



Induced pluripotent Stem cell (iPSC) derivation pipeline



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Quick introduction to the processes

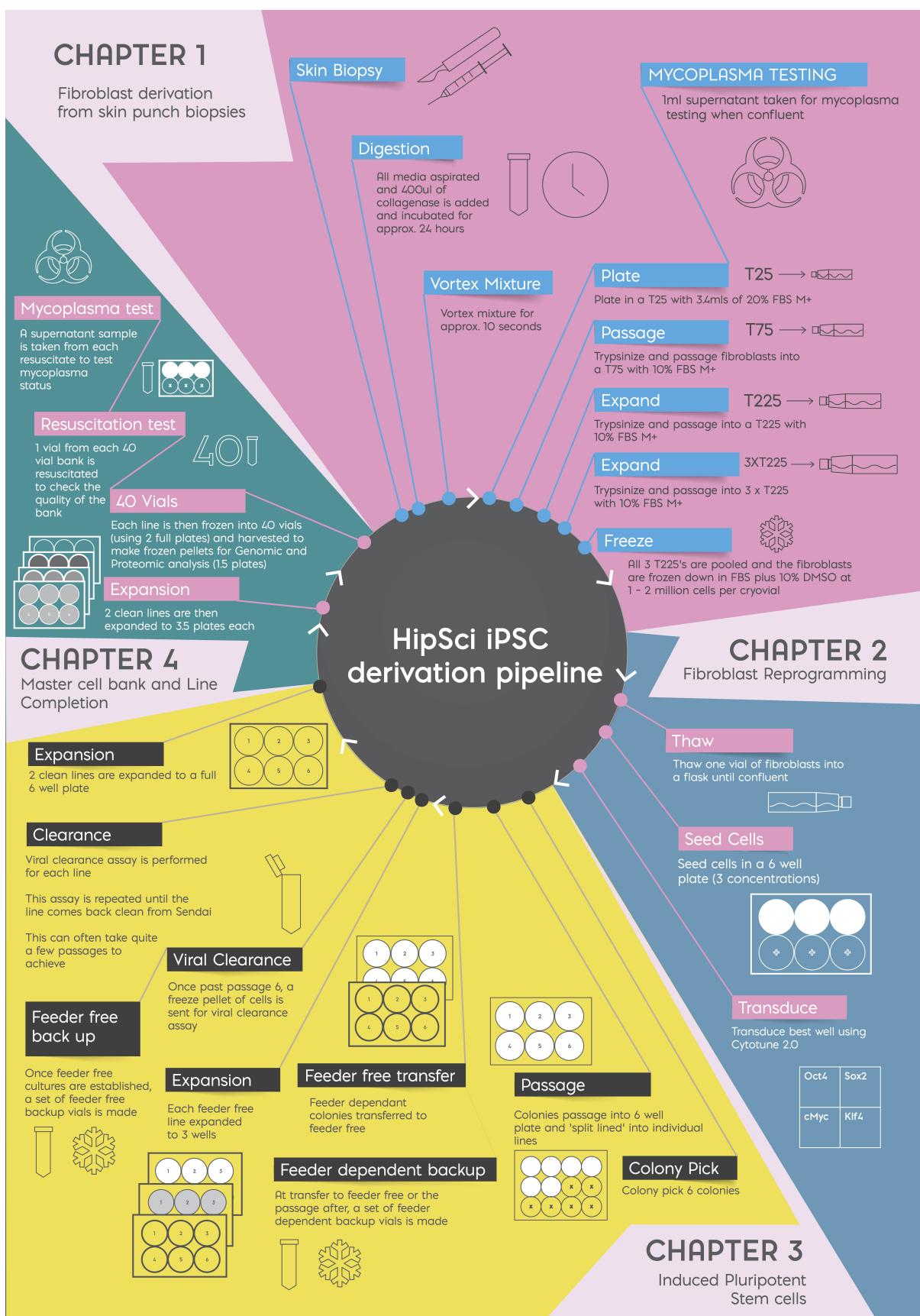
The main goal of the HipSci team within the Cellular Generation and Phenotyping department (CGaP) (<http://www.sanger.ac.uk/science/groups/cellular-generation-and-phenotyping>) is to reprogram primary fibroblasts derived from skin punch biopsies, to functioning induced pluripotent Stem cells (iPSCs). In some rare cases, we have taken receipt of blood samples, and in a similar way reprogrammed the erythroblasts obtained from the blood to iPSCs (in depth procedures and processes using blood are not covered in this document as the main focus of the HipSci pipeline is iPSC derivation from skin biopsies).

A biological sample enters the pipeline by receipt of a small skin punch biopsy (usually ~2 mm in size). This biopsy is dissected, originally using a manual process, but in the last year we have moved to an enzymatic method. These samples are then plated into a tissue culture dish and within a matter of days, fibroblasts are observed. The fibroblasts are then cultured until sufficient numbers are obtained to cryogenically freeze stocks.

In the rare cases we have received blood, we extract peripheral blood mononuclear cells (PBMCs). Using these PBMCs we enrich for erythroblasts, which are the cell type used in the reprogramming step. All primary samples received are screened for mycoplasma before progressing any further through the pipeline.

Fibroblasts or PBMCs are thawed, transduced using Cytotune 2.0 Sendai virus (containing the Yamanaka genes encoding transcription factors Oct4, Sox2, cMyc and Klf4) and maintained until iPSC colony formation. Colonies are then picked and cultured to obtain a sizable yield of IPS cells, which are banked to a commercial grade standard. These banks then undergo quality checks to ensure the banks pass resuscitation tests and are free of mycoplasma.

All processes performed in the pipeline, except media change, are logged in a Laboratory Information Management System (LIMS). LIMS is a database to help report the pipeline metrics and is vital for sample tracking and ethical compliance.



Chapter one

Fibroblast derivation from skin punch biopsies

Receiving sample

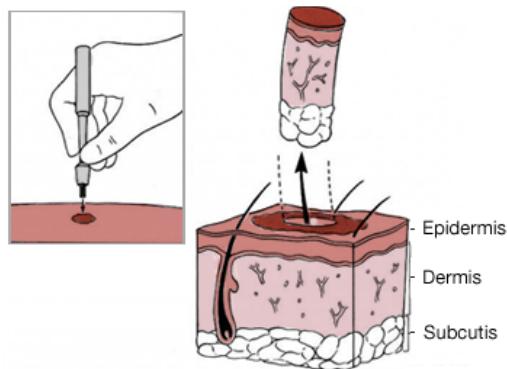


Figure 1 – Schematic Representation of a skin punch biopsy
(<http://www.dermatology.ucsf.edu/skincancer/general/detection/biopsy.aspx>)

A ~2 mm skin punch biopsy from an anonymised patient is received and registered into the LIMS before proceeding into the quarantine laboratory. All samples of genetic material (including blood) coming into the CGaP department must have a mycoplasma test performed on them in quarantine before being released into the main laboratory areas.

Dissection of Biopsy

The biopsy is placed within a sterile class II microbiological cabinet, which has been deep cleaned prior to use. Either, the biopsy is removed from the vessel, or the media, in which the biopsy was transported in, is removed from the vessel and the biopsy is incubated in collagenase overnight. The following day the biopsy is vortexed to break up the biopsy as much as possible before resuspending in fibroblast culture media and transferring to a T25 flask. The flask is then kept in a quarantine incubator set at 37°C and 5% CO₂.

The morphology and growth of the cell population must be closely monitored and screened for the presence of any microbiological contaminants regularly. Care must also be taken in the first few days to allow time for the cells to attach.

Fibroblast Derivation

We would hope to observe fibroblast attachment within the first 3-4 days. If a healthy level of cells is observed, the media can be replenished or changed. Approximately 1-2 weeks after dissection, the cells are usually sufficiently confluent to passage.

These cells are transferred to a larger vessel with each passage, and this process of expansion is repeated until three T225 flasks are confluent with healthy fibroblasts. From these three flasks at

least six vials are frozen down at $1-2 \times 10^6$ cells per vial in Foetal Bovine Serum (FBS) and 10% DMSO. These are cryogenically stored until required.

Chapter two

Fibroblast Reprogramming

One vial from the primary cell stock is thawed into a T75 - T150 flask, depending on the cell count and viability obtained from the frozen vial. Medium is changed on a regular basis (every 3-4 days) until the fibroblasts are healthy and around 70-80% confluent.

The fibroblasts are seeded across 3 wells of a 6 well plate at a density of 0.5, 0.75 and 1×10^5 and incubated at 37°C and 5% CO₂ for 48-72hrs (the timing of this is dependent on what makes the most logistical sense in terms of when other samples in the department are ready). The remaining cell suspension is pelleted (the minimum pellet size required is 2×10^6 , if too few cells are obtained they are re-seeded into a relevant sized flask and another attempt is made when confluent). The pellet is snap frozen and stored at -80°C until required for shipment to DNA Pipelines. This pellet functions as the control sample for comparison in the PluriTest and in the identity checks to ensure that no samples swaps have occurred during the life of the donor in the pipeline.

Transduction

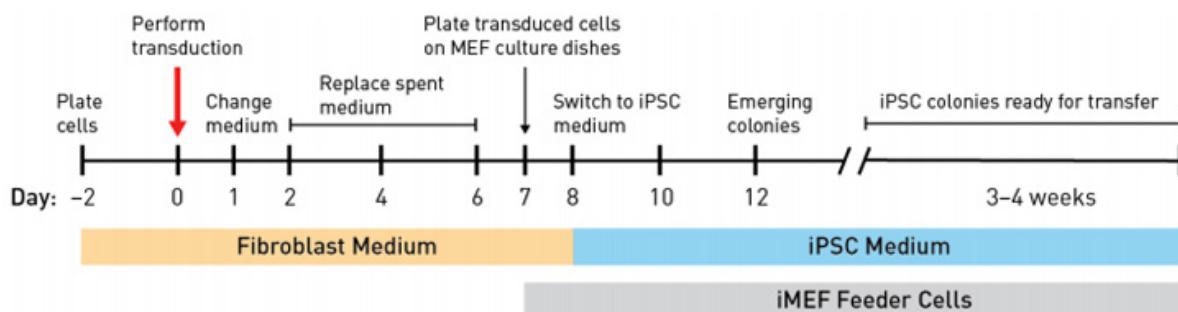


Figure 2 – Timeline for Cytotune 2.0 Sendai reprogramming of fibroblasts (Thermo Fisher Scientific CytoTune 2.0 manual, https://tools.thermofisher.com/content/sfs/manuals/cytotune_ipsc_2_0_sendai_reprog_kit_man.pdf).

On the day of reprogramming, using the Thermo Fisher Scientific product; CytoTune 2.0™, a master mix of the Sendai vectors is made up in an appropriate volume of medium to allow for the multiplicity of infection (MOI) of 5:5:3 as given below:

hKOS is used at a MOI of 5

cMyc is used at an MOI of 5

Klf4 is used at an MOI of 3

One well out of the three is selected, and selection is based on a confluence between 50-80%. The medium is aspirated and the master mix added to the selected well. This remains in place for

approximately 24 hour, after which it is removed and fresh standard fibroblast medium is replaced. Medium is changed every two days until day seven.

Seven days after reprogramming the transduced fibroblasts are removed and plated onto a 10 cm Mouse Embryonic Fibroblast (MEF) feeder plate at 1.5×10^5 cells (the remaining cells are discarded). Medium is changed to iPSC medium after 24 hours and changed daily from this point on.

Chapter Three

Induced Pluripotent Stem cells

Colony Picking

From around day twelve colonies start to form and after a total of 3 – 4 weeks these colonies should be ready to pick (See Figure 3 below for an example colony). Colonies must not be picked before day 21, as they will not be established enough to produce healthy clones. For each donor, up to six good quality colonies are picked, where possible.

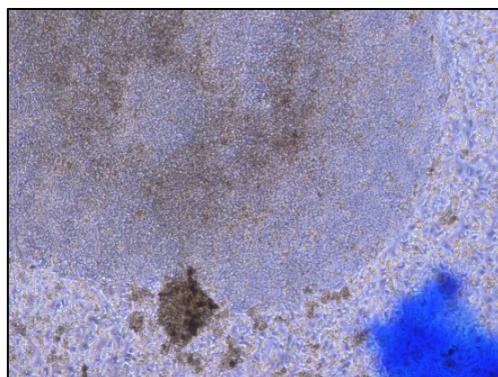


Figure 3 – Colony marked and ready for picking. (Taken at 5x magnification under phase contrast microscopy)

The colony is then carefully cut into smaller pieces using a pipette tip (see figure 4 below).

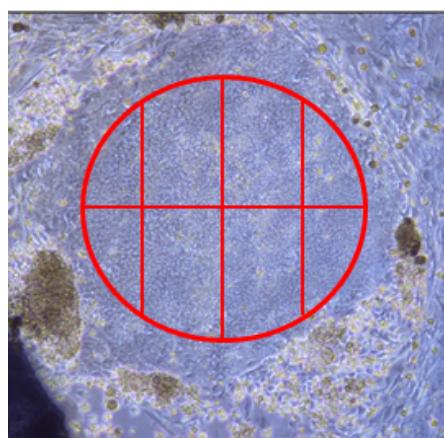




Figure 4 – Example of areas to divide within a colony. (Taken at 5x magnification under phase contrast microscopy)

These pieces are collected into a vessel and, if required, some of the fragments maybe broken down further before plating into a 12 well MEF feeder plate with iPSC medium and ROCK inhibitor. Medium is changed every day (without ROCK inhibitor) until colonies are large enough to passage, which is expected to take 6 – 8 days (see appendix 1 for feeder dependant grading system).

The original 10cm dish is disposed of either, after we are sure the colonies that have been picked are healthy and stable, or after 40 days. The quality of the 10cm dishes can be highly variable and when plates are over grown or colonies are merging, rescue methods can be deployed.

Feeder dependant passaging

When colonies are of an adequate size to passage, a mixture of equal parts collagenase and dispase is added to the feeder dependant cells and incubated for 45-60 minutes at 37°C until the good quality colonies detach and move freely in solution. The solution and colonies are collected into a vessel and allowed to pellet out of suspension without centrifugation. Once pelleted, the colonies are washed with fresh iPSC medium. The colonies are gently broken up into smaller pieces and suspended in a volume appropriate to the density of the pieces.

Only pieces of medium size are plated, larger pieces will settle out quickly and are left in the vessel, single cells and very small colonies will stay in the top portion of the medium and these are discarded. This is a very subjective process and can take time to master.

Split line

Once colonies are established in one well of a 6 well plate, we perform what we call a ‘split line’. This is mainly an administrative task where, at the point of passage, we give each of the clones a unique numerical identifier in our LIMS software. At this point there should be no more than 6 lines in culture. There is the potential that split line can occur when passaging from the initial 12 well plate if we are confident in what is obtained. This can help speed up the transfer to feeder free step, as it is preferable to split line prior to transfer.

Transfer to Feeder Free

Once individual lines are of good quality (at least a B grade, see appendix 1) and confluent on three wells of a 6 well plate, the cells are transitioned onto feeder free medium for at least two days prior to passaging (even if the transition of media doesn’t occur, a transfer can still be attempted). Once transitioned, the colonies are lifted, washed, broken down gently and plated onto vitronectin coated 6 well plates (a feeder dependant plate is kept as a backup in case the transfer needs to be repeated). Cell lines do not often acclimatise well to a transfer to feeder free, but nothing drastic is attempted at this point as it can take 2-4 standard passages for the line to adjust. If this fails it is simplest to go back and try and transfer from the feeders again. There are numerous variations that can be tried at transfer to feeder free and no singular option has been found to work best, it very

much varies from line to line. The transfer is attempted up to a maximum of four times, after which the cell line is considered for feeder dependant culture or it does not progress any further.

We make a feeder dependant backup frozen vial(s) as soon as possible in case the line is lost from culture for any reason, or if the quality of the line begins to decline. If the new feeder free line establishes well (with little spontaneous differentiation) then the feeder dependant backup plate is frozen or disposed of (if sufficient backups are already made).

Medium change is performed daily up until the cells are ready for passaging, which for feeder free cultures should be every 4 - 5 days. These colonies should be medium to large in size, highly compacted, with smooth defined edges (see figure 5 for examples of colonies ready to passage). Once feeder free cultures are looking healthy again, a backup frozen stock should be made as soon as possible.

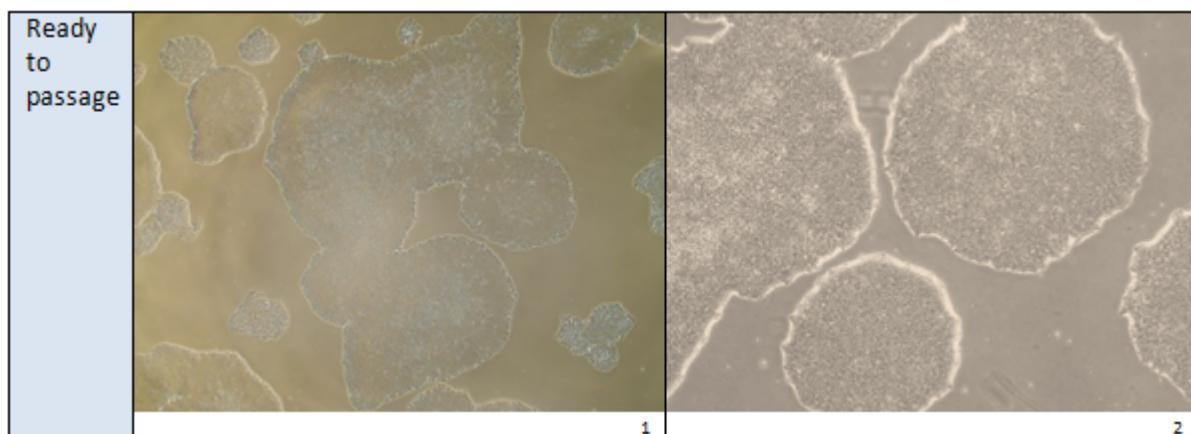


Figure 5 – Examples of ideal colonies ready to passage. (Taken at 4x magnification under phase contrast microscopy)

Feeder free passaging

0.5 mM EDTA solution is added to the cultures. After a four minute incubation at room temperature the colonies should exhibit these characteristics;

- Shiny, bright 'halos' around the outside of the colonies as they begin to lift.
- Small 'holes' appearing in the colonies as the bonds between the cells are weakened.

Once these have been observed, the EDTA is removed and the wells are gently washed with fresh medium up to 3 times. The colonies will come off the plate in small pieces. The pieces are then plated straight onto pre-coated 6 well vitronectin plates using a split ratio of up to 1:8 depending on confluence and grade (see appendix 2 for feeder free grading system).



Chapter Four

Master cell bank and Line Completion

Master Cell Bank

Two lines from the original 6 colonies picked will be chosen to carry on through the pipeline to master cell bank, the remaining lines will be frozen as backups (unless already frozen earlier on in the process). The two lines are selected based on the absence of Sendai virus (tested by reverse transcription polymerase chain reaction (RT-PCR)) and being rated a grade A-B with less than 10% differentiation. After this analysis, 3.5-4 confluent 6 well plates will be prepared for each line, from which 40 vials are master cell banked (2 full plates are used for this process as well as making a maintenance plate if no previous frozen backup were made). The other 1.5 – 2 plates are used in a process we refer to as a ‘harvest’; this is where snap frozen pellets for DNA Pipelines and Proteomics analysis are produced.

Quality Control

Once the master cell bank has been in liquid nitrogen for at least 24 hours, one vial will be resuscitated to make sure that the bank is viable. Once colonies are established, a sample of the supernatant will be taken from this culture to test for mycoplasma contamination. Once all the analysis has passed (resuscitation and mycoplasma testing) the maintenance (if one was made) and the resuscitation plate will be discarded and the line will be signed off as complete.

Once two lines per donor are master cell banked (in some cases only one line might make it through to bank if the quality of the donor is poor), one of the DNA Pipeline pellets for each line (as well as the fibroblast pellet) are submitted to DNA Pipelines for analysis. RNA and DNA are extracted from the pellets, the grade of which is checked and if any samples fail, CGaP are informed and backup pellets are submitted. The lines are identity checked to ensure they match the original parent donor before being run through the PluriTest:

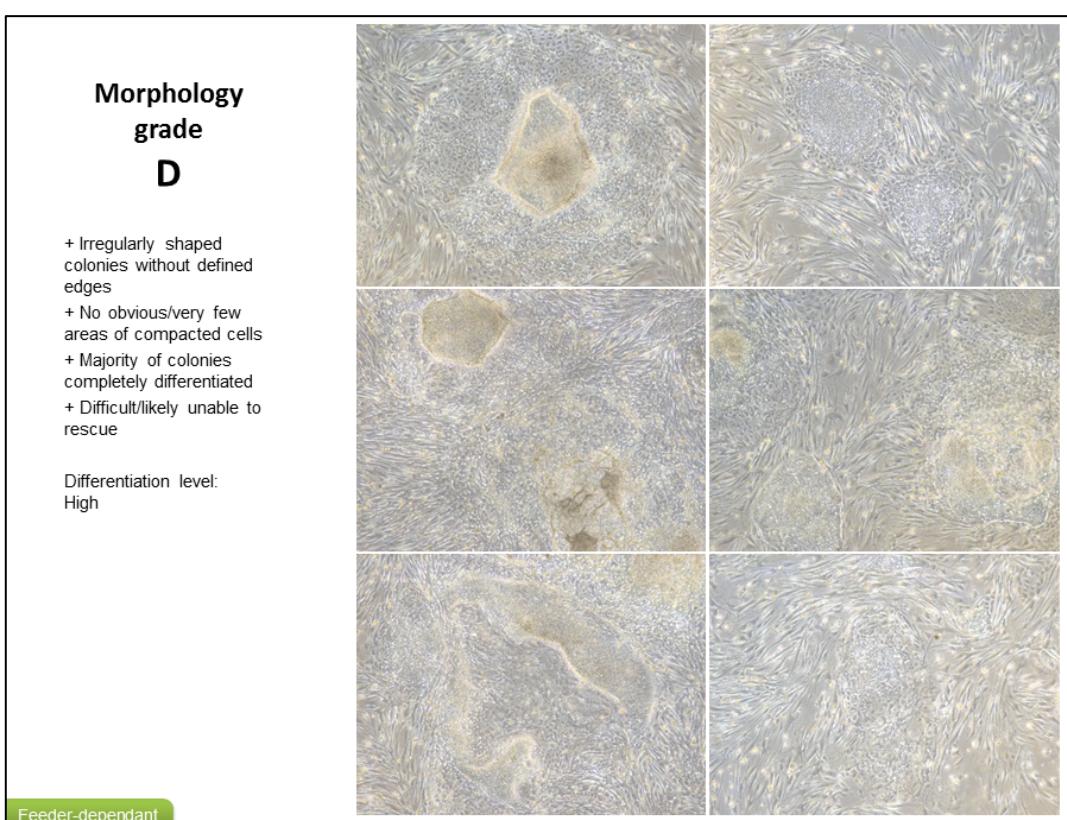
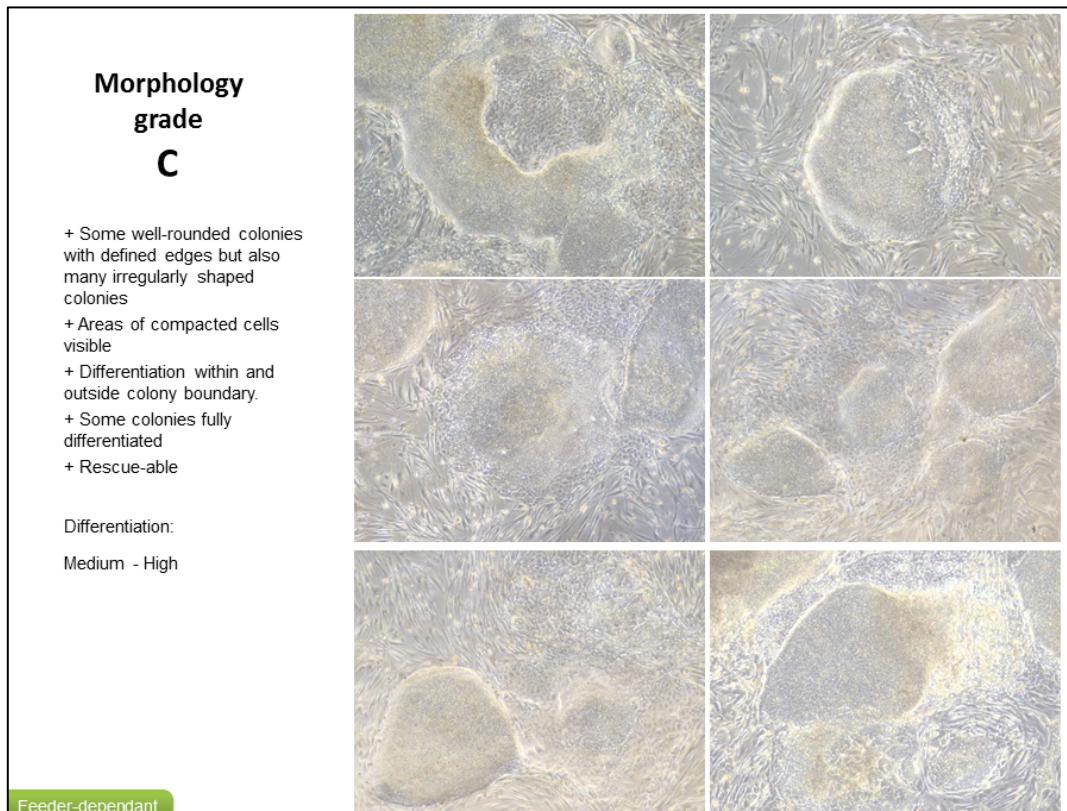
(<http://www.nature.com/nmeth/journal/v8/n4/full/nmeth.1580.html>).

This data is reported back to the Project Manager where it is reviewed and used to officially ‘select’ lines that are pluripotent and void of acquired genetic abnormalities in culture. These selected lines are requested for shipment to collaborators; Kings College London and The University of Dundee (Dundee receive both the snap frozen pellet which is used for proteomics analysis as well a vial from the master cell bank). 20 vials from the master cell banks are then prepared and shipped to ECACC along with a certificate of analysis (CofA) for further distribution to any interested researchers. 216 lines have also been sent to the cell bank EBiSC for distribution to the research community.

Appendix 1

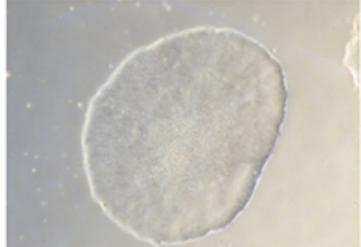
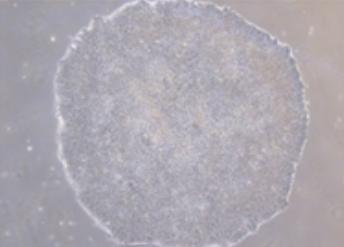
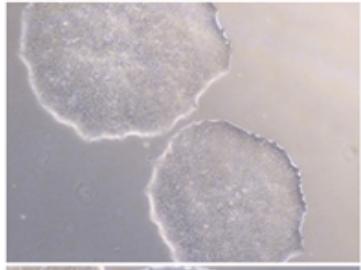
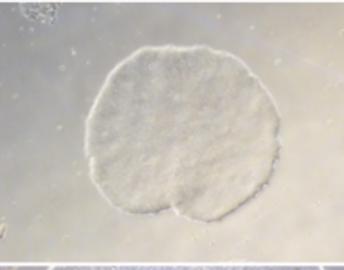
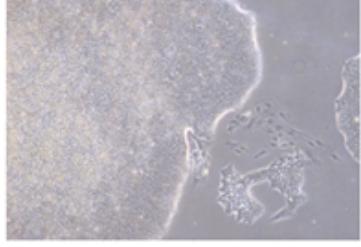
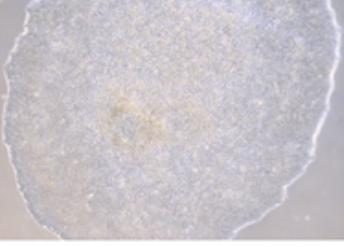
Feeder Dependant Grading System

Morphology grade				
A				
+ 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.				
Feeder-dependant				
Morphology grade				
B				
+ 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				
Feeder-dependant				



Appendix 2

Feeder Free Grading System

Morphology grade A <ul style="list-style-type: none"> + Well-rounded colonies + Smooth, defined edges + Compacted cells. + May see slightly uneven/speckled colony surface (stippling-type effect), mostly due to overgrowth + Minimum or very low levels of overgrowth <p>Differentiation: None - Low.</p>	     
Morphology grade B <ul style="list-style-type: none"> + Well-rounded colonies + Most colonies have smooth, defined edges + Mostly compacted cells + Overgrowth /differentiation that has led to some ring-shaped or 'egg' like colonies + Rescuable <p>Differentiation: Low - Medium</p>	