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1. Preface

This booklet of protocols is intended to serve as a primer for culturing pluripotent stem cells using MEF Conditioned Medium. MEF Conditioned Medium can be used in combination with Matrigel™ for the undifferentiated proliferation of human embryonic stem (ES) and induced pluripotent stem (iPS) cells. Protocols for preparation of both matrices have been included in this pack for convenience. The specific matrix used in the production of your cell line is included in the information provided with the cells at shipping

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. The protocols contained in this document are the protocols that were used to produce the cells we have sent you. 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.

2. Protocols

Section 1: Preparing Conditioned Medium

Required Equipment

1. Sterile Biosafety cabinet
2. Incubator set to 37°C with 5% CO2
3. Pipet-Aid
4. 4°C Refrigerator
5. 200µl Pipetman

Required Supplies

1. 0.1% Gelatin-coated T75 flask (Falcon, 353023) or equivalent
2. Sterilized Pasteur pipettes (Fisherbrand, 13-678-20D) or equivalent
3. 10 and 25ml sterile serological pipettes (Fisher: 13-67827F, 13-678-36D) or equivalent
4. Sterile 200µl Pipette tips (Molecular Bio, 2770)

Required Reagents

1. DMEM/F-12 Medium (Invitrogen, 11330-032)
2. Knockout Serum Replacer (KOSR) (Invitrogen, 10828-028)
3. L-glutamine, non-animal, cell culture tested (Sigma, G-8540)

4. MEM Non-Essential Amino acid solution (Invitrogen, 11140-050)
5. Basic Fibroblast Growth Factor (β-FGF) (Invitrogen, PHG0021) or equivalent
6. PBS without CaCl or MgCl (Invitrogen, 14190-250)
7. Bovine Serum Albumin (Sigma, A2153)
8. 2-Mercaptoethanol (14.3M) (Sigma, M7522)

Prepare Media and Reagents

1. **Stem Cell Culture Medium without bFGF: (250ml)**
    1. To make Stem Cell Culture Medium without bFGF combine the following components. Filter sterilize. Medium can be stored up to 14 days at 4°C.
        1. 200ml DMEM/F-12 Medium
        2. 50ml Knockout Serum Replacer
        3. 2.5ml 100mM L-Glutamine +BME Solution (See below)
        4. 0.5ml Non-Essential Amino Acids
2. **L-Glutamine +BME Solution (for Stem Cell Culture Medium)**
    1. To make L-Glutamine +BME Solution combine the following components, mix well and use immediately.
        1. 73mg L-Glutamine
        2. 5ml PBS without CaCl and MgCl
        3. 3.5µl 2-Mercaptoethanol
3. **2µg/ml Basic FGF Solution (to be added after conditioning)**
    1. To make 2µg/ml Basic FGF Solution, combine the following components, aliquot and store at -20°C for up to 6 months.
        1. 10µg Basic FGF
        2. 5ml 0.1% BSA in PBS without $CaCl_2$ and $MgCl_2$
4. **Plate Inactivated MEFs**

    Note: WiCell recommends CF1 MEFs for use in conditioning medium.

    1. Flasks containing inactivated MEFs are used to condition medium for pluripotent stem cells grown on Matrigel™.
        1. Flasks should be coated with 0.1% gelatin solution and stored in the 37°C incubator at least one day before plating MEFs. Gelatin-coated flasks/plates may be stored in the incubator for up to one week prior to plating MEFs for use.
    2. If freshly inactivated MEFs are used, 20 ml of MEFs at a density of 2.12×10^5 MEF cells/ml (or 4.24×10^6 cells total) should be plated into each T75 flask to be used for conditioning medium (note that this is a different density than is generally used when plating pluripotent stem cells directly onto MEFs).
    3. If frozen inactivated MEFs are used, 20 ml of MEFs at a density of 2.42×10^5 MEF cells/ml (or 4.84×10^6 cells total).
    4. Return the flask to the incubator so cells can attach overnight, being sure to distribute them evenly across the plate. Vent the cap to allow proper CO_2 exchange.

5. Flasks can be stored or used up to 2 weeks at 37°C.

5. **Condition Medium**

   "Conditioning" medium is the act of culturing it with cells. In this case, once medium has been in contact with MEF cells for approximately 24 hours, it is "Conditioned Medium". The steps below outline the conditioning process.

   1. Retrieve a T75 flask of plated inactivated MEFs from incubator and move to biosafety cabinet.
   2. Aspirate medium and rinse with DMEM/F-12 basal medium.
   3. Using a 25ml pipette, add 20ml Stem Cell Culture Medium without bFGF to flask. Replace cap and return to incubator. Once the flask is positioned in the incubator, vent cap to allow proper CO_2 exchange. Allow medium to incubate on MEFs overnight.
   4. The next day, retrieve flask and collect Conditioned Medium. Transfer to a sterile receiving unit using a 10ml pipet.
      Note: Conditioned Medium is not complete. bFGF needs to be added before it can be used to feed pluripotent stem cells.
   5. Add a fresh 20ml of Stem Cell Culture Medium without bFGF to the flask to condition additional medium. The flask does not need to be rinsed between exchanges of Stem Cell Culture Medium. This medium will be ready to collect as described above the following day.
   6. Prior to use, add bFGF to achieve a final concentration of 4ng/ml. (for 250 ml medium, 0.5ml of 2µg/ml Basic FGF solution is required) and filter. Completed medium can now be used to feed cells cultured on Matrigel.
   7. Medium, once conditioned, can be used the same day, or stored for future use. Medium can be store at 4°C for up to 2 weeks from the date of the initial medium preparation (meaning that medium prepared on the 1^{st}, and conditioned on the 10^{th} can be stored only until the 14^{th}). Alternatively, medium can be frozen at stored at -80°C for up to one year.
   8. T75 flasks plated with inactivated MEFs can be used for 1 week to condition medium (collecting medium daily).

Section 2: Aliquoting and Plating Matrigel™

Required Equipment

1. For aliquoting
   1. Sterile biosafety cabinet
   2. -20°C freezer
   3. 4°C refrigerator
   4. 200µl or 1000µl Pipetman
2. For plating
   1. Sterile biosafety cabinet
   2. Pipet-Aid

Required Supplies

1. For aliquoting
   1. 1.5ml Microcentrifuge tubes, sterilized (Fisher, 05-408-129), labeled

2. Microfuge tube holders
3. Ice Bucket with lid filled with ice
4. 2 small containers filled with ice
5. Freezer Boxes
6. Sterile 200µl or 1000µl Pipette tips (Molecular Bio, 2770)

2. For plating
    1. 15ml conical tube (Corning, 430052)
    2. 10ml sterile serological pipettes (Fisher, 13-678-27F) or equivalent
    3. 6-well tissue culture plates (Nunc, 140675)
    4. Parafilm

Required Reagents

1. For aliquoting
    1. Matrigel™, Growth Factor Reduced (BD Biosciences®, 354230) or equivalent
2. For plating
    1. DMEM/F-12 medium (Invitrogen, 11330-057)
    2. Matrigel™, Growth Factor Reduced aliquot (as prepared in section 1).

Preparing to Aliquot Matrigel™

1. Matrigel™ is frozen at -20˚C to -80˚C, liquid at 4˚C, and gels rapidly at room temperature. It is crucial to keep the Matrigel™ frozen until you are ready to aliquot it.
2. Calculate the volume of Matrigel™ needed per 1mg tube. 1mg is enough for two 6-well plates (WiCell recommends using the product at 0.5mg/6 well plate. If smaller volume aliquots are more appropriate for your use, adjust calculations appropriately).

$$\frac{1\text{ml}}{X\text{ml}} = \frac{\text{given concentration}}{1\text{mg}}$$

X = ml of Matrigel required per tube

Example:
Concentration of lot: 8.6mg/ml

$$\frac{1\text{ml}}{X\text{ml}} = \frac{8.6\text{mg}}{1\text{mg}}$$

X=0.1163ml = **116µl per tube for 1mg per tube**

Note: While the example above is for 1mg per aliquot, depending on volume of cell culture performed in the laboratory, different sized aliquots may be preferred. Aliquots of 0.5mg (enough for one plate), 1.0mg (two plates), and 2.0mg (four plates) are useful. If several different sized aliquots are used, make sure the concentration is clearly labeled on the tube.

Thaw the Matrigel™

1. Thaw overnight on ice at 4˚C (in a refrigerator). Ensure that the neck of the bottle is not submerged in the ice to reduce risk of contamination.
2. Place centrifuge tube racks and sterile containers of 1.5ml tubes in the -20 ˚C or -80˚C freezer. Place an unopened box of the appropriate sized pipette tips in the -20˚C freezer. These will be used for aliquoting

the following day.

Aliquoting Matrigel™

1. Fill two small containers with ice and place both in the sterile biosafety cabinet. One of containers will hold the Matrigel™ bottle; the other one will hold the box of pipette tips.
2. In the sterile biosafety cabinet, open the metal seal on the Matrigel™ bottle and carefully remove the rubber cover of the Matrigel™. Place bottle on ice. Retrieve one tube rack from the freezer, place in sterile biosafety cabinet and add sterile microtubes to it.
3. Aliquot the Matrigel™ ™ into each tube according to the calculations based on the lot concentration. Switch tips every 5-7 tubes to ensure sterility and prevent clogging of the tip.
4. Transfer tubes in batches to the -20 °C or -80 °C freezer as soon as the rack is filled. Work quickly – if the Matrigel™ is allowed to warm at all, it will congeal and will not be appropriate for plating. Retrieve a new rack from the freezer the same time you place the aliquot in the freezer.

Thawing Aliquots and Coating Plates with Matrigel™

Note: Each Matrigel™ aliquot is intended for one use. It cannot be thawed and re-frozen. Excess Matrigel™ may be plated, and used within 7-10 days.

1. Place a sterile, 15ml conical tube and a cold, sterile bottle of DMEM/F-12 medium into the sterile biosafety cabinet. Add 11ml cold DMEM/F-12 medium to the conical tube.
2. Remove one 1.0 mg Matrigel™ aliquot from the freezer, and add 1ml of cold DMEM/F-12 medium to it.
3. Gently pipette up and down to thaw and dissolve the Matrigel™. Immediately transfer it to the 15ml conical tube with 11 ml DMEM/F-12 medium and pipet to mix.
4. Immediately plate 1ml/well into each well of a 6-well plate. This will be enough for 2 full 6-well plates.
5. Allow to set one hour at room temperature before use or storage.
6. If the plates will not be used immediately after preparation, add an additional 1ml of DMEM/F-12 medium to each well to prevent drying. If any portion of the well dries out, do not use the well. Store plates as is in a 37°C incubator OR wrapped in parafilm in at 2-8°C. Both storage techniques require using the plates within 7-10 days after preparation. If stored at 2-8°C, plates should be warmed before use.

Section 3: Thawing Pluripotent Stem Cells Protocol

Note: Before thawing, check the certificate of analysis 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 appropriate attachment and expansion in conditioned medium for most cell lines. Please consult the certificate of analysis to determine if ROCK inhibitor is recommended for use with a specific lot. If ROCK inhibitor is not recommended, it will not be referenced on the certificate of analysis.

Required Equipment

1. Sterile biosafety cabinet
2. 37°C / 5% CO_2 incubator
3. 37°C water bath
4. Centrifuge
5. 100µl or 200µl Pipetman

Required Supplies

1. Forceps
2. Cryogenic handling gloves and eye protection
3. 5ml sterile serological pipettes (Fisher,13-678-27E) or equivalent
4. 95% Ethanol
5. Sterilized Pasteur pipettes (Fisher, 13-678-20D)
6. 1.5ml Microcentrifuge tubes, sterilized (Fisher, 05-408-129)
7. Microcentrifuge tube holders
8. Freezer Boxes
9. Sterile 100µl or 200µl Pipette tips (Molecular Bio, 2770)

Required Reagents

1. Matrigel™, Growth Factor Reduced (BD Biosciences®, 354230) coated 6-well plate (Nunc, 140675), prepared as indicated previously.
2. Conditioned Medium (as prepared in section 1)
3. DMEM/F-12 Basal Medium
4. ROCK Inhibitor (Y-27632 dihydrochloride; Tocris, 1254)
5. Sterile water (Sigma, W3500)

Reconstitute ROCK Inhibitor and Aliquot Working Stock Solution

Note: Perform work sterilely.

1. Make 10mM working stock solution by diluting 1mg ROCK inhibitor (FW 320.26) into 295µl water to achieve a 10mM solution, filter sterilize. Note: If FW of material is not 320.26, dilute appropriately to achieve a 10mM solution.
2. ROCK inhibitor working stock solution will be used at 1µl to 1ml final medium volume. Working stock solution alliquoted into working volumes (recommended at 20-50µl). and stored at -80°C for up to 1 year, or at 4°C up to 2 months.

Prepare Plate to Receive Cells

1. If prepared Matrigel™ coated plate has been stored at 4°C, allow to warm to room temperature for one hour. If plates have been stored at room temperature for one hour or have been stored at 37°C, they are ready to use.
2. 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.
3. Remove excess plating medium from prepared Matrigel™ coated plate from wells which will receive cells, and add 1.5 ml Conditioned Medium to every well that will receive cells.
4. Label plate appropriately (WiCell recommends at minimum the cell line name, passage number on the vial, date and technician initials).

Remove Vial and Thaw

Note: As always in the laboratory, but especially when handling frozen vials, wear appropriate personal protective equipment including cryo gloves and eye protection as vials stored in the liquid phase of liquid nitrogen may explode when warmed.

1. Remove the pluripotent stem cell vial from the liquid nitrogen storage tank using forceps.
2. Roll the vial between your gloved hands until the outside is free of frost. This should take between 10-15 seconds.
3. Using long forceps immerse the vial in a 37°C water bath without submerging the cap. Swirl the vial gently.
4. When only an ice crystal remains, remove the vial from the water bath.
5. Ensure the cap is tight 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.
6. Removal of Cryoprotectant and Re-suspension of Pluripotent Stem Cells
    1. Transfer the cells gently into a sterile 15ml conical tube using a 1ml glass pipette.
    2. Slowly, add 11ml of Conditioned 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.
    3. Centrifuge the cells at 200 x g for 5 minutes.
    4. Aspirate and discard the supernatant with a sterilized Pasteur Pipette.
    5. Re-suspend the cell pellet in 2.0ml of warmed Conditioned Medium for every well which will receive cells. Consult the CoA that was included in the shipping package for the recommended number of wells for thaw. For example, if the CoA indicates a 1:1 thawing ratio, thaw 1 vial into 1 well (re-suspend pellet in 2 mls). If it indicates a 1:3 thaw ratio, thaw 1 vial into 3 wells (re-suspend pellet in 6 mls).
    6. Gently pipette cells up and down in the tube a few times.

Plate Pluripotent Stem Cells

1. Slowly add the cell suspension drop-wise into the well(s).
2. 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.
3. The next day, remove the spent medium with debris using a sterile 5ml serological pipette and transfer it into a second prepared 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 fed with separate medium and maintained in a separate incubator.
4. Add 2.0ml of warmed Conditioned Medium to each of the original wells in the initial plate. Place both plates gently into an incubator overnight.
5. Feed daily as outlined below.

Section 4: Feeding Pluripotent Stem Cells Protocol

Required Equipment

1. Biosafety cabinet
2. 37°C / 5% CO_2 incubator

Required Supplies

1. 5ml and 10ml sterile serological pipettes (Fisher: 13-678-27E, 13-67827F) or equivalent

Required Reagents

1. Conditioned Medium (see Preparing Conditioned Medium in section 1)

Feeding Pluripotent Stem Cells

1. Observe the pluripotent stem cells using a microscope. If they require passaging, follow the passaging protocol below.
2. Aspirate the spent medium with a sterilized Pasture pipette. If feeding more than one plate, use a different pipette for each to reduce risk of contamination.
3. Add 2.0ml of warmed Conditioned Medium to each well. After pipettes are used once, they must be disposed of to reduce the contamination potential. Do not reinsert a used pipette into sterile medium for any reason.
4. Return the 6-well plate to the 37°C incubator.
5. Repeat procedure daily until cells are ready to be passaged or harvested.

Section 5: Passaging Pluripotent Stem Cells 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.

Required Equipment

1. Biosafety cabinet
2. Hood (biosafety or static) equipped with stereomicroscope
3. Colony marker
4. 37°C / 5% CO_2 incubator
5. 37°C water bath

Required Supplies

1. Matrigel™-coated 6-well plate, (0.5mgMatrigel/plate)
2. 5ml sterile serological pipettes (Fisher, 13-678-27E) or equivalent

Required Reagents

1. Conditioned Medium (as prepared in section 1)
2. DMEM/F-12 Basal Medium (Invitrogen, 11330-057) or equivalent
3. Dispase

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

1. In general, split cells when the first of the following occur:
    1. Pluripotent stem cell colonies are becoming too dense or too large.
    2. 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.

Prepare Dispase Solution

1. **Dispase Solution Recipe**
    1. To make 2mg/ml Dispase Solution, combine the following components, filter sterilize the following, store at 4^{o}C for up to 14 days.
        1. 100mg Dispase
        2. 50ml DMEM/F-12

Prepare Culture for Passaging using Dispase

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 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

2. **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 modified pipette or 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.
3. **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.

Passaging Cells with Dispase

1. Remove culture plate from incubator and place it in the biosafety cabinet.
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.).
3. Add 1ml room temperature Dispase Solution to each well to be passaged.
4. Incubate for 7 minutes at 37°C.
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.
6. While incubating, calculate the amount of medium that will be used to wash off the cells. Ideally, enough medium would be used to plate 0.5ml of cells into to each new well. EXAMPLE: 1:4 split of 1 well into 4

wells—scrape with 2ml and plate 0.5ml into each new well. 1:3 split of 2 wells into 6 wells—scrape with 3ml and plate 0.5ml into each new well. If calculated volume is less than 3ml, double the scraping volume so that each new well gets 1ml/well. EXAMPLE: 1:2 split of 1 well into 2 wells—scrape with 2ml and plate 1ml into each new well.

7. Aspirate the Dispase Solution with a Pasteur pipette. Remove the Dispase Solution carefully without disturbing the attached cell layer.
8. Gently wash the attached cells three times with 1ml of DMEM/F-12 per wash.
   Note: Do not dispense the medium in a continuous stream in one spot because the cells in that area will peel off.
9. If removing differentiation by suction, ensure the pipette tip is intact and not chipped, and carefully press the pipette tip to the marked area and suction off the differentiated cells. Confirm the complete removal under the microscope.
10. Add the calculated volume of warmed Conditioned Medium to each well.
11. 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.
12. 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.
13. Leave the contents in the wells until all wells are scraped. After the pluripotent stem cells are removed from the surface of the well, pool the contents of the scraped wells into a sterile conical tube.
14. 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.
15. Determine how much medium is required to add to each new well so that the total volume in the well is 2ml once the cells are added, usually 0.5ml of cell suspension can be added to each new well (0.5ml of cell suspension + 1.5ml of pre-plated culture medium). Add the medium to each well and set aside.

Plating Cells

1. Gently re-suspend the cells using a 5ml pipette.
2. Add the determined volume (usually 0.5 or 1ml) of cell suspension to each well of the new plate.
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. Incubate cells overnight to allow colonies to attach.
5. Feed culture as previously described until ready to passage or freeze.

Non-Enzymatic Manual Passaging Protocol

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.

1. **Manual Passaging**
    1. Transfer the plate of cells to be passaged into a hood equipped with a stereomicroscope.
    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 modified pipette, pipetman tip, needle or SweMed™ instrument.
    3. Transfer the pieces into the readied Matrigel™ coated plate. Transfer up to 50 colony pieces into each prepared well.
    4. Return the plate to the incubator. 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.
    5. Incubate cells overnight to allow colonies to attach. If they do not appear to be attached the following day, do not feed and allow an additional day to attach.
    6. Feed culture as previously described until ready to passage or freeze.

Section 6: Freezing Pluripotent Stem Cells Protocol

Required Equipment

1. Biosafety cabinet
2. 37°C / 5% CO_2 Incubator
3. 37°C water bath
4. Centrifuge

Required Supplies

1. Metal forceps
2. Cryogenic handling gloves and eye protection
3. Isopropanol freezing containers (Fisher, 15-350-50) and isopropanol
4. Plastic cryovial holders
5. 5ml and 10ml sterile serological pipettes (Fisher: 13-678-27E, 13-67827F) or equivalent

Required Reagents

1. 95% Ethanol
2. Conditioned Medium (as prepared in section 1)
3. DMEM/F-12 Basal Medium (Invitrogen, 11330-057) or equivalent
4. Dispase Solution (as prepared in section 5)
5. Defined FBS (Hyclone, SH30070.01)
6. Dimethyl Sulfoxide (DMSO) 10ml ampoules (Sigma Aldrich, D2438)

Prepare for Freeze

1. Label cryovials appropriately using ethanol and liquid nitrogen resistant methods, and maintaining sterility of the vials. WiCell recommends that vials be labeled with the cell line, passage number (WiCell recommends adding one passage to the plate number so that the passage number on the vial represents

the overall passage number of the culture after thaw), the freeze date, and your initials. Place vials in biosafety cabinet.

2. Prepare cryopreservation medium
   Note: Cryopreservation medium should be made immediately before freezing cells, and should be maintained on ice. Discard excess medium.
   1. Filter sterilize the following
      1. 6ml Defined FBS
      2. 2ml Conditioned Medium
   2. Add 2ml Sterile DMSO
      2.1. Do not filter medium once it contains DMSO
3. 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.
4. Obtain a recharged isopropanol freezing container. Each container can hold 18 vials. The isopropanol must be replaced every 5 uses.
5. Prepare 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.
6. View all cells under the microscope. Discard any contaminated plates and mark areas of differentiation.
7. Identify and remove differentiation as described above if necessary.
8. Remove spent medium and add 1ml of Dispase Solution to each well of each 6-well plate.
9. Incubate cells for 5-7 minutes at 37°C.
10. 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.

Harvest and Wash Cells

1. Aspirate Dispase solution from each well, taking care not to remove any floating colonies.
2. Wash each adherent well with 1ml of warmed sterile DMEM/F-12. Repeat two more times for a total of three washes, completely removing medium while leaving colonies attached at each wash.
3. Add 1ml of warmed Conditioned Medium to each well.
4. Take up 1ml of medium from the well in the 5ml pipette and scrape the surface of the plate while simultaneously slowly pipetting the Conditioned Medium up and down to wash the cells off the surface. Be careful to keep cells in small clumps. Cells will recover from the thaw more efficiently if frozen in aggregates.
5. Pool the cells in a sterile 50ml centrifuge tube per plate.
6. Wash each plate with 3ml of warmed Conditioned Medium, transferring the medium from well to well and add the medium to the 50ml conical tube.
7. Centrifuge cells at 200 x g for 5 minutes.
8. Aspirate the supernatant being careful not to disturb the cell pellet. Gently re-suspend each cell pellet by adding 0.5ml Conditioned Medium per well very slowly drop wise. Freezing 1 plate at 1 well/cryovial will require 3ml of Conditioned Medium. If there is more than one conical tube, you may want to pool them at this point for a uniform lot. **Note:** If freezing more than 6 plates of cells, either alter the size of the pool

vessel, or freeze in batches.

9. Retrieve the Cryopreservation Medium from the ice bath and place in biosafety cabinet. Ensure the medium is evenly mixed by using a 10ml pipet, otherwise the DMSO will remain settled on the bottom.
10. While gently tapping the tube of cells, very slowly and drop-wise, add an equal volume of Cryopreservation Medium. For example, for 6 vials, add 3ml 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.
11. Pipette pooled cells very gently to evenly mix suspension.
12. With the same pipette, distribute 1ml of cell suspension to each prepared vial. Mix cell pool every 6-10 vials for even distribution.
13. Quickly, tighten caps and place cryovials into an isopropanol containing freezing container. Place the freezing containers in the -80°C freezer overnight.
14. Transfer cell vials to liquid nitrogen storage the following day.

Section 7: 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. When transferring cultures from one feeder-free platform to another or to a feeder-independent platform, follow the manufacturer's instructions for transitioning cultures to the new platform. When transferring feeder-free cultures to a murine embryonic fibroblast (MEF) based culture platform, passage cells as described here, re-suspending the cells prior to plating in the appropriate medium for feeder-based culture. If using WiCell's MEF based protocols following transition, no adaptation time is required, and cells should be re-suspended in standard KOSR containing Stem Cell Culture Medium prior to plating on MEFs. However, if using alternate protocols, depending on platform (media, feeders) transitioning medium over the course of days or passages may be appropriate. Regardless of final platform, we recommend maintaining the initial cultures in the original conditions in parallel until the user can be confident that the transition is successful.

3. Version History

Version	Change Description	Effective Date
A	Document Initiation. CC00126.	05-May-2011
B	CC00285, Made changes to make consistent with other shipping protocols, specifically to the layout of supplies, reagents, and materials sections. Minor procedural edits for clarity.	09-July-2013
C	CC00544, added copyright	28-May-2015
D	CC00635, Added details about Rock Inhibitor use thawing section. Clarified how to break up cells in harvest and spin section.	03/17/2017
1.0	CC-319, Transitioned SOP-SH-003 to Qualio as SH-3.	
2.0	CC-364, Updated numbering and title.	See Qualio or Controlled Copy

4. Attachments

No attachments.