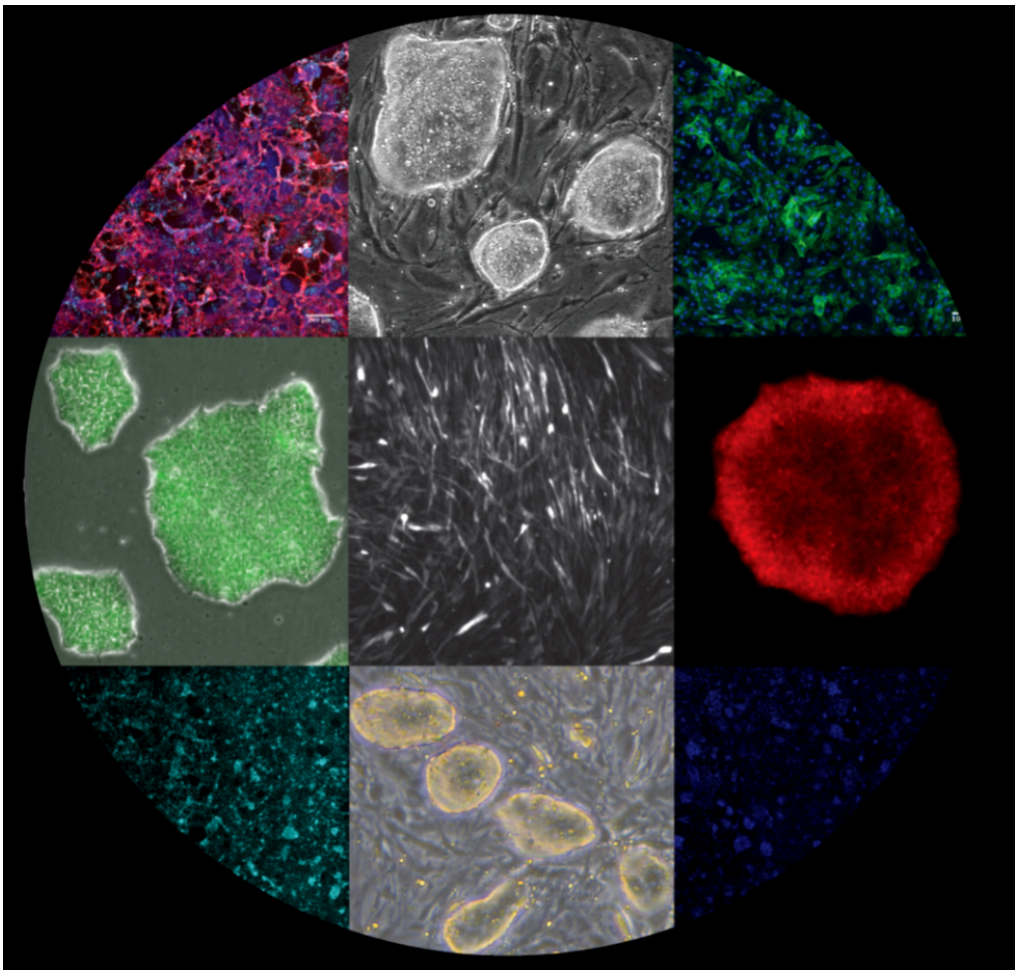
ÖAW AUSTRIAN ACADEMY OF SCIENCES

Stem Cell Core Facility

IMBA Institute of Molecular Biotechnology of the Austrian Academy of Sciences

IMBA iPSC Biobank Guide



General Guidelines for Handling Human Stem Cells

The protocols herein provide guidance on culture, expansion, cryopreservation and resuscitation of IMBA iPSC Biobank cell lines.

All cell culture activities including media and reagent preparation, 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.

Since 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, iPSC lines are routinely cultured without Penicillin/Streptomycin.

CONTENT

1 – Media and Reagents

Media

• StemFlex™ Medium	Gibco™	Cat# A3349401 *

Reagents

• RevitaCell™ Supplement (100X)	Gibco™	Cat# A2644501 *
• CloneR™	STEMCELL Technologies	Cat# 05888 *
• DPBS, no calcium, no magnesium	Gibco™	Cat# 14190144
• Matrigel® hESC-Qualified Matrix, LDEV-free	Corning®	Cat# 354277 *
• ReLeSR™	STEMCELL Technologies	Cat# 100-0483 *
• DMSO	Sigma-Aldrich	Cat# D-8418
• KnockOut™ Serum Replacement	Gibco™	Cat# 10828028

*Please refer to manufacturer's instructions before usage.

Material

• 6-well tissue culture treated dish	e. g. Corning®	Cat# 3516
• Cryogenic Tubes	e. g. Thermo Scientific™	Cat# 374081
• 15mL Conical Centrifuge Tubes	e. g. Falcon™	Cat# 352095
• Cell freezing container	e. g. Corning® CoolCell™	Cat# 432000

2 – Thawing and Maintenance of Human iPS Cells

One IMBA iPSC Biobank cryovial contains about $0.5x10^6$ cells and can be thawed on up to 4 wells of a 6-well tissue culture treated dish. Cells are cultured (plated, passaged and frozen) in clusters. Upon thawing it is recommended to seed different densities.

1. Prior to starting, prepare for each clone to be thawed:
   - 16-20mL StemFlex medium (depending on how many wells are used) supplemented with RevitaCell (1:100 dilution of 100x RevitaCell stock) and allow to warm to room temperature.
   - Up to 4 wells of a 6-well tissue culture treated dish coated with Matrigel.
     Note: The diluting instructions for Matrigel are provided in the Product Insert. The dilution factor is calculated for each lot of Matrigel, based on the protein concentration. Please refer to the lot-specific Certificate of Analysis, which can be requested on the Corning website.
2. Prepare a 15mL Falcon tube containing 3mL StemFlex + RevitaCell.
3. Partially thaw the frozen vial of iPS cells at 37°C, using a water bath, until there is a small ice crystal remaining.

4. Add 1mL of StemFlex + RevitaCell solution dropwise to the cryovial, then gently collect and transfer the entire cell suspension to the 15mL Falcon tube containing 3mL StemFlex + RevitaCell.
5. Centrifuge at 120 xg for 5 minutes.
6. During centrifugation, aspirate Matrigel solution from wells of the prepared 6-well coated culture dish and replace with 2mL StemFlex + RevitaCell solution per well.
7. After centrifugation, aspirate the supernatant from the cell pellet and, using a 5mL pipette, gently re-suspend in up to 6mL of StemFlex + RevitaCell solution. Close tube, invert gently and allow a gradient from large to small cell clusters develop (20-30"). Take 1-2mL cell suspension from the lower third of the tube and distribute to prepared wells. Ideally, this suspension contains cell clusters with 5-10 cells per cluster.
8. Agitate plate gently to ensure even distribution of cells across the well. Transfer plate to a tissue culture incubator set at 37°C, 5% CO_2.
9. Check cell attachment under a phase contrast microscope after 24 hours, wash once with 2mL DPBS and change medium to 2mL StemFlex medium supplemented with CloneR (1:10 dilution of 10x Cloning Supplement stock).
10. Change medium to StemFlex without supplement 48 hours post thawing. Change medium daily thereafter.

3 – Culture and Expansion of Human iPS Cells

iPS cells should be observed every day for assessment of morphology (see SECTION 5 for morphology rating) and should be passaged after reaching approximately 70% of confluency, with well compacted colonies showing well-defined edges (SECTION 5: A-B).

1. Prepare a 6-well tissue culture treated dish coated with Matrigel.
2. Allow StemFlex medium to warm to room temperature.
3. Aspirate Matrigel solution after incubation and add 2mL StemFlex per well.
4. Aspirate spent medium from wells to be passaged, wash each well to be passaged with 2mL of DPBS and aspirate.
5. Add 300µL of ReLeSR per well, rock plate to cover whole well surface.
6. Aspirate ReLeSR after 30-40".
7. Incubate at room temperature for up to 5 minutes, until colonies display bright 'halos' around the edges and small holes start to appear throughout the colonies.
8. Add 1mL StemFlex medium to the well and gently wash the cells from the plate by pipetting medium around the well to dislodge cell clusters, repeat several times to break cell clusters into smaller pieces. Note: do not over pipette the cells as this will result in generating single cells rather than cell clusters.
9. Transfer the cell suspension into in a 15mL Falcon tube containing up to 5mL StemFlex medium, close tube, invert and allow a gradient to develop. Take 1-2mL cell suspension from the lower third of the tube and distribute to prepared wells.
10. Gently rock the plate, observe desired seeding density and incubate at 37°C, 5% CO_2.

4 – Cryopreservation of Human iPS Cells

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

1. Prepare appropriate volume of freezing medium (10% DMSO in KnockOut Serum Replacement) to freeze 1mL cell suspension per vial.
2. Prepare a cell freezing container.
3. Aspirate spent medium, wash wells with 2mL of DPBS per well and aspirate.
4. Add 300µL of ReLeSR per well, rock plate to cover whole well surface.
5. Aspirate ReLeSR after 30-40".
6. Incubate at room temperature for up to 5 minutes, until colonies display bright 'halos' around the edges and small holes start to appear throughout the colonies.
7. Immediately add 1mL of StemFlex media to each well and gently wash the cells from the plate by pipetting the medium around the well, approximately three times, using a 5mL pipette. Keep attention to not over pipette the cells as this will result in generating single cells rather than cell clusters.
8. Pool cell suspension into a 15mL / 50mL Falcon tube and centrifuge at 120 xg for 5 minutes.
9. Aspirate the supernatant, tap the falcon tube to dislodge the compacted pellet.
10. Re-suspend in the required volume of freezing medium.
11. Dispense 1mL of cell cluster suspension into each cryovial and seal tightly.
12. 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).
13. Once frozen, transfer the cells, to an Ultra-Low Temperature storage vessel (LN2 or -150°C freezer).

5 – Evaluation of Human iPS Colonies and Morphology Ratings

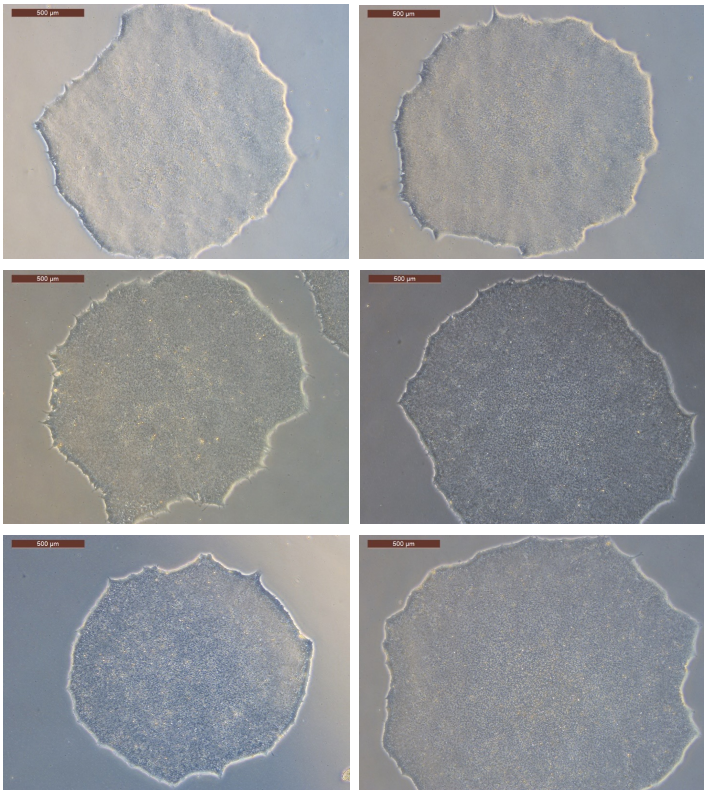
A

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



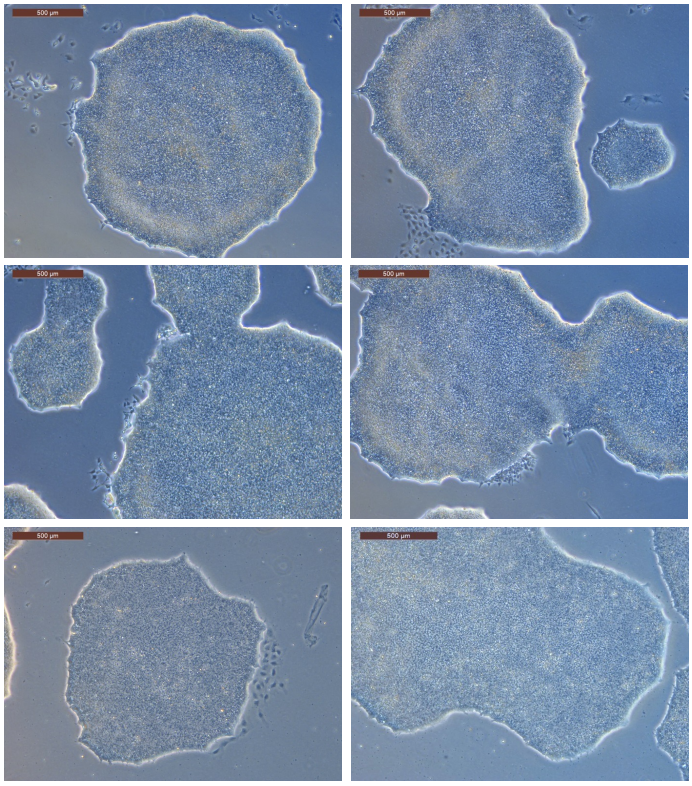
B

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



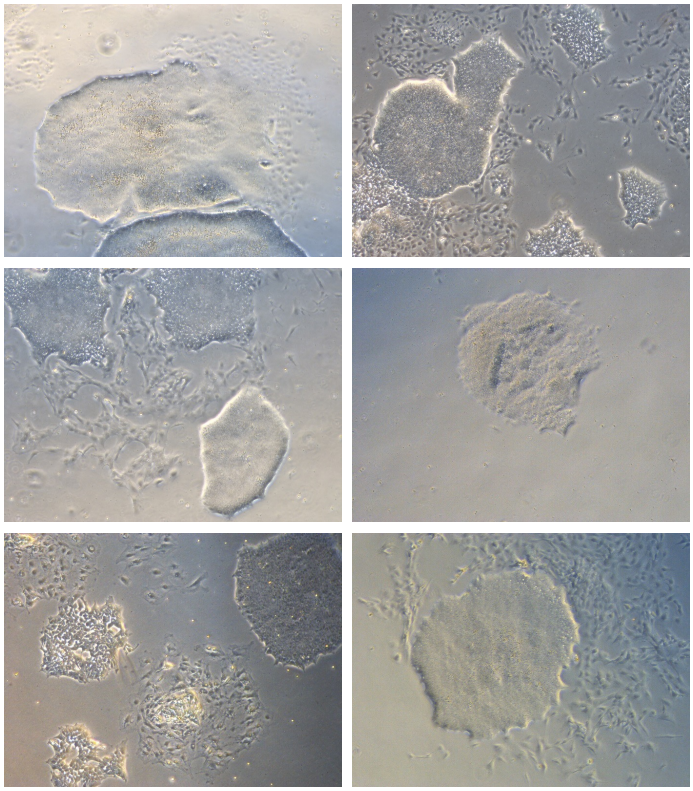
C

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
- Rescue-able

Differentiation: Medium - High



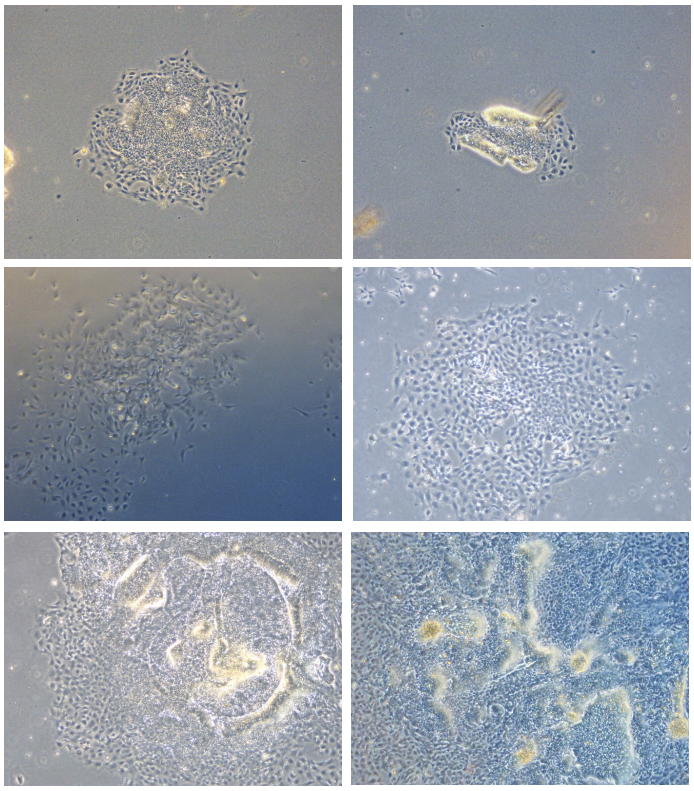
D

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: High



6 – References

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

Standards for Human Stem Cell Use in Research. June 2023 https://www.isscr.org/standards-document

http://assets.thermofisher.com/TFS-Assets/LSG/brochures/pluripotent-stem-cell-guidebook.pdf