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1. Preface

This booklet includes protocols for culturing pluripotent stem cells in a Feeder Independent system using either mTeSR™1, mTeSR™ Plus or TeSR-E8™ culture medium, and basement membrane extract (BME) culture matrices or rhVitronectin. We recommend that you read through these protocols prior to thawing your cells.

Please refer to the "Thaw and Culture Recommendations" section of the Certificate of Analysis provided in your shipment to determine the specific culture medium and matrix that should be used with the lot of cells provided (see highlighted sections in Figure 1 example), as these are the conditions used to verify quality and characterization of the lot you received.

Thaw and Culture Details

Cell Line Name	Example Cell Line
WiCell Lot Number	WB12345
Provider	Example Institution
Banked By	WiCell
Thaw and Culture Recommendations	WiCell recommends thawing 1 vial into 1 well of a 6 well plate using mTeSR™1 and Matrigel®.
Protocol	WiCell Feeder Independent Pluripotent Stem Cell Protocol
Culture Platform Prior to Freeze	Feeder Independent
	Medium: mTeSR™1
	Matrix: Recombinant Human Vitronectin
Passage Number	p12 These cells were cultured for 11 passages prior to freeze and post reprogramming or colony selection. WiCell adds +1 to the passage number at freeze to best represent the overall passage number of the cells at thaw. Plated cells at thaw should be labeled passage 12.
Date Vialed	24-June-2018
Vial Label	Example Cell Line WB12345 24Jun2018
Biosafety and Use Information	Appropriate biosafety precautions should be followed when working with these cells. The end user is responsible for ensuring that the cells are handled and stored in an appropriate manner. WiCell is not responsible for damages or injuries that may result from the use of these cells. Cells distributed by WiCell are intended for research purposes only and are not intended for use in humans.

Figure 1.

WiCell recommends that pluripotent stem cells (PSCs) should be first thawed and established in the recommended culture conditions (medium and matrix) found on the Certificate of Analysis prior to any attempt to transfer the cells to alternate culture platforms. Follow the recommended culture protocol (found on the Certificate of Analysis) until you have established your own cell bank of frozen vials that can be successfully recovered. As you thaw and expand cells from your initial 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 few vials as early as possible, continue your culture, and freeze again when you have enough cells for a 20 vial bank or larger. Pool the harvested cells during each freeze. This will ensure you have an adequate stock of uniform 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 mycoplasma, and assayed for karyotype and identity via Short Tandem Repeat (STR) post thaw to ensure that the stability and identity of the banked material is as expected. These and other tests are available from WiCell's full-service Characterization Laboratory; to learn more about WiCell's Characterization services 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: Aliquoting and Plating Basement Membrane Extract (BME)

1. Note: Refer to the Certificate of Analysis specific to the lot of cells being thawed to know which specific matrix should be used to initially thaw the vial, found in the "Thaw and Culture Recommendations" section.

Required Equipment

1. For aliquoting
    1. Sterile biosafety cabinet
    2. -20ºC freezer
    3. 4ºC 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. Microcentrifuge 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)
    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)
        2. Cultrex® Reduced Growth Factor Basement Membrane Extract (R&D Systems, 3433, 3434 or 3445)
2. For plating
    1. DMEM/F-12 (Invitrogen, 11330-057)
    2. Matrigel™, Growth Factor Reduced aliquot (as prepared in following section)
    3. Cultrex® Reduced Growth Factor Basement Membrane Extract (as prepared in following section)

Preparing to Aliquot BME

1. BME gels rapidly at room temperature. It is crucial to keep the BME stored per the manufacturers 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. 1mg is enough for two 6-well plates (WiCell recommends using the product at 0.5mg/6 well plate. If smaller or larger volume aliquots are more appropriate for your use, adjust calculations appropriately.)

   1ml = given concentration

   Xml 1mg

   X = ml of BME required per tube

   Example:

   Concentration of lot: 8.6mg/ml

   1ml = 8.6mg

   Xml 1mg

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

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 4ºC (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 biosafety cabinet, carefully open BME container, being careful to maintain the 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 microcentrifuge tubes 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 -20ºC or -80°C freezer as soon as the rack is filled. Work quickly – if the 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 aliquots 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 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 15ml conical tube with 11 ml DMEM/F-12 medium and pipet to mix.
6. Immediately plate 1ml/well of a 6-well plate. This will be enough for 2 full 6-well plates.
7. Allow to set for at least 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 two 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 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 2: Preparing and Plating rhVitronectin

1. **Note:** Refer to the Certificate of Analysis specific to the lot of cells being thawed to know which specific matrix should be used to initially thaw the vial.

Required Equipment

1. Sterile biosafety cabinet
2. -80°C freezer
3. 100µl or 200µl micropipette

Required Supplies

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. Sterile 100 or 200µl pipette tips (Molecular Bio, 2770)
5. 1.5ml microcentrifuge tubes, sterilized (Fisher, 05-408-129)
6. Microcentrifuge tube holders
7. Freezer boxes

Required Reagents

1. rhVitronectin (R&D Systems, 2308-VN) or equivalent
2. Sterile PBS without $CaCl_2$ and $MgCl_2$ (Invitrogen, 14190-250) or equivalent

Aliquoting rhVitronectin

1. The following is sufficient for coating 3 wells of a 6 well plate. Adjust aliquots for your own use as appropriate.
2. Reconstitute 50µg rhVitronectin in 200µl sterile PBS without CaCl2 and MgCl2.
3. Aliquot 60µl per tube into sterile microcentrifuge tubes. This will be 15µg of rhVitronectin, or enough to coat 3 wells at 5µg/well.
4. Store tubes at -80°C until ready to use.

Plating rhVitronectin

1. Remove aliquot from freezer and allow to thaw at room temperature.
2. Dilute aliquot with 6ml of sterile PBS without $CaCl_2$ and $MgCl_2$ into a 15ml conical tube, pipet to mix, and immediately plate at 2ml diluted rhVitronectin per well.
3. Store in 37°C incubator. It can be used after one hour of plating and may be stored for up to 7 days before use. Prior to use, ensure there are no dry areas in the well; if there are, the well should not be used. Ensure the incubator has the appropriate humidity level, or the rhVitronectin plates will dry out faster, check plates before using.

Section 3: Thawing Pluripotent Stem Cells: Feeder Independent –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. Biosafety cabinet
2. 37°C / 5% CO_2 incubator
3. 37°C water bath
4. Centrifuge
5. 100µl or 200µl micropipette

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 or rhVitronectin (R&D Systems, 2308-VN) coated 6-well plate (Nunc, 140675), prepared as indicated previously
2. Cell culture medium specified on the Certificate of Analysis
    1. mTeSR™1 Medium (Stem Cell Technologies, 85850)
    2. mTeSR™ Plus (Stem Cell Technologies, 05825)
    3. TeSR-E8™ (Stem Cell Technologies, 05990)
3. ROCK Inhibitor (Y-27632 dihydrochloride; BD Biosciences, 562822), if needed (check the Certificate of Analysis in the shipment packet)
4. 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. 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.

Prepare Plate to Receive Cells

1. Retrieve prepared BME or rhVitronectin 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 or rhVitronectin coated plates. Add 1.5 ml culture medium to every well that will receive cells.
4. Label plate appropriately (WiCell recommends at minimum including 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 a small 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 culture 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 culture 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 cells from 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 2ml/well culture medium to cells daily as described below until ready to be passaged or harvested.

Section 4: Feeding Pluripotent Stem Cells: Feeder Independent –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. Culture medium specified in the Certificate of Analysis
    1. mTeSR™1 Medium (Stem Cell Technologies, 85850)

2. mTeSR™ Plus (Stem Cell Technologies, 05825)
3. TeSR-E8™ (Stem Cell Technologies, 05990)

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 culture 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: Feeder Independent -Protocol

There are two methods for passaging.

1. A reagent-based method of passaging is recommended for cultures with greater than 10 colonies and is suitable for standard passaging using either EDTA (Versene®) or Dispase. While for best results WiCell currently recommends passaging with EDTA (Versene®), Dispase based-passaging protocols have been included for convenience for those laboratories choosing to use this method.
2. A manual passaging method is used when there is significant differentiation present and the culture must be managed to remove this differentiation.

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 or rhVitronectin (R&D Systems, 2308-VN) coated 6-well plate (Nunc, 140675), prepared as indicated previously
2. Cell culture medium specified in the Certificate of Analysis
    1. mTeSR™1 Medium (Stem Cell Technologies, 85850)
    2. mTeSR™ Plus (Stem Cell Technologies, 05825)
    3. TeSR-E8™ (Stem Cell Technologies, 05990)
3. DMEM/-F-12 Medium (Invitrogen, 11330), if using Dispase

4. Dispase (Invitrogen, 17105-041) or EDTA 0.02% ® Solution (Versene) (Lonza 17-711E) or equivalent

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 1:6 when using Dispase for passaging and between 1:8 and 1:20 when using EDTA for passaging. 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 4-6 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 Culture for Passaging

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

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 protocols detailed below as this occurs during the procedure.
    1. EDTA Passaging: Just prior to removing the EDTA ® wash step, 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. Add 1ml/well of EDTA® and begin treatment time. Continue the passage procedure as detailed below.
    2. Dispase Passaging: 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.
    3. Make sure not to dry out the culture well, add more wash reagent if needed.

Passaging Cells Using Reagent-based Method (use either EDTA or Dispase)

1. **Passaging Cells Using EDTA®**
    1. **Harvest Cells**

1. Place plate of cells to be passaged in the sterile 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. Rinse each well with 1ml room temperature EDTA ®. Aspirate removing marked areas of differentiation as previously described (removal by suction).
4. Add 1ml room temperature EDTA ® to each well, set aside in the BSC.
5. Treat with EDTA® for 6-9 minutes (depending on cell line) undisturbed at room temperature.
6. While treating with EDTA®, aspirate the plating medium from a plate pre-coated with BME or rhVitronectin (as described previously), add 1.5ml/well of culture medium per well, set aside.
7. Also, while treating with EDTA®, determine volume of cells to add to each new well by dividing total volume of cell suspension by the number of possible wells based on the split ratio.

   EXAMPLE 1: 1 well collected in a total of 3ml at a split ratio of 1:20 à 3ml ÷ 20 wells = 0.150ml/well = 150ul per well.

   EXAMPLE 2: 4 wells collected in a total of 6ml at a split ratio of 1:15 à 6ml ÷ 60 wells = 0.1ml/well = 100ul per well.
8. Aspirate the EDTA® with a Pasteur pipette carefully without disturbing the attached cell layer.
    1. If cells become free-floating, collect and spin down. Re-suspend gently in 3-6ml, depending on your culture. Use this re-suspension volume to calculate volume of cells/well to add.
9. Use 3ml of culture medium, hold a 5ml glass pipette perpendicular to the plate. Wash the cells off of the first well with culture medium using a back and forth motion and starting at the bottom of the well and working to the top of the well. Repeat if necessary. 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).
    1. **Note:** Minimize bubbles by pipetting gently. Cells should wash off without touching the pipette to the bottom of the plate. If cells stay adhered after rinsing plate, gently scrape cells, and increase the incubation time at the next passage to allow cells to wash off.
10. After the cells are removed from the surface of the well, pool the contents of the scraped wells into a sterile conical tube.

2. Check final volume of cell suspension, and correct to approximately 3ml/well passaged if needed.
3. **Plate Cells**
    1. Gently re-suspend the cells using a 5ml pipette.
    2. Add determined volume of cell suspension to each well of the prepared 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.

2. **Passaging Cells Using Dispase**

    1. **Prepare 2mg/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 4ºC for up to 14 days.
    2. Remove pluripotent stem cell plate from incubator and place it in the biosafety cabinet.
    3. **Harvest Cells**
        1. 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.).
        2. Add 1ml room temperature Dispase Solution to each well to be passaged.
        3. Incubate for 7 minutes at 37°C.
        4. While incubating, aspirate the plating medium from a plate pre-coated with BME or rhVitronectin (as described previously), add 1.5ml/well of culture medium per well, set aside.
        5. Aspirate the Dispase Solution with a Pasteur pipette. Remove the Dispase carefully without disturbing the attached cell layer.
        6. Gently wash the attached cells three times with 1ml/well 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.
        7. 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).
        8. 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.
        9. Pipette the medium slowly up and down to wash the cells off the surface. Be careful not to create bubbles.
        10. Leave the contents in the wells until all wells are scraped.
        11. 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.
        12. 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.
        13. 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 of medium and cells in each of the new wells (0.5ml of cell suspension + 1.5ml of pre-

plated culture medium)

4. **Plate 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.

3. **Manual Passaging**
    1. **Note:** Manual passaging puts a very high selection pressure on cultures, and therefore we recommend only using when absolutely necessary. Occasionally, cultures may be very sparse after thaw, requiring manual passaging. Other than directly out of thaw, we recommend manual passaging only when necessary to save an irreplaceable culture.
    2. Aspirate the plating medium from a plate pre-coated with BME or rhVitronectin (as described previously), add 1.0ml/well of culture 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: Feeder Independent -Protocol

Required Equipment

1. Biosafety cabinet
2. 37°C / 5% CO2 Incubator
3. 37°C water bath
4. Centrifuge
5. Light microscope
6. Liquid Nitrogen storage tank, and liquid nitrogen

Required Supplies

1. Metal forceps
2. Cryogenic handling gloves and eye protection
3. Isopropanol freezing containers, and isopropanol
4. 1.5ml Cryovials, (Nunc, 5000-1020)
5. Plastic cryovial holders
6. 5ml and 10ml sterile serological pipettes (Fisher,13-678-27E, 13-67827F) or equivalent
7. 95% Ethanol

Required Reagents

1. Cell culture medium specified in the Certificate of Analysis
    1. mTeSR™1 Medium (Stem Cell Technologies, 85850)
    2. mTeSR™ Plus (Stem Cell Technologies, 05825)
    3. TeSR-E8™ (Stem Cell Technologies, 05990)
2. mFreSR™ Cryopreservation Medium (Stem Cell Technologies, 05854/05855)
3. DMEM-F12 Medium (Invitrogen, 11330-057), if using Dispase
4. EDTA 0.02% solution® or Dispase Solution (2mg/ml) as required

Prepare for Freeze

1. One well of cells will be frozen into 2 vials. 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. View all cells under the microscope. Discard any contaminated plates. Identify and remove differentiation as described above if necessary.
5. **Freezing Using EDTA® Harvest**
    1. If cells from more than one plate are being frozen, stagger the EDTA ® wash and treatment by 3-4 minutes between plates to avoid overexposure. Overexposure to EDTA® will lead to the cells detaching from the plate.
    2. Remove spent medium and add 1ml of EDTA ® to each well of each 6-well plate. Aspirate. Again, add 1ml of EDTA® to each well, set plate aside.
    3. Treat pluripotent stem cells with EDTA® for 6-8 minutes (depending on cell line) at room temperature.
    4. **Harvest and Wash Cells**
        1. Aspirate EDTA® from each well, taking care not to remove any floating colonies.
        2. If harvesting 6 wells of cells, gently wash the cells off with 3ml of mFreSR ™ Medium for each plate using a 5ml glass pipette. Do not work with more than 3ml/well (overfilling wells may lead to contamination). Hold the pipette perpendicular to the plate. Wash cells off of the first well with medium using a back and forth motion and starting at the bottom of the well and working to the top of the well. Repeat if necessary, transferring the medium from well to well, add the cells to the 50ml conical tube.
        3. Wash each plate again with 3ml of mFreSR ™ Medium to collect any remaining cells, transferring the medium from well to well and add the medium to the 50ml conical tube.
        4. Repeat harvest for any remaining plates. Continue to pool all cells to create a uniform lot.
    5. Add mFreSR ™Medium to the pooled cells reach desired freezing density. Vials frozen using EDTA ® can be less dense than other standard methods. Freezing 1 plate at 2 cryovials/well will require a total of 12ml of mFreSR™1 Medium
    6. Pipette pooled cells very gently to evenly mix suspension.
    7. With the same pipette, distribute 1ml of cell suspension to each prepared vial. Mix cell pool every 6-10 vials for even distribution.
    8. Quickly, place cryovials into an isopropanol containing freezing container. Place the freezing containers in the -80°C freezer overnight.
    9. Transfer cell vials to liquid nitrogen storage the following day.
6. **Freeze Using Dispase Harvest**
    1. Remove spent medium and add 1ml of Dispase Solution to each well of each 6-well plate.
    2. Incubate pluripotent stem cells for 5 minutes at 37°C.
    3. Confirm colony separation by viewing the plate under the microscope. Look for the perimeter of the colony to appear folded back.
    4. **Harvest Cells**
        1. Aspirate Dispase Solution from each well, taking care not to remove any floating colonies.
        2. Wash each adherent well with 1ml of 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 culture medium to each well.
4. When harvesting 6 wells of a 6-well plate, take up 1ml of medium from the first three wells in a 5ml glass pipette. Hold the pipette perpendicular to the plate and gently scrape the surface of the plate while simultaneously dispensing medium 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. Transfer the cells in a sterile 50ml centrifuge tube per plate.
6. Repeat harvest process for remaining 3 wells, add cell to same conical tube to create a cell pool.
7. Wash each plate with 3ml of culture medium to collect any residual cells, transfer the medium from well to well and add the medium to the 50ml conical tube.
8. Centrifuge cells at 200xg for 5 minutes
9. Aspirate the supernatant being careful not to disturb the cell pellet. Very slowly and gently re-suspend cell pellet by adding 1ml mFreSR™ Medium per every well harvested. Freezing 1 plate at 1 well/cryovial will require 6ml of mFreSR™ Medium. If there is more than one conical tube, you should pool them at this point for a uniform lot.

5. Pipette pooled cells very gently to evenly mix suspension.
6. With the same pipette, distribute 1ml of cell suspension to each prepared vial. Mix cell pool every 6-10 vials for even distribution.
7. Quickly, place cryovials into an isopropanol containing freezing container. Place the freezing containers in the -80°C freezer overnight.
8. Transfer cell vials to liquid nitrogen storage the following day.

Section 7: Transitioning Between Culture Platforms

Transfer between culture 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 independent platform to another, follow the manufacturer's instructions for transitioning cultures to the new platform. When transferring feeder independent cultures to a murine embryonic fibroblast (MEF) based culture platform, passage cells as using Dispase as described above, 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 (medium, feeders) transitioning medium over the course of days or passages may be appropriate. Regardless of final platform, WiCell recommends maintaining the initial cultures in the original conditions in parallel until the user can be confident that the transition is successful.

Section 8: Photographs of Pluripotent Stem cells grown on Matrigel™ in mTeSR™1

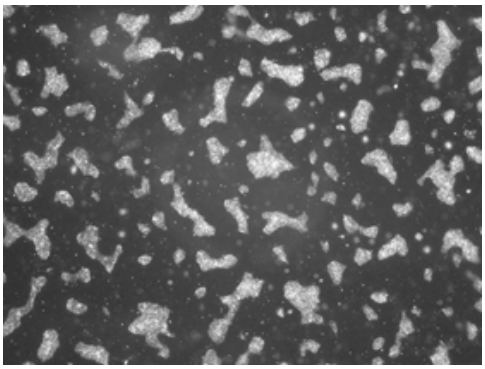

Fig 1: Pluripotent Stem cells mid split cycle (2.5x)

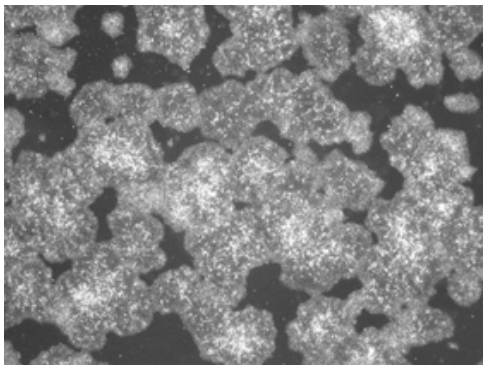

Fig 2: Pluripotent Stem cells, minimal differentiation, ready to be split (2.5x)

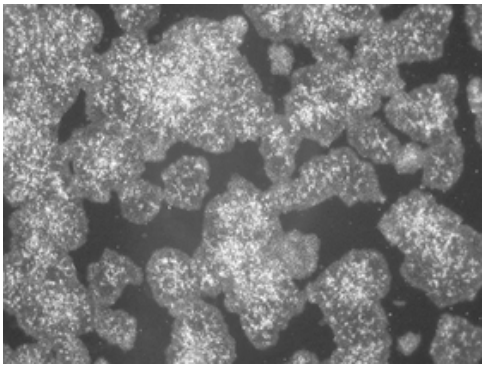

Fig 3: Pluripotent Stem cells, minimal differentiation, ready to be split (2.5x)

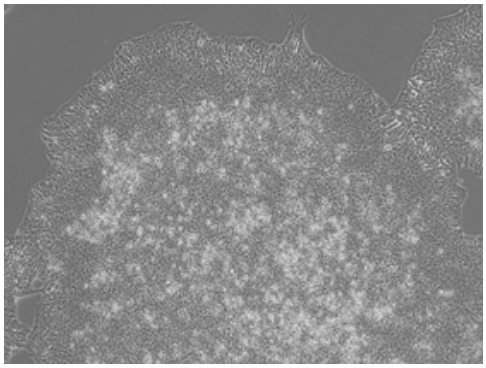

Fig 4: Pluripotent Stem cell colony pre-split (10x)

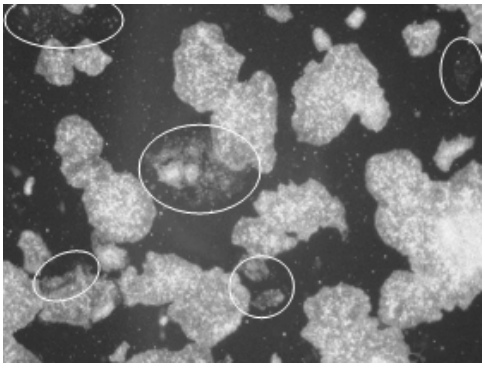

Fig 5: Differentiation, see circled areas (2.5x)

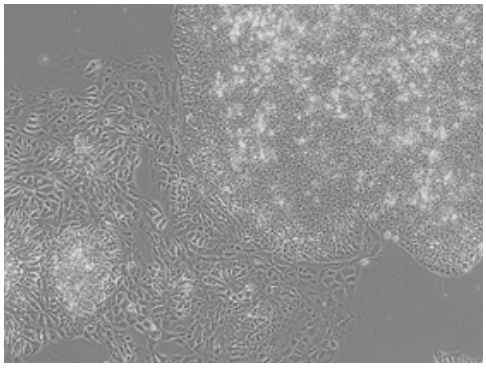

Fig 6: Differentiation (10x) between colonies

3. Version History

Version	Version History	Effective Date
A	This document was previously not part of WiCell's document control system.	Not applicable
B	Updated protocol to represent current procedures, incorporated into the WiCell Document Control System	08Apr10
C	Added attachment 2: MSDS to be included with WiCell produced cells only	13Aug10
D	CC00127. Changed to reflect passage and freezing techniques using EDTA in place of Dispase. Referenced shipping packet insert in the thaw protocol to acquire recommended thaw ratio. Removed transfer of spent medium on day one after a thaw. MSDS was removed.	24Aug11
E	CC00211, made changes to layout and format to be consistent with other shipping protocols, specifically the ordering of the supplies and reagents within sections. Changed EDTA to Versene, added BSC sterilization of prepared vials to Dispase freezing section, reduced Versene treatment time to 6-9 minutes	09Jul13
F	CC00389. Added use of Rock Inhibitor note to section 3, and amended E8 reference to mTeSR™1.	09Oct13
G	CC00392, Corrected spelling and grammatical errors.	30Jan14
H	CC00445, reordered sections to allow for better flow and added copyright.	28May15
I	CC00617, Added more references to checking the CoA for ROCK inhibitor use recommendations	19Aug16
J	CC00635, Added details about Rock Inhibitor use in thawing section. Removed section for ROCK inhibitor prep, added to thaw protocol. Clarified how to break up cells in harvest and spin section. Changed Vitronectin plating concentration to 5ug/well.	07Jul17

K	CC00771, Updated vendor info for mTeSR1, Matrigel, Dispase and EDTA. Corrected ROCK inhibitor prep, removed directive to store prepared Matrigel plates at 4^{o}C, removed directive to roll vial between hands while thawing, removed directive to transfer spent medium after thaw to new well, added more description for how to remove cells using EDTA, reduced the volume of dispase prepared, added freezing container must be room temperature, made harvesting sections consistent among methods, corrected volume for passaging examples, added that cells should not flow down the side of the tube when thawing, updated guidelines for removing differentiation, updated cell culture images. Changed “no-spin” passage method to reagent-based. Removed unnecessary DMEM/F-12 from reagent lists.	23Apr19
1.0	CC01257 & CC-318, Made inclusive to multiple types of culture media. Included an example CoA to show where to find culture platform info. Added example CoA to highlight culture platform fields. Transitioned SOP-SH-002 to Qualio as SH-2.	12Aug20
2.0	CC-363, Updated numbering.	17Aug20
3.0	CC-903, Change from Matrigel to Basement Membrane Extract (BME). Remove "or equivalent" in equipment & materials sections.	See Qualio or Controlled Copy

4. Attachments

No attachments.