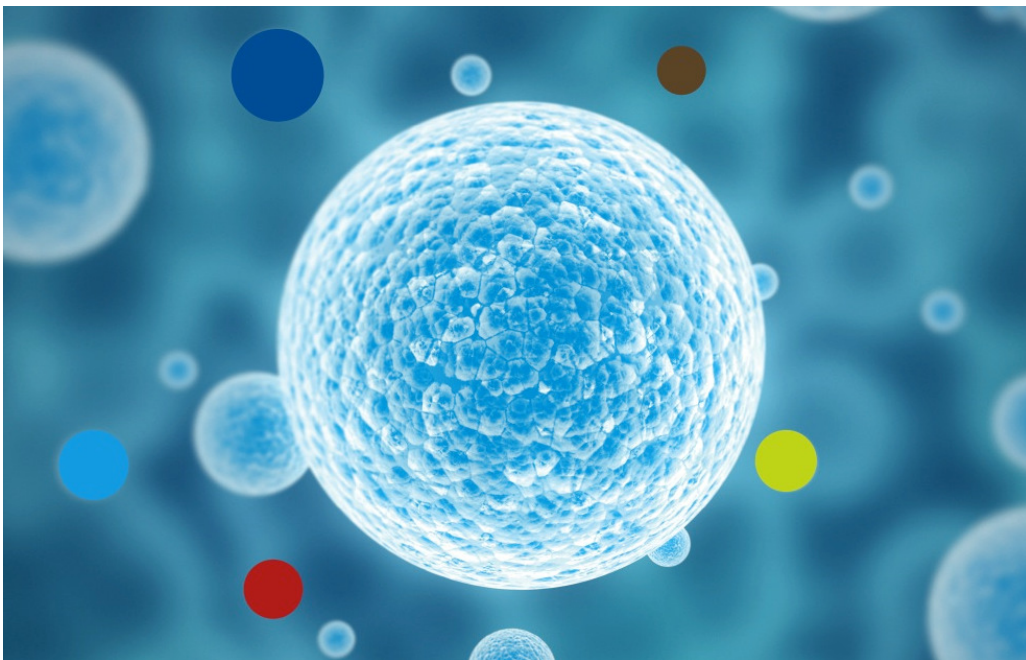


PROTOCOL FOR THE USE OF INDUCED PLURIPOTENT STEM CELLS



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www.imi.europa.eu

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User Protocol

for human induced pluripotent stem cells



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. iPSCs do not grow in standard media such as DMEM.
- Use the correct media and matrix combination (information found on Certificate of Analysis).
- 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 well (9.5 cm²) of a 6-well plate.

All cell manipulations, tissue culture vessel preparations and medium preparations should be performed under aseptic conditions within a Class II Microbiology Safety Cabinet (MSC). 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 an MSC 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

6 well tissue culture treated plate (Greiner Bio One Ltd Cat. No. 657160 or similar)

- Culture matrix and diluents
 - Matrigel (BD Cat. No. 354230 (10ml)) and DMEM (Lonza Cat. No. BE12-719F-1-12) **or**
 - Geltrex (Life Technologies Cat. No. A1413302 (5ml)) and DMEM/F12 (Life Technologies Cat. No. 11320-074) **or**
 - Vitronectin (Life Technologies Cat. No. A14700 1ml at 0.5mg/ml) and PBS (-/-) (Lonza Cat. No. BE17-516F)
- Culture medium
 - mTeSR1 culture medium (StemCell Technologies Cat. No. 05850) **or**
 - Essential 8 medium kit (Life Technologies Cat. No. A1517001)
- DMEM (for example Lonza Cat. No. BE12-719F-1-12)
- DMEM/F12 (for example Life Technologies Cat. No. 11320-074)
- PBS (-/-) (for example Lonza Cat. No. BE17-516F)
- EDTA (for example Life Technologies Cat. No. AM9260G (100ml) AM9261 (500ml) AM9262 (1L))
- Cryostor Cryopreservation Medium (Sigma-Aldrich, Cat. No. C2874)
- DMSO (for example Sigma-Aldrich Cat. No. D2438 (50ml))
- FBS of US or Australian origin (Sigma-Aldrich, Cat. No. F2442 (500ml))
- ROCK inhibitor (for example Sigma-Aldrich Cat. No. Y0503-1mg)

4. Matrix preparation

Stock vials of Matrigel and Geltrex should be thawed overnight on ice or within a refrigerator (5°C) prior to use. Culture vessels, tubes and stripettes™ should be pre-chilled prior to making aliquots or coating. All manipulations of Matrigel and Geltrex must be carried out on ice-packs to avoid premature gelling. Do not repeatedly freeze thaw stock or working vials of Matrigel or Geltrex.

Preparation of Matrigel

1. Upon receipt, store Matrigel at -20°C.
2. Protein concentration within the Matrigel stock vial is batch dependent and must be obtained from its accompanying certificate of analysis. Use this concentration to calculate the volume of Matrigel required for 2mg protein / working vial.
3. Transfer 2mg of protein into pre-chilled 15ml tubes. These tubes are working aliquots and should be stored at -20°C until required.
4. When required, thaw the working Matrigel aliquot in the fridge (5°C) overnight. Add 6ml of cold (5°C) DMEM to the vial and mix by pipetting up and down thoroughly. This is enough for a whole 6-well plate as 1 ml of the Matrigel/DMEM mix is required for one well of a 6-well plate. Chilled, diluted Matrigel must be used immediately to coat ice-cold tissue culture vessels. Then the vessel should be incubated at 37°C / 5% CO₂ for 1 hour and equilibrated to room temperature 30 minutes prior to use. Alternatively, vessels can be sealed with Parafilm™ after the incubation at 37°C / 5% CO₂ and stored at 5°C for a maximum of one week. Ensure stored vessels are equilibrated to room temperature 30 minutes prior to use.
5. Prior to use aspirate Matrigel from the vessel using a stripette™ or similar, wash vessel with 1ml of DMEM and replace with an appropriate volume of culture medium (2ml of medium per well of a 6-well plate). Vessels are now ready for cell culture use.

Preparation of Geltrex

1. Upon receipt Geltrex should be stored at -80°C. After thawing, mix 5ml of Geltrex stock solution with 5ml of ice-cold DMEM-F12. Make 180µl aliquots of diluted Geltrex in pre-chilled 15ml tubes. These tubes are working stocks and should be stored at -20°C.
2. When required, thaw the working Geltrex aliquot at 5°C overnight. To dilute the working stock for use, add 8.82ml of ice-cold DMEM-F12 to the vial using a chilled stripette™ and mix by pipetting up and down thoroughly. This is enough for a whole 6-well plate as 1.5 ml of chilled, diluted Geltrex is required for one well of a 6-well plate.
3. After adding the diluted Geltrex to the required wells the vessel should be incubated at 37°C / 5% CO₂ for 1 hour and equilibrated to room temperature 30 minutes prior to use. Alternatively, vessels can be sealed with Parafilm™ after the incubation at 37°C / 5% CO₂ and stored at 5°C for a maximum of two weeks. Ensure stored vessels are equilibrated to room temperature 30 minutes prior to use.

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4. Prior to use aspirate the solidified Geltrex from the vessel, wash the vessel with 1ml of DMEM-F12 and replace with an appropriate volume of culture medium (for example 2ml of medium per well). Vessels are now ready for tissue culture use.

Preparation of Vitronectin

1. Upon receipt, store Vitronectin at -80°C . Prior to use, thaw the stock vial of Vitronectin at room temperature and prepare 60 μl aliquots in sterile polypropylene tubes and freeze the aliquots at -80°C or use immediately. One 60 μl aliquot is sufficient for coating all wells of a 6-well plate.
2. To prepare Vitronectin at a working concentration of $0.5\mu\text{g}/\text{cm}^2$, dilute the Vitronectin 1:100 by gently mixing 6ml of room temperature PBS (-/-) with 60 μl of Vitronectin. Add 1ml of diluted Vitronectin to each well of a 6-well plate.
3. Incubate the coated culture vessels at room temperature for 1 hour. If storage is required, vessels can be sealed with ParafilmTM and stored at 5°C for up to 3 days. Allow the vessel to equilibrate to room temperature for 1 hour prior to use.
4. To prepare the vessel for culture, remove the excess Vitronectin from the culture vessel and discard. It is not necessary to wash the culture vessel after the removal of Vitronectin.

5. Medium preparation

mTeSR1

1. When required, remove the mTeSR1 supplement (5x) from the freezer and thaw overnight at 5°C prior to use. Do not thaw at 37°C.
2. Aseptically add 100ml of mTeSR1 supplement (5x) to 400ml of cold (5°C) basal medium.
3. Aliquot medium into volumes required for 1 week of culture work.
4. Complete mTeSR1 may be stored at 5°C for 1 week or at -20°C for 6 months. Frozen complete mTeSR1 may be thawed once. Do not repeatedly freeze thaw medium. Prior to use, warm mTeSR1 to room temperature, do not leave medium at room temperature for longer than 2 hours per day and avoid exposure to light to avoid degradation of medium components.

Essential 8 (E8)

1. When required, remove the E8 supplement (50x) from the freezer and thaw overnight at 5°C prior to use. Do not thaw at 37°C.
2. Aseptically remove 10ml of E8 basal medium to leave 490ml.
3. Add 10ml of E8 supplement (50x) to the 490ml of basal cold (5°C) medium.
4. Aliquot medium into volumes required for 1 week of culture work.
5. Complete E8 may be stored at 5°C for 1 week or at -20°C for 6 months. Frozen complete E8 may be thawed once. Do not repeatedly freeze thaw medium. Prior to use, warm E8 to room temperature, do not leave medium at room temperature for longer than 2 hours per day and avoid exposure to light to avoid degradation of medium components.

6. Thawing human iPSCs

1. Cells should be thawed rapidly by placing the cryovial in a water bath set to maintain 37°C. Swirl the cryovial gently in the water bath to ensure rapid thaw but do not submerge the cap of the cryovial. Disinfect the cryovial with 70% alcohol or an equivalent disinfectant before opening.
2. Using a 5ml sterile stripette™, transfer the cryoprotectant/cells mix from the cryovial into a 15ml centrifuge tube. Care should be taken not to physically damage cells.
3. Slowly, drop by drop, add 10ml of appropriate medium at room temperature to the cells in the 15ml centrifuge tube. Gently rock the 15ml centrifuge tube back and forth while adding drops to minimise osmotic shock to the cells. This is a crucial step and cells should be treated as gently as possible.
4. Check tube to ensure all cell contents are removed and if not, rinse with 1ml of appropriate medium.
5. A small amount of cells can be used for performing a cell count. A single cell suspension should be created using trypsin or similar. As a general guideline the seeding density range for one well of a 6-well plate is between 2×10^5 - 1×10^6 viable cells. Refer to CoA for guidelines for a specific EBiSC cell line lot number.
6. Centrifuge the cells at $200 \times g$ for 2 minutes. Remove and discard the supernatant.
7. Prepare culture vessels by adding an appropriate amount of medium (for example 1.5 - 2ml per one well of a 6-well plate)
8. Gently tap the 15ml centrifuge tube to dislodge the cell pellet then gently add 1ml of appropriate medium and seed into 2 wells of a coated 6-well plate (adjust if using other culture formats or if advised differently in the Certificate of Analysis). Do not over aspirate the cells as this will lead to decreased viability due to generation of a single cell suspension.
9. Gently rock plate side to side, back and forth to spread the cells evenly across the well.
10. It is advisable to record images of cells immediately post-thaw, at 48 hours and at approximately 70-80% of confluence.

7. 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 (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, cells 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 95% of the medium from the wells using an aspirator pipette. Do not completely remove the medium; a thin film of medium should cover the cell layer to avoid drying out the cells.
3. Aseptically add 2ml of fresh medium per 1 well of a 6-well plate by gently adding to the side of the well. Incubate cells at 37°C / 5 % CO₂.
4. Typically, medium exchanges occur daily on six of seven days with increased volume of media (1.5x - 2x the normal amount; cell density dependent) if cells need to be left for longer periods between media changing. Do not leave more than two days between medium exchanges.

8. Passaging human iPSCs using EDTA

NOTE: Passaging method is determined by growth matrix. Read explanation below carefully.

Typically established cultures can be split 1:4 to 1:8 (i.e. transferring all colonies from one well to four or up to eight) but the exact split ratio can vary for each cell line. Refer to the Certificate of Analysis for recommended split ratios for your cell line. Split ratios can be adjusted to ensure cells are passaged within 4-5 days of culture in order to maintain log phase of growth. Cell lines should be passaged when the cells are approximately 70-80 % confluent 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. Passage cells with 0.5mM EDTA by first removing spent medium from each vessel requiring passage.
2. Cells growing on Geltrex or Matrigel should be washed with 1ml of 0.5mM EDTA per well. For cells growing on Vitronectin, wash each well with 1ml of PBS (-/-). Quickly aspirate the EDTA or PBS from the well and repeat the wash step.
3. Add 1ml of 0.5mM EDTA to each culture vessel and incubate at 37°C / 5.% CO₂ for 4 minutes with Geltrex or Matrigel and for 5 minutes at room temperature with Vitronectin. Ambient temperature can influence EDTA efficacy and incubation time should be optimized
4. On completion of incubation time, check the cell layer under an inverted microscope to see if the colonies have feathered edges and holes throughout. This indicates that cells have detached from each other but not from the culture vessel and are ready to be removed from the 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. Take care as the cells are loosely attached. Immediately add 1ml of desired media to 1 well in order to neutralise the EDTA.
5. Gently wash the cells from the culture vessel using a 1ml sterile pipette. Three gentle aspirations of the well or flask should suffice. These aspirations will dislodge cell clusters, ideally leaving a proportion of differentiated cells attached. Excessive aspiration 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.
6. 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 plate coated with the matrix of choice containing an appropriate volume of desired medium.
7. To ensure even distribution of cell clusters, gently disperse the clusters by carefully moving the vessel side to side, back and forth several times before placing the vessel in an incubator maintained at 37°C / 5% CO₂.

9. Thawing and passaging with ROCK inhibitor

1. Normally we do not recommend the use of selective Rho-associated kinase (ROCK) inhibitor (Y27632) inhibitor. However, in a case of poor cell survival after dissociation ROCK inhibitor can be used to offset the effect of apoptosis. Additionally ROCK inhibitor enhances cell survivor after cryopreservation and can therefore be used during the initial thaw. This could be an option for inexperienced users to enhance their chances of success.
2. When thawing or passaging cells supplement appropriate medium with ROCK inhibitor to maintain 10 μ M final concentration. To obtain the required concentration dilute 10mM of stock ROCK inhibitor 1:1000 in cell culture medium, for example for 1ml of medium add 1 μ l of 10mM ROCK inhibitor.

Reconstituting ROCK inhibitor

1. ROCK inhibitor is light sensitive hence special care should be taken to avoid and minimize potential exposure to light.
2. Centrifuge stock vial for a few seconds to bring down the powder to the bottom of the vial, spray and wipe the vial with 70% ethanol and place it in the microbiological safety cabinet.
3. Add appropriate volume of sterile molecular grade water to lyophilized ROCK inhibitor. Calculate the required volume as per formula below
4.
$$\text{Mass [g]} = \text{Molarity} \left[\frac{\text{mol}}{\text{l}} \right] \times \text{Volume [l]} \times \text{Molecular Weight} \left[\frac{\text{g}}{\text{mol}} \right]$$
5. Mix thoroughly, incubate for 3 minutes at room temperature and aliquot into appropriate size aliquots.
6. Store aliquots at -20 °C and avoid repeated thawing and freezing.
7. Once thawed aliquots may be kept at 5°C for a week.

10. Cryopreserving human iPSCs

1. Keep reagents and freezing container (e.g. Mr. Frosty™) chilled during the cryopreservation procedure.
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. The type of cryoprotectant medium used depends on culture conditions and laboratory preferences. Use either commercially available Cryostor CS10 or DMSO based freeze mix (10% DMSO in FBS and culture medium). Cryostor is supplied ready to use and is stored at 5°C. To prepare DMSO based cryoprotectant, mix 40% FBS with 10% DMSO, then mix with 50% appropriate medium.
4. Remove spent medium from the tissue culture vessel and wash the vessel twice with the recommended volume of wash buffer depending on culture conditions (Wash buffer for Geltrex/Matrigel is 0.5mM EDTA, wash buffer for Vitronectin is PBS -/-).
5. To lift the cells from the tissue culture plastic, add 1ml of 0.5mM EDTA to the tissue culture vessel. Incubate the cells for the recommended time and temperature, depending on matrix used. Aspirate the EDTA from the well. Care must be taken as the colonies are very loosely attached to the plastic.
6. Afterwards add 1 ml of cryoprotectant per 1 well. Gently wash the cryoprotectant over the vessel with a 1ml sterile pipette to dislodge the cells from the plastic. Do not aspirate more than 3 times to avoid breaking the cell clumps into single cells. Place the cryoprotectant and cell mix into an appropriately labelled cryovial.
7. If cryopreservation of more wells is desired, cells from the same passage number and culture condition should be pooled together. An aliquot of pooled cells can be used for a cell count. Centrifuge harvested cells at 200 x g for 2 minutes, aspirate spent medium and gently re-suspend the cell pellet in an appropriate volume of cryoprotectant. One well of a 6-well plate gives rise to approximately $1-2 \times 10^6$ cells. It is recommended to freeze around $1-2 \times 10^6$ cells per cryovial. Use 1ml of cryoprotectant-cell mix per cryovial.
8. Immediately place the cryovials into a pre-chilled Mr Frosty tub (5°C) then immediately transfer the Mr Frosty tub 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).

11. 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 tissue culture system. No guarantees can be given regarding cell viability or quality where the advised tissue culture system is not used.

12. 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 vector clearance 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.

13. Precautions and troubleshooting tips

Problem	Observation	Possible Solution/Precaution
Low viability of iPSCs after thaw	<ul style="list-style-type: none"> • Little to no colonies visible within 4 days after recovery 	<ul style="list-style-type: none"> • Ensure that cryovials are thawed quickly and that medium is added to the cells very slowly (drop-wise while gently swirling the tube) • Add 10µm ROCK inhibitor at thaw but do not use routinely • 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	<ul style="list-style-type: none"> • Cells do not attach properly • Non-typical morphology • High levels of cell death • Cells do not proliferate 	<ul style="list-style-type: none"> • Use lower split ratio and maintain a more confluent culture • 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 an 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 • Check if plates were coated correctly, matrix is within expiry date and check batch with manufacturer if this issue occurs on a regular basis and other reasons have been excluded
Spontaneous differentiation	<ul style="list-style-type: none"> • Colonies do not have defined edges • Cells within the colonies are less compact • Cells appear flattened and bigger or fibroblastic 	<ul style="list-style-type: none"> • 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 plates outside the incubator to decrease temperature fluctuation and exposure to light. • Decrease colony density by plating fewer cell aggregates per cm² during passaging

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Problem	Observation	Possible Solution/Precaution
		<ul style="list-style-type: none"> 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 1: 6 well. 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.
Non-uniform distribution of colonies within plate	<ul style="list-style-type: none"> 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 	<ul style="list-style-type: none"> Make sure that the whole surface area of the tissue culture vessel is 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 Take care when placing plate into the incubator and leave undisturbed for 24 hours
Significant scraping is required to dislodge cells	<ul style="list-style-type: none"> Colonies do not come off the plate with 2- 3 rinses with a 1ml pipette 	<ul style="list-style-type: none"> 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	<ul style="list-style-type: none"> Cells start to lift off even though they seemed to attach after passage 	<ul style="list-style-type: none"> Rather than exchanging medium top up wells 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

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14. Contact details

In case of queries, please contact:

European Collection of Authenticated Cell Cultures (ECACC)
Culture Collections, Public Health England, Porton Down, Salisbury, SP4 0JG, UK

Telephone: +44 (0) 1980 612684

Email: culturecollections.technical@phe.gov.uk

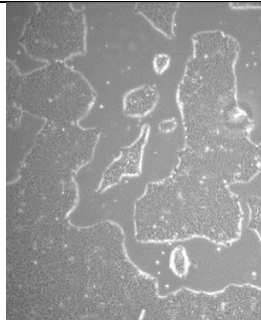
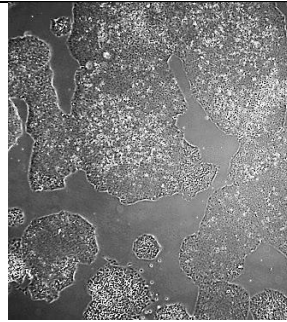
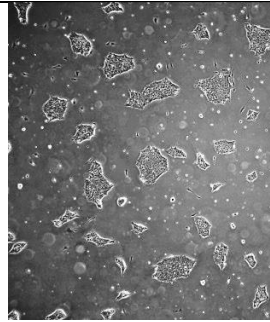
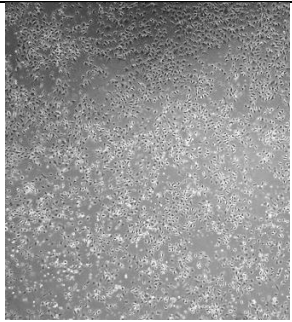
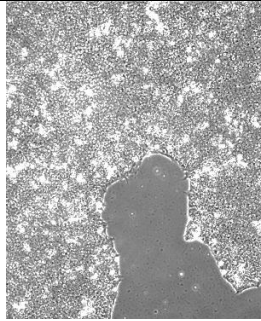
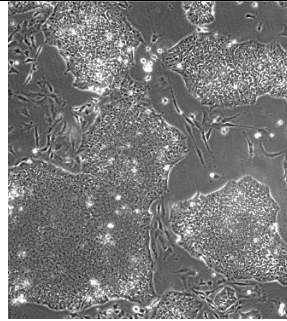
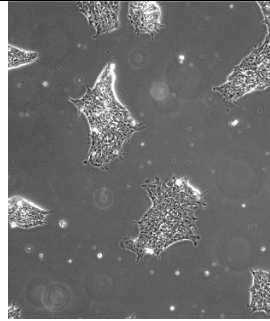
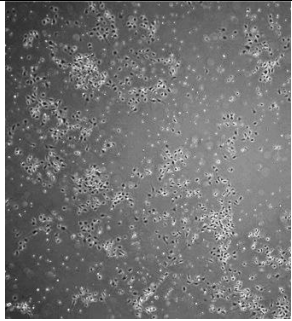
Website: www.phe-culturecollections.org.uk/ebisc

15. Sources of documents / related information

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

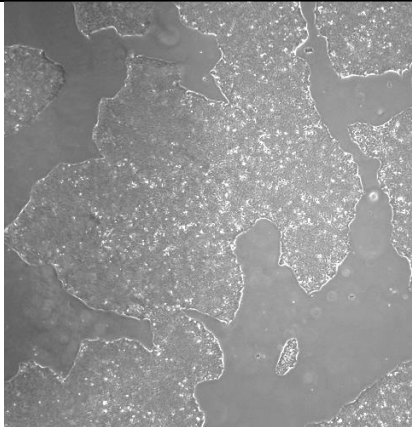
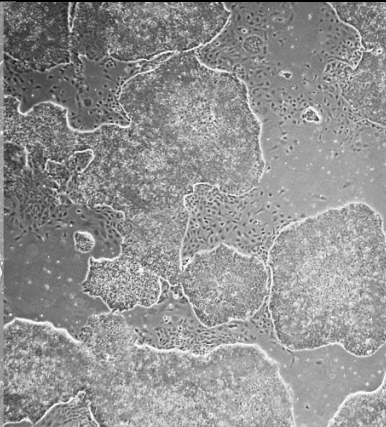
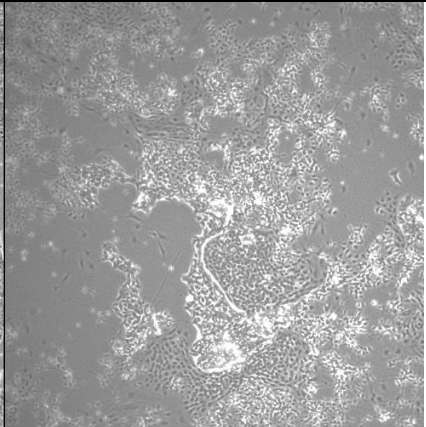
16. Appendix A

Examples of scoring of iPSC colonies

	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				

17. Appendix B

Examples of different levels of differentiated cells present in culture

	L	M	H
	Low levels of differentiated cells in culture	Medium levels of differentiated cells in culture	High levels of differentiated cells in culture
X4			
X10	