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 Human Induced Pluripotent Stem Cells Initiative (HipSci).
All recommendations refer to the culture of iPSCs in 6-well plates. This is not to say that iPSCs cannot be cultured in other sizes of tissue culture vessel but we would not recommend the use of 10 cm dishes with Feeder Free cultures, as with a vitronectin and E8 culture system we found the colonies would easily dissociate when confluent.

All cell manipulations, tissue culture vessel preparations and medium preparations 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 Trigene/Distel or equivalent disinfectant and 70% ethanol. Each cell line should be handled separately to avoid mislabelling or cross-contamination between cell lines. It is advisable that a small number of vials are cryopreserved as a master stock, as soon as possible.

Cells provided were cultured in the presence of Penicillin and Streptomycin.

Materials	
6 well tissue culture treated plate	Corning 3516
500 ml 0.2 µM filter unit	Thermo Scientific 450-0020
1000 ml 0.2 µM filter unit	Thermo Scientific 127-0020
0.22 µM filter	Thermo Scientific 190-2520
Syringe	VWR International 613-3931
Mouse Embryo Fibroblasts (MEF) cells	AMSBIO GSC-6001G
Gelatin powder (Porcine derived)	Sigma G1890
Vitronectin	Stemcell Technologies 07180
Essential 8 medium	Life Technologies (50 X 10 ml) A1517001
Advanced DMEM F12	Invitrogen 12634028
Knockout Serum Replacer (KSR)	Invitrogen (100 ml) 10828028
L-Glutamine	Invitrogen (5 ml) 25030024
2-Mercaptoethanol (2-ME)	Sigma (25 ml) M3148
Zebrafish Fibroblast Growth Factor (FGF)	Undiluted stock - CSCR (0.5 mg)
Dispase	Gibco – 17105-041
Collagenase IV	Invitrogen 17104019
1.8 ml Cryovials	Scientific Laboratory Supplies (x450) 375418K
DPBS (no calcium, no magnesium)	Life Technologies 14190144
Ethylenediaminetetraacetic acid (EDTA)	Life Technologies (100 ml) AM9260G
Dimethyl sulfoxide (DMSO)	Sigma-Aldrich (50 ml) D2438
Foetal Bovine Serum (FBS)	Sigma-Aldrich (500 ml) F2442
ROCK inhibitor (ROCK)	Sigma-Aldrich (1 mg) Y0503
Sterile filtered water	Sigma Aldrich (500 ml) W1503
15 ml / 50 ml Falcon Tubes	Falcon 352097/352098
Penicillin Streptomycin (10,000 U/ml)	Invitrogen (100 ml) 15140122

Equipment required
Class II Microbiology Safety Cabinet
Incubator set at 37°C / 5% CO_2
Waterbath set at 37°C
-80°C Storage
Liquid Nitrogen or appropriate Cryo storage unit
Cell freezing containers (also known as 'Mr Frosty')
phase contrast microscope (4x, 10x, 40x magnification)
'Pipette boy' and selection of stripettes (5 ml / 10 ml)
Gilson Pipettes (P1000 / P200 / P100 / P20) and corresponding sterile tips

ParafilmTM

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SECTION 1 - Medium Preparation

0.5 mM EDTA Solution

- Prepare fresh 0.5 mM EDTA by diluting Ultrapure 0.5 M EDTA, pH8.0 with DPBS using a 1:1000 dilution (for example, 10µl Ultrapure 0.5M EDTA in 10 ml DPBS).
- Store at room temperature.
- Use on day of preparation only.

Freeze Medium

- Prepare a 10% DMSO in Knock-out Serum Replacement (KSR) solution (for example 1 ml DMSO to 9 ml KSR).
- Store at 4°C until use.
- Use on day of preparation only.

ROCK inhibitor

- Reconstitute ROCK inhibitor by diluting 5 mg in 1.5 ml of sterile filtered water to make 10 mM stock solution.
- Aliquot and store at -20°C for up to 6 months; aliquots can be thawed once and should then be discarded.

2-ME Working stock

- It is essential the preparation of 2-ME is performed within a fume hood.
- Calculate how much working stock of 2-ME is required (each bottle requires 350 µl working solution).
- Perform a 1:100 dilution of the original stock solution using filter sterile water.
- Filter sterilise working solution using a 0.22 µM and syringe.
- Working solution should be used on day of production and any excess should be disposed of according to company's chemical safety procedures.

0.1% Gelatin preparation

- Weigh 1 g of Gelatin powder and add to 500 ml sterile filtered water.
- Heat in a water bath to 56°C until Gelatin is fully dissolved (approximately 30 minutes).
- Pour 500 ml of sterile filtered water into a 1000 ml 0.2 µM filter unit and filter through.
- To the same filter unit add the dissolved Gelatin solution and filter through.
- Perform a sterility check before use (optional).

Collagenase IV

- Thaw aliquots of frozen KSR and L-Glutamine at 4°C overnight or on day of use at 37°C (do not overheat the components).
- Weigh 500 mg of Collagenase IV powder.
- Dissolve collagenase IV in 50 ml of Advance DMEM at 37°C (for approximately 30 minutes).
- Add the following to a 500 ml 0.2 µM filter unit:
  - 350 ml of Advanced DMEM,
  - 50 ml of dissolved collagenase solution,
  - 100 ml of thawed KSR,
  - 5 ml of L-Glutamine,
  - 350 µl of 2-ME working solution,

- Label with preparation date, and store at 4°C for up to 14 days.
- Perform a sterility check before use (optional).

Allow to warm to room temperature before use, cold aliquots can be placed in a 37°C waterbath to warm for no more than 10 minutes.

Dispase

- Weigh 0.5g of Dispase powder.
- Dissolve Dispase in 50 ml of Advance DMEM at 37°C for approximately 30 minutes.
- Add the following to a 500 ml 0.2 µM filter unit:
  450 ml of Advanced DMEM,
  50 ml of dissolved Dispase solution,
- Label with preparation date, and store at 4°C for up to 14 days.
- Perform a sterility check before use (optional).

Allow to warm to room temperature before use, cold aliquots can be placed in a 37°C waterbath to warm for no more than 10 minutes.

MEF Medium

- Thaw aliquots of frozen reagents (FBS and L-Glutamine) overnight at 4°C or on day of use in a water bath at 37°C.
- Remove 50 ml of Advanced DMEM F12 from stock bottle.
- To the remaining 450 ml of Advanced DMEM F12 add:
  50 ml of FBS
  5 ml of L-Glutamine
  350 µl of 2-ME working solution (x100 stock dilution)
  5 ml of Penicillin Streptomycin (10,000 U/ml stock solution) (optional)

- Label with preparation date, and store at 4°C for up to 14 days.
- Perform a sterility check on complete medium before use (optional)

Allow complete medium to warm to room temperature before use, cold bottles can be placed in a 37°C waterbath to warm.

Advanced DMEM F12 for Feeder Dependant cultures

- Thaw aliquots of frozen reagents (KSR and L-Glutamine) overnight at 4°C or on day of use in a water bath at 37°C.
- Remove 100 ml of Advanced DMEM F12.
- To the remaining 400 ml of Advanced DMEM F12:
  Add 100 ml of KSR.
  Add 5 ml of L-Glutamine.
  Add 350 µl of 2-ME working solution
  5 ml of Penicillin Streptomycin (optional)

- Label with preparation date and store at 4°C for up to 14 days.
- Perform a sterility check on complete medium before use (optional).

Allow complete medium to warm to room temperature before use, new, cold bottles, without FGF, can be placed in a 37°C waterbath to warm for no more than 10 minutes.

Note: Before use in IPS derivation, add 1 ml FGF (4 µg/ml) to 500 ml of medium for a final concentration of 8 ng/ml. Label bottle with date the FGF was added.
Storage: +4°C (once FGF is added do not warm in waterbath) Expires: 3 days after addition of FGF.

Complete Essential 8 Medium (E8)

- Thaw aliquots of frozen E8 Supplement at 4°C overnight (do not thaw at 37°C as this will degrade the FGF)
- Add 10 ml of thawed E8 supplement to 500 ml of E8 basal medium.
- Add 5 ml of Penicillin Streptomycin (optional)
- Swirl bottle to mix (avoid creating air bubbles).
- Label with preparation date and store at 4°C for up to 14 days.
- Perform a sterility check on complete medium before use (optional).

Allow complete medium to warm to room temperature before use, new, cold bottles can be placed in a 37°C waterbath to warm for no more than 10 minutes.

SECTION 2 - Preparation of 6 well culture plates

Vitronectin coating

- Upon receipt, store vitronectin at -80°C.
- Prior to use, thaw the stock vial of vitronectin at room temperature or overnight at 4°C.
- Dilute the Vitronectin in DPBS to a final concentration of 10 µg/ml (example: 2 ml vitronectin to 48 ml DPBS).
- Gently mix the solution by inverting or 'swirling' the container.

Note: Do not vortex or pipette to mix

- Immediately dispense 1 ml of the Vitronectin solution to as many wells of a 6 well pate as required.
- Gently rock the 6 well plate back and forward to spread the matrix across the whole surface of the well.
- Incubate at Room Temperature for one hour before use.
- Prepared plates can be sealed with ParafilmTM then stored at 4°C for up to 3 days. Allow the vessel to equilibrate to room temperature for 1 hour prior to use.

MEF 'feeder' layer

- Upon receipt, store MEF cells cryogenically.
- Warm MEF medium to room temperature.
- Coat the required number of plates in 0.1% gelatin.
- Dispense 1.5 ml of the 0.1% gelatin solution to as many wells of a 6 well pate as required.
- Gently rock the 6 well plate back and forward to spread the solution across the whole surface of the well.
- Incubate at room temperature for a minimum of 20 minutes in the flow hood.
- Calculate the number of MEF cells needed:
  6 well plate = 1.14×10^6 per plate.
- Calculate the number of vials to be thawed depending on the cell density per vial.
- Aliquot 5 ml of warm MEF medium for each vial to be thawed into a falcon tube.
- Thaw cells in the water bath or dry bath at 37°C until a small ice crystal remains.
- Add cells dropwise using a 1 ml Gilson to the medium.
- Centrifuge at 200 x *g* for 3 minutes and aspirate supernatant.
- Re-suspend in 1 ml MEF medium using a P1000 pipette.
- Top up cell suspension with 5 ml of MEF medium for each vial.
- Perform two viability cell counts using Trypan blue.
- Calculate the viable cell density and calculate the total volume of medium required to dilute the cells to a density of 7.6×10^4 per ml.
- Aspirate gelatin from plates.

- Swirl MEF cell suspension regularly to homogenise cells and aliquot:
  6 well plate = 2.5 ml per well / 15 ml per plate.
- Transfer plates to incubator and agitate in stacks of four plates or less (do not swirl). Incubate overnight at 37°C and 5% CO_2.
- Store at 37°C, 5% CO_2 for up to three days. Plates are best used the next day.

SECTION 3 - Thawing Human iPS Cells

Feeder Free

- Prior to starting, prepare a stock of E8 + ROCK by adding 10 mM ROCK to an aliquot of E8 to a final concentration of 10 µM (1:1000 dilution) and allow to warm to room temperature.
- Partially thaw the frozen vial of iPS cells at 37°C, using a waterbath, until there is a small ice crystal remaining.
- Dry and spray the vial with 70% Ethanol before placing in the culture hood.
- Add 1 ml of E8 + ROCK solution drop-wise to the cryovial with a 5 ml strippete, then gently collect and transfer the entire cell suspension to a 15 ml Falcon tube.
- Add 8 ml of E8 + ROCK solution to the cell suspension.
- Centrifuge at 120 g for 3 minutes.
- During centrifugation aspirate Vitronectin from one well of a prepared 6 well coated culture plate, replace lid and leave to dry for no more than 4 minutes.
- Add 1 ml of E8 + ROCK solution to one well ready for use.
- Aspirate supernatant from the cell pellet and using a 5 ml stripette gently re-suspend in 1 ml of E8 + ROCK solution (pipette gently up and down once to mix). Transfer cell suspension to the prepared well of a 6 well plate.
- Agitate plate gently within a tissue culture incubator set at 37°C and 5% CO_2 to ensure even distribution of cells across the well.
- Check cell attachment under a phase contrast microscope after 24 hours.
- If attachment is good, change medium to 2 ml E8. If there are more cells floating than attached top up with 1 ml freshly made E8 + ROCK solution.

Feeder Dependant

- Prior to starting, prepare a stock of Feeder Dependant medium + ROCK by adding 10 mM ROCK inhibitor to an aliquot of Feeder Dependant medium (12 ml per line being thawed) to a final concentration of 10 µM (1:1000 dilution) and allow to warm to room temperature.
- Partially thaw the frozen vial of iPS cells at 37°C, using a waterbath, until there is a small ice crystal remaining.
- Dry and spray the vial with 70% Ethanol before placing in the culture hood.
- Add 1 ml of the Feeder Dependant medium + ROCK solution drop-wise to the cryovial with a 5 ml strippete, then gently collect and transfer the entire cell suspension to a 15 ml Falcon tube.
- Add 8 ml of the Feeder Dependant medium + ROCK solution to tube and let the visible colonies sediment.
- Aspirate medium from 1 well of the pre-prepared feeder plate and wash with 1 ml of DPBS.
- Aspirate DPBS and add 1 ml Feeder Dependant medium + ROCK solution to the well.
- Aspirate supernatant from the cell pellet and gently re-suspend the pellet using a 5 ml stripette in 1 ml of Feeder Dependant medium + ROCK solution (pipette slowly once or twice maximum taking care not to break colonies) and transfer to one well of a 6 well plate.
- Agitate plate gently (do not swirl) within a tissue culture incubator set at 37°C and 5% CO_2 to ensure even distribution of cells across well.
- Top up with 1 ml of fresh Feeder Dependant medium + ROCK solution after 24 hours.

SECTION 4 - Culturing of Human iPS cells

Feeder Free and Feeder Dependant

Note: It is good practice to observe iPSC lines daily under phase contrast microscope (4x, 10x, 40x magnification) for iPSC-like morphology, the presence of differentiated cells and confluence (see appendix 1 and 2 for grading systems)

- Cells are fed by removing 95% of the medium from the wells using an aspirator pipette.
- Aseptically add 2 ml of fresh medium (Feeder Dependant medium or Complete E8 for Feeder Free) per 1 well of a 6 well plate by gently adding to the side of the well. Incubate cells at 37°C, 5% CO_2.
- Medium should be changed daily except for on the day of passaging.

SECTION 5 – Passaging Human iPS cells

Feeder Free passaging with EDTA

Note: Feeder Free iPSC should be observed every day; refer to grading system to assess morphology and confluency (see appendix 1 for Feeder Free grading system).
Cell lines should be passaged when the cells are approximately 70% confluent, well compacted and when the colonies have well defined edges (grade A-B). Cells may also require passaging if levels of differentiation start to exceed that of iPSC or colonies start to look overgrown or unhealthy (grade C-D).

- Established cultures can be split 1:4 to 1:6 (i.e. transferring all colonies from one well to four or six wells).
- Aspirate spent medium for wells to be passaged.
- Wash wells with 2 ml of DPBS per well and aspirate.
- Add 1 ml of 0.5 mM EDTA solution to wells to be passaged, rock plate to cover whole well surface.
- Incubate at room temperature for 4 – 8 minutes, observing under phase contrast microscopy until colonies display bright 'halos' around the edges and small holes start to appear throughout the colonies (see figure 1 below).

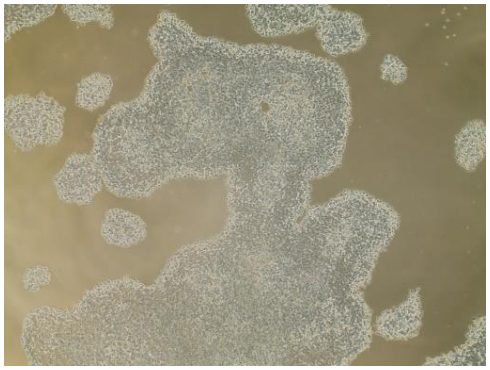


Figure 1 – EDTA effects on iPSC colonies after 4 minutes
(Taken at 4x magnification under phase contrast Microscopy)

- Aspirate the 0.5 mM EDTA by tilting the plate forward slightly to collect the EDTA in the bottom edge of the wells. Take care as the cells are loosely attached.
- Immediately add 2 ml of Complete E8 medium to the wells.
- Using this 2 ml of medium gently wash the cells from the plate by pipetting the medium around the well, approximately three times, using a 5 ml / 10 ml stripette. This should dislodge cell clusters without dislodging any differentiated cells.

Note: Do not over pipette the cells as this will result in single cells rather than cell clusters.
Note: It is possible that occasionally not all cells will lift, there may be 'rings' left behind on the plate (see figure 2 below)

Figure 2 – 'Rings' left behind after passaging
(Taken at 4x magnification under phase contrast Microscopy)

- Dilute the cell suspension with Complete E8 medium in a 15 ml / 50 ml falcon tube at an appropriate cell density (in accordance with your desired split ratio).
- Aspirate Vitronectin solution from pre-prepared coated 6 well plates, allow to dry for no more than 4 minutes.
- Seed cell solution into as many wells as required.
- Agitate plate gently within a tissue culture incubator set at 37°C and 5% CO_2 to ensure even distribution of cells across the well.

Feeder Dependant passaging with Collagenase and Dispase

Note: Feeder Dependant iPSC should be observed on Day 4-7 after previous passage; refer to grading system to assess morphology and confluency (see appendix 2 for Feeder Dependant grading system).

Note: Passaging should be performed as below on Day 5-7 after previous passage when colonies are compacted and have well defined edges (grade A-B). Cells may also require passaging if levels of differentiation start to exceed that of iPSC or colonies start to look overgrown or unhealthy (grade C-D).

- Aspirate the spent medium from the cells and wash with 2 ml of DPBS per well.
- Aspirate DPBS and add 1 ml of both Collagenase and Dispase per well.
- Rock the dish to cover the surface of the cells and set a timer for 45 minutes.
- Incubate in a tissue culture incubator set at 37°C, 5% CO_2 for 45 minutes.

Note: If after 45 minutes no colonies appear to be lifting, incubate for a further 10-15 minutes. Do not exceed 60 minutes. Differentiated cells should remain attached.

- Collect suspension of floating colonies and gently dispense into a falcon tube, taking care not to break up the colonies.
- Leave the colonies to sediment out of suspension and form a pellet. Do not centrifuge.
- Aspirate the supernatant from the pellet and re-suspend in 5-10 ml of medium (depending on pellet size).
- Leave the colonies to sediment out of suspension again and repeat the previous step.
- Remove MEF medium from feeder plate(s), wash with 2 ml DPBS per well.
- Add 1.5 ml of warm Feeder Dependant medium (containing FGF) per well.
- Aspirate as much spent medium as possible from the falcon tube so only the cell pellet is left.
- Re-suspend cell pellet in 1 ml of fresh Feeder Dependant medium (containing FGF).
- Using a P1000 Gilson pipette, collect and dispense the cell suspension up to 3 times to gently break

up the colonies to medium/small fragments.

Note: Be careful not to over pipette, or pipette too harshly as this will result in single cells.

- Add the appropriate volume of Feeder Dependant medium (containing FGF) to the cell suspension (between 3 – 10 ml depending on original pellet size).
- Make sure the suspension is fairly homogenous (by gentle agitation); assess by eye the 'gradient' of the fragment sizes. Take 1 ml of cell suspension from around the middle of the tube (to collect only medium sized fragments).
- Dispense 'drop-wise' into each well, distributing evenly across the well.
- Assess confluence of fragments (by phase contrast microscopy). Add more suspension if required (remember to agitate suspension again before re-plating).

Note: Feeder Dependant cultures can sustain a maximum split ratio of approximately 1:3.

- Agitate plate gently (do not swirl) within a tissue culture incubator set at 37^{o}C and 5% CO_2, to spread the fragments equally around the well, leave to settle overnight.

SECTION 6 - Cryopreservation of Human iPS cells

Feeder Free

Note: Colonies should be large enough to freeze 4-5 days after passaging when cells are approximately 70-80% confluent. A confluent well of a 6 well plate will have enough cells to generate 5-6 frozen vials. Cells are usually frozen when their morphology lies between grade A-B (refer to the Feeder Free grading system to assess morphology in appendix 1).

- Prepare appropriate volume of freeze medium to freeze 1 ml cell suspension per vial.
- Prepare a cell freezing container (if required make sure to use appropriate volume of Iso-propanol) and store at 4^{o}C until use.
- Aspirate spent medium and wash wells with 2 ml of DPBS per well and aspirate.
- Add 1 ml of 0.5 mM EDTA solution to wells, rock plate to cover whole well surface.
- Incubate at room temperature for 4 – 8 minutes, observing under phase contrast microscopy until colonies display bright 'halos' around the edges and small holes start to appear throughout the colonies (see figure 1).
- Aspirate the 0.5 mM EDTA by tilting the plate forward slightly to collect the EDTA in the bottom edge of the wells. Take care as the cells are loosely attached.
- Immediately add 2 ml of Complete E8 medium to the wells.
- Using this 2 ml of medium gently wash the cells from the plate by pipetting the medium around the well, approximately three times, using a 5 ml / 10 ml stripette. This should dislodge cell clusters without dislodging any differentiated cells.

Note: Do not over pipette the cells as this will result in single cells rather than cell clusters.

- Pool cell suspension into a 15 ml / 50 ml Falcon tube and centrifuge at 120 x *g* for 1 minute.
- Aspirate the supernatant, tap the falcon tube to dislodge the compacted pellet.
- Re-suspend in the required volume of freeze medium (1 ml per vial).
- Dispense 1 ml of cell colony suspension into each cryovial and seal tightly.
- 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).
- Once frozen transfer the cells, on dry ice, to an Ultra-Low Temperature storage vessel (LN2 or -150°C freezer).

Feeder Dependant

Note: Colonies should be large enough to freeze 5-7 days after passaging when cells are approximately 70-80% confluent. A confluent well of a 6 well plate will have enough cells to generate 1 frozen vial. Cells are usually frozen when their morphology lies between grade A-B (refer to the Feeder Dependant grading system to assess morphology in appendix 2).

- Aspirate the spent medium from the cells and wash with 2 ml of DPBS per well.
- Aspirate DPBS and add 1 ml of both Collagenase and Dispase per well.
- Rock the dish to cover the surface of the cells and set a timer for 45 minutes.
- Incubate in a tissue culture incubator set at 37°C, 5% CO_2 for 45 minutes.
- Prepare appropriate volume of freeze medium to freeze 1 ml cell suspension per vial.
- Prepare a cell freezing container (if required make sure to use appropriate volume of Iso-propanol) and store at 4°C until use.

Note: If after 45 minutes no colonies appear to be lifting, incubate for a further 10-15 minutes. Do not exceed 60 minutes. Differentiated cells should remain attached.

- Collect suspension of floating colonies and gently dispense into a falcon tube, taking care not to break up the colonies.
- Leave the colonies to sediment out of suspension and form a pellet. Do not centrifuge.
- Aspirate the supernatant from the pellet and re-suspend in 5-10 ml of medium (depending on pellet size).
- Leave the colonies to sediment out of suspension again and repeat the previous step.
- Leave the colonies to sediment out of suspension once more.
- Aspirate as much medium as possible so only the cell pellet is left.
- Re-suspend in appropriate volume of freezing medium, giving a total volume of 1 ml per cryovial.

Note: Do not over aspirate the cells as this will result in single cells rather than cell clusters.

- Dispense 1 ml of cell colony suspension into each cryovial and seal tightly.
- 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).
- Once frozen transfer the cells, on dry ice, to an Ultra-Low Temperature storage vessel (LN2 or -150°C freezer).

SECTION 7 – Transfer to Feeder Free

Note: Prior to attempting to transfer a Feeder Dependant culture to Feeder Free ensure the quality of the line is of grade A-B (refer to the Feeder Dependant grading system to assess morphology in appendix 2) and that confluence is around 70%.

- 2-3 days post passaging the Feeder Dependant culture replace the Feeder Dependant medium with E8 and continue to media change using E8 until the culture is ready to passage.

Note: If you have not had a chance to change medium prior to passage but feel your culture is of good enough quality to transfer you can still attempt transfer but maybe seed with a Feeder Dependant, E8 medium 50:50 mix.

- On day of passage aspirate the spent medium from ½ a 6 well plate and wash with 2 ml of DPBS per well.
- Aspirate DPBS and add 1 ml of both Collagenase and Dispase per well.
- Rock the dish to cover the surface of the cells and set a timer for 45 minutes.
- Incubate in a tissue culture incubator set at 37°C, 5% CO_2 for 45 minutes.

Note: If after 45 minutes no colonies appear to be lifting, incubate for a further 10-15 minutes. Do not exceed 60 minutes. Differentiated cells should remain attached.

- Collect suspension of floating colonies using E8 medium and gently dispense into a falcon tube, taking care not to break up the colonies.
- Leave the colonies to sediment out of suspension and form a pellet. Do not centrifuge.
- Aspirate the supernatant from the pellet and re-suspend in 5-10 ml of medium (depending on pellet size).
- Leave the colonies to sediment out of suspension again and repeat the previous step.
- Aspirate Vitronectin solution from pre-prepared coated ½ 6 well plate, allow to dry for no more than 4 minutes.
- Add 1 ml of E8 medium to each well.

Note: At this point you may also want to prep a MEF feeder plate and create a Feeder Dependant 'maintenance' plate in case the transfer doesn't work.

- Aspirate as much spent medium as possible from the falcon tube so only the cell pellet is left.
- Re-suspend cell pellet in 1 ml of fresh E8 medium.
- Using a P1000 Gilson pipette, collect and dispense the cell suspension up to 3 times to gently break up the colonies to medium/small fragments.

Note: Be careful not to over pipette, or pipette too harshly as this will result in single cells.

- Add the appropriate volume of E8 medium to the cell suspension (between 3 – 6 ml depending on original pellet size).
- Make sure the suspension is fairly homogenous (by gentle agitation); assess by eye the 'gradient' of the fragment sizes. Take 1 ml of cell suspension from around the middle of the tube (to collect only medium sized fragments).
- Dispense 'drop-wise' into each well, distributing evenly across the well.
- Assess confluence of fragments (by phase contrast microscopy), seed the vitronectin plate at a higher density to a normal passage. Add more suspension if required (remember to agitate suspension again before re-plating).
- Agitate plate gently within a tissue culture incubator set at 37^{o}C and 5% CO_2 to ensure even distribution of cells across the well.

Section 8 - Troubleshooting

Problem	Observation	Possible Solution/Precaution
Low viability after thaw	Little to no colonies visible within 4 days after recovery	• Feeder Dependant lines can take up to 2 weeks for colonies appear. • 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 but do not use in routine culture of iPSC. • Ensure that cells were banked at log phase of growth. • Try thawing cells into a smaller tissue culture vessel.
Low Viability after Passage	• Cells do not attach properly • Non-typical morphology • High levels of cell death	• Use lower split ratio and maintain a more confluent culture. • Ensure cells are in log phase of growth at

Problem	Observation	Possible Solution/Precaution
	• Cells do not proliferate	passaging. • Work quickly or reduce incubation time of EDTA as cells might become more sensitive. • 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.
Spontaneous differentiation	• Colonies do not have defined edges • Cells within the colonies are less compact • Cells appear flattened and bigger	• Ensure the plates used have coating that is not older than 3 days. • Avoid leaving plates outside the incubator for more than 15 minutes. • Decrease colony density by plating less cell aggregates during passaging, split ratio as high as 1:20 can aid in removing differentiated cells. • Feed cells daily.
Non-uniform distribution of colonies within plate	Areas with too high 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 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 24h.
Significant scraping is required to dislodge cells	Colonies do not come off the plate with 2- 3 rinses with a P1000 pipette / 5 ml stripette	• 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 80 % confluent.
Cells detach after 1st medium change post passage	Cells start to lift off even though they seemed to settle fine after passage	• Exchange medium very gently, do not drop medium rapidly onto the cells, tilt the plate and pipette medium down the side of the well. • Change medium only 48 hours post passage to allow aggregates to fully attach, top up medium 24 hours post passage instead.

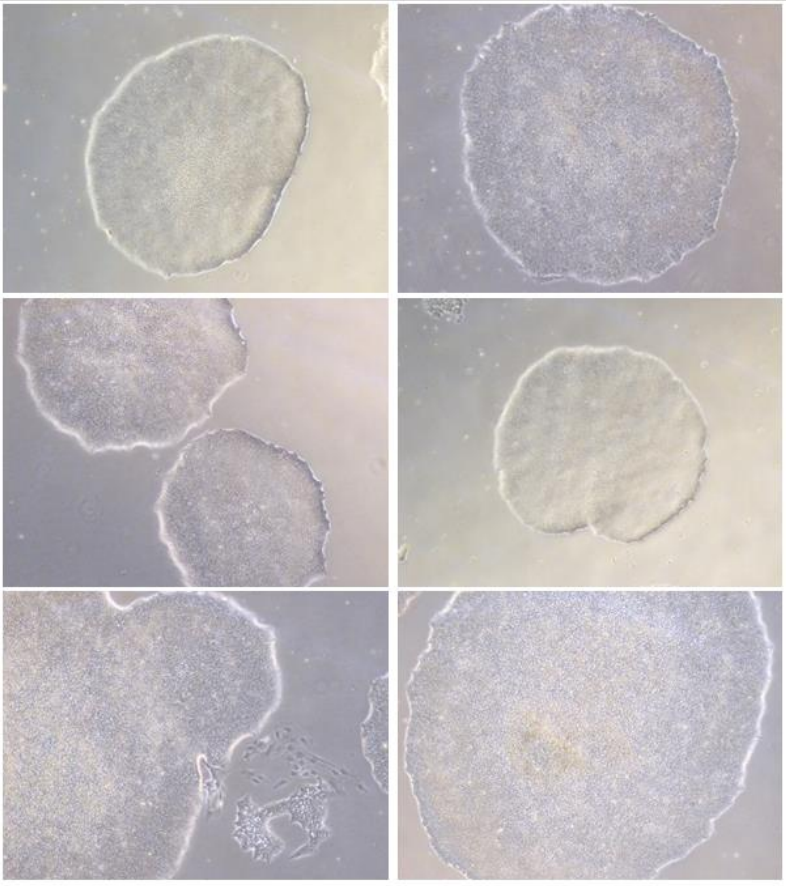
APPENDIX 1 – FEEDER FREE GRADING SYSTEM

Morphology grade

A

+ 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

Differentiation: None - Low.



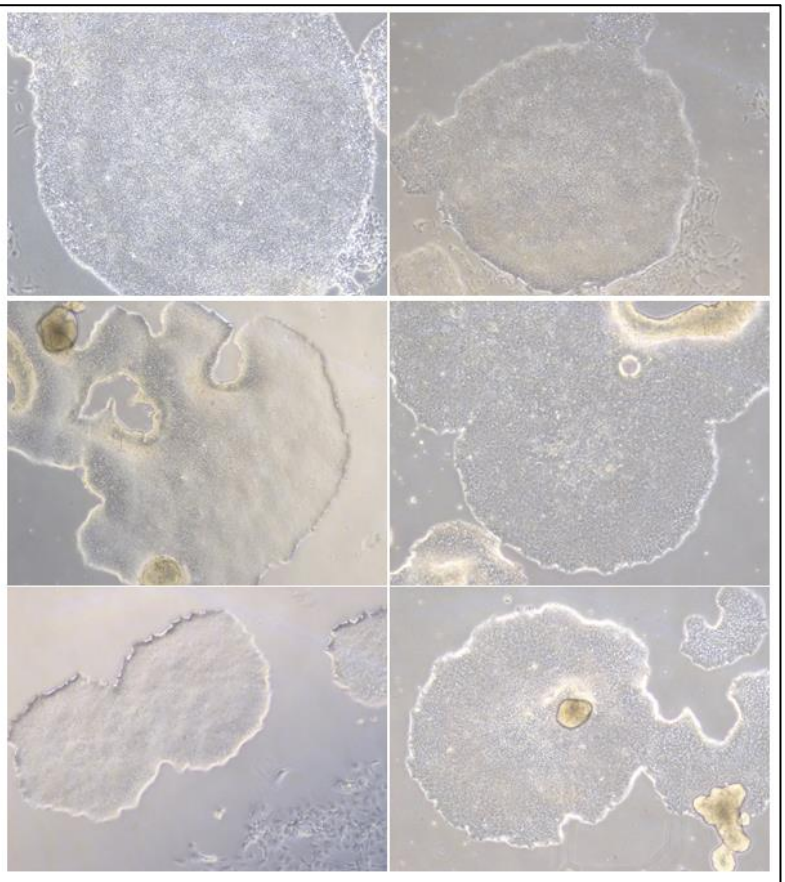
Feeder-free

Morphology grade

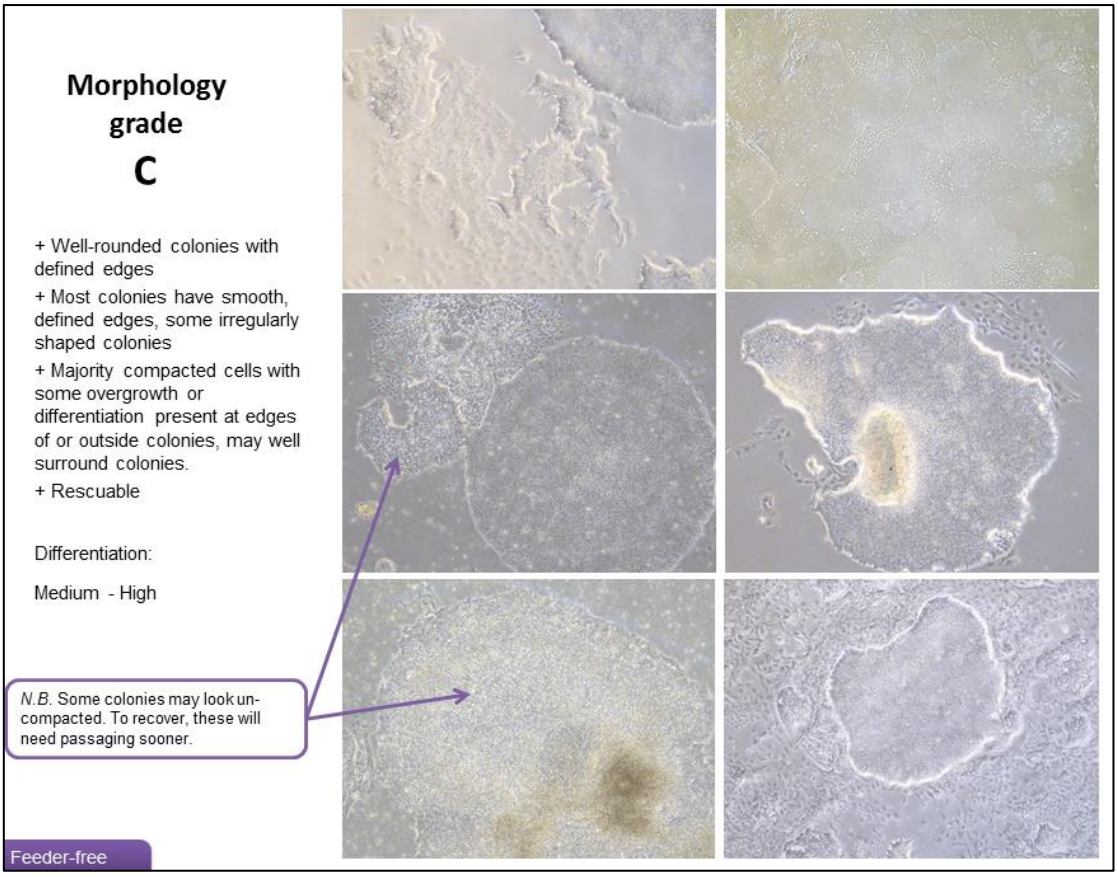
B

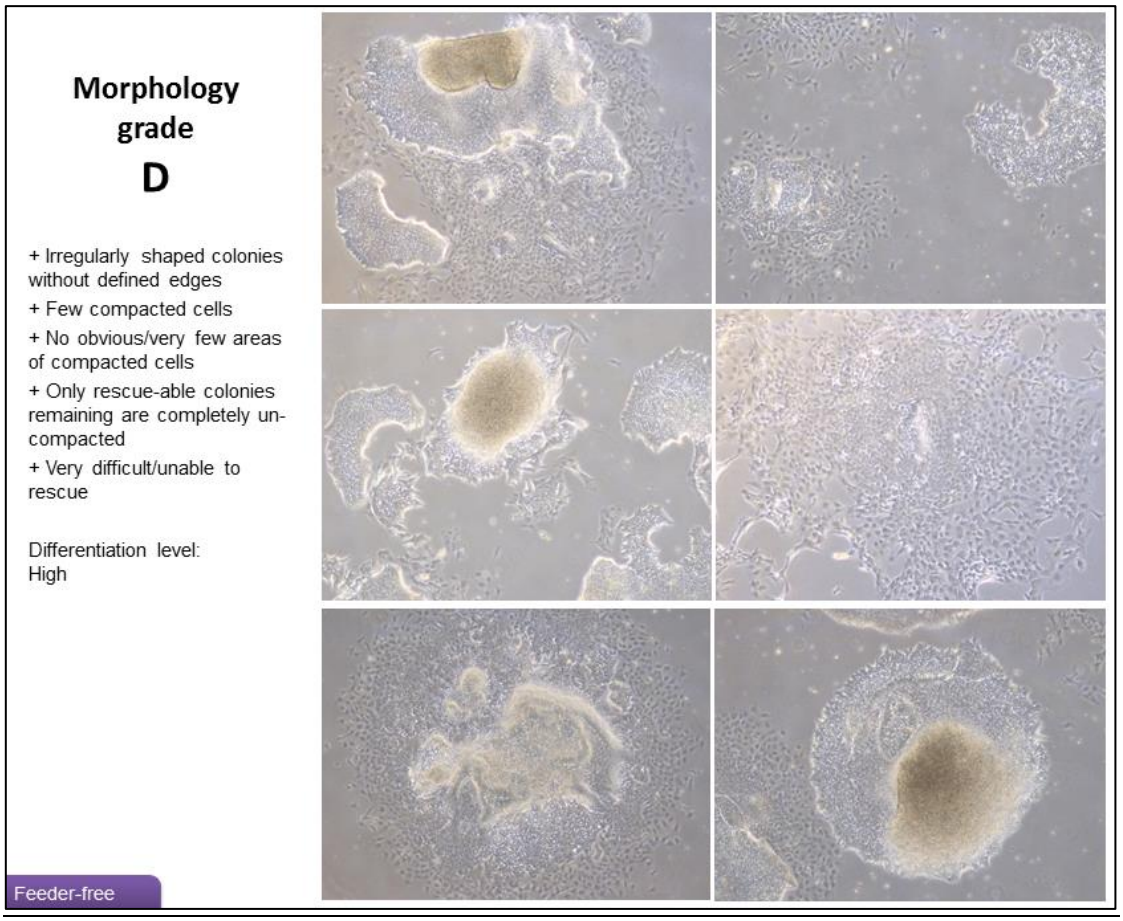
+ Well-rounded colonies
+ Most colonies have smooth, defined edges
+ Mostly compacted cells
+ Overgrowth /differentiation that has led to some ring-shaped or 'egg' like colonies
+ Rescuable

Differentiation: Low - Medium



Feeder-free


Morphology grade
C
+ Well-rounded colonies with defined edges
+ Most colonies have smooth, defined edges, some irregularly shaped colonies
+ Majority compacted cells with some overgrowth or differentiation present at edges of or outside colonies, may well surround colonies.
+ Rescuable
Differentiation:
Medium - High
N.B. Some colonies may look un-compacted. To recover, these will need passaging sooner.
Feeder-free


Morphology grade
D
+ Irregularly shaped colonies without defined edges
+ Few compacted cells
+ No obvious/very few areas of compacted cells
+ Only rescue-able colonies remaining are completely un-compacted
+ Very difficult/unable to rescue
Differentiation level:
High
Feeder-free

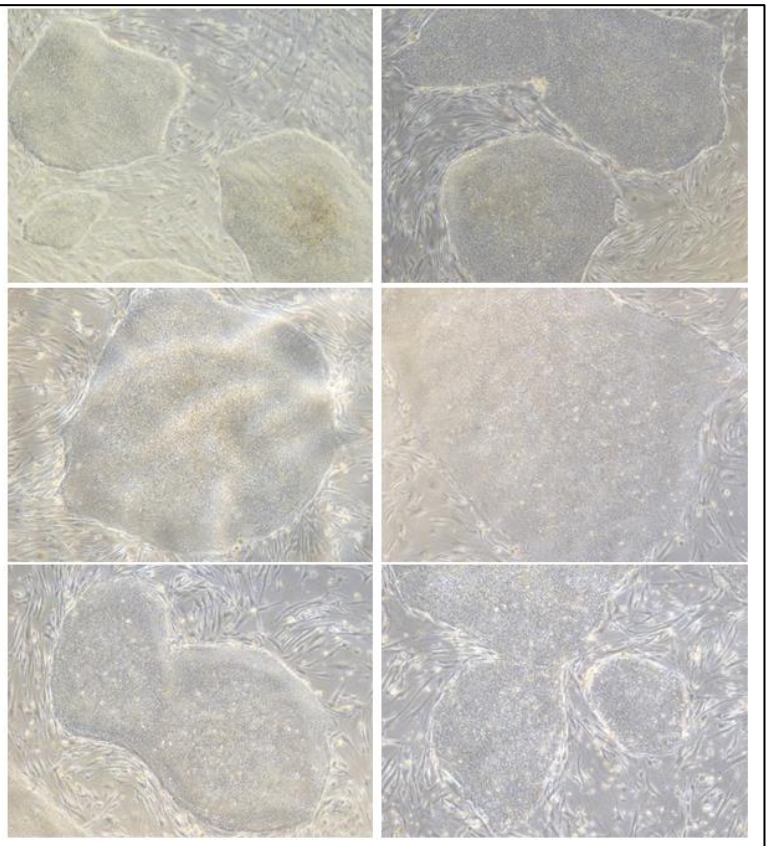
APPENDIX 2 – FEEDER DEPENDANT GRADING SYSTEM

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.



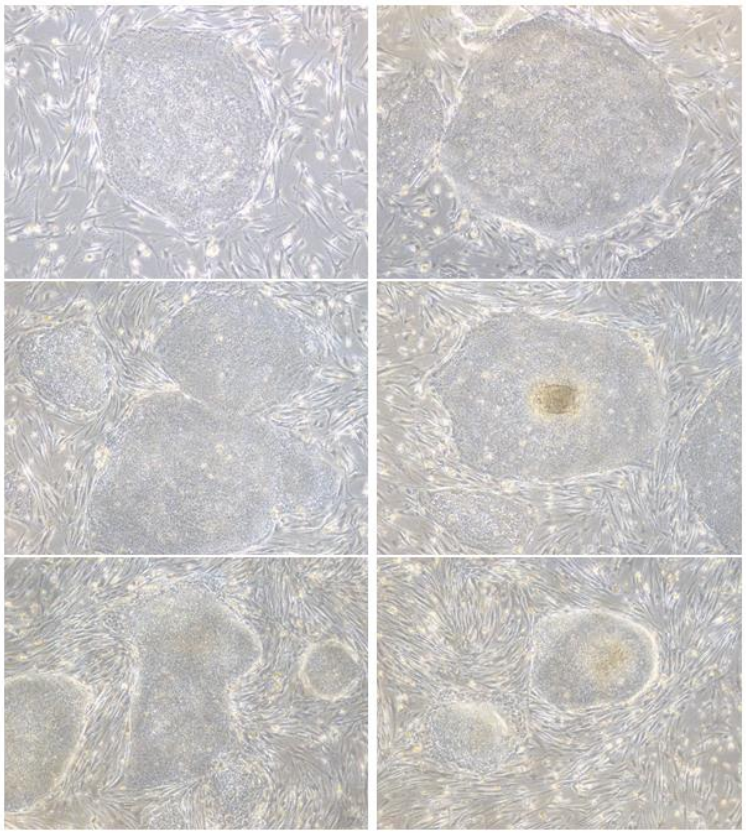
Feeder-dependant

Morphology grade

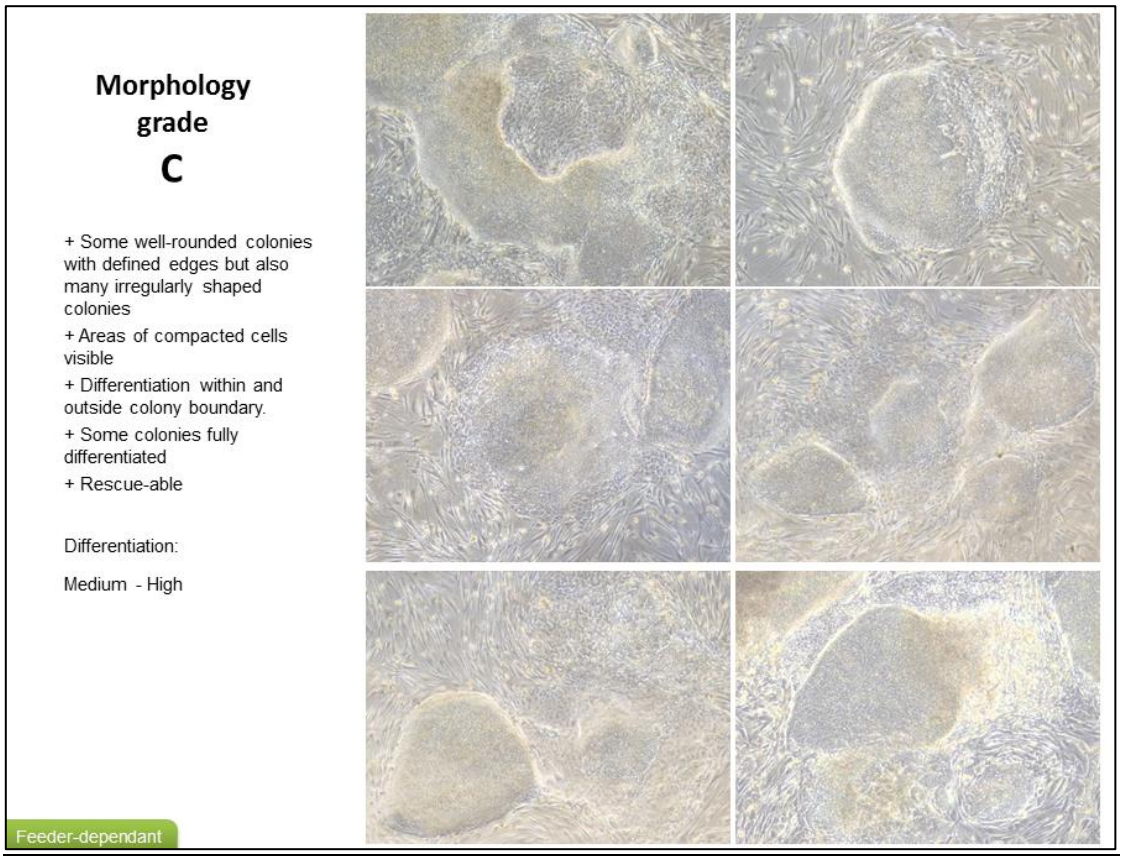
B

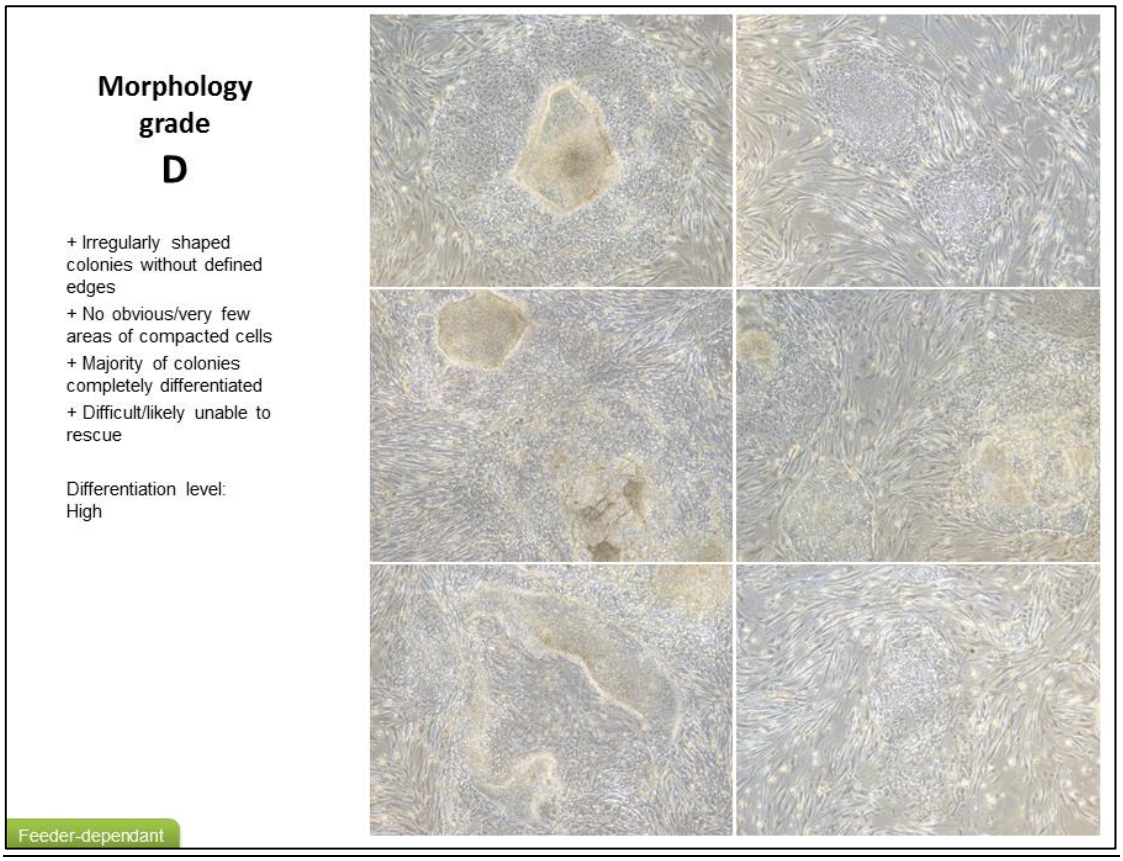
+ Well-rounded colonies
+ Most colonies have smooth, defined edges
+ Compacted cells with some overgrowth or slightly uneven colony surface (stippling-type effect)
+ Differentiation present at edges of or outside colonies

Differentiation: Low - Medium



Feeder-dependant





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