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 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 Cytogenetics Laboratory; to learn more about WiCell Cytogenetic's offerings and how to submit samples visit www.wicell.org/cytogenetics.

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 $0.75x10^5$ cells/ml at 2.5 ml per well in a 6 well plate ($1.87x10^5$ cells/well). If inactivated MEFs have been frozen and thawed before use, WiCell recommends plating at a density of $0.90x10^5$ cells/ml at 2.5 ml per well in a 6 well plate ($2.25x10^5$ cells/well) 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.

Section 2: Thawing Pluripotent Cells Protocol

Note: 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). 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. 37ºC / 5% CO_2 incubator

2.1.3. 37ºC water bath

2.1.4. Centrifuge

2.1.5. Microscope

2.1.6. 100µl or 200µl Pipetman

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 (1or 2 wells of MEFs 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)

2.2.7. Microcentrifuge tube holders

2.2.8. Freezer Boxes

2.2.9. Sterile 100µl or 200µl Pipette tips (Molecular Bio, 2770)

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, PHG0021) 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, M7522)

2.3.10. ROCK Inhibitor (Y-27632 dihydrochloride; Tocris, 1254)

2.3.11. Sterile water (Sigma, W3500)

2.3.12. **Stem Cell Culture 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)**

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.

2.3.13.1.1. 73.0mg L-Glutamine

2.3.13.1.2. 5.0ml PBS without $CaCl_2$ and $MgCl_2$

2.3.13.1.3. 3.5µl 2-Mercaptoethanol (14.3M)

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

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 just prior to making Stem Cell Culture Medium. Do not re-freeze aliquots.

2.3.14.1.1. 10µg Basic FGF

2.3.14.1.2. 5ml 0.1% BSA in PBS with $CaCl_2$ and $MgCl_2$

2.3.15. **Reconstitute ROCK Inhibitor and Aliquot Working Stock Solution**

Note: Perform work sterilely.

2.3.15.1. Make 10mM working stock solution by diluting 1mg ROCK inhibitor (FW 320.26) into 295µl sterile water to achieve a 10mM solution. Note: if FW of material is not 320.26, 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. Aliquots can be stored long term at -80°C for up to 1 year and up to 2 months at 4°C.

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 cryo gloves. Remove the pluripotent stem cell vial from the liquid nitrogen storage tank using forceps.

2.4.4. Roll the vial between your gloved hands until the outside is free of frost. This should take between 10-15 seconds.

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

2.4.6. Immerse the vial in a 37ºC 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. Ensure the vial cap is tightly closed and 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 glass pipette.

2.5.2. Slowly, add 4ml 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 pluripotent stem 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. Re-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.

2.6. Plate 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 wells which will receive cells (number of wells determined by the thaw recommendation found in the certificate of analysis). Rinse the well with 1ml sterile DMEM/F-12. Aspirate the DMEM/F-12 rinse medium prior to plating.

2.6.3. Slowly add the 2.5 ml stem cell suspension drop-wise into the well.

2.6.4. Place plate into the incubator and gently shake 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.

2.6.5. The next day, remove the spent medium with debris using a sterile 5ml serological pipette and transfer it into a prepared (as previously described) well of a second 6-well plate. This will serve as the backup for the thaw and should be given to a second technician if possible. This back up should be feed with separate medium and maintained in a separate incubator.

2.6.6. Gently add 2.5ml of Stem Cell Culture Medium to the first original plate. Place both plates into an incubator overnight.

2.6.7. Feed daily until ready to passage or freeze.

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

3.2. Required Supplies:

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

3.3. Required Reagents:

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

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

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

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

3.3.5. Basic Fibroblast Growth Factor (β-FGF) (Invitrogen, PHG0021) or equivalent

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

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

3.3.8. Bovine Serum Albumin (Sigma, A2153)

3.3.9. 2-Mercaptoethanol (Sigma, M7522)

3.3.10. Stem Cell Culture Medium as in section 2.

3.4. Feeding Pluripotent Stem Cells

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

3.4.2. 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 6-well plate to the 37ºC 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 recommended for thaws with greater than 10-20 colonies and is suitable for standard passaging. A non-enzymatic manual passaging method is recommended for sparse (fewer than 10-20 colonies) thaws or splits. This is also the method when there is significant differentiation present and the culture must be maintained.

4.1. Required Equipment:

4.1.1. Biosafety cabinet

4.1.2. 37ºC / 5% CO_2 incubator

4.1.3. 37ºC water bath

4.1.4. Centrifuge (not required for no-spin method)

4.1.5. Microscope with colony marker

4.1.6. Hood (biosafety or static) equipped with stereomicroscope (for "pick-to-remove")

4.2. Required Supplies:

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

4.2.2. Modified pipette or micropipette tip (for "pick-to-remove")

4.3. Required Reagents

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

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

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

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

4.3.5. Basic Fibroblast Growth Factor (β-FGF) (Invitrogen, PHG0021) or equivalent

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

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

4.3.8. Bovine Serum Albumin (Sigma, A2153)

4.3.9. 2-Mercaptoethanol (Sigma, M7522)

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

4.3.11. Defined FBS (Hyclone, SH30070.01)

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

4.3.13. Stem Cell Culture Medium as in section 2.

4.3.14. **1mg/ml Collagenase Solution**

4.3.14.1. To make 1mg/ml Collagenase Solution combine the following components filter sterilize and store at 4ºC for up to 14 days:

4.3.14.2. 25mg Collagenase Type IV

4.3.14.3. 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.

Note: 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. Cells will need to be split every 4-10 days based upon appearance.

4.5. Prepare Plate to Receive Cells

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

4.5.2. Add 1.5 ml of warmed Stem Cell Culture Medium to each well if the split ratio is 1:3 or more. Use 0.5ml is the split ratio is less than 1:3.

4.5.3. 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.

4.6. 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.

4.6.1. Examine the cultures to be passaged and remove differentiation if necessary. Use the colony marker on the microscope to mark all areas of differentiation on the plate of pluripotent stem cells. Determine which method of differentiation removal (selection method) is required based on the level of differentiation:

% Differentiation	Selection Method
<10%	No selection required
10-20%, or isolated differentiated colonies	Removal by suction
20-50+% or partially differentiated colonies	Pick-to-remove

>50-60%	Manual Passage

4.6.2. **Removal by suction**: If removing differentiation by suction, on the last aspiration of the passaging procedure, confirm the Pasteur pipette tip is in 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. Passage immediately after removal of differentiation.

4.6.3. **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.

Note: Picking-to-remove involves significant selection. Increasing selection events can drive a cell line toward karyotypical abnormalities, so frequent karyotyping is a necessity if picking is done routinely. In general, WiCell only recommends this procedure when absolutely necessary (precious cultures that cannot be replicated and are not backed-up). It should be used as a "last resort" and not a routine procedure.

4.3.1. **Passaging Cells with Collagenase**

4.3.1.1. Remove culture plate from incubator and place it in the biosafety cabinet.

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

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

4.3.1.4. Incubate for 5-7 minutes at 37ºC.

4.3.1.5. 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.

4.3.1.6. Aspirate the Collagenase Solution with a Pasteur pipette. Remove the collagenase carefully without disturbing the attached cell layer.

4.3.1.7. 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 off the medium.

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

4.3.1.8. 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.

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

4.3.1.10. Using a sterile 5 ml pipette, bring up medium into the pipette. Hold the pipette perpendicular to the plate and gently scrape the surface of the plate while simultaneously dispensing medium. Repeat if necessary.

Note: Minimize bubbles by scraping and pipetting gently.

4.3.1.11. 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. 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.

4.3.1.12. Leave the contents in the wells until all wells are scraped. After the cells are removed from the surface of the well, pool the contents of the scraped wells into a sterile conical tube.

4.3.1.13. 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.

4.3.1.14. Take up 1-2ml 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.

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

4.3.1.16. 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)

4.3.2. **Plate Cells**

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

4.3.2.2. Add 1ml of cell suspension to each well of the new plate.

4.3.2.3. Return the 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, 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.

4.3.2.4. Incubate cells overnight to allow colonies to attach.

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

4.4. Manual Passaging

Note: Manual Passaging is recommended only for very sparse cultures. It is common for cells coming out of thaws to be sparse and need to be manually passaged. Do not regularly manually passage as this may select for karyotypically abnormal cells. Very occasionally and only if a culture is highly differentiated, is this method used for passaging.

4.4.1. Transfer the plate of cells to be passaged and the prepared MEF plate into a hood equipped with a stereomicroscope.

4.4.2. 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, pipetman tip, needle, or SweMed™ instrument.

4.4.3. Transfer the pieces into the readied MEF plate. Transfer up to 50 colony pieces into the prepared well.

4.4.4. Add additional medium to the well of the new plate to equal approximately 2.5 ml culture medium total.

4.4.5. Feed the old plate to use as a back-up plate, and return to the incubator overnight. If the passage was successful and free of contamination, this plate can be discarded the day following passage.

4.4.6. 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.

4.4.7. Incubate cells overnight to allow colonies to attach.

4.4.8. 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. 37°C / 5% CO_2 Incubator

5.1.3. 37°C 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

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

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

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

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

5.3.5. Basic Fibroblast Growth Factor (β-FGF) (Invitrogen, PHG0021) or equivalent

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

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

5.3.8. Bovine Serum Albumin (Sigma, A2153)

5.3.9. 2-Mercaptoethanol (Sigma, M7522)

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

5.3.11. Defined FBS (Hyclone, SH30070.01)

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

5.3.13. Stem Cell Culture Medium as in section 2

5.3.14. 1mg/ml Collagenase Solution as in section 4

5.3.15. **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.

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

5.3.15.2. After filtering, add 2ml Sterile DMSO.

5.3.15.3. Do not filter DMSO, it will degrade the filter membrane.

5.4. **Prepare for Freeze**

5.4.1. Label cryovials with the cell line, passage number (add 1 to the passage number on the plate), the freeze date, and your initials. Use an alcohol proof pen or labels that resist liquid nitrogen and ethanol.

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 isopropanol freezing container. Each container can hold 18 vials. The isopropanol must be replaced every 5 uses.

5.4.4. Prepare the required amount of Cryopreservation Medium, 0.5ml will be needed for every vial plus a little extra to account for pipet error, keep on ice until ready to use.

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

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

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

5.4.8. 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.

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. Working across the top three wells, take up 1ml of medium from each well in a 5ml serological pipette and scrape the surface of the plate while slowly expelling the medium to wash the cells off the surface. Repeat once or twice per well to remove the majority of the cells from the well surface. 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 a sterile 50ml centrifuge 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 each plate with 3ml of Stem Cell Culture Medium, transferring the medium from well to well and add the medium to the 50ml conical tube.

5.5.7. Centrifuge at 200 x g for five minutes.

5.5.8. Aspirate the supernatant being careful not to disturb the cell pellet. Gently re-suspend each cell pellet in 0.5ml Stem Cell Culture Medium per well. Freezing 1 plate at 1 well/cryovial will require 3ml of Stem Cell Culture Medium.

Note: If freezing more than 6 plates of cells, either alter the size of the pool vessel, or freeze in batches.

5.5.9. 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.

5.5.10. While gently tapping the tube of cells, very slowly and drop-wise, add an equal volume of Cryopreservation Medium. For example, for 3 vials, add 1.5ml of Cryopreservation Medium. Pipette up and down two times to mix, though do not break up the colonies.

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.

5.5.11. Pipette pooled cells very gently to evenly mix suspension.

5.5.12. 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.

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

5.5.14. Transfer cell vials to liquid nitrogen storage the following day.

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 mTeSR™1/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

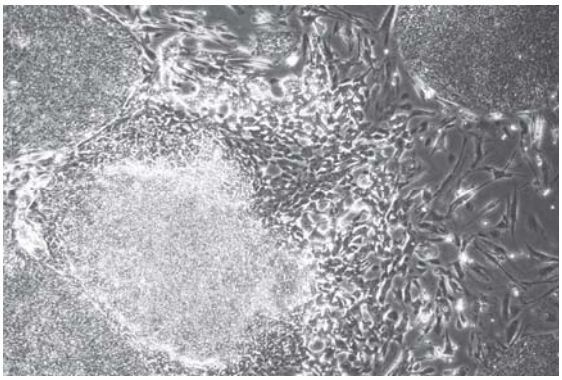
B	Updated protocol to represent current procedures, incorporated into WiCell Document Control System	08-April-2010
C	Added attachment 2 MSDS included for WiCell produced cells only	13-August-2010
D	CC00128. Added MEF Plating Density Recommendations and Transitioning Between Feeder Dependent and Feeder Independent Platforms.	24-August-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.	09-July-2013
F	CC00361, corrected page break on page 1.	20-August-2013
G	CC00543, added copyright.	28-May-2015
H	CC00635, updated note about ROCK inhibitor in CoA, added ROCK inhibitor prep, clarify language in harvest and spin during freeze procedure	See SPDC

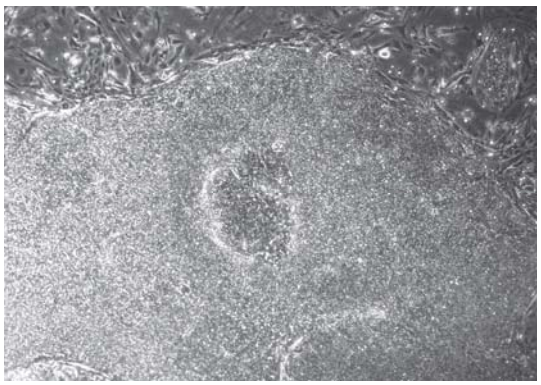
7.2. Approvals

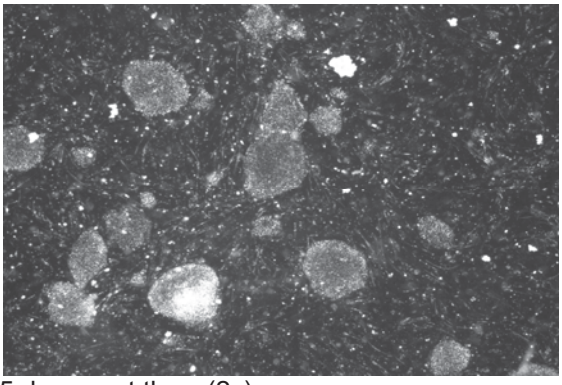
5/4/2017 X JKG JKG Senior Quality Assurance Specialist Signed by: Gay, Jenna	5/25/2017 X TEL TEL Director - WiCell Stem Cell Bank Signed by: Ludwig, Tenneille

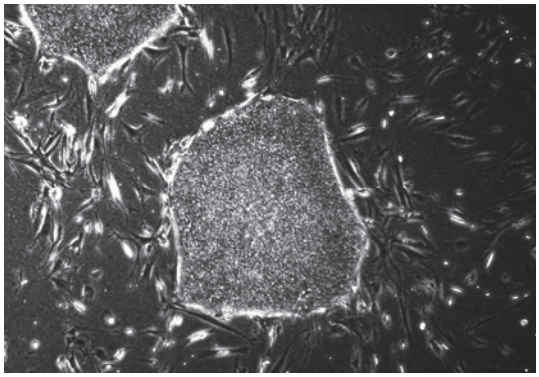
Attachment 1: Photographs of hES Cells

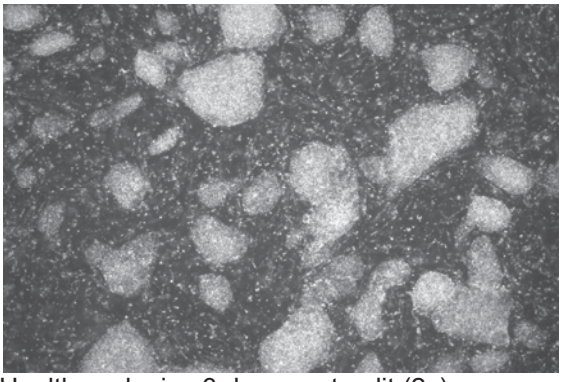
These are a few photos to give you an idea of how to assess differentiation levels in your cultures. We recommend removing differentiation when more than 10% is present in a culture. Magnification is noted for each photograph

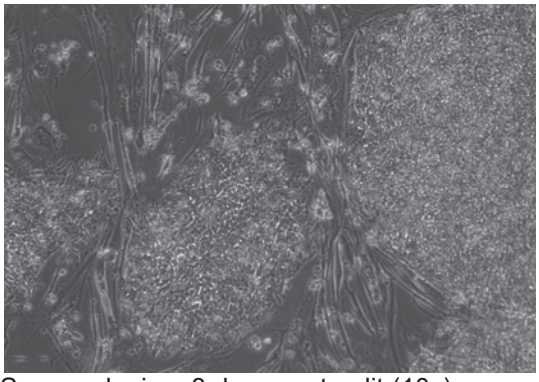

Differentiation around colony (10x)


Differentiation in colony center (10x)


5 days post thaw (2x)


Healthy colony on MEF Cells (10x)


Healthy colonies 3 days post-split (2x)


Same colonies- 3 days post-split (10x)