General Guidelines for Handling Feeder Free Human iPSCs

This document provides guidance on how to resuscitate, culture and cryopreserve human induced pluripotent stem cells (iPSCs) supplied by the Human Induced Pluripotent Stem Cells Initiative (HipSci).
All recommendations refer to the culture of iPSCs in 6-well plates.

All cell manipulations, tissue culture vessel preparations and medium preparations should be performed under aseptic conditions within a Class II Microbiology Safety Cabinet. The cabinet should be cleaned thoroughly before use, and after processing each cell line by wiping all surfaces with Trigene/Distel or equivalent disinfectant and 70% ethanol. Each cell line should be handled separately to avoid mislabelling or cross-contamination between cell lines. It is advisable that a small number of vials are cryopreserved as a master stock, as soon as possible.

Cells provided were cultured in the presence of Penicillin and Streptomycin.

Contents

Materials	
6 well tissue culture treated plate	Corning 3516
0.22 µM filter	Thermo Scientific 190-2520
Syringe	VWR International 613-3931
Vitronectin	Stemcell Technologies 07180
Essential 8 medium	Life Technologies (50 X 10 ml) A1517001
Knockout Serum Replacer (KSR)	Invitrogen (100 ml) 10828028
1.8 ml Cryovials	Scientific Laboratory Supplies (x450) 375418K
DPBS (no calcium, no magnesium)	Life Technologies 14190144
Ethylenediaminetetraacetic acid (EDTA)	Life Technologies (100 ml) AM9260G
Dimethyl sulfoxide (DMSO)	Sigma-Aldrich (50 ml) D2438
ROCK inhibitor (ROCK)	Sigma-Aldrich (1 mg) Y0503
Sterile filtered water	Sigma Aldrich (500 ml) W1503
15 ml / 50 ml Falcon Tubes	Falcon 352097/352098
Penicillin Streptomycin (10,000 U/ml)	Invitrogen (100 ml) 15140122

Equipment
Class II Microbiology Safety Cabinet
Incubator set at 37°C / 5% CO_2
Water bath set at 37°C
-80°C Storage
Liquid Nitrogen or appropriate Cryo storage unit
Cell freezing containers (also known as 'Mr Frosty')
Phase contrast microscope (4x, 10x, 40x magnification)
'Pipette boy' and selection of stripettes (5 ml / 10 ml)
Pipettes (P1000 / P200 / P100 / P20) and corresponding sterile tips
ParafilmTM

Reagent Preparation

Medium

0.5 mM EDTA Solution

- Prepare fresh 0.5 mM EDTA by diluting Ultrapure 0.5 M EDTA, pH 8.0 with DPBS using a 1:1000 dilution (for example, 10 µl Ultrapure 0.5 M EDTA in 10 ml DPBS).
- Store at room temperature.
- Use on day of preparation only.

Freeze Medium

- Prepare a 10% DMSO in Knock-out Serum Replacement (KSR) solution (for example 1 ml DMSO to 9 ml KSR).
- Store at 4°C until use.
- Use on day of preparation only.

ROCK inhibitor

- Reconstitute ROCK inhibitor by diluting 5 mg in 1.5 ml of sterile filtered water to make 10 mM stock solution.
- Aliquot and store at -20^{o}C for up to 6 months; aliquots can be thawed once and should then be discarded.

Complete Essential 8 Medium (E8)

- Thaw aliquots of frozen E8 Supplement at 4^{o}C overnight (do not thaw at 37^{o}C as this will degrade the FGF).
- Add 10 ml of thawed E8 supplement to 500 ml of E8 basal medium.
- Add 5 ml of Penicillin Streptomycin (optional).
- Swirl bottle to mix (avoid creating air bubbles).
- Label with preparation date and store at 4^{o}C for up to 14 days.
- Perform a sterility check on complete medium before use (optional).

Allow complete medium to warm to room temperature before use, new, cold bottles can be placed in a 37^{o}C water bath to warm.

6 well culture plates

Vitronectin coating

- Upon receipt, store vitronectin at -80°C.
- Prior to use, thaw the stock vial of vitronectin at room temperature or overnight at 4°C.
- Dilute the vitronectin in DPBS to a final concentration of 10 µg/ml (example: 2 ml vitronectin to 48 ml DPBS).
- Gently mix the solution by inverting or swirling the container.

Do not vortex

- Immediately dispense 1 ml of the Vitronectin solution per well to as many wells of a 6 well pate as required.
- Gently rock the 6 well plate back and forth to spread the matrix across the whole surface of the well.
- Incubate at room temperature for one hour before use.
- Prepared plates can be sealed with ParafilmTM then stored at 4°C for up to 7 days. Allow the vessel to equilibrate to room temperature for 1 hour prior to use.

Culture Methods

We strongly recommend that you thaw HipSci vials in accordance with this protocol, using the reagents stated above. We also recommend you make a master bank of the lines as soon as possible to avoid any risk of losing the lines. Once the lines have thawed and are established, after 1-2 passages you can consider transitioning them to a different culture system if you wish. Bear in mind the cells may not respond well to this change and a maintenance plate using the original culture system is recommended until the new method stabilises.

Thawing Human iPSCs

- Prior to starting, prepare a stock of E8 + ROCK by adding 10 mM ROCK to an aliquot of E8 to a final concentration of 10 µM (1:1000 dilution), and allow to warm to room temperature.
- Partially thaw the frozen vial of iPS cells at 37°C, using a water bath, until there are small ice crystals remaining.
- Dry and spray the vial with 70% Ethanol before placing in the culture hood.
- Add 1 ml of E8 + ROCK solution drop-wise to the cryovial with a 5 ml strippete, then gently collect and transfer the entire cell suspension to a 15 ml Falcon tube.
- Add 8 ml of E8 + ROCK solution to the cell suspension.
- Centrifuge at 120 g for 3 minutes.
- During centrifugation aspirate vitronectin from one well of a prepared 6 well coated culture plate.
- Add 1 ml of E8 + ROCK solution to one well ready for use.
- Aspirate supernatant from the cell pellet and using a 5 ml stripette gently re-suspend in 1 ml of E8 + ROCK solution (pipette gently up and down once to mix). Transfer cell suspension to the prepared well of a 6 well plate.
- Agitate plate gently within a tissue culture incubator set at 37°C and 5% CO_2 to ensure even distribution of cells across the well.
- Check cell attachment under a phase contrast microscope after 24 hours.
- If attachment is good, change medium to 2 ml E8. If there are more cells floating than attached top up with 1 ml freshly made E8 + ROCK solution.

Culturing of Human iPSCs

It is good practice to observe iPSC lines daily under phase contrast microscope (4x, 10x, 40x magnification) for iPSC-like morphology, the presence of differentiated cells and confluence (see appendix 1 for grading systems)

- Cells are media changed by removing 95% of the medium from the wells using an aspirator pipette.
- Aseptically add 2 ml of complete E8 medium per 1 well of a 6 well plate by gently adding to the side of the well. Incubate cells at 37°C, 5% CO_2.
- Medium should be changed daily except for on the day of passaging.

Passaging Human iPSCs

Feeder Free iPSC should be observed every day; refer to grading system to assess morphology and confluency (see appendix 1 for Feeder Free grading system).

Cell lines should be passaged when the cells are approximately 70% confluent, well compacted and when the colonies have well defined edges (grade A-B). Cells may also require passaging if levels of differentiation start to exceed that of iPSC or colonies start to look overgrown or unhealthy (grade C-D).

- Established cultures can be split 1:4 to 1:6 (i.e. transferring all colonies from one well to four or six wells).
- Aspirate spent medium from wells to be passaged.
- Wash wells with 2 ml of DPBS per well and aspirate.
- Add 1 ml of 0.5 mM EDTA solution to wells to be passaged, rock plate to cover whole well surface.
- Incubate at room temperature for 4 – 8 minutes, observing under phase contrast microscopy until colonies display bright halos around the edges and small holes start to appear throughout the colonies (see Figure 1 below).

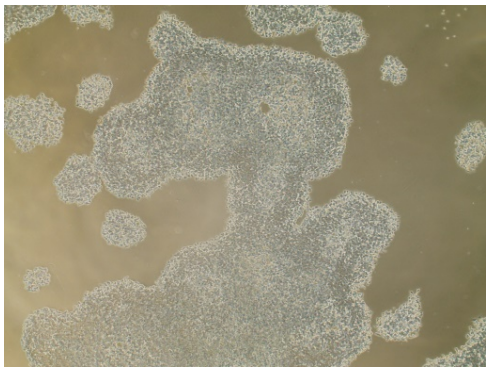


Figure 1 – EDTA effects on iPSC colonies after 4 minutes (taken at 4x magnification under phase contrast microscopy).

- Aspirate the 0.5 mM EDTA by tilting the plate forward slightly to collect the EDTA in the bottom edge of the wells. Take care as the cells are loosely attached.
- Immediately add 2 ml of Complete E8 medium to the wells.
- Using this 2 ml of medium gently wash the cells from the plate by pipetting the medium around the well, approximately three times, using a 5 ml / 10 ml stripette. This should dislodge cell clusters without dislodging any differentiated cells.

Do not over pipette the cells as this will result in single cells rather than cell clusters.

It is possible that occasionally not all cells will lift, there may be rings left behind on the plate (see Figure 2 below).

Figure 2 – Rings left behind after passaging (taken at 4x magnification under phase contrast Microscopy).

- Dilute the cell suspension with Complete E8 medium in a 15 ml / 50 ml falcon tube at an appropriate cell density (in accordance with your desired split ratio).
- Aspirate vitronectin solution from pre-prepared coated 6 well plates.
- Seed cell solution into as many wells as required.
- Agitate plate gently within a tissue culture incubator set at 37^{o}C and 5% CO_2 to ensure even distribution of cells across the well.

Cryopreservation of Human iPSCs

Colonies should be large enough to freeze 4-5 days after passaging when cells are approximately 70-80% confluent. A confluent well of a 6 well plate will have enough cells to generate 5-6 frozen vials per well.

Cells are usually frozen when their morphology lies between grade A-B (refer to the Feeder Free grading system to assess morphology in appendix 1).

- Prepare appropriate volume of freeze medium to freeze 1 ml cell suspension per vial.
- Prepare a cell freezing container (if required make sure to use appropriate volume of replenished Iso-propanol) and store at 4^{o}C until use.
- Aspirate spent medium and wash wells with 2 ml of DPBS per well and aspirate.
- Add 1 ml of 0.5 mM EDTA solution to wells, rock plate to cover whole well surface.
- Incubate at room temperature for 4 – 8 minutes, observing under phase contrast microscopy until colonies display bright halos around the edges and small holes start to appear throughout the colonies (see figure 1).
- Aspirate the 0.5 mM EDTA by tilting the plate forward slightly to collect the EDTA in the bottom edge of the wells. Take care as the cells are loosely attached.
- Immediately add 2 ml of Complete E8 medium to the wells.
- Using this 2 ml of medium gently wash the cells from the plate by pipetting the medium around the well, approximately three times, using a 5 ml / 10 ml stripette. This should dislodge cell clusters without dislodging any differentiated cells.

Do not over pipette the cells as this will result in single cells rather than cell clusters.

- Pool cell suspension into a 15 ml / 50 ml Falcon tube and centrifuge at 120 x g for 1 minute.

- Aspirate the supernatant, tap the falcon tube to dislodge the compacted pellet.
- Re-suspend in the required volume of freeze medium (1 ml per vial).
- Dispense 1 ml of cell colony suspension into each cryovial and seal tightly.
- Immediately place the cryovials into a pre-chilled cell freezing container (4°C) then immediately transfer the container to a -80°C freezer. Allow the cells to remain at -80°C overnight (16-36 hours).
- Once frozen transfer the cells, on dry ice, to an Ultra-Low Temperature storage vessel (LN2 or -150°C freezer).

Troubleshooting

Problem	Possible Solutions/Precautions
Low viability after thaw • Little to no colonies visible within 4 days after recovery	• Ensure you screen round the edges of the well, cells have a tendency to congregate at the sides of a well. • It is advisable to leave plates for a maximum of 2 weeks as colonies can appear within this time. This is not ideal for feeder free cultures. To ensure quality of future vials: • Ensure that cryovials are thawed quickly and that medium is added to the cells very slowly (drop-wise while gently swirling the tube). • Ensure that cells were banked at log phase of growth. • Try thawing cells into a smaller tissue culture vessel. • Ensure 10 µm ROCK inhibitor is added at thaw – if 24 hours post thaw nothing appears to have attached you can top up with 1 ml of medium plus 10 µm ROCK inhibitor, but after this point cells should be media changed daily and without ROCK inhibitor.
Low viability after passage • Cells do not attach properly • Non-typical morphology • High levels of cell death • Cells do not proliferate	• Use lower split ratio and maintain a more confluent culture. • Ensure cells are in log phase of growth at passaging. • Avoid too long an incubation time in EDTA as cells might become more sensitive and colonies will disaggregate to single cells, which often die or differentiate when plated. • If cells do not come off easily Increase incubation time of EDTA. This is to avoid having to harshly rinse cells off thereby creating too small aggregates/ single cell suspension.
Non-uniform distribution of colonies within plate • Areas with too high density of iPS cells • In addition some areas may have fewer colonies	• Make sure that the whole surface area of the tissue culture vessel is evenly coated with the appropriate matrix. • Ensure that the cell aggregates are evenly distributed by gently rocking the plate back and forth and side to side. Not in a circular motion. • Take care when placing plate into the incubator and leave undisturbed for 24h. • Ensure incubators are level and free from vibrations.
Colonies are not coming off the plate	• Ensure that incubation time and temperature of EDTA are in accordance with matrix. • Increase incubation time of EDTA without producing a single cell suspension. • Do not let cells become more than 80 % confluent. • Ensure the EDTA has not gone over its use by date or precipitated in the stock solution
Cells detach after 1st medium change post passage. • Cells start to lift off even	• Exchange medium very gently, do not drop medium rapidly onto the cells, tilt the plate and pipette medium down the side of the well.

though they seemed to attach fine after passage	• If colonies appear to be falling apart once cells compact then investigate the matrix, is it still in date, when were the plates made, were they made up correct, were they stored correctly, are the stocks being stored correctly.
Spontaneous differentiation • Colonies do not have defined edges • Cells within the colonies are less compact • Cells appear flattened and bigger • Morphology as depicted in C and D of the grading system (appendix 1)	In a bid to avoid in the first instance: • Ensure the plates used have coating that is not older than 7 days. • Avoid leaving plates outside the incubator for more than 15 minutes. • Ensure media is within date and has not been left in temperatures of room temperature or higher for extended periods (1-2 hours). • iPSCs are extremely sensitive and any changes in reagent type or user-to-use technique variability can cause an increase in spontaneous differentiation. • Fragment size at seeding can have an impact, too small or single cells, can spontaneous differentiate, too large and differentiation can form in the centre of the colony. To tackle differentiation once it sets in: • Standard passaging is often the safest first option for 3-4 passages, especially if you are transitioning culture systems, as the cells need time to adapt. • Passaging early i.e. before the dish has reached its usual confluence, as long as the iPSC colonies are compacted and of a reasonable size. • Modifying the incubation time in EDTA, leaving it on slightly longer, replacing the EDTA with media and taping the plate as opposed to washing it in the hope that enough healthy colonies dislodge. This may require scale down of culture rather than usual split ratios. • Manually scrapping the differentiation off the dish with a sterile tip. This can be hard to perform if you do not have a microscope in a hood, to get around this you can mark with a pen where the healthy colonies are and then take your plate into the hood and scrap off everywhere except the pen marks. • Alternative to scrapping the differentiated cells off, you can colony pick the healthy colonies into a new 6 or 12 well dish. • Split ratios as high as 1:20 can aid in removing differentiated cells, but you may risk karyotypic abnormalities especially if you persistently use high split ratios.

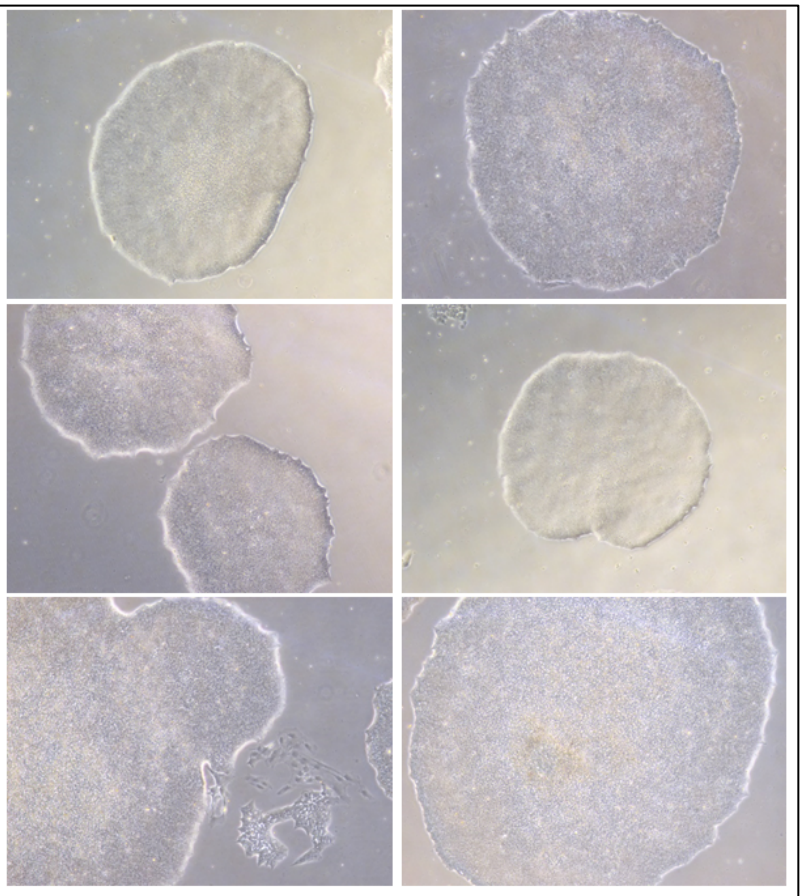
Appendix 1 – Feeder Free Grading System

Morphology grade

A

+ Well-rounded colonies
+ Smooth, defined edges
+ Compacted cells.
+ May see slightly uneven/speckled colony surface (stippling-type effect), mostly due to overgrowth
+ Minimum or very low levels of overgrowth

Differentiation: None - Low.



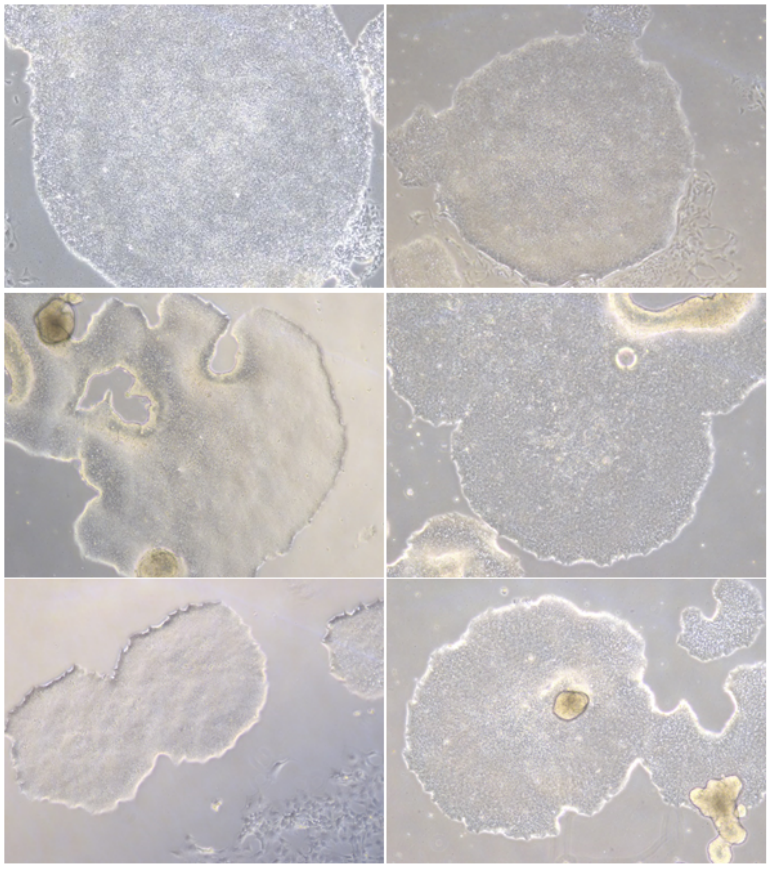
Feeder-free

Morphology grade

B

+ Well-rounded colonies
+ Most colonies have smooth, defined edges
+ Mostly compacted cells
+ Overgrowth /differentiation that has led to some ring-shaped or 'egg' like colonies
+ Rescuable

Differentiation: Low - Medium



Feeder-free

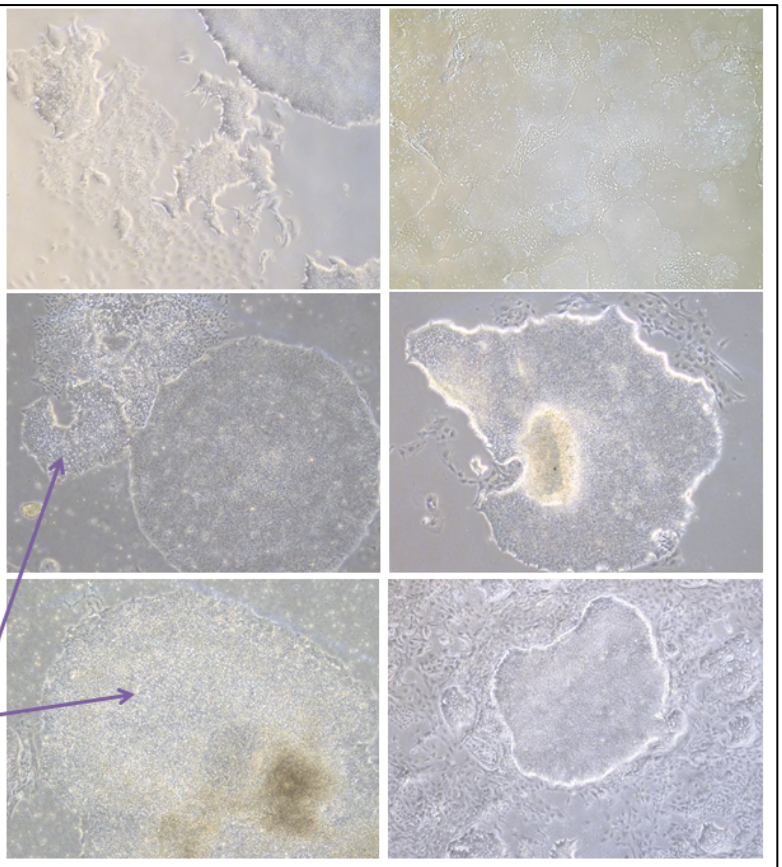
Morphology grade C

+ Well-rounded colonies with defined edges
+ Most colonies have smooth, defined edges, some irregularly shaped colonies
+ Majority compacted cells with some overgrowth or differentiation present at edges of or outside colonies, may well surround colonies.
+ Rescuable

Differentiation:

Medium - High

N.B. Some colonies may look un-compacted. To recover, these will need passaging sooner.

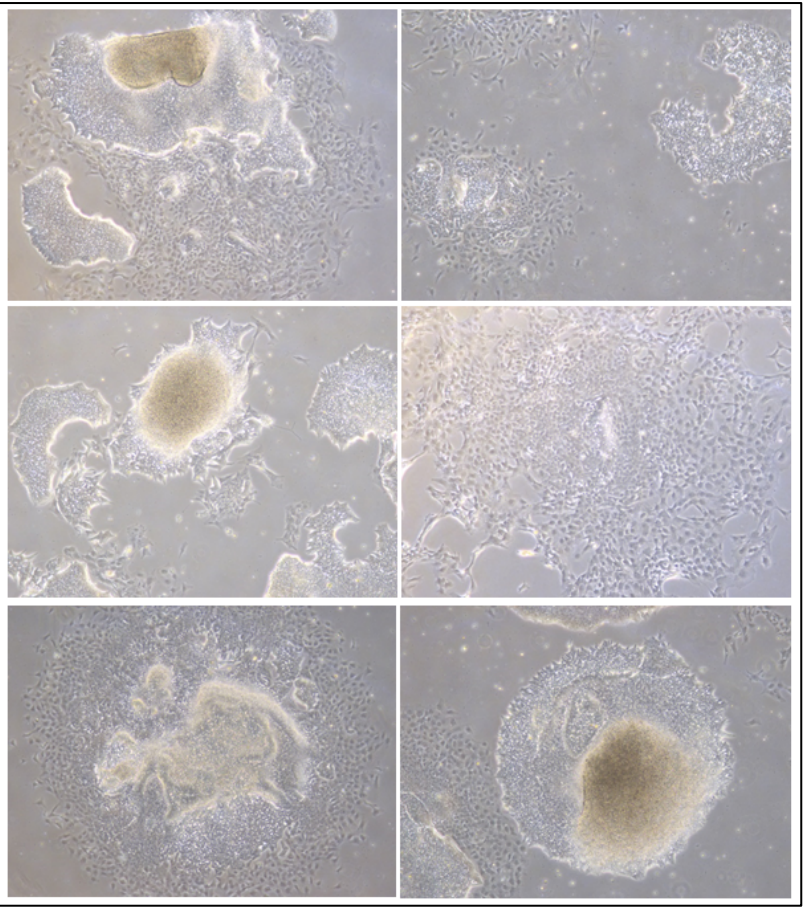


Feeder-free

Morphology grade D

+ Irregularly shaped colonies without defined edges
+ Few compacted cells
+ No obvious/very few areas of compacted cells
+ Only rescue-able colonies remaining are completely un-compacted
+ Very difficult/unable to rescue

Differentiation level:
High



Feeder-free

Acknowledgements

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