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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

Notice for Matrigel Use: Matrigel is a Basement Membrane Extract (BME) and equivalent BMEs include Cultrex and Geltrex. If Matrigel is not available for your use, we have found the Cultrex can be used interchangeably in our protocols with Matrigel. Use of Geltrex, however would need to be optimized to suit your application. Alternatively, some cultures will work with Vitronectin, Laminin, or other protein matrices, but may need ROCK inhibitors for attachment. We suggest you test any matrix for support prior to your experiments or use a Human PSC qualified product.

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, taking into account the Notice for Matrigel Use found in this protocol. 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 Characterization's offerings and how to submit samples visit www.wicell.org/characterization.

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 x10 ^{5} MEF cells/ml (or 4.84x10 ^{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 1st, and conditioned on the 10th 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 Basement Membrane Extract (BME)

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Required Equipment

1. For aliquoting
    1. Sterile biosafety cabinet
    2. -20°C freezer

3. 4°C refrigerator
4. 200µl or 1000µl micropipette

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)

Required Reagents

1. For aliquoting
    1. Basement Membrane Extract (BME)
        1. Growth Factor Reduced Matrigel (Corning, 354230) or
        2. Cultrex® Reduced Growth Factor Basement Membrane Extract (R&D Systems, 3433, 3434 or 3445)
2. For plating
    1. DMEM/F-12 medium (Invitrogen, 11330-057)
    2. Matrigel™, Growth Factor Reduced aliquot (as prepared in the following section) or
    3. Cultrex® Reduced Growth Factor Basement Membrane Extract (as prepared in the following section)

Preparing to Aliquot BME

1. BME gels rapidly at room temperature. It is crucial to keep the BME stored per the manufacturer's instructions until you are ready to aliquot it.
2. Confirm the concentration of the BME lot. the concentration can vary significantly between lots.
3. Calculate the volume of BME needed per 1mg tube (see Figure 1.). 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).

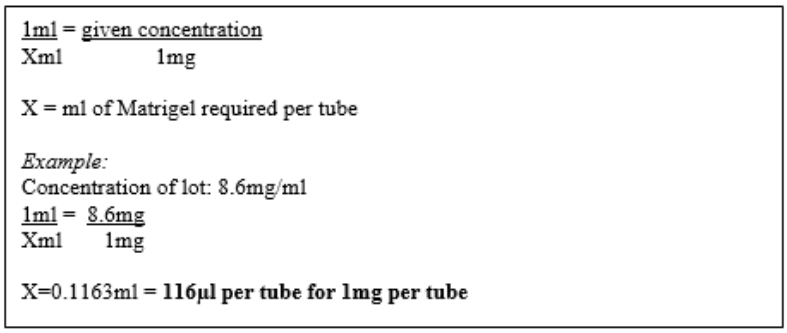

Figure 1.

4. The day before aliquoting, place microcentrifuge tube racks and containers of sterile 1.5ml microcentrifuge tubes in the -20°C or -80°C freezer. Place an unopened box of the appropriate sized pipette tips (based on calculation in previous step) in the -20ºC freezer.

Thaw the BME

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.

Aliquoting BME

1. **Note:** 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.
2. Fill two small containers with ice and place both in the sterile biosafety cabinet. One of containers will hold the BME bottle; the other one will hold the box of pipette tips.
3. In the sterile biosafety cabinet, carefully open BME container, being careful to maintain sterility of the rim of the container. Place bottle on ice. Retrieve one tube rack from the freezer, place in sterile biosafety cabinet and add sterile microtubes to it.
4. Aliquot the BME 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.
5. Close tubes and transfer them in batches to the -20 °C or -80 °C freezer as soon as the rack is filled. Work quickly – if BME 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 BME

1. **Note:** Each BME aliquot is intended for one use. It cannot be thawed and re-frozen. Excess BME may be plated, and used within 7-10 days.
2. **Note:** 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. The instructions below are based on 1.0mg aliquots used to prepare two plates at one time. Make adjustments for different sized aliquots as appropriate.
3. Place a sterile, 15ml or 50ml 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.
4. Remove one 1.0 mg BME aliquot from the freezer. Use a micropipette with a 1000ul tip to add 1ml of cold DMEM/F-12 medium to it.
5. Gently pipette up and down to thaw and dissolve the BME. Immediately transfer it to the conical tube

with 11 ml DMEM/F-12 medium and pipet to mix.

6. Immediately plate 1ml/well into each well of a 6-well plate. This will be enough for 2 full 6-well plates.
7. Allow to set one or two hours in a 37°C incubator before use. For optimal results, plate the day before using and store overnight in 37°C incubator.
8. If the plates will not be used within 2 hours 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, use the plates within 7-10 days after preparation. Ensure the incubator has the appropriate humidity level, or the BME plates will dry out faster, check plates before using.

Section 3: Thawing Pluripotent Stem Cells Protocol

1. Note: Before thawing check the Certificate of Analysis in the shipping packet insert to determine the following.
    1. Recommended number of wells one vial should be thawed into (this can vary among lots).
    2. If ROCK inhibitor is recommended to be used.
        1. In general, ROCK inhibitor is not required for appropriate attachment and expansion for most cell lines.
        2. Please consult the Certificate of Analysis to determine if ROCK inhibitor is recommended for use with a specific lot.
        3. If ROCK inhibitor is recommended, it will be referenced on the Certificate of Analysis. If you do not see a recommendation for the use of ROCK inhibitor, WiCell does not recommend ROCK inhibitor for successful recovery.

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. BME coated 6-well plate (Nunc, 140675), prepared as indicated previously.
2. Conditioned Medium (prepared as indicated previously)
3. DMEM/F-12 Basal Medium
4. ROCK Inhibitor (Y-27632 dihydrochloride; Tocris, 1254), if needed (check the Certificate of Analysis in the shipment packet)
5. Sterile water (Sigma, W3500) if ROCK Inhibitor will be used

Reconstitute ROCK Inhibitor and Aliquot Working Stock Solution, if needed

1. **Note:** Perform work sterilely.
2. 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.
3. Aliquote into appropriate working volumes (recommended at 20-50µl). ROCK inhibitor working stock solution will be used at 1ul to 1ml final culture medium volume. Aliquotes can be stored long term at -80°C for up to 1 year and up to 2 months at 4°C.

Prepare Plate to Receive Cells

1. Retrieve prepared BME-coated plate from 37°C incubator.
2. Acquire the lot-specific 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 BME plate. Add 1.5ml 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

1. **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.
2. Remove the pluripotent stem cell vial from the liquid nitrogen storage tank using forceps.
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.

Removal of Cryoprotectant and Re-suspension of Pluripotent Stem Cells

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 shear the cells and reduce attachment.
2. To decrease osmotic shock add 11ml of Conditioned Medium slowly and 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. If ROCK inhibitor has been recommended on the lot-specific Certificate of Analysis to increase attachment, prepare plating medium while centrifuging. Add 1ul of ROCK inhibitor working stock solution to every 1ml

of culture medium to achieve a final concentration of 10uM (as previously described). You will need 0.5ml of culture medium for every well which will receive cells. Set medium aside.

5. Aspirate and discard the supernatant with a sterilized Pasteur Pipette.
6. Re-suspend the cell pellet in 0.5ml of warmed Conditioned Medium for every well that will receive cells (number of wells receiving cells is based on the thaw recommendations 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 4 wells, re-suspend the pellet in 2ml, 0.5ml will be plated into each well. If using ROCK Inhibitor, use the prepared plating medium described above.
7. Very gently pipette cells up and down in the tube a few times.

Plate Pluripotent Stem Cells

1. Slowly add 0.5ml of the cell suspension into each well.
2. Place plate gently into the incubator and 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.
    1. **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 across the well.
3. Feed 2.0ml of warmed Conditioned Medium to cells daily as described below until ready to be passaged or harvested.

Section 4: Feeding Pluripotent Stem Cells Protocol

Required Equipment

1. Biosafety cabinet
2. 37°C / 5% CO_2 incubator
3. Light microscope (a camera is recommended)

Required Supplies

1. 5ml and 10ml sterile serological pipettes (Fisher: 13-678-27E, 13-67827F) or equivalent
2. Sterilized Pasteur pipettes (Fisher, 13-678-20D)

Required Reagents

1. Conditioned Medium (prepared as described 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. If they do not require passaging, aspirate the spent medium with a sterilized Pasture pipette. If feeding more than one plate, use a different pipette for each plate in order 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. Return the 6-well plate to the 37°C incubator.
4. Repeat procedure daily until cells are ready to be passaged or harvested.

Section 5: Passaging Pluripotent Stem Cells Protocols

There are two methods for passaging.

1. An enzymatic method recommended for cultures with greater than 10 colonies and is suitable for standard passaging.
2. A manual passaging method is used when there is significant differentiation present and the culture must be maintained, or there are fewer than 10 colonies.

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. 5ml sterile serological pipettes (Fisher, 13-678-27E) or equivalent
2. Sterilized Pasteur pipettes (Fisher, 13-678-20D)
3. Cutting tool (for manual passaging only: this can be a needle, modified Pasteur pipette, micropipette tip, SweMed™ instrument, etc.)

Required Reagents

1. BME-coated 6-well plate (Nunc, 140675), prepared as indicated previously
2. Conditioned Medium (prepared as indicated previously)
3. DMEM/F-12 Basal Medium (Invitrogen, 11330-057) or equivalent
4. Dispase (Invitrogen, 17105-041)

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

Note: 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 generally need to be split every 4-10 days based upon appearance, but decisions on passaging should be made by evaluating the appearance of the cells. Occasionally cells will need to be passaged earlier or later than usual.

Prepare Dispase Solution

1. **Prepare 2ml/ml Dispase Solution**
    1. Scale up or down based on need.
    2. Combine 50mg Dispase and 25ml DMEM/F-12.
    3. Filter sterilize, store at 4 °C for up to 14 days.

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. Use Table 1 to determine which method of differentiation removal 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 of Undifferentiated colonies

Table 1.

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.
3. **Removal by Suction**: If removing differentiation by suction, become familiar with the passaging protocol detailed below as this occurs during the procedure.
    1. Just prior to removing the third wash step after treating with Dispase, confirm the Pasteur pipette tip in intact and not chipped. Carefully press the pipette tip to the circled area and suction off the differentiated cells. Collect cells immediately after removal of differentiation.
    2. Make sure not to dry out the culture well, add more wash reagent if needed.

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 fed separately and left unpassaged to be 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. While incubating, aspirate the plating medium from a plate pre-coated with BME or (as described previously), add 1.5ml/well of warmed Conditioned Medium per well, set aside.
6. Aspirate the Dispase Solution with a Pasteur pipette. Remove the Dispase Solution carefully without disturbing the attached cell layer.
7. Gently wash the attached cells three times with 1ml of DMEM/F-12 per wash. If removing by suction, aspirate marked areas of differentiation as previously described when removing the third rinse.
    1. **Note:** Do not dispense the medium in a continuous stream in one spot because the cells in that area will peel off.
8. Add 1-2ml of culture medium to each well (depending on determined passage ratio: If 1:4 or greater use 2ml/well. If <1:4 use less medium). Do not work with more than 3ml/well (overfilling wells may lead to contamination).
9. Hold a 5ml pipette perpendicular to the plate and gently scrape the surface of the plate while simultaneously dispensing medium. Repeat if necessary.
    1. **Note:** Minimize bubbles by scraping and pipetting gently.
10. Pipette the medium slowly up and down to wash the cells off the surface. Be careful not to create bubbles.
11. Leave the contents in the wells until all wells are scraped.

12. 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.
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.
14. Determine how much additional medium is required so 0.5ml 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 2.0ml to 2.5ml of medium and cells in each of the new wells (0.5ml of cell suspension + 1.5ml of pre-plated culture medium)

Plating Cells

1. Gently re-suspend the cells using a 5ml pipette.
2. Add 0.5ml of cell suspension to each well of the new plate.
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 wells.
    1. **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. If passage was successful and free of contamination, the backup well can be discarded the day following passage.
6. Feed culture as previously described until ready to passage or freeze.

Manual Passaging

1. **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.
2. Aspirate the plating medium from a plate pre-coated with BME (as described previously), add 1.0ml/well of warmed Conditioned Medium per well, set aside. If using ROCK inhibitor, add 1µl/ml ROCK inhibitor working stock solution to each well that will receive cells.
3. Remove pluripotent stem cell 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 of cells should be fed separately and left to be used as a backup 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 have only one well to passage) be sure to leave some material in the well to serve as a back-up.
4. Add 1.0ml culture medium to each well to be passaged plus 1µl/ml/well ROCK inhibitor working stock solution, if using.
5. Transfer the plate of pluripotent stem cells to be passaged into a sterile hood equipped with a stereomicroscope (a PCR enclosure may be used if extreme care is taken).
6. Remove the undifferentiated colonies from the culture 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 Pasteur pipette, micropipette tip, needle or SweMed™ instrument.
7. Take plate back to the biosafety cabinet, use a 5ml pipette to transfer all of the medium containing the

pieces into the prepared wells. Transfer up to 50 colony pieces into each prepared well.

8. Return the plate to the incubator. 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 wells.
    1. **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.
9. Rinse and feed the plate that cells were harvested from, and return it to the incubator as well.
10. 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. Additional medium may be added to the well.
11. If passage was successful and free of contamination, the backup well can be discarded the day following passage.
12. 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 (prepared as previously described)
3. DMEM/F-12 Basal Medium (Invitrogen, 11330-057) or equivalent
4. Dispase Solution (prepared as previously described)
5. Fetal Bovine Serum (R&D Systems, S12450), non-heat-inactivated
6. Dimethyl Sulfoxide (DMSO) 10ml ampoules (Sigma Aldrich, D2438)

Prepare for Freeze

1. One well of cells will be frozen into 1 vial. Label cryovials with the cell line, passage number (increase the passage number on the plate by 1 to label the vial so that the passage number on the vial is reflective of the passage number at thaw), the freeze date, and your initials. Use an alcohol-proof pen or labels that resist liquid nitrogen and ethanol. Place in biosafety cabinet.
2. Sterilize the vials in the biosafety cabinet 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.

3. Obtain a recharged, room temperature isopropanol freezing container. The isopropanol must be replaced every 5 uses.
4. Prepare cryopreservation medium
    1. Cryopreservation medium is required to be made fresh just before the PSCs are frozen.
    2. Determine the amount of Cryopreservation Medium required by following the formula in Figure 2. **Note:** The volume may be rounded up to the next whole number for easier preparation.
    3. DMSO cannot be filtered, this means that all components must begin sterile and be added sterilely.

(0.5ml/vial **x** # of vials)
+ extra ~1-5% for pipette error
total amount cryo medium needed

Example →

(0.5ml/vial **x** 36 vials)
+ extra 1-5% for pipette error
20ml cryo medium needed

Figure 2.

    4. Prepare as described in Table 2. Combine all components sterility, keep sterile, keep on ice.

Component	%	Example for a 66-vial lot, including the extra 1-5%
FBS (non-heat-inactivated)	60%	12ml
Conditioned Medium	20%	4ml
DMSO	20%	4ml

Table 2.

5. View all cells under the microscope. Discard any contaminated plates and mark areas of differentiation.
6. Identify and remove differentiation as described above if necessary.
7. Remove spent medium and add 1ml of Dispase Solution to each well of each 6-well plate.
8. Incubate cells for 5-7 minutes at 37°C.
9. Confirm colony separation 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	Version History	Effective Date
A	CC00126, Document Initiation.	05May11
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.	09Jul13
C	CC00544, added copyright	28May15

D	CC00635, Added details about Rock Inhibitor use thawing section. Clarified how to break up cells in harvest and spin section.	17Mar17
1.0	CC-319, Transitioned SOP-SH-003 to Qualio as SH-3.	12Aug20
2.0	CC-364, Updated numbering and title.	17Aug20
3.0	CC-1068, Added that Cultrex may be used interchangeably with Matrigel. Removed storage option of 4°C for BME plates. Updated vendor information for Matrigel, FBS, dispase and Rock inhibitor. Removed directive to roll frozen vial between hands. Added directive to not allow cells to flow down the side of conical tube.	See Qualio or Controlled Copy

4. Attachments

No attachments.