>80%	Proceed to step 7.
<30%, big colonies	Proceed to step 7, dissociating for 5 minutes at 37°C.
Only a few colonies	Proceed to step 7. Aspirate Gentle Cell Dissociation Reagent, add complete medium (10 mL if passaging to a 10-cm dish or 3 mL if passaging to a 60-mm dish) gently detach the colonies with a cell scraper, and transfer the colonies to new Matrigel-coated 10-cm or 60-mm dish (proceed to step 11).

7. Aspirate media from cells and rinse with 5 mL of DMEM/F12 containing 1x Antibiotic-Antimycotic. Add 5 mL Gentle Cell Dissociation Reagent and incubate at room temperature for 4 to 6 minutes or at 37°C for 3 to 5 minutes.

    - Ensure that regions of differentiation have been removed by aspiration prior to passaging.

8. Aspirate the Gentle Cell Dissociation Reagent and wash cells with 5 mL of DMEM/F12 containing 1x Antibiotic-Antimycotic. Add 5 mL of DMEM/F12 containing 1x Antibiotic-Antimycotic to cells and gently detach the colonies with a cell scraper.
9. Transfer the detached cell aggregates to a 15-mL conical tube. Rinse the dish with an additional 5 mL of DMEM/F12 containing 1x Antibiotic-Antimycotic to collect any remaining cell aggregates and transfer to the tube. Pellet cell aggregates by centrifuging at 1200 rpm (200 g) for 3 minutes.

    - Take care to avoid breaking up cell aggregates.

10. Gently aspirate supernatant. Carefully pipette the cell aggregate mixture up and down 3 to 5 times with a glass pipet in complete media to break up the cell aggregates.

    - Do not create a single-cell suspension.

11. Plate the cell aggregate mixture at the desired density onto a Matrigel-coated 10-cm dish in complete media containing 10 µM ROCK inhibitor. Recommended passage ratios to a 10-cm or 60-mm dish depending on confluency of the 10-cm dish when ready to passage (step 6) are outlined in Table 3. Place the cells in a 37°C incubator. Move the culture vessel in several quick back-and-forth and side-to-side motions to distribute the cell aggregates. Do not disturb the cells for 24 hours.

    - Uneven distribution of aggregates may result in increased differentiation of iPSCs.

Table 3. Recommended passage ratios to a 10-cm or 60-mm dish depending on confluency of 10-cm dish when ready to passage.

Confluency	Passage to 10-cm dish	Passage to 60-mm dish
60%–70% (ideal)	1: 10–12	1:20–30
70%–80%	1:12–15	1:30–40
>80%	1:20–30	1:50–60
<30%, big colonies	1:3–5	1:5–10
Only a few colonies	1:1	1:1–2

12. After 24 hours, change media daily. Monitor colonies for growth and differentiation until next passage. Increase the volume of media in the dish from 8 mL (days 2 and 3) to 10 mL (days 4 and 5) and then to 12 mL (days 6 and 7).

    - If the colonies are at an optimal density, the cultures can be split 1:10 to 1:20 every 7 days.
    - If the colonies are too dense or too sparse, adjust the split ratio accordingly.
    - Freeze iPSCs ideally at a low passage number.

3.3 Freezing iPSCs

Materials:

- iPSC culture ready to passage (10-cm or 60-mm dish)
- Cryovials
- 15-mL tube
- 5-mL glass pipet
- Cell scraper
- Gentle Cell Dissociation Reagent
- DMEM/F12
- Antibiotic-Antimycotic
- 20% DMSO in FBS
- Centrifuge
- 37°C incubator
- Cell counter and cell counting slide

Procedure:

1. Detach colonies from culture vessel using Gentle Cell Dissociation Reagent and pellet cell aggregates as described in Section 3.2, steps 6 to 10. Prior to pelleting cell aggregates, determine cell and/or colony number using cell counter.

    - Freeze 1.5 million to 2 million cells or >50 colonies per cryovial (4–6 cryovials per 10-cm dish; 2–3 cryovials per 60-mm dish).
    - Label cryovials while centrifuging cells. Label should include cell line name, passage number, and date.

2. Gently aspirate supernatant. Gently resuspend the cell pellet in FBS in half the volume needed to result in 1 mL of cell aggregate mixture per cryovial.

    - For example, to freeze 5 cryovials (1 mL x 5 cryovials = 5 mL), resuspend cell pellet in 2.5 mL FBS.
    - Take care to keep the cell pellet intact when aspirating supernatant.
    - Take care to avoid breaking up cell aggregates when dislodging the cell pellet.

3. Add an equal volume of 20% DMSO/FBS to the cell aggregate mixture to obtain a final DMSO concentration of 10%. Mix well.
4. Transfer 1 mL of cell aggregate mixture to each cryovial.
5. Place cryovials in a cryo-box and store at –20°C for 3 to 4 hours. Transfer cryo-box to –80°C and store overnight. Transfer cryovials to liquid nitrogen.

    - Do not store cryovials at –80°C for more than 3 days. iPSCs are sensitive to changes in temperature. Long-term storage at –80°C reduces cell survival after thawing.