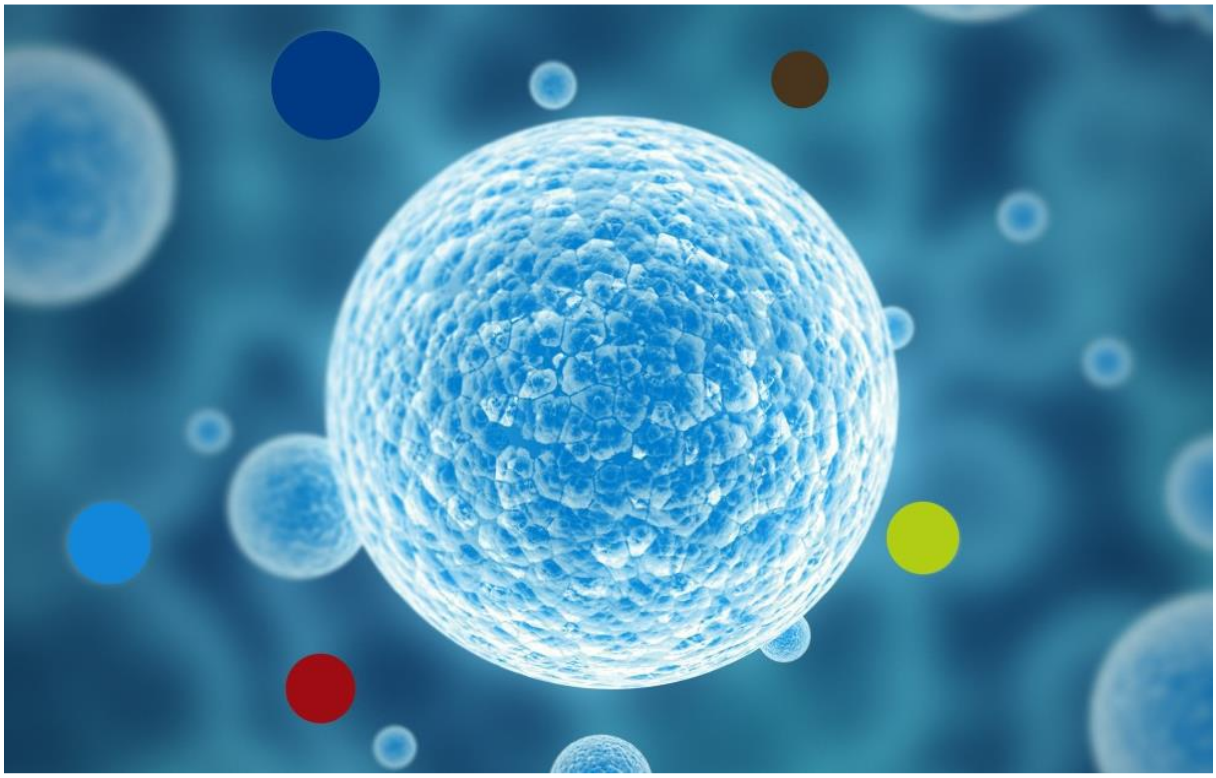
PROTOCOL FOR THE USE OF INDUCED PLURIPOTENT STEM CELLS



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User Protocol for Human induced Pluripotent Stem Cells

Version 4

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1. Important information: Read prior to commencing any work

Human induced pluripotent stem cell (iPSC) lines are different to any other established cell line. If you are not familiar with culturing iPSCs make sure you read these instructions carefully.

Key points for success

- Read these instructions carefully, including the sections on required reagents, thawing and passaging with ROCK inhibitor and precautions and troubleshooting tips.
- Make sure all necessary reagents are available prior to thawing the cells. iPSC lines do not grow in standard media such as DMEM.
- Use the appropriate media and matrix combination for your iPSC lines. Information found on Certificate of Analysis indicates the reagents in which each iPSC line has been expanded and cryopreserved.
- Make sure your equipment is calibrated regularly and no reagents have expired.

2. General guidelines for handling human iPSCs

This document provides guidance on how to resuscitate, culture and cryopreserve human induced pluripotent stem cells (iPSCs) supplied by the European Bank of induced pluripotent Stem Cells (EBiSC). All recommendations refer to the culture of iPSCs in one 60mm cell culture dish.

All cell manipulations, cell culture vessel preparations and medium preparations should be performed under aseptic conditions within a class II biosafety cabinets (BSC). The cabinet should be cleaned thoroughly before use and after processing each cell line by wiping all base surfaces with 70% alcohol. No more than one cell line should be handled in a BSC at any one time to avoid mislabeling or cross-contamination between cell lines. It is advisable that a small number of vials are cryopreserved as a master stock. It is recommended to perform regular checks on equipment used to culture iPSCs and to store reagents to ensure they are working within specifications.

For recommendations on culture systems refer to the Certificate of Analysis (CoA) specific for the cell line and lot number.

3. Materials and reagents

- 60 mm cell culture treated dish (for example VWR Cat. No. 734-2040)
- Matrigel, growth factor reduced basement membrane matrix (for example Corning Cat. No. 356231 (10ml)) and DMEM/F-12 (for example ThermoFisher Scientific Cat. No. 21331020)
- mTeSR™1 culture medium (StemCell Technologies Cat. No. 85850)
- DMEM/F-12 (for example ThermoFisher Scientific Cat. No. 21331020)
- 1x PBS without Ca^{2+}/Mg^{2+} (for example ThermoFisher Scientific Cat. No 14190144)
- EDTA (for example ThermoFisher Scientific Cat. No. AM9260G (100 ml), AM9261 (500 ml), AM9262 (1L))
- Cryostor®CS10 Cryopreservation Medium (for example StemCell Technologies Cat. No. 07930)
- ROCK inhibitor (for example Avantor/VWR Cat. No. CAYM10005583-1 or Sigma-Aldrich Cat. No. Y0503-1 mg)

All materials and reagents should be prepared, stored and handled according to Manufacturer's instructions.

EBiSC iPSCs can be adapted to alternative culture matrix and medium if required. See section 8 and get in touch with EBiSC for additional guidance via Contact@EBiSC.org.

4. Thawing and seeding of human iPSCs with Rock inhibitor

When thawing cells, supplement mTeSR™1 with ROCK inhibitor to maintain 10µM final concentration. To obtain the required concentration and a sufficient medium volume for thawing, we recommend diluting 16µL 5mM of stock ROCK inhibitor in 8ml medium.

1. Provide two Matrigel-coated 60mm dishes
2. Cells have to be thawed rapidly by placing the cryovial in a water bath set to maintain +37°C. Ensure a rapid thaw but do not submerge the cap of the cryovial. Disinfect the cryovial with 80% ethanol or an equivalent disinfectant before opening.
3. Using a 5ml sterile serological pipette, transfer the cryoprotectant/cells mix from the cryovial into a 15ml centrifuge tube.
   Take care not to physically damage cells! Avoid vigorous pipetting of the cell suspension!
4. Slowly add droplet-wise 10ml of mTeSR™1 complete medium at room temperature to the cell suspension in the 15ml centrifuge tube. Gently rock the 15ml centrifuge tube back and forth while adding droplets to minimise osmotic shock to the cells. This is a crucial step and cells have to be treated as gently as possible.
5. Check the cryovial to ensure that the entire cell suspension was transferred and rinse the cryovial with 1 ml of mTeSR™1 afterwards.
6. A small amount of cells can be used to determine cell numbers.
   Since hiPS cells were dissociated into small clumps before freezing, apply trypsin or a similar enzyme to achieve a single cell suspension before counting, if your counting method needs single cells.
   As a general guideline, the seeding density range is 1x 10^6viable cells per 60mm dish.
7. Centrifuge the cells at 200 x *g* for 2 minutes. Remove and discard the supernatant.
8. Prepare culture vessels by adding an appropriate amount of mTeSR™1 containing 10µM Rock inhibitor (for example 3 ml per 60 mm plate, depending on the resuspention volume after centrifugation). The final culture volume shoud be between 4 - 5ml.
9. Gently tap the 15 ml centrifuge tube to dislodge the cell pellet. Then gently add 2 ml of mTeSR™1 containing 10 µM Rock inhibitor with a 5ml steril serological pipette and seed into 2 coated 60mm plate (1 ml per plate, adjust if using other culture formats or if advised differently in the Certificate of Analysis). Make sure to pipette cell gently. Too harsh pipetting will lead to decreased viability. Do not use a standard 1000µl pipette tip, as the small clumps will be damaged/dislocked.
10. Gently move the plate in a 8-shaping pattern followd by side to side, back and forth to spread the cells evenly across the plate.
11. It is advisable to take phase contrast microscopical images of cells immediately post-thaw, at 24 hours, at 48 hours and at approximately 70 - 80% of confluence.

5. Culturing human iPSCs

1. It is good practice to observe iPSC lines daily under phase contrast microscope (4x, 10x, 20x and 40x magnification) to check for iPSC-like morphology, the presence of differentiated cells and confluence. A typical scoring method could look like this (for examples of iPSC colonies and different differentiation levels see appendix A and B):

A	**Optimal, compacted iPSC colonies with defined edges; morphology uniform across colonies**
B	**Acceptable iPSC colonies with some differentiation around the edges, cell more loosely packed within colonies**
C	**Good adherence with iPSC colonies emerging**
D	**Poor adherence and no obvious iPSC**

2. Cells are fed by removing the medium from the dish using an aspirator pipette. Make sure to perform the medium change as quickly as possible to avoid drying.
3. Aseptically add 4 ml of fresh mTeSR™1 per 60mm dish by gently adding to the side of the dish. Incubate cells at +37°C / 5% CO_2.
4. Typically, medium exchanges have to be done daily on five of seven days with increased volume of media (recommended are 5ml medium) if cells need to be left for longer periods between media changing (for example over the weekend). Do not leave more than two days between medium changes.

6. Passaging human iPSCs using EDTA

Typically, established cultures have to be split 1:4 to 1:8 (i.e. transferring all colonies from one plate to four or up to eight new matrigel-coated plates) but the exact split ratio can vary for each cell line depending on its proliferation capacity. Split ratios have to be adjusted to ensure cells are passaged within 4 - 5 days of culture in order to maintain log phase of growth. Cell lines have to be passaged when the cell culture reaches approximately 70 - 80% confluency or if colonies have been growing on the same plate for more than 7 days. Colonies must not become too dense or display enhanced differentiation.

1. Aspirate the medium from each vessel requiring passage.
2. Wash the cells with 2ml of 0.5mM EDTA per well. Quickly aspirate the EDTA from the well and repeat the wash step.
3. Add 2ml of 0.5mM EDTA to each culture vessel and incubate at +37°C / 5% CO_2 for 5 minutes. Ambient temperature can influence EDTA efficacy and incubation time should be optimized.
4. After 5 minutes of incubation time, check the cell layer under an inverted microscope to see if the colonies begin to have feathered edges and holes throughout. Cells treated with EDTA should appear white under the inverted microscope. This indicates that cells have started to detach from each other but not from the culture vessel. Remove the 0.5mM EDTA from the vessel by tilting the vessel forward slightly to collect the EDTA in the bottom edge of the vessel. A small liquid film should be kept to avoid drying of the cells. Take care as the cells could already be loosely attached. Incubate for another 1 - 11 minutes at room temperature or at +37°C / 5% CO_2 depending on the cell line.
5. Recommendation: Incubate for up to 5 more minutes at +37°C / 5% CO_2, then check the state of detachment under the microscope and extend the incubation time if needed or switch to room temperature incubation.
6. ATTENTION: The incubation time must not exceed 16 minutes overall.
7. If necessary, carefully tap the vessel on the bench to ensure a complete detachment of the cells. If the vessel gets tilted after tapping, the detachment should be visible even without a microscope. The detachment of the cells can also be checked under an inverted microscope. After detaching, immediately add 2 ml of mTeSR™1 to the 60mm plate in order to neutralise EDTA.
8. Gently wash the cells from the culture vessel using a 5ml sterile pipette. Three gentle aspirations of the plate is sufficient. These aspirations will dislodge cell clusters, ideally leaving potentially differentiated cells attached. Excessive pipetting of the cells will result in single cell suspension rather than cell clusters, which will decrease cell viability. It can be beneficial to leave some cell material behind rather than trying to remove all cells and compromising clump size (up to 10% - 20% of remaining material is normal).
9. Seed the cells at an appropriate cell density by transferring the required volume of cells (in accordance with your desired split ratio) to a new dish coated with Matrigel containing a sufficient volume of mTeSR™1 depending on the split ration. The final culture volume should be between 4 to 5 ml.
10. To ensure even distribution of cell clusters, gently disperse the clusters by carefully moving the vessel in a 8-shaping pattern followed byside to side, back and forth several times before placing the vessel in an incubator maintained at +37°C / 5% CO_2.

7. Cryopreserving human iPSCs

1. Keep reagents and freezing container chilled during the cryopreservation procedure. If using an isopropanol-based cryopreserving container such as Mr. Frosty™, ensure that isopropanol is exchanged regularily.
1. *Recommendation: Replace the isopropanol in the freezer container after every third use.*
2. Cells must be cryopreserved when in their log phase of growth to enhance survival upon thaw. The optimal time for harvest is normally when cells are approximately 70 - 80% confluent.
3. Use commercially available Cryostor CS10. Cryostor is supplied ready to use and is stored at +5°C.
4. Remove spent medium from the cell culture vessel and wash the vessel twice with 2 ml of 0.5 mM EDTA depending.
5. To lift the cells from the cell culture plastic, add 2ml of 0.5mM EDTA to the cell culture vessel. Incubate the cells for the recommended time and temperature (view point 8)
6. If cryopreservation of cells from more dishes is desired, cells from the same passage number and culture condition should be pooled together. Use an aliquot of pooled cells for the cell count. Centrifuge harvested cells at 200 x *g* for 2 minutes, aspirate the supernatant and gently re-suspend the cell pellet in 1ml cryoprotectant per 1x 10^6 – 2x 10^6 cells.
7. Immediately, place the cryovials into an isopropanol-containing freezing container such as Mr. Frosty™ that was pre-chilled to +5°C. Then immediately transfer the container into a -80°C freezer to ensure that the cell suspension cools down with a cooling rate of -1°C per minute. Allow the cryovials to remain at -80°C overnight (16 - 36 hours). Once frozen, transfer the cryovials into the gas phase of liquid nitrogen or a -150°C freezer for long-term storage.

8. Adaptation of EBiSC cell lines to alternative matrices and media

Where required, the matrix and media used for a specific cell line can be changed to an appropriate alternative during passage or at thaw. This process is carried out in one step by simply passaging or thawing into the new cell culture system. No guarantees can be given regarding cell viability or quality where the advised cell culture system is not used. Where possible, for example if transitioning culture conditions during passage, it is advisable to keep some cells in the original media/matrix combination until sure that iPSCs have adapted well into new conditions.

iPSC lines deposited into EBiSC may have originally been cultured under conditions differing from this protocol. Based on our experience, iPSCs can be aligned with this protocol by simply applying the procedures for thawing and culturing the iPSCs according to this protocol. If you need any additional advice, please get in touch via Contact@EBiSC.org.

9. Promoting Vector Clearance

In some occasions it is possible that the vectors used for reprogramming the primary material have not cleared. In order to promote clearance of reprogramming vectors the split ratio at passaging can be increased. Higher split ratios are used to dilute the vectors out of the cells and after a few passages this should be successful. Split ratios of up to 1:50 are possible but this is cell line dependent and it is advisable to have a parallel well with a normal split ratio as back-up. Manual passaging or selective passaging can be used as an alternative. However, there is no guarantee that every cell line can be cleared.

10. Precautions and troubleshooting tips

Problem	Observation	Possible Solution/Precaution
Low viability of iPSCs after thaw	• Little to no colonies visible within 4 days after recovery	• Ensure that cryovials are thawed quickly and that medium is added to the cells very slowly (drop-wise while gently swirling the tube) • Add 10 µm ROCK inhibitor at thaw • Ensure that cells were banked at log phase of growth with low levels of differentiation • Let small colonies grow until robust and passage with low split ratio (1:1 or 1:2)
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 • Test another culture treated dish brand • Ensure cells are in log phase of growth at passaging • Work quickly or reduce incubation time of EDTA as clump size could be influenced by too long exposure to EDTA • Increase incubation time of EDTA if cells do not come off easily. This is to avoid having to harshly rinse cells off thereby creating too small aggregates/ single cell suspension. Make sure to tap the plates carefully on the bench to ensure a sufficent detachment from the surface. • Check if plates were coated correctly, Matrigel stocks are within expiry date and check batch with manufacturer if this issue occurs on a regular basis and other reasons have been excluded • Test another Matrigel lot

Problem	Observation	Possible Solution/Precaution
Spontaneous differentiation	• Colonies do not have defined edges • Cells within the colonies are less compact • Cells appear flattened and bigger or fibroblastic	• Ensure cells are being cultured using recommendations given here (i.e. daily feeding of cells) • Ensure that reagents are freshly prepared (i.e. used within two weeks) • Avoid leaving dishes outside the incubator to decrease temperature fluctuation and exposure to light. • Decrease colony density by plating fewer cell aggregates per cm^2 during passaging • If good iPSC colonies persist between differentiated areas manual picking of colonies with good iPSC morphology using a pipette tip can be considered. It is recommended to select several colonies and cut them in pieces with a pipette tip, lift them, aspirate them and then pass them to a fresh 60mm plate. • Removal of differentiated cells by scraping the differentiation away with a pipette tip leaving iPSC colonies intact can be considered. Care must be taken not to disturb the iPSC colonies and not to scrape away too much of the matrix layer in this process.

Problem	Observation	Possible Solution/Precaution
Non-uniform distribution of colonies within plate	• Areas with too high a density of iPS cells and where cells start to differentiate from the middle. Additionally to areas with hardly any colonies	• Make sure that the whole surface area of the cell culture vessel is coated with Matrigel. • Ensure that the cell aggregates are evenly distributed by gently rocking the plate back and forth and side to side • Take care when placing plate into the incubator and leave undisturbed for 24 hours • Passage the plate and seed to a new plate to avoid further differentiaton of the dense areas.
Significant scraping is required to dislodge cells	• Colonies do not come off the plate with 2 - 3 rinses with a 5ml pipette	• Ensure that incubation time and temperature of EDTA are in accordance with matrix • Increase incubation time of EDTA • Do not let cells become more than 70% confluent • Do not let colonies become overgrown in the centers, sometimes it is necessary to passage a less confluent plate with fewer but robust colonies, using lower split ratio
Poor attachment and significant increase in cell death post-passage	• Cells start to lift off even though they seemed to attach after passage	• Rather than exchanging medium top up plates with fresh medium to ensure sufficient amount of nutrients and leave cells undisturbed for an additional 24 hours to allow aggregates to fully attach • Exchange medium very gently, do not subject colonies to excessive shear forces by rapid addition of medium

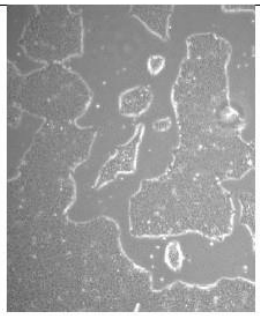
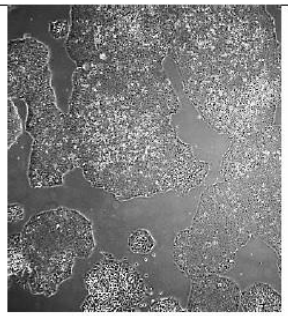
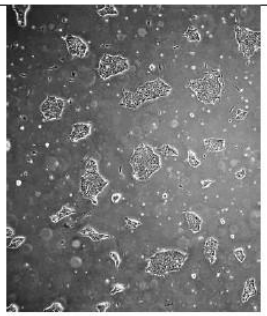
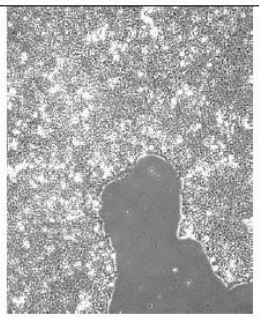
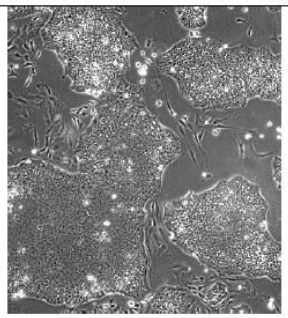
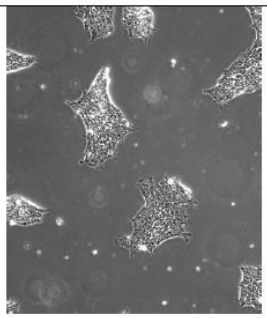
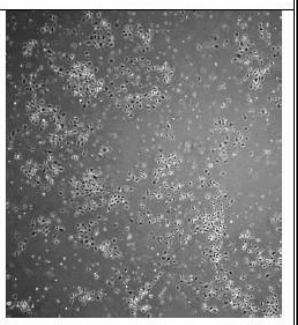
11. Contact details

In case of queries, please contact:
European Collection of Authenticated Cell Cultures (ECACC)
Culture Collections, UK Health Security Agency, Porton Down, Salisbury, SP4 0JG, UK
Telephone: +44 (0) 1980 612684
Website: www.culturecollections.org.uk\ebisc
Technical support: www.culturecollections.org.uk/contactus/technical-enquiries.aspx

12. Sources of documents / related information

1. **Material Safety Data Sheet (MSDS)** for EBiSC iPSCs – available online on the specific cell line detail webpage at: www.culturecollections.org.uk
2. **Protocol for the Use of EBiSC induced Pluripotent Stem Cells** (this document) - available online on the specific cell line webpage at: www.culturecollections.org.uk
3. **Certificate of Analysis** – download online by visiting the specific cell line webpage at www.culturecollections.org.uk then clicking the Certificate of Analysis icon and entering the batch number of the cells, found on the EBiSC vial label.

13. Appendix A

	A	B	C	D
	Optimal, compacted iPSC colonies with defined edges; morphology uniform across colonies	Acceptable iPSC colonies with some differentiation around the edges, cells more loosely packed within colonies	Good adherence with iPSC colonies emerging	Poor adherence and no obvious iPSC
X4				
X10				

14. Appendix B