	

Standard Operating Procedure	Number: UOXFSOP19

Title: **Culture of iPSC from cryopreserved samples, and expansion to create Masterstock**

Approval	Date Implemented:		
	Name	Signature	Date
Author:	Sally Cowley	SA Cowley	02.2013
User Review			
Work Package Lead			

Change History Version No/Date Issued	**Reason for Issue/Change Summary**
v2 02.2014	Expansion of SOP19 v1 – more detail, cross-referencing to related SOPs, and specific instructions for generation of Master frozen stocks by StemBANCC partners

1.0 Purpose

StemBANCC partners will receive vials of iPSc frozen at ~passage 20. To ensure consistency across different laboratories, thawing, culture and creation of Master frozen stocks within individual labs should be as standardised as possible. Locally-produced Master iPSC stocks should undergo key QC checks.

2.0 Scope

1) Thawing iPSc from vials received from StemBANCC
2) Culture in mTeSR-1 on matrigel
3) Passaging using EDTA
4) Standardised Expansion and freezing to create Master frozen stock
5) Quality Control of cells to ensure stocks are suitable for subsequent use.

3.0 Related Documents

StemDB database
BD Matrigel hESC-qualified Matrix guidelines for use
SOP TRA-1-60 characterisation by Flow cytometry
SOP Nanog characterisation by Flow cytometry
SOP Preparation of DNA and RNA samples for Illumina arrays
Genotyping sample spreadsheet template

4.0 Responsibility

All StemBANCC staff handling iPSCS

5.0 Health Safety & Environment

See Materials notes below

6.0 Materials & Equipment

Product name	Supplier	Catalogue number
For thawing cells:		
6-well tissue culture plate	Corning (suggested)	3516
BD Matrigel™ hESC-qualified Matrix	BD Biosciences/Corning Also Fisher Scientific	354277 08-774-552
Alternative to matrigel – Geltrex® LDEV-Free hESC-qualified Reduced Growth Factor Basement Membrane Matrix	Life Tech	A1413302
KO-DMEM (for matrigel preparation)	Life Technologies	10829-018
Dulbecco's PBS	Sigma (suggested)	D8537
ROCK inhibitor Y-27632 **	AbCam	Ab 120-129
mTeSR™1 Medium	Stem Cell Technologies	05850
Penicillin/Streptomycin***	Life Technologies	15140-122
Additional reagents for passaging cells:		
10cm diameter Tissue culture dish [100 X 15mm]	Corning (suggested)	430167
EDTA (Versene, 0.02%)	Scientific Laboratory Suppliers Ltd	LZB17-711E
Alternatively, 0.5M EDTA pH8.0 (dilute 1000x to 0.5mM in PBS for working stock)	Life Technologies	15575-038

Additional reagents for freezing cells:		
HybriMax Dimethyl sulphoxide (DMSO)	Sigma	D2650
ES-qualified FCS	Life Technologies	10439-024/16141-079
COOLCELL-1C/Min	Various suppliers	BCS-136
Alternatively, Nalgene Mr Frosty	Various supplier	5100-0001
Cryovials for nitrogen vapour storage *	NUNC	37726

Materials NOTES:

- *** Vapour-phase nitrogen storage is recommended. Externally threaded cryovials are suitable for nitrogen vapour storage only. If used for liquid nitrogen storage, they should be equipped with a protective Cryoflex sleeve to prevent LN2 seepage into the vial and subsequent explosion. Preferably, use internally threaded vials with good O-ring seals. Personal Protective Equipment, including face mask should be used when handling frozen vials to minimise risk of injury, especially to face.**
- **** NB we have found that some cheap sources of ROCK inhibitor are not reliable.**
- *****Add Penicillin/Streptomycin to mTeSR before use if desired. It is supplied 100x, so add 5ml/500ml bottle. If using pen/strep routinely, periodic culture an aliquot of each cell line without antibiotics is suggested to check for any 'subclinical' infection.**

2x Freeze medium
20% DMSO
60% ES-qualified FBS
20% KO-DMEM
Store at -20 ^{O}C in 40ml aliquots; thaw completely, mix and keep on ice before use; can be refrozen several times.

7.0 Procedure

7.1 Receiving frozen iPSc

- Place cells in nitrogen storage facilities immediately. Storage at 80^{o}C should be avoided since they will rapidly deteriorate and result in no viable cells. If nitrogen storage unavailable, thaw on receipt.

7.2 Preparing Matrigel aliquots

- Thaw the Matrigel vial on ice (overnight is advisable)

NOTE: Matrigel will gel rapidly at 22°C to 35°C. Gelled Matrigel can be re-liquified if placed on ice for 24-48hours.

- Label and precool sterile cryotubes or 1.5ml eppendorfs in a rack, and pipette tips at -20^{o}C.
- Once thawed, swirl the vial to ensure the Matrigel is mixed. Spray with 70% ethanol then air dry. Keep vial on ice and always handle using sterile technique in a laminar flow cabinet.
- Dispense into appropriate aliquots using the precooled tubes. Work in small batches, to prevent the matrigel from warming up. Refreeze aliquots immediately, at -20 ^{o}C for frequent use, or -80 ^{o}C for longer-term storage. Multiple freeze thaws should be avoided. The volume of the aliquots is determined by the dilution factor

provided on the Certificate of Analysis for each batch. This is typically 270-350µl per aliquot for diluting to 25ml with medium when needed for coating plates. Other size aliquots can be made if desired, so long as they are subsequently diluted with an appropriately scaled volume of medium.

7.3 Coating plates with Matrigel

- Work quickly to keep everything cold
- Prepare a tube with the required volume of cold KO-DMEM
- Remove matrigel aliquot from freezer, spray with 70% ethanol, wipe dry
- Add 1ml KO-DMEM from the measured volume, pipette up and down to thaw and dissolve simultaneously, transfer to the prepared tube, mix.
- Plate 1ml matrigel per well of a 6-well plate.
- Return plate to 37^{O}C incubator for at least 1 hour. It may be left overnight at 37^{O}C. For longer storage, it should be removed to fridge and the plate sealed with parafilm to prevent drying out – warm before use.

NOTE: if the matrigel dries out on the plate, iPSc will not adhere to it

- Any unused diluted matrigel may be stored in the fridge for 1 -2 weeks.

7.4 Thawing iPSC

- Prepare two wells of a 6-well plate with matrigel
- Transport the cryovial to the lab on dry ice
- Prepare a 15ml centrifuge tube with 9mls PBS

NOTE: KO-DMEM may be preferable to enhance viability here, or better still mTeSR, though costly

- Defrost the cryovial in 37^{O}C water bath, swirling tube until only a small piece of ice is still visible
- Spray the outside of the tube with 70% ethanol, dry well
- Remove the cells from the vial into the PBS
- Immediately centrifuge at 400g for 5 minutes
- Prepare 4ml mTeSR/ROCKi mix (10µl 100x ROCKi stock/ml mTeSR; 100x ROCKi at 1mM = 10µM final concentration)
- Aspirate matrigel from wells, add 1.6ml mTeSR/ROCKi to 2nd well
- Aspirate supernatant from centrifuged cells
- Tap tube to resuspend cells, and add 2.4mls mTeSR/ROCKi mix to resuspend cells, but don't over pipette
- Add 2ml cells to 1st well, 0.4ml cells to second well, labelling plate with full cell-line name, passage number, date and operator initials

NOTE: Thawing to 2 wells at a 80:20 ratio is designed so that at least one well will have a high density in the case cell recovery is poor, whilst the second well serves as a back-up in case the first passage from the high-density well fails

- Feed daily 100% change of mTeSR medium. Lift any areas of gross differentiation, if necessary, using a p1000 tip

NOTE: Bring mTeSR to room temperature but don't prewarm whole bottle to 37°C, as bFGF has short half-life at 37°C

- Passage 1 well when nearly confluent

7.5 Passaging iPSC using EDTA to create a Masterstock

- Precoat a 10cm dish with 6ml matrigel
- Prewarm 0.5mM EDTA to 37°C

- Lift any areas of gross differentiation, if necessary, using a p1000 tip
- Aspirate media from nearly confluent well of iPSc, wash with 2mls of PBS/well (room temp)
- Aspirate PBS and add 1ml prewarmed EDTA/well then immediately remove
- Add 1ml EDTA, incubate 37˚C 4 – 6 min
- Remove matrigel from 10cm dish (NB don't allow to dry)
- Check cells have start to detach from each other but not from the plastic

NOTE: if cells have lifted from plastic, the supernatant can be centrifuged to retrieve cells, and ROCKi added to the medium

- Carefully aspirate EDTA and flush the loosened cells with 7 x 1ml mTeSR, using a p1000 tip, moving around the well to ensure even flushing. Don't pipette up and down multiple times, as this will result in the patches disintegrating too much.
- Transfer all to the 10cm dish

NOTE: It is recommended to allow cells to sit in the cabinet for 10 mins to allow them to settle and start to adhere evenly across the dish, before transferring back to the incubator. Moving them immediately risks them all swirling into the middle

- Transfer carefully to incubator
- Change 100% medium daily, and increase the volume to 10ml as cells start to become more confluent
- Passage to 6-8 10cm dishes when nearly confluent. These will yield ~120 -160 million cells when nearly confluent. This is sufficient to freeze at least 30 vials of cells at ~2 million cells/vial, and have sufficient left over for QC analysis.

7.6 Freezing a Masterstock of iPSc

- Prelabel enough cryovials with printed labels including cell line name, passage number, date and operator initials, and chill in cryovial racks at -20°C
- Prewarm 0.5mM EDTA to 37°C
- Remove 1ml medium from batch of cells for mycoplasma testing
- Lift any areas of gross differentiation, if necessary, using a p1000 tip
- Aspirate media from nearly confluent wells of iPSc, wash with 10mls of PBS/10cm dish (room temp)
- Aspirate PBS and add 5ml prewarmed EDTA/dish then immediately remove
- Add 5ml EDTA, incubate 37˚C 4 min
- Check cells have start to detach from each other but not from the plastic
- Carefully aspirate EDTA and flush the loosened cells with 3 x 1ml **COLD** mTeSR/dish using a p1000 tip, moving around the well to ensure even flushing
- Pool cells from all dishes. Remove enough for QC analysis (and put on ice; see section 7.7). Mix remainder with an equal volume of ice-cold 2x freeze medium and aliquot 1ml/vial. For example, 6 plates creates 18ml cells @~ 2 million/ml; remove 3ml for QC, add 15ml freeze medium to remainder.
- Transfer to chilled Mr Frosty (prepared according to manufacturer's instructions) or COOLCELL and place in -80°C freezer (if freezing less than 12 vials in COOLCELL, fill each empty well with COOLCELL filler vials). This allows controlled reduction of temperature at -1°C/min. Cells should be transferred to nitrogen storage the next day. If the cells are left at -80°C for more than a couple

of days, they will deteriorate, and extended storage at -80°C will result in no viable cells.

7.7 Quality Control analyses

- The 3ml aliquot of cells removed during freezing Masterstock of cells can be used for QC analyses. See other SOPs on how to carry out the analyses.
- Remove 50ul cells for counting.
- Spin remaining 3ml cells at 400g 5 mins, resuspend in PBS then aliquot to 3 sterile, DNase/RNase-free eppendorf tubes and spin at 400G for 3 mins to pellet cells. Remove supernatant completely.
- Freeze two tubes at -80°C: 1 for DNA/RNA extraction later, and 1 as a backup. DNA can be submitted for SNP analysis to check for any chromosomal changes.

NOTE: G-banding on fixed metaphase cells is an alternative to SNP analysis. There are advantages and disadvantages to both – ideally both would be performed, but budgets do not always allow for this.

- Resuspend the cells in the 3rd tube in 4% Paraformaldehyde to fix the cells for FACs analysis, incubate at RT for 10 mins, then spin and resuspend in 0.5ml sterile PBS. Store at 4°C until Tra-1-60 FACs staining is performed (0.1% sodium azide [note: hazardous chemical] should be added if staining will be delayed for more than a couple of days).

NOTE: If FACs facilities are not available, Tra-1-60 staining of cells in situ is advisable as an alternative QC.

- Thaw a vial of the frozen batch of iPSc to assess viability. Count an aliquot of the thawed cells before plating (include live/dead assessment and % in clumps), and assess the % confluence at 24h post-thaw.

8.0 Definitions

N/A

9.0 Troubleshooting

N/A

10.0 Appendices

N/A