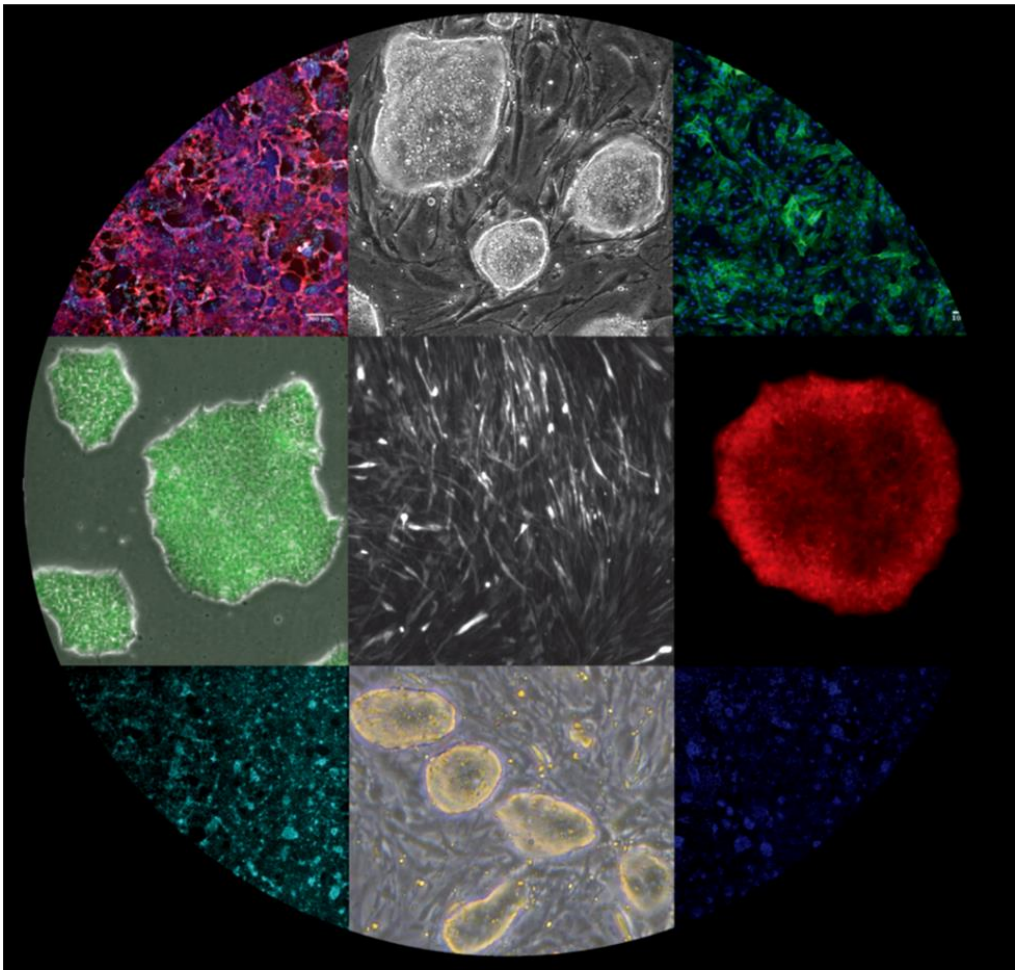
ÖAW AUSTRIAN ACADEMY OF SCIENCES

Stem Cell Core Facility

IMBA Institute of Molecular Biotechnology of the Austrian Academy of Sciences

iPSC Biobank Guide



General Guidelines for Handling Human Stem Cells

The protocols herein provide guidance on culture and maintenance of human pluripotent stem cells (iPSCs), cryopreservation and resuscitation.

All cell culture activities including media preparation, colony picking, passaging, thawing and cryopreservation 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 Sekusept (or equivalent disinfectant) and 70% ethanol. Each cell line should be handled separately to avoid mislabeling or cross-contamination between cell lines.

We routinely culture cell lines without Penicillin/Streptomycin in our facility with exception of colony picking. Prolonged use of antibiotics may lead to deterioration in aseptic technique, selection of drug-resistant organisms, and delayed detection of low-level infection by either mycoplasma or other bacteria.

Content

SECTION 1 - Medium and Reagents Preparation

0.5mM EDTA Solution for passaging of human iPS cells

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

Freezing Medium

1. For human iPS cells: prepare a 10% DMSO in Knock-out Serum Replacement (KoSR) solution (for example 1mL DMSO to 9mL KoSR).
2. Store at 4°C until use.
3. Use on day of preparation only.

Y-27632 (ROCK inhibitor)

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

Complete Essential 8 Medium (E8)

1. Thaw aliquots of frozen E8 Supplement at 4°C overnight (do not thaw at 37°C as this will degrade the FGF).
2. Add 10mL of thawed E8 supplement to 500mL of E8 basal medium.
3. Swirl bottle to mix (avoid creating air bubbles).
4. Label with preparation date and store at 4°C for up to 14 days.

Note: Allow complete medium to warm to room temperature before use.

Vitronectin coating

1. Always store vitronectin stocks at -80°C.
2. Prior to use, thaw the stock vial of vitronectin at room temperature (or overnight at 4°C).
3. Dilute the Vitronectin in DPBS to a final concentration of 10µg/mL (example: 2mL vitronectin to 48mL DPBS).
4. Gently mix the solution by inverting or pipetting.
5. Immediately dispense 1mL of the Vitronectin solution to as many wells of a 6 well plate as required.
6. Gently rock the 6 well plate back and forward to spread the matrix across the whole surface of the well.
7. Incubate at room temperature for one hour before use.
8. Prepared plates can be sealed with Parafilm then stored at 4°C for up to 3 days. Allow the vessel to equilibrate to room temperature for 1 hour prior to use.

Note: Do not vortex to mix.

Matrigel coating

1. Thaw one aliquot of Corning® Matrigel® on ice.
2. Dispense 24mL of cold DMEM/F12 into a 50mL conical tube and keep on ice.
3. Add thawed Corning® Matrigel® to the cold DMEM/F12 (in the 50mL tube) and mix well. The vial may be washed with cold medium if desired.
4. Immediately use the diluted Corning® Matrigel® solution to coat tissue culture treated culture ware.
5. Swirl the culture ware to spread the Corning® Matrigel® solution evenly across the surface.

> Note: If the culture ware's surface is not fully coated by the Corning® Matrigel® solution, it should not be used for human iPS cell culture.

6. Incubate at room temperature (15-25°C) for at least 1 hour before use. Do not let the Corning® Matrigel® solution evaporate.
7. Gently tilt the culture ware onto one side and allow the excess Corning® Matrigel® solution to collect at the edge. Remove the excess Corning® Matrigel® solution using a serological pipette or by aspiration. Ensure that the coated surface is not scratched.
8. Immediately add E8 medium (e.g., 2 mL/well if using a 6-well plate).

> Note: If not used immediately, the culture ware must be sealed to prevent evaporation of the Corning® Matrigel® solution (e.g. with Parafilm®) and can be stored at 2-8°C for up to 1 week after coating. Allow stored coated culture ware to come to room temperature (15 - 25°C) for 30 minutes before moving onto the next step.

SECTION 2 - Thawing Human iPS cells

1. Prior to starting, prepare a stock of E8 + ROCK by adding 10mM ROCK inhibitor to an aliquot of E8 to a final concentration of 10µM (1:1000 dilution) and allow to warm to room temperature.
2. Partially thaw the frozen vial of iPS cells at 37^{O}C, using a water bath, until there is only a small ice crystal remaining.
3. Dry and spray the vial with 70% Ethanol before placing in it the culture hood.
4. Add 1mL of the E8 + ROCK solution dropwise to the cryovial using a 5mL pipette, then gently collect and transfer the entire cell suspension to a 15mL Falcon tube.
5. Add 8mL of E8 + ROCK solution to the cell suspension.
6. Centrifuge at 120g for 3 minutes.
7. During centrifugation, aspirate Vitronectin from one well of a prepared 6 well coated culture plate and leave to dry for no more than 4 minutes.
8. Add 1mL of E8 + ROCK solution to one well ready for use.
9. Aspirate supernatant from the cell pellet and using a 5mL pipette gently re-suspend in 1mL of E8 + ROCK solution (pipette gently up and down once to mix). Transfer cell suspension to the prepared well of a 6 well plate.
10. 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.
11. Check cell attachment under a phase contrast microscope after 24 hours.
12. If attachment is good, change medium to 2mL E8. If there are more cells floating than attached top up with 1mL freshly made E8 + ROCK solution.

SECTION 3 - Culturing of Human iPS cells

We routinely culture cells in 6-well plates and 10cm dishes for practical reasons.
It is a good practice to observe iPSC lines daily under phase contrast microscope (4x, 10x, 40x magnification) for stem cell-like morphology, the presence of differentiated cells and confluence (see SECTION 8 for morphology rating).

1. Cells are fed by removing 95% of the medium from the wells using an aspirator pipette.
2. Aseptically add 2mL of fresh medium per 1 well of a 6 well plate by gently adding to the side of the well. Use 10mL of media for a 10cm dish. Incubate cells at 37°C, 5% CO2.
3. Medium should be changed daily except on the day of passaging.

SECTION 4 - Passaging of Human iPS cells

Feeder-free passaging with EDTA (clumps)

iPS cells should be observed every day; see the rating system on section 8 for assessment of their morphology and confluency.
iPS cells should be passaged when they are approximately 70% confluent, and their colonies well compacted with well-defined edges (section 8: A-B).
If levels of differentiation start to exceed that of compact iPS colonies start to look overgrown or unhealthy (section 8 C), the colonies may also be handpicked.

1. Subconfluent cultures can be split 1:4 to 1:6 (i.e., harvesting all colonies from one well and transfer to four or six wells).
2. Aspirate spent medium from wells to be passaged.
3. Wash wells with 2mL of DPBS per well and aspirate.
4. Add 1mL of 0.5mM EDTA solution to wells to be passaged, rock plate to cover whole well surface.
5. 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).
6. Aspirate the 0.5mM 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.
6. Immediately add 2mL of Complete E8 media to the wells.
7. Using this 2mL of medium gently wash the cells from the plate by pipetting the medium around the well, approximately three times, using a 5mL / 10mL pipette. This should dislodge cell clusters without dislodging any differentiated cells.

Note: Cells might leave behind rings of still attached cells after rinsing them off (see figure 2)

8. Dilute the cell suspension with Complete E8 media in a 15mL / 50mL falcon tube at an appropriate cell density (in accordance with your desired split ratio).
9. Aspirate Vitronectin (or Matrigel) solution from pre-prepared coated 6 well plates; allow to dry for no more than 4 minutes.
10. Seed cells into as many wells as required (see figure 3).
11. Move plate gently to a tissue culture incubator set at 37°C and 5%.

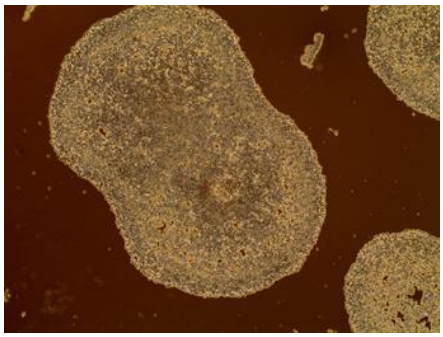


Figure 1 – EDTA effects on iPSC colonies after 4 minutes (4x original magnification)

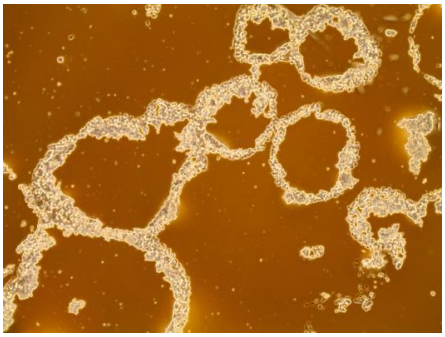


Figure 2 – 'Rings' left behind after passaging (4x original magnification)

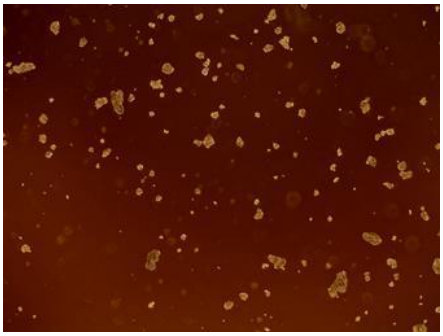


Figure 3 – colonies seeded on 6-well plate (4x original magnification)

Feeder-free passaging with accutase for single cell seeding

1. Aspirate spent media from wells to be passaged.
2. Wash wells with 2mL of DPBS per well and aspirate.
3. Add 1mL of accutase to wells to be passaged, rock plate to cover the whole well surface.
4. Incubate for 5-7 minutes at 37°C, observing under phase contrast microscopy until the colonies are in a single cell suspension.
5. Add 1mL of Complete E8 media + rock inhibitor (1:1000) to dilute accutase action.
6. Collect cell suspension into Falcon tube.
7. Count cells, if necessary.
8. Centrifuge cell suspension at 120g for 5 minutes.
9. Aspirate supernatant and gently resuspend cell pellet with 1mL of Complete E8 media + rock inhibitor (1:1000).
10. Seed cells accordingly.

SECTION 5 – Alkaline Phosphatase staining

The Vector Blue AP Substrate Kit produces a blue, heat stable reaction product. This chromogen can be used singly or in combination with other alkaline phosphatase or peroxidase substrates for multiple label applications. With the aid of imaging systems and software, the spectral profile of this substrate can be distinguished from other enzyme substrates in applications where antigens are co-localized. Vector Blue is also fluorescent.

Preparation of substrate working solution:

To 5 ml of 100 mM - 200 mM Tris-HCl, pH 8.2 - 8.5 buffer*
Add 2 drops (≈80 µl) of Vector Blue Reagent 1
Add 2 drops (≈80 µl) of Vector Blue Reagent 2
Add 2 drops (≈45 µl) of Vector Blue Reagent 3

Staining procedure:

1. Aspirate media from the well.
2. Wash plate with 2 ml DPBS/ well of a 6 well plate.
3. Aspirate DPBS.
4. Add 1ml of substrate working solution (enough to completely cover the surface).
5. Incubate 30 min in the dark at RT.
6. Aspirate the substrate working solution
7. Add 2ml DPBS/well.
8. Assess cells under the microscope.

Note: Increasing the incubation time in the substrate solution up to four hours may increase staining sensitivity 2-4 times, however background toning may also increase.
Plates can be kept at 4°C in PBS and sealed with Parafilm.

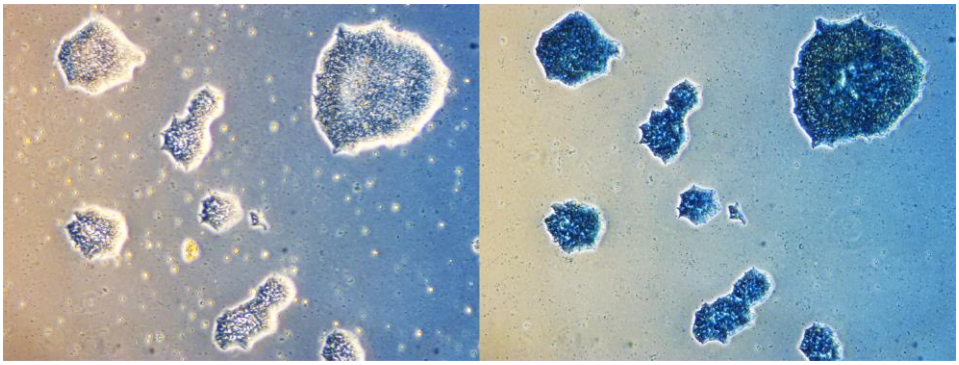


Figure 4 – Unstained (left) and AP-stained cells (right)

SECTION 6 - Picking of human iPS colonies

1. Prepare plates: coat 12 well plates with vitronectin as described previously
2. Prepare 3mL E8 media per colony: prewarm E8 to room temperature, thaw ROCK stock solution and add 1µL ROCK stock solution per mL media.
3. Prepare a 6 well intermediate washing plate (not coated with vitronectin): dispense 1mL of the media prepared in step 2.
4. Assess cells under a phase contrast microscope to identify good iPSC colonies and mark them with a microscope object marker. Colonies which have grown into each other should be avoided.
5. Take the dish into the hood and center the dish onto one of the marked colonies using the appropriate microscope (we routinely use Evos® microscope).
6. Using a 200µl pipette encircle a colony to clear the surrounding surface and ensure not to disturb any surrounding colonies (see figure 6).
7. Subsequently, using the pipette, gently divide the colony into approximately 4-12 fragments depending on its size (see example image above).
8. Scrape the fragments off the dish, collect with the pipette and transfer them into the intermediate washing plate prepared in step 2.
9. Repeat from step 5 for up to 6 colonies (using a different well in the intermediate washing plate for each colony).
10. Put the picking plate back into the incubator.
11. Place the target well of the intermediate plate on the Evos microscope, collect all colony pieces and transfer to the vitronectin coated plate. Make sure not to transfer any single cells.
12. Repeat step 11 for all picked colonies (using a different well in the feeder plate for each colony). Check if you forgot any colony pieces by swirling the plate and discard if colonies in all wells got transferred.
13. Gently shake the plate back and forth to evenly distribute the cells. Transfer the plate into the incubator and gently shake the plate back and forth again.
14. Repeat steps 5 to 13 for all the cell lines.
15. Leave the plates undisturbed for 24 hours. Then top up with 1mL E8, followed by daily medium changes until colonies become large enough to passage (around 7 days post picking, cells might be due for passaging).
16. Repeat all the steps above the next few days until at least 6 colonies per cell line have been picked.
17. When all possible/enough colonies were picked, discard the picking plate.
18. Expand the lines as quickly as possible to freeze them for backup.

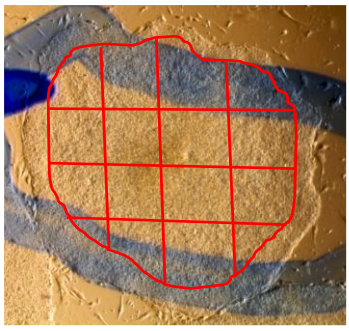


Figure 6 – Marked iPSC colonies prior to picking (4x original magnification)

SECTION 7 - Cryopreservation of Human iPS cells

Cells should be frozen when wells are approximately 70-80% confluent (usually 4-5 days after passaging). A confluent well of a 6 well plate will have enough cells to generate 5-6 frozen vials. Cells are usually frozen when their morphology are within ratings A and B (Section 12).

1. Prepare appropriate volume of freezing medium to freeze 1mL cell suspension per vial.
2. Prepare a cell freezing container.

Note: if you are using the Nalgene Mr Frosty cooling container make sure to use appropriate volume of Isopropanol and store at 4°C until use. We routinely use Isopropanol-free cell freezing containers (CoolCell, Biocision).

3. Aspirate spent medium and wash wells with 2mL of DPBS per well and aspirate.
4. Add 1mL of 0.5mM EDTA solution to wells, rock plate to cover whole well surface.
5. 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).
6. Aspirate the 0.5mM 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.
7. Immediately add 2mL of Complete E8 media to the wells.
8. Using this 2mL of medium gently wash the cells from the plate by pipetting the medium around the well, approximately three times, using a 5mL / 10mL pipette. This should dislodge cell clusters without lifting any differentiated cells.

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

9. Pool cell suspension into a 15mL / 50mL Falcon tube and centrifuge at 120g for 4 minutes.
10. Aspirate the supernatant, tap the falcon tube to dislodge the compacted pellet.
11. Re-suspend in the required volume of freezing medium (1mL per vial).
12. Dispense 1mL of cell colony suspension into each cryovial and seal tightly.
13. 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).
14. Once frozen transfer the cells, on dry ice, to an Ultra-Low Temperature storage vessel (LN2 or -150°C freezer).

SECTION 8 - Evaluation of human iPS colonies and morphology ratings

A

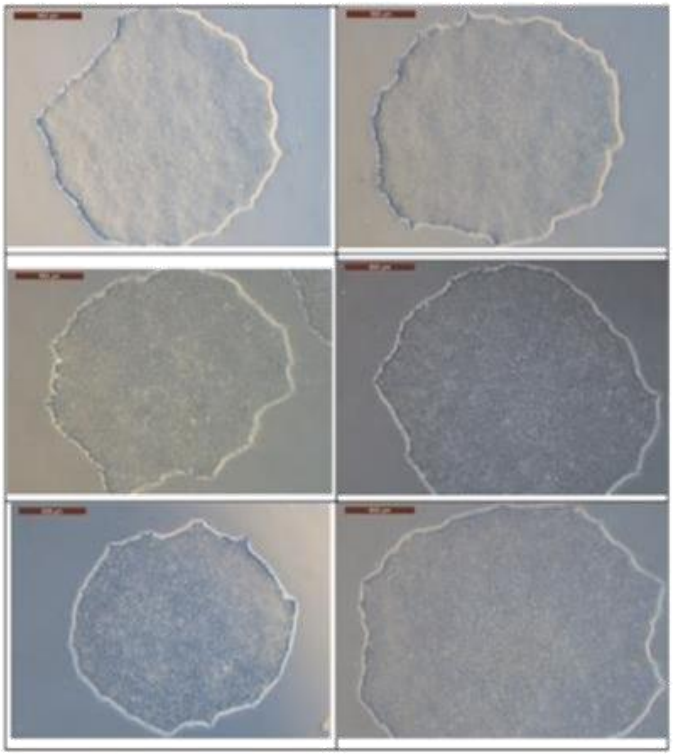
Feeder-free

Morphology rating

★★★★

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

Differentiation: None - Low.



B

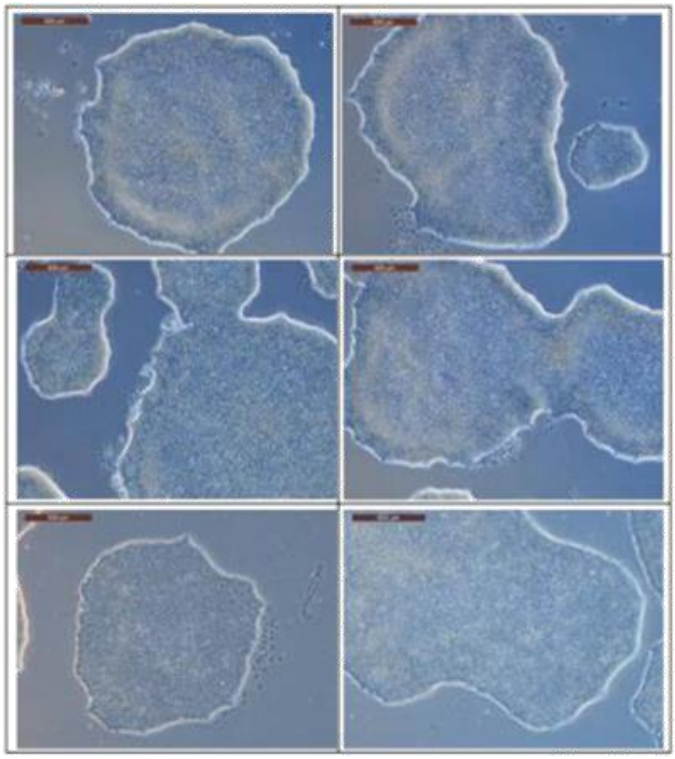
Feeder-free

Morphology rating

★★★

- Well-rounded colonies
- Most colonies have smooth, defined edges
- Compacted cells with some overgrowth or slightly uneven colony surface (stippling-type effect)
- Differentiation present at edges of or outside colonies

Differentiation: Low - Medium



C

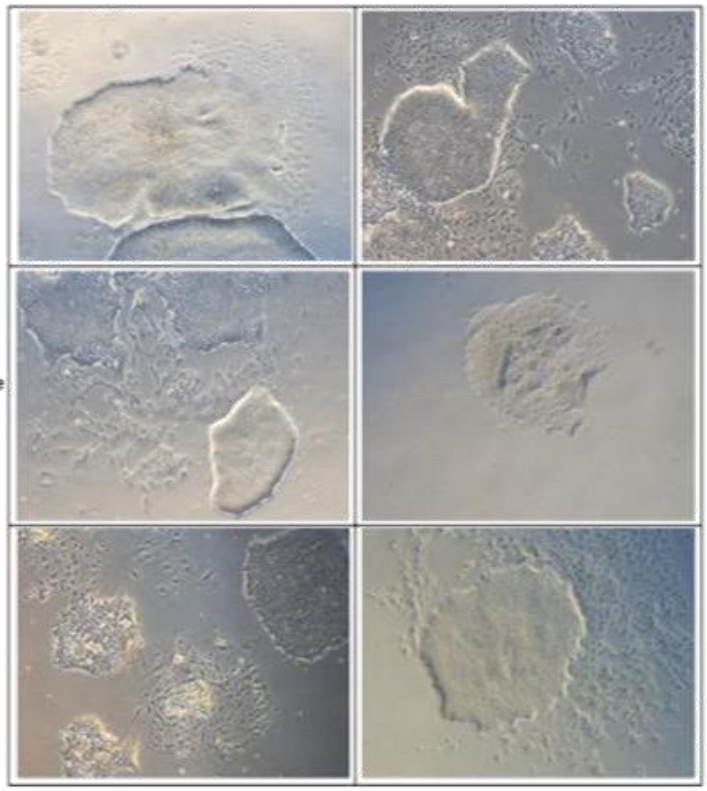
Feeder-free

Morphology rating

★★

- Some well-rounded colonies with defined edges but also many irregularly shaped colonies
- Areas of compacted cells visible
- Differentiation within and outside colony boundary.
- Some colonies fully differentiated
- Could be rescued by colony picking

Differentiation: Medium - High



D

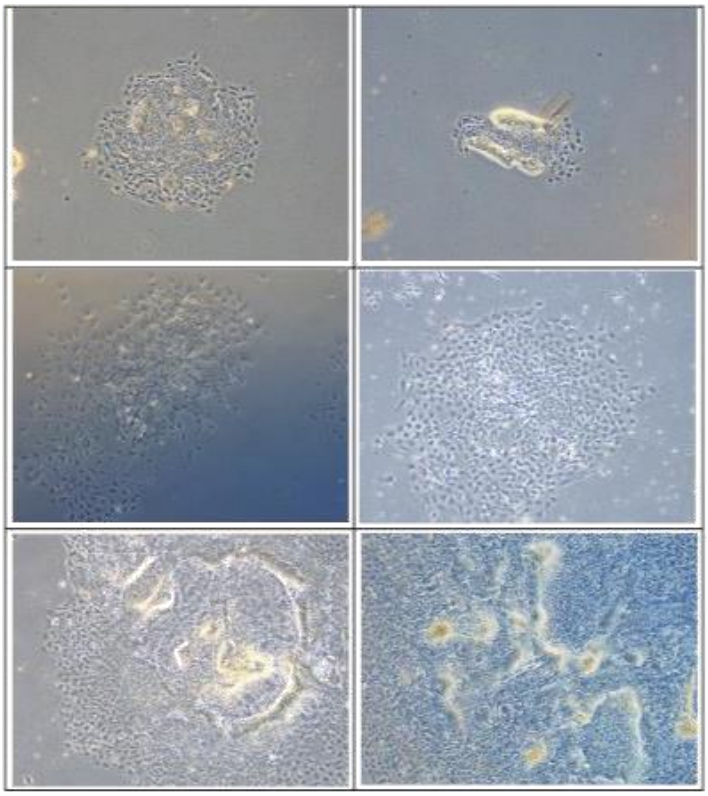
Feeder-free

Morphology rating

★

- Irregularly shaped colonies without defined edges
- No obvious/very few areas of compacted cells
- Majority of colonies completely differentiated
- Difficult/likely unable to rescue

Differentiation level: High



SECTION 9 - List of Reagents and Equipment used

Materials & Vendor		Catalogue number
6 well tissue culture treated plate	Corning	3516/10578911
0.05% Trypsin/EDTA	Thermo Fisher Scientific	25300054
15mL / 50mL Falcon Tubes	Falcon	352070/188271
Accutase	Stem Cell Technologies	7920
Advanced DMEM/F-12, with HEPES	Thermo Fisher Scientific	42430025
Advanced DMEM/F-12, without HEPES	Thermo Fisher Scientific	12634010
AO/PI staining solution	Nexcelom Bioscience	CS2-0106-5mL
Cellometer Counting Chamber	Nexcelom Bioscience	CHT4-PD100-003
CloneR	StemCell Technologies	5889
Cryotube Vial	Thermo Scientific (Nunc)	366656
Dimethyl sulfoxide (DMSO)	Sigma Aldrich	D276855
DPBS (no calcium, no magnesium) (DPBS (-/-))	Thermo Fisher Scientific	14190094
Essential 8™ medium	Thermo Fisher Scientific	A1517001
Matrigel	Stem Cell Technologies	354230
Penicillin-Streptomycin solution	Sigma Aldrich	P-0781
Sterile filtered water	Sigma Aldrich	W1503
Trypsin-EDTA 0.5% (10X)	Gibco	15400-054
Ultrapure 0.5M EDTA, pH 8.0	Thermo Fisher Scientific	15575020
VECTOR Blue Alkaline Phosphatase Substrate Kit	Vector Laboratories	SK-5300
Vitronectin XFTM	Stemcell Technologies	7180
Water for embryo transfer	Sigma Aldrich	W1503-500ML
Y-27632 (ROCK inhibitor)	Sigma Aldrich	Y0503

Equipment required	Vendor
Cell freezing containers (also known as 'CoolCell')	BioCision
Cell freezing containers (also known as 'Mr Frosty') or	Nalgene
Cellometer Auto 2000 (or other method for cell counting)	Nexcelom Bioscience
Class II Microbiology Safety Cabinet	SZABO SCANDIC
Incubator set at 37°C / 5% CO2	PHCbi (Panasonic)
Liquid Nitrogen or appropriate Cryo storage unit	Cryo-Therm
Ultra-low temperature freezer (-80°C storage)	PHCbi (Panasonic)
Water bath set at 27°C	Grant
Water bath set at 37°C	Grant

SECTION 10 - References

Kilpinen H et al., (2017) Common genetic variation drives molecular heterogeneity in human iPSCs. Nature. 2017 Jun 29;546(7660):686

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Takahashi, K., et al., (2007) Induction of Pluripotent Stem Cells from Adult Human Fibroblasts by Defined Factors. Cell, 131(5): p. 861-872.

Ludwig TE et al. (2006) Feeder independent culture of human embryonic stem cells. Nat Methods 3(8):637–46.