WiCell Feeder–Dependent Pluripotent Stem Cell Protocols

Supplement: Culture of Elf1 Cells

WiCell	**Feeder-Dependent Pluripotent Stem Cell Culture Protocols Supplement: Culture of Elf1 Cells**	
	SOP Number: SOP-SH-009	**Version: D**

1. PURPOSE

1.1. This Standard Operating Procedure (SOP) provides additional information regarding the propagation of Elf1 cells using WiCell's Feeder-Dependent Pluripotent Stem Cell Protocols. The formula for a modified cKOSR medium for growing Elf1 cells (referred to as Elf1 cKOSR) and the recommended density for MEFs are provided. If you have any additional questions, please contact technical support on the WiCell website at www.wicell.org.

2. REFERENCES

2.1. WiCell SOP-SH-001

3. DEFINITIONS

3.1. **Elf1 cKOSR:** the cell culture medium used for culturing Elf1 embryonic stem cells

4. REQUIRED MATERIAL

4.1. DMEM/F12, high glucose, containing GlutaMax (10565-018, Invitrogen)

4.2. KnockOut serum replacer (KOSR) (10828-028, Invitrogen)

4.3. 100 mM sodium pyruvate (11360-070, Invitrogen)

4.4. 100X nonessential amino acids (11140-050, Invitrogen)

4.5. 100X Penicillin-Streptomycin (15140-122, Invitrogen)

4.6. 14.3M β-mercaptoethanol (M6250, Sigma–Aldrich)

4.7. human LIF (LIF1005, Millipore)

4.8. IGF1 (13769, Sigma-Aldrich) (Optional)

4.9. 0.5mg/mL FGF2 (PHG6015, Invitrogen)

4.10. 1 mM PD0325901 (S1036, Selleck)

4.11. 1 mM CHIR99021 (S2924, Selleck)

4.12. 0.05% Trypsin/EDTA (25300-054, Invitrogen)

5. PROCEDURE

5.1. **MEF Plating Density Recommendations**

5.1.1. Plate inactivated MEFs at a modified density of $3.5x10^4$ cells per cm^2 according to the Feeder-Dependent Pluripotent Stem Cell Protocol. This translates into a plating density for freshly inactivated MEFs of $1.34x10^5$ cells/mL at 2.5 mL per well in a 6 well plate (3.36×10^5 cells/well).

5.1.2. On the day cells are to be thawed or passaged, prepare MEF plate(s) for use by aspirating MEF plating medium and rinsing with 1ml/well of DMEM/F-12+Glutamax. Add 1.5ml Elf1 cKOSR medium to each well, and store in incubator until ready to use.

5.2. **Elf1 cKOSR Medium Preparation**

5.2.1. Preparation of Stock Solutions for Elf1 cKOSR

Component	Dilution Procedure
1mM CHIR99021	Dilute 5mg CHIR99021 in 9.96 mL sterile DMSO
1mM PD0325901	Dilute 5mg PD0325901 in 10.37 mL sterile DMSO
1mg/mL human LIF	Dilute 25µg in 25µL sterile water
0.1 mg/mL 2-FGF	Dilute 50µg in 0.5mL sterile water

5.2.2. **Elf1 cKOSR Medium Formulation**

Reagent	For ~500ml Elf1 cKOSR	For ~250ml Elf1 cKOSR
DMEM/F12, high glucose, w/ Glutamax	400ml	200ml
KOSR	100ml	50ml
100X Pen/Strep	1ml	500ul
100x NEAA	5ml	2.5ml
100 mM sodium pyruvate	5ml	2.5ml
0.1 mg/mL 2-FGF	60µl	30µl
CHIR99021 Stock Solution	750 µl	375µl
PD03296501Stock Solution	200µl	100µl
human LIF Stock Solution	5µl	2.5µl
14.3 M BME	3.5µl	1.75µl
IFG1*	10ng/ml final conc.	

***** IGF1 is optional and can be removed from culture medium without affecting cell morphology per the cell line provider.

5.3. **Thaw and Culture**

Note: Before thawing, check the certificate of analysis included in the shipping packet insert to determine the recommended thaw ratio (number of wells into which a single vial of cells should be thawed).

5.3.1. Thaw according to WiCell Feeder-Dependent Pluripotent Stem Cell Protocol (SOP-SH-001) using the Elf1 cKOSR medium instead of the standard cKOSR medium.

5.3.2. Observe the cells daily after thaw. Colonies will be difficult to see initially but will form small mounds over time. Continue to observe and feed with Elf1 cKOSR medium daily until the cells need to be passaged.

5.4. **Passage**

5.4.1. Rinse each well to be passaged with 1ml warmed Trypsin/EDTA. Aspirate and add 1ml/well Trypsin/EDTA to each well to be passaged. Incubate at 37°C for 5 minutes.

5.4.2. After incubation, use a micropipette to disassociate cells into a single cell suspension, and transfer to a conical tube.

5.4.3. Collect any residual cells remaining in the wells with 1ml/well 10% FBS in DMEM/F-12 + GlutaMax (neutralization medium), and add to the conical tube containing cells.

5.4.4. Spin for 5 minutes at 200 x g. Aspirate supernatant and suspend pellet in an appropriate volume of Elf1 cKOSR medium to achieve the desired passaging ratio (a typical passaging ratio is between 1:5 and 1:10). For example, if 2 wells were being split 1:6, suspend the pellet in 6ml and seed each new well with 0.5ml of cells.

5.5. **Culture Notes**

5.5.1. After passaging, it will be several days before colonies are visible. Feed daily.

5.5.2. The Elf1 cell line has always been cultured on MEF feeders, but can be cultured on Matrigel. Culture on Matrigel results in a flatter colony morphology.

5.6. **Cryopreservation**

5.6.1. Follow cryopreservation techniques in the WiCell Feeder Dependent Pluripotent Stem Cell Protocol (SOP-SH-001) with the following modifications:

5.6.1.1. Use Trypsin/EDTA as the harvest reagent instead of collagenase. Harvest as directed above.

5.6.1.2. Use Elf1 cKOSR medium instead of the standard cKOSR medium, including when preparing the cryopreservation medium.

6. REVISION HISTORY

Version	Change Description	Effective Date
A	CC00526, Document Initiation.	04/09/2015
B	CC00527, Added that Elf1 cKOSR and trypsin should also be used in the cryopreservation, removed aliquot directives. Changed text regarding confirming thaw ratio to red. Added copyright	07/07/2015
C	CC00565, Added optional addition of IGF1 to the culture medium per provider.	01/21/2016
D	CC00803, Addressed formatting/font issue in the purpose section. Updated 2-FGF concentration.	See SPDC

7. APPROVALS

1/31/2018 X JKG JKG Quality Assurnace Manager Signed by: Gay, Jenna	2/16/2018 X TEL TEL Director - WiCell Stem Cell Bank Signed by: Ludwig, Tenneille

WiCell Feeder Based (MEF) Pluripotent Stem Cell Protocols

Preface

This booklet of protocols is intended to serve as a primer for culturing pluripotent stem cells on mouse embryonic fibroblast (MEF) feeder cells. These protocols are representative of how the cells were cultured and banked. WiCell recommends that pluripotent stem cells (PSCs) should be first thawed and established in the conditions in which they were initially frozen prior to transfer to alternate culture platforms. We recommend that you read through these protocols prior to thawing your cells, and follow them until you have established your own cell bank of frozen vials. As you thaw and expand your initial cell vials, we strongly encourage you to establish your own bank of frozen vials as soon as possible. Once the culture is established, freeze back a portion of the material, and freeze again when you have enough cells for 20 vials. This will ensure you have an adequate stock of material to expand from as you conduct experiments.

Because this bank will be the basis of all future work with this cell line, WiCell recommends that it be screened for karyotype and STR post thaw to ensure that the stability and identity of the banked material is as expected. These and other tests are available from WiCell's full-service Characterization Laboratory; to learn more about WiCell Characterization's offerings and how to submit samples visit www.wicell.org/characterizations .

If you have any additional questions, please contact us through technical support on the WiCell website at www.wicell.org.

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Section 1: MEF Plating Density Recommendations

For MEF based culture systems, WiCell recommends using only CF1 strain MEFs. Standard plating density for freshly inactivated MEFs is 75,000 cells/ml at 2.5 ml per well in a 6 well plate (187,500 cells/well, which is equal to 19,530 cells/cm^2). If inactivated MEFs have been frozen and thawed before use, WiCell recommends plating at a density of 90,000 cells/ml at 2.5 ml per well in a 6 well plate (225,000 cells/well, which is equal to 23,440 cells/cm^2) to compensate for the loss of viability following the freeze thaw process. There can be significant variation in MEF quality from lot to lot, and therefore WiCell recommends qualifying all MEFs used to assure that they will support pluripotent stem cell culture before use with critical cell lines.

For MEFs to adhere properly, vessels must be coated with a sterile 0.1% gelatin (Sigma G1890-100G) in water solution. It is recommended that gelatin be allowed to coat plates for 24 hours in a 37°C incubator prior to use. In cases of extreme urgency, a minimum of one hour of coating time in a 37°C incubator is required. Use the MEFs plates within 7 days of plating, with a total life span of 14 days. The cells do not require medium exchange, they will maintain in the same plating medium until they are used.

Section 2: Thawing Pluripotent Cells Protocol

Note 1: Before thawing, check the Certificate of Analysis included in the shipping packet insert to acquire the recommended number of wells one vial should be thawed into (this can vary among different lots).

Note 2: In general, ROCK inhibitor is not required for most cell lines. Please consult the Certificate of Analysis to see if ROCK inhibitor is recommended. If ROCK inhibitor is not recommended, it will not be mentioned on the Certificate of Analysis.

2.1. Required Equipment

2.1.1. Biosafety cabinet

2.1.2. 37°C / 5% CO_2 incubator

2.1.3. 37°C water bath

2.1.4. Centrifuge

2.1.5. Microscope

2.1.6. 10µl, 200µl and 1000µl micropipettes

2.2. Required Supplies

2.2.1. Forceps

2.2.2. Cryogenic handling gloves and eye protection

2.2.3. Two 6-well plates with freshly inactivated MEF cells prepared at least the day before use (only 1or 2 wells of MEFs are needed on each plate)

2.2.4. 5ml sterile serological pipettes (Fisher, 13-678-27E) or equivalent

2.2.5. 95% Ethanol

2.2.6. 1.5ml Microcentrifuge tubes, sterilized (Fisher, 05-408-129), or equivalent

2.2.7. Microcentrifuge tube holders

2.2.8. Freezer storage boxes

2.2.9. Sterile 10µl, 200µl and 1000µl micropipette tips (Fisher, 21-236-35, 21-402-185, 21-236-85) or equivalent

2.2.10. Sterile Pasteur Pipettes (Fischer, 13-678-20D) or equivalent

2.3. Required Reagents

2.3.1. DMEM/F-12 Medium (Invitrogen, 11330-032)

2.3.2. Knockout Serum Replacer (KOSR) (Invitrogen, 10828-028)

2.3.3. L-glutamine, non-animal, cell culture tested (Sigma, G-8540)

2.3.4. MEM Non-Essential Amino acid solution (Invitrogen, 11140-050)

2.3.5. Basic Fibroblast Growth Factor (β-FGF) (Invitrogen, PHG0024) or equivalent

2.3.6. PBS without $CaCl_2$ or $MgCl_2$ (Invitrogen, 14190-250)

2.3.7. PBS with $CaCl_2$ and $MgCl_2$ (Invitrogen, 14040-141)

2.3.8. Bovine Serum Albumin (Sigma, A2153)

2.3.9. 2-Mercaptoethanol (Sigma, M3148)

2.3.10. ROCK Inhibitor (Y-27632 dihydrochloride; BD Biosciences, 562822), <u>if needed</u> (check the certificate of analysis in shipment packet)

2.3.11. Sterile water (Sigma, W3500)

2.3.12. **Stem Cell Culture Medium (may also be referred to as hES medium or hESC medium) (250ml)**

2.3.12.1. To make Stem Cell Culture Medium, combine following components, filter sterilize, store at 4ºC for up to 14 days, or for up to one year at -20ºC. If frozen, use within 14 days after thaw.

2.3.12.2. 200ml DMEM/F-12 Medium

2.3.12.3. 50ml Knockout Serum Replacer

2.3.12.4. 2.5ml 100mM L-Glutamine +BME Solution (See below)

2.3.12.5. 2.5ml MEM Non-Essential Amino Acids

2.3.12.6. 0.5ml 2µg/ml Basic FGF solution (See below)

2.3.13. **100mM L-Glutamine +BME Solution (for Stem Cell Culture Medium)**

2.3.13.1. To make 100mM L-Glutamine +BME Solution, combine the following components just prior to making Stem Cell Culture Medium, ensure solution is well mixed. Discard any extra. May be scaled up or down based on need.

2.3.13.2. 73.0mg L-Glutamine

2.3.13.3. 5.0ml PBS without $CaCl_2$ and $MgCl_2$

2.3.13.4. 3.5µl 2-Mercaptoethanol (14.3M)

2.3.14. **2µg/ml Basic FGF Solution (for Stem Cell Culture Medium)**

2.3.14.1. To make 2µg/ml Basic FGF Solution, combine the following components. Aliquot 0.5ml/tube and store at -20°C for up to 6 months. Each aliquot is enough to make 250ml of Stem Cell Culture Medium. Thaw aliquot at room temperature (or at 4°C) just prior to making Stem Cell Culture Medium. Do not re-freeze aliquots.

2.3.14.2. 10µg Basic FGF

2.3.14.3. 5ml 0.1% BSA in PBS with $CaCl_2$ and $MgCl_2$

2.3.15. **Reconstitute ROCK Inhibitor and Aliquot Working Stock Solution, if needed.**
Before preparing, check the certificate of analysis included in the shipping packet insert to see if ROCK Inhibitor is recommended for thawing, this can vary among lots. If ROCK inhibitor is not recommended, it will not be mentioned on the Certificate of Analysis.

Note: Perform work sterilely.

2.3.15.1. Make 10mM working stock solution by diluting 1mg ROCK inhibitor (FW 338.3) into 295µl sterile water to achieve a 10mM solution. Note: if FW of material is not 338.3, dilute appropriately to achieve a 10mM solution.

2.3.15.2. Aliquot into appropriate working volumes (recommended at 20-50µl). ROCK inhibitor working stock solution will be used at 1µl to 1ml final medium volume to achieve a final concentration of 10µlM (for example: Add 250µl of 10mM working stock solution to 250ml Stem Cell Culture Medium). Aliquots can be stored long term at -80°C for up to 1 year and up to 2 months at 4°C.

2.3.15.3. Filtering the medium after addition of ROCK inhibitor is not necessary if working with sterile stock solution. However, filtering may be performed to ensure sterility of final product if desired.

2.4. Remove vial and thaw

2.4.1. Prior to removing vial from storage, acquire the thaw recommendation (number of wells one vial should be thawed into) found in the Certificate of Analysis included in the shipping packet insert.

2.4.2. Wear eye protection as vials stored in liquid nitrogen may accidentally explode when warmed.

2.4.3. Wear ultra-low temperature cryogenic gloves. Remove the cell vial from the liquid nitrogen storage tank using forceps.

2.4.4. Quickly remove the label or copy the information written on the tube in your notebook. The writing or printed information may come off the vial in the ethanol bath. This should take no longer than 10 seconds.

2.4.5. Ensure the vial cap is tightly closed, grasp vial with forceps

2.4.6. Immerse the vial in a 37°C water bath without submerging the cap. Swirl the vial gently.

2.4.7. When only a small ice crystal remains, remove the vial from the water bath.

2.4.8. Immerse the vial into a 95% ethanol bath to sterilize the outside of the tube. Briefly (15-30 seconds) air-dry the vial in the sterile biosafety cabinet.

2.5. Removal of Cryoprotectant and Re-suspension of Pluripotent Stem Cells

2.5.1. Transfer the cells gently into a sterile 15ml conical tube using a 1ml or 5ml glass pipette. Do not allow cells to flow down the side of the tube, as this will cause shearing and reduce attachment.

2.5.2. Slowly, add 10ml of warmed Stem Cell Culture Medium drop-wise to cells in the 15ml conical tube. While adding the medium, gently move the tube back and forth to mix the cells. This reduces osmotic shock to the cells.

2.5.3. Centrifuge the cells at 200 x g for 5 minutes.

2.5.4. Aspirate and discard the supernatant with a sterilized Pasteur pipette.

2.5.5. Suspend the cell pellet in 2.5ml Stem Cell Culture Medium for every well that will receive cells (number of wells receiving cells is based on the thaw recommendation found in the certificate of analysis which is included in the shipping packet insert). *For example*: When the thaw recommendation is to thaw 1 vial into 1 well, re-suspend the pellet in 2.5ml.

2.5.6. Gently pipette cells up and down in the tube a few times.

2.5.7. Set aside while preparing the MEF plate to receive cells.

2.6. Prepare MEF Plate and Transfer Stem Cells

2.6.1. Label a 6-well plate containing inactivated MEF cells with the passage number from the vial, the date and your initials.

2.6.2. Aspirate the MEF medium from well(s) that will receive cells (thaw recommendations vary among lots, refer to the Certificate of Analysis to know how many wells one vial should be seeded into). Rinse the well(s) with 1ml sterile DMEM/F-12.

2.6.3. Aspirate the DMEM/F-12 rinse medium.

2.6.4. Slowly add the 2.5 ml stem cell suspension into the well (if plating into more than 1 well, gently mix the cell suspension prior to adding to plate).

2.6.5. Place plate into the incubator and gently move it back and forth and side to side to evenly distribute the cells—avoid circular motions to prevent pooling in the center of the well.

2.7. Create Culture Back-Up for Thawed Material

2.7.1. The next day, prepare 1 well of a second 6-well MEF plate to receive the floating cell material from the thaw well. This will serve as the backup culture for the thaw because the floating cellular material usually attaches and remains viable.

2.7.1.1. Aspirate MEF medium from 1 well of a second 6-well plate.

2.7.1.2. Rinse with 1ml sterile DMEM/F-12, aspirate.

2.7.2. From the thaw well, transfer the spent medium with debris using a sterile 5ml serological pipette into the prepared second MEF plate.

2.7.3. This back-up culture should be given to a second technician if possible.

2.7.4. Feed back-up culture daily with separate medium from the primary thawed material and maintain in a separate incubator.

2.8. Maintain Cells

2.8.1. Gently add 2.5ml of Stem Cell Culture Medium to the first original thaw well(s) and return to incubator.

2.8.2. Feed and monitor all cells daily until ready to passage or freeze. Cells are typically ready to passage for the first time out of freeze between day 6 and 14.

Section 3: Feeding Pluripotent Stem Cells: Feeder-Dependent (MEF) Protocol

3.1. Required Equipment:

3.1.1. Biosafety cabinet

3.1.2. 37˚C / 5% CO_2 incubator

3.1.3. Microscope

3.1.4. Water bath

3.2. Required Supplies:

3.2.1. 5ml and 10ml sterile serological pipettes (Fisher: 13-678-27E, 13-67827F) or equivalent

3.2.2. Sterile Pasteur Pipettes (Fischer, 13-678-20D) or equivalent

3.3. Required Reagents:

3.3.1. Stem Cell Culture Medium as in section 2

3.4. Feeding Pluripotent Stem Cells

3.4.1. Observe the stem cells using a microscope. If they require passaging, follow the passaging protocol below.

3.4.2. Use a 37°C water bath to warm enough medium to feed 2-2.5ml for each well that will be fed.

3.4.3. Aspirate the spent medium with a sterilized Pasteur pipette. If feeding more than one plate, use a different pipette for each plate to reduce risk of contamination.

3.4.4. Add 2-2.5ml of warmed Stem Cell Culture Medium to each well. After pipettes are used once, they must be disposed to reduce the contamination potential. Do not reinsert a used pipette into sterile medium for any reason.

3.4.5. Return the cells to the 37°C incubator.

3.4.6. Repeat procedure daily until ready to passage or freeze.

Section 4: Passaging Pluripotent Stem Cells: Feeder-Dependent (MEF) Protocols

Note: There are two methods for passaging: An enzymatic "no-spin" method suitable for standard passaging. A non-enzymatic manual passaging method is recommended when there is significant differentiation present and the culture must be maintained.

4.1. Required Equipment:

4.1.1. Biosafety cabinet

4.1.2. 37°C / 5% CO_2 incubator

4.1.3. 37°C water bath

4.1.4. Microscope with colony marker

4.1.5. Hood (biosafety or static) equipped with stereomicroscope

4.2. Required Supplies:

4.2.1. 5ml sterile serological pipettes (Fisher, 13-678-27E) or equivalent

4.2.2. Sterile Pasteur Pipettes (Fisher, 13-678-20D) or equivalent

4.2.3. Modified Pasteur pipette or micropipette tip

4.3. Required Reagents

4.3.1. DMEM/F-12 Medium (Invitrogen, 11330-032)

4.3.2. Collagenase Type IV (Invitrogen, 17104-019)

4.3.3. Stem Cell Culture Medium as in Section 2

4.3.4. **1mg/ml Collagenase Solution**

4.3.4.1. To make 1mg/ml Collagenase Solution, combine the following components, filter sterilize and store at 4ºC for up to 14 days. Scale up or down based on your needs.

4.3.4.2. 25mg Collagenase Type IV

4.3.4.3. 25ml DMEM/F-12

4.4. Determine When to Passage (Split) Cells and the Correct Density

4.4.1. In general, split cells when the first of the following occur:

4.4.1.1. Mouse Embryonic Fibroblasts (MEF) feeder layer is two weeks old.

4.4.1.2. Pluripotent stem cell colonies are becoming too dense or too large.

4.4.1.3. Increased differentiation occurs.

4.4.2. The split ratio is variable, though generally between 1:2 and 1:4. Occasionally cells will grow at a different rate and the split ratio will need to be adjusted. A general rule is to observe the last split ratio and adjust the ratio according to the appearance of the pluripotent stem cell colonies. If the cells look healthy and colonies have enough space, split using the same ratio, if they are overly dense and crowding, increase the ratio, and if the cells are sparse, decrease the ratio.

4.4.3. Cells will need to be split every 4-6 days based upon appearance.

4.5. Warm enough Stem Cell Culture medium in a 37ºC water bath.

4.6. Prepare MEF Plate to Receive Cells

4.6.1. Ready the prepared MEF plate for culture by aspirating the MEF medium from the wells, and rinsing with 1ml/well sterile DMEM/F-12.

4.6.2. Aspirate rinse medium and add 1.5 ml of warmed Stem Cell Culture Medium to each well if the split ratio is 1:3 or more. Add 0.5ml/well if the split ratio is less than 1:3.

4.6.3. Label plate appropriately (WiCell recommends at minimum the cell line name, passage number on the vial, date and technician initials), and place in incubator until ready to plate passaged cells.

4.7. Prepare Culture for Passaging with Collagenase

Note: Determine total number of wells you are passaging into. The volumes in this section may need to be adjusted based on culture conditions. The final plating volume should be 2.0ml/well.

4.7.1. Examine the cultures to be passaged and remove differentiation if necessary. Determine which method of differentiation removal (selection method) is required based on the level of differentiation:

% Differentiation	Selection Method
<5%, or isolated differentiated colonies	Removal by suction
5-30% or partially differentiated colonies	Pick-to-remove
>30%	Manual Passage

4.7.2. **Removal by suction**: Use the colony marker on the microscope to mark all areas of differentiation on the plate of pluripotent stem cells. If removing differentiation by suction, on the last aspiration of the passaging procedure after treatment with Collagenase Solution, confirm the Pasteur pipette tip is intact and not chipped. Carefully press the pipette tip to the circled area and suction off the differentiated cells. Confirm the complete removal under the microscope. Finish passage immediately after removal of differentiation. When removing multiple areas of differentiation, take care not to dry out the well by gently moving the plate to allow rinse liquid to hydrate the well.

4.7.3. **Pick-to-Remove:** If removing differentiation by the pick-to-remove method, transfer the plate into a hood equipped with a stereomicroscope. Remove all areas of differentiation with a sterile modified pipette or sterile micropipette tip. Aspirate medium containing removed differentiation from well and continue passaging with Collagenase as below.

4.7.4. **Manual passage:** See "Manual Passaging" section below.

4.7.5. **Passaging Cells with Collagenase**

4.7.5.1. Place plate in the biosafety cabinet.

4.7.5.2. Aspirate the spent medium from the wells to be passaged with a Pasteur pipette. If possible, at least one well of cells should be fed independently and left unpassaged to be used as a backup to protect against problems with the split that would otherwise jeopardize the culture (contamination, etc.).

4.7.5.3. Add 1ml room temperature Collagenase Solution to each well to be passaged.

4.7.5.4. Incubate for 5-7 minutes at 37°C.

4.7.5.5. To confirm appropriate incubation time, view the surface under a microscope. Look for the perimeter of the colony to appear highlighted or just slightly folded back. The colonies will not be coming completely off the plate. If the perimeters are not folding back yet, continue to incubate, checking the surface periodically.

4.7.5.6. Aspirate the Collagenase Solution with a Pasteur pipette, being careful not to disturb the attached cell layer.

4.7.5.7. Gently add 1ml of warmed DMEM/F-12 to each well with a 5ml pipette. Check to be sure that the cells remain adhered to the plate. Aspirate the medium.

Note: Do not dispense the medium in a continuous stream in one spot since the cells in that area will peel off.

4.7.5.8. If removing differentiation by suction, ensure the pipette tip is intact (free of chips) and carefully press the pipette tip to the marked area and suction off the differentiated cells. Confirm the complete removal under the microscope.

4.7.5.9. Add 2ml of Stem Cell Culture Medium to each well.

4.7.5.10. Using a sterile 5 ml pipette, bring up the medium from the well(s) into the pipette. Hold the pipette perpendicular to the plate and gently scrape the surface of the plate back and forth horizontally while simultaneously dispensing medium. It may be helpful to tilt the plate slightly forwards to see the colonies better, being careful not to tilt too far and spill any medium present in the plate. Repeat if necessary until all cells are removed.

Note: Minimize bubbles by scraping and pipetting gently.

4.7.5.11. Pipette the medium slowly up and down to wash the cells off the surface. Be careful not to create bubbles. You may move between wells with the same medium to remove cells or remove each well individually. Do not work with more than 3ml/well (overfilling wells may lead to contamination); use a conical tube for the remainder if more volume is needed.

4.7.5.12. Return the removed cell material back to the well(s) until all wells are scraped to prevent the wells drying out. After the all the cells are removed from the surface of the wells, pool the contents of the scraped wells into a sterile conical tube.

4.7.5.13. Pipette cells up and down gently a few times in the conical tube to further break-up cell colonies if needed. Pipette carefully to reduce foaming.

4.7.5.14. Take up 1-2ml of fresh Stem Cell Culture Medium in a 5ml pipette and add it to the first well to wash and collect residual cells. Take up the medium and transfer it into each subsequent well to collect cells.

4.7.5.15. Transfer the Stem Cell Culture Medium wash to the conical tube containing the cells.

4.7.5.16. Determine how much additional medium is required to so 1ml of cell suspension can be added to each new well. This is dependent on the split ratio and the number of wells used. There should be a total of 2-2.5ml of Stem Cell Culture Medium and cells in each of the new wells (1ml of cell suspension + 1-1.5ml of pre-plated Stem Cell Culture Medium).

4.3.1.1.1. In the case 1 well is split 1:1 into 1 well, a final total volume in the well may be up to 3ml.

4.7.6. **Plate Cells**

4.7.6.1. Gently re-suspend the cells using a 5ml pipette.

4.7.6.2. Add 1ml of cell suspension to each well of the prepared MEF plate.

4.7.6.3. Return the plate to the incubator after plating the cells. Gently move the plate back and forth and side to side to evenly distribute the cells—avoid circular motions to prevent pooling in the center of the well.

Note: While cells are attaching, try to limit opening and closing the incubator doors, and if you need to access the incubator, open and close the doors carefully. This will prevent disturbing the even distribution of cells to the surface of the well.

4.7.6.4. Incubate cells overnight to allow colonies to attach.

4.7.6.5. If passage was successful and free of contamination, the backup well can be discarded the following day.

4.7.6.6. Feed culture as previously described until ready to passage or freeze.

4.8. Manual Passaging

Note: Manual passaging puts a very high selection pressure on cultures, and therefore we recommend only using when absolutely necessary. Occasionally, cultures may be very sparse after thaw, requiring manual passaging. Other than directly out of thaw, we recommend manual passaging only when necessary to save an irreplaceable culture.

4.8.1. Have a prepared MEF plate readied with 1ml/well of culture medium as described above, set aside in the biosafety cabinet.

4.8.2. Remove culture plate from incubator and place it in the biosafety cabinet. Aspirate the spent medium from the wells to be passaged with a Pasteur pipette. If possible, one well should be left and fed separately to be used as a back-up to protect against problems with the split that would otherwise jeopardize the culture (contamination, etc.). If it is not possible to leave an entire well (if you only have one well to passage) be sure to leave some material in the well and add 2.5ml fresh medium after manual passage to serve as a back-up.

4.8.3. Add 1.0 ml of Stem Cell Culture Medium to each well to be passaged.

4.8.4. Transfer the plate of cells to be passaged into a hood equipped with a stereomicroscope.

4.8.5. Remove the undifferentiated colonies from the pluripotent stem cell plate by cutting each colony into several (4-5) pieces and nudge them so they are floating in the medium. This can be done with a sterile modified pipette, micropipette tip, needle, or other cutting tool.

4.8.6. Take the plate back to the biosafety cabinet, use a 5ml pipette to transfer all of the medium containing the pieces into the prepared well(s). Transfer up to 50 colony pieces into the prepared well.

4.8.7. Add additional medium to the well of the new plate to equal 2-2.5 ml culture medium total.

4.8.8. If maintaining the well that was manually passed from, add 2.5ml of fresh Stem Cell Culture Medium.

4.8.9. Return the new plate to the incubator after plating the cells. Move the plate in several quick, short, back-and-forth and side-to-side motions to further disperse cells across the surface of the wells.

Note: While cells are attaching, open and close the incubator doors carefully. This will prevent disturbing the even distribution of cells to the surface of the well.

4.8.10. Incubate cells overnight to allow colonies to attach.

4.8.11. If the passage was successful and free of contamination, the backup well can be discarded the day following passage.

4.8.12. Feed culture as previously described until ready to passage or freeze.

Section 5: Freezing Stem Cells: Feeder-Dependent (MEF) Protocol

5.1. **Required Equipment**

5.1.1. Biosafety cabinet

5.1.2. 37ºC / 5% CO_2 Incubator

5.1.3. 37ºC water bath

5.1.4. Centrifuge

5.2. **Required Supplies**

5.2.1. Metal forceps

5.2.2. Cryogenic handling gloves and eye protection

5.2.3. Isopropanol freezing containers (Fisher, 15-350-50) or equivalent

5.2.4. Plastic cryovial holders

5.2.5. 5ml and 10ml sterile serological pipettes (Fisher: 13-678-27E, 13-67827F) or equivalent

5.2.6. 95% Ethanol

5.3. **Required Reagents**

5.3.1. Fetal Bovine Serum (FBS) (Omega, FB-11), do not heat-inactivate

5.3.2. DMEM/F-12 Medium (Invitrogen, 11330-032)

5.3.3. Dimethyl Sulfoxide (DMSO) 10ml ampoules (Sigma Aldrich, D2438)

5.3.4. Stem Cell Culture Medium as in Section 2

5.3.5. 1mg/ml Collagenase Solution as in Section 4

5.3.6. **Cryopreservation Medium**

Note: Make Cryopreservation Medium as indicated below. Medium should be made fresh before use and kept on ice. Discard any medium remaining after freeze.

5.3.6.1. Prepare 0.5ml of cryopreservation medium for every vial to be frozen, plus an additional 1-5 ml to account for pipetting error. The following is to prepare 10ml of Cryopreservation medium, adjust volumes as necessary.

5.3.6.2. Add 6ml FBS to 2ml Stem Cell Culture Medium, filter sterilize.

5.3.6.3. After filtering, add 2ml sterile DMSO (do not filter DMSO, it will degrade the filter membrane).

5.3.6.4. Store on ice until ready to use.

5.4. **Prepare for Freeze**

5.4.1. Label cryovials with the cell line, passage number (increase the passage number on the vial label by 1 from what was on the culture plate at time of harvest), the freeze date, and your initials. Use an alcohol proof pen or labels that resist liquid nitrogen and ethanol. Place in biosafety cabinet.

5.4.2. Sterilize the biosafety cabinet (with the labeled vials in it) for 20 minutes with UV light. Turn on the blower and open the sash. Spray down the whole surface with ethanol and allow it to evaporate for 20 minutes prior to initiating cryopreservation.

5.4.3. Obtain a recharged, room-temperature isopropanol freezing container. Each container can hold 18 vials. The isopropanol must be replaced every 5 uses.

5.4.4. View all cells under the microscope. Discard any contaminated plates and circle areas of differentiation.

5.4.5. Remove spent medium and add 1ml of Collagenase Solution to each well of each 6-well plate.

5.4.6. Incubate cells for 5-7 minutes at 37ºC.

5.4.7. Confirm appropriate incubation time by viewing the plate under the microscope. Look for the perimeter of the colony to appear highlighted and slightly folded back. The colonies will not be coming completely off the plate. If the perimeters are not folding back yet, continue to incubate, checking the surface periodically.

5.5. **Harvest and Spin Cells**

5.5.1. Aspirate Collagenase Solution from each well, taking care not to remove any floating colonies.

5.5.2. Add 1ml of Stem Cell Culture Medium to each well.

5.5.3. When harvesting 6 wells of a 6-well plate, work across the top three wells as a group. Take up 1ml of medium from each well in a 5ml serological pipette, hold the pipette perpendicular to the plate and gently scrape the surface of the plate back and forth horizontally while simultaneously dispensing medium. It may be helpful to tilt the plate slightly forwards to see the colonies better, being careful not to tilt too far and spill any medium present in the plate. Repeat if necessary until all cells are removed. Transfer cells and medium to the next well and repeat until all three wells have been harvested. Work quickly to prevent wells from drying out before harvest. Be careful to keep cells in small clumps. Cells will recover from the thaw more efficiently if frozen in aggregates.

5.5.4. Pool the cells in one sterile 15ml centrifuge tube, 1 tube per plate.

5.5.5. Repeat the above step with the remaining bottom three wells; add cells to same conical tube to create a pool of cells.

5.5.6. Wash the plate with 3ml of fresh Stem Cell Culture Medium, transferring the medium from well to well and add the medium to the 15ml conical tube.

5.5.7. Repeat this process if harvesting from more than one plate until all cells are harvested.

5.5.8. Centrifuge at 200 x g for five minutes.

5.6. **Suspend Pellet(s) and Vial Cells**

5.6.1. Aspirate the supernatant being careful not to disturb the cell pellet(s). Gently re-suspend each cell pellet in 0.5ml Stem Cell Culture Medium per each well harvested. Freezing 5 plates (6 wells x 5 plates) at 1 well/cryovial will require 15ml of Stem Cell Culture Medium.

Note: If freezing more than 6 plates of cells, alter the size of the pool vessel.

5.6.2. Transfer 9ml of cells to a new 50ml conical tube. Do not work with more than 9ml of cell suspension at a time. If cell pool is larger than 9ml, perform the next steps in batches. This is because one freezing container can only hold 18 vials (9ml cells + 9ml cryo medium = 18 vials).

5.6.3. Retrieve the Cryopreservation Medium from the ice bath and place in the biosafety cabinet. Ensure the medium is evenly mixed by using a 10ml pipet, otherwise the DMSO will remain settled on the bottom.

5.6.4. While gently tapping the tube of cells, very slowly and drop-wise add an equal volume of Cryopreservation Medium. For example, add 9ml of Cryopreservation Medium to 9ml of cell suspension. **Note:** At this point, cells are in contact with DMSO, and work must be performed efficiently. Once cells are in contact with DMSO, they should be aliquoted and frozen within 2-3 minutes.

5.6.5. Pipette the cells very gently to evenly mix suspension.

5.6.6. With the same pipette, distribute 1ml of cell suspension to each of the prepared cryovials. Mix the cell pool every 6-10 vials for even distribution.

5.6.7. Quickly, tighten caps and place cryovials into an isopropanol containing freezing container. Place the freezing container in the -80ºC freezer overnight.

5.6.8. The next day, transfer cell vials to liquid nitrogen storage.

Section 6: Transitioning Between Culture Platforms

Transfer between platforms should be done only after an initial bank of material has been frozen back in the original conditions. If transferring into mTeSR1/Matrigel or E8/Matrigel platform, WiCell generally recommends that the first passage into a Feeder-Independent platform be more dense (for example, if you would generally split 1:3, your first passage into a Feeder-Independent platform from MEFs should be done at 1:2 or 1:2.5). After the first passage, normal passage densities should be used. If transferring to alternate feeder free or feeder independent culture platforms, follow the manufacturer's guidelines for transfer. Regardless of final platform, we recommend maintaining the initial cultures in the original conditions parallel until the user can be confident that the transition is successful.

Section 7: Revision History and Protocol Approvals

7.1. Revision History

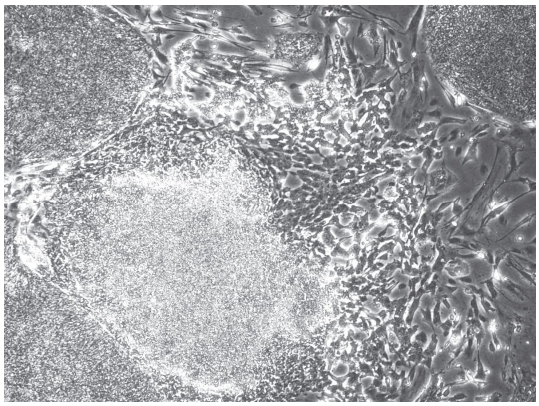
Version	Change Description	Effective Date
A	This document was previously not included in WiCell's document control system.	Not applicable
B	Updated protocol to represent current procedures, incorporated into WiCell Document Control System	04/08/2010
C	Added attachment 2 MSDS included for WiCell produced cells only	08/13/2010
D	CC00128. Added MEF Plating Density Recommendations and Transitioning Between Feeder Dependent and Feeder Independent Platforms.	08/24/2011
E	CC00284, updated format to be consistent will all shipping protocols, specifically the layout of supplies and materials, minor procedural edits for clarity, corrected formulas for magnesium chloride and calcium chloride, added instruction to confirm the thaw recommendation on certificate of analysis, added to warm culture medium before use.	07/09/2013
F	CC00361, corrected page break on page 1.	08/20/2013
G	CC00543, added copyright.	05/28/2015
H	CC00635, updated note about ROCK inhibitor in CoA, added ROCK inhibitor prep, clarify language in harvest and spin during freeze procedure	06/06/2017
I	CC00754, Clarified alternative names for stem cell culture medium, updated ROCK inhibitor vendor info, updated 2-Mercaptoethanol catalog number, updated FBS vendor info, removed directive to roll vial between hands while thawing vial, corrected volume of culture medium to add to cells while thawing, added to feed well prior to performing pick-to-keep, added description about why passage number is increased by 1 at freeze, added that freezing container must be room temperature, added definition of MEF to preface, removed FBS from passaging reagent list, added that cells should not travel down side of tube while thawing, updated differentiation removal guidelines.	02/19/2018
J,	CC01069, Added gelatin coating of vessels. Added to tilt plate while harvesting. Added freezing container catalog info. General update made to add more details.	See SPDC

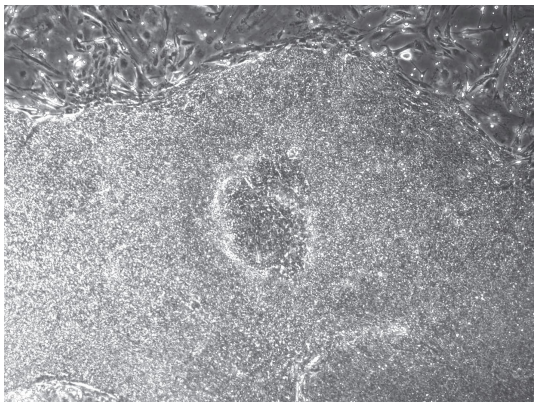
7.2. Approvals

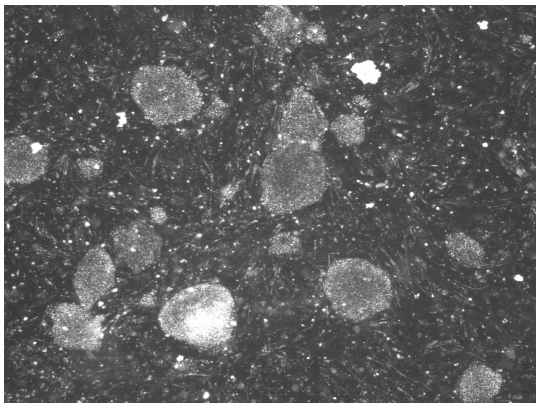
4/24/2020 X JKG JKG Quality Assurance Manager Signed by: Gay, Jenna	4/24/2020 X TEL TEL Director - WiCell Stem Cell Bank Signed by: Ludwig, Tenneille

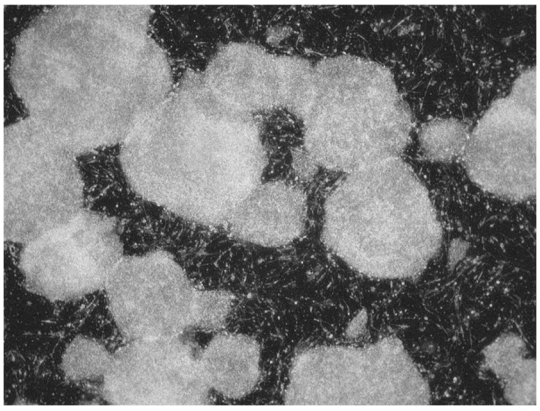
Attachment 1: Photographs of hES Cells on MEF feeder layer

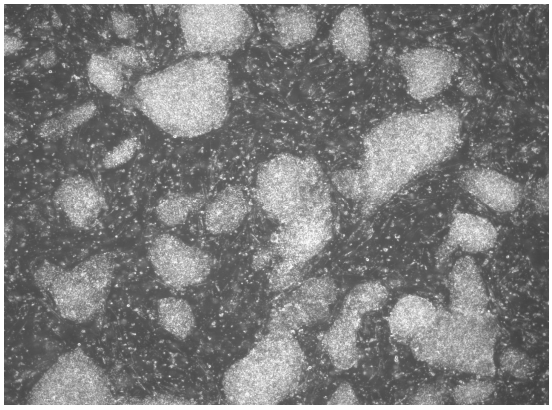
These are a few photos to give you an idea of how to assess differentiation levels in your cultures.

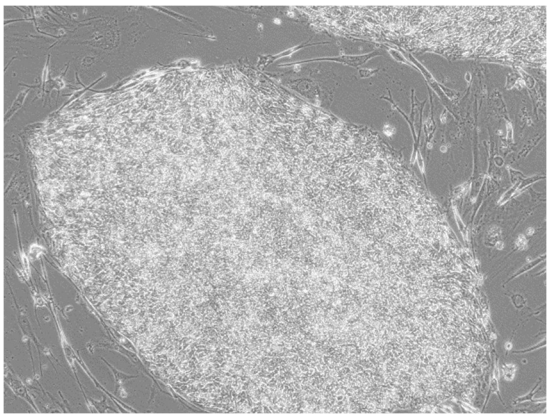

Differentiation around colony (10x)


Differentiation in colony center (10x)


5 days post thaw (2x)


Colonies ready to split (2x)


Healthy colonies 3 days post-split (2x)


Healthy colony on MEF Cells (10x)