Feeder-Independent Pluripotent Stem Cell Protocols

E8 Medium

Preface

This booklet of protocols is intended to serve as a primer for culturing pluripotent stem cells using E8 medium. E8 medium can be used in combination with either Matrigel™ or rhVitronectin for the undifferentiated proliferation of human embryonic stem (ES) and induced pluripotent stem (iPS) cells. While 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. These 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.

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1.4. Preparing to Aliquot Matrigel™

1.4.1. 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.

1.4.2. 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 or

larger volume aliquots are more appropriate for your use, adjust calculations appropriately).

1ml = given concentration
Xml 1mg

X = ml of Matrigel 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**

1.4.3. The day before aliquoting, 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 (based on calculation in previous step) in the -20°C freezer.

1.5. Thaw the Matrigel™

1.5.1. 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.

1.6. Aliquoting Matrigel™

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.

1.6.1. 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.

1.6.2. 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.

1.6.3. 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.

1.6.4. 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.

1.7. 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.

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.

1.7.1. 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.

1.7.2. Remove one 1.0 mg Matrigel™ aliquot from the freezer, and add 1ml of cold DMEM/F-12 medium to it.

1.7.3. 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.

1.7.4. Immediately plate 1ml/well of a 6-well plate. This will be enough for 2 full 6-well plates.

1.7.5. Allow to set one hour at room temperature before use or storage.

1.7.6. 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 2: Preparing and Plating rhVitronectin

2.1. Required Equipment

2.1.1. Sterile biosafety cabinet

2.1.1. -80˚C freezer

2.1.2. 100µl or 200µl Pipetman

2.2. Required Supplies

2.2.1. 15ml conical tube (Corning, 430052)

2.2.2. 10ml sterile serological pipettes (Fisher, 13-678-27F) or equivalent

2.2.3. 6-well tissue culture plates (Nunc, 140675)

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

2.2.5. DMEM/F-12 medium (Invitrogen, 11330-057)

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

2.2.7. Microcentrifuge tube holders

2.2.8. Freezer Boxes

2.3. Required Reagents

2.3.1. rhVitronectin (R&D Systems, 2308-VN) or equivalent

2.3.2. Sterile PBS without $CaCl_2$ and $MgCl_2$ (Invitrogen, 14190-250) or equivalent

2.4. Aliquoting rhVitronectin

2.4.1. Reconstitute 125µg rhVitronectin in 500µl sterile PBS without $CaCl_2$ and $MgCl_2$.

2.4.2. Aliquot 60µl per tube into sterile microcentrifuge tubes. This will be 15µg of rhVitronectin, or enough to coat 3 wells at 5µg/well.

2.4.3. Store tubes at -80°C until ready to use.

2.5. Plating rhVitronectin

2.5.1. Remove aliquot from freezer and allow to thaw at room temperature.

2.5.2. Dilute aliquot with 6ml of DMEM/F-12 medium into a 15ml conical tube, pipet to mix, and immediately plate at 2ml diluted rhVitronectin per well.

2.5.3. 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.

Section 3: Preparing ROCK Inhibitor Working Stock Solution

3.1. Required Equipment

3.1.1. Sterile biosafety cabinet

3.1.2. 100µl or 200µl Pipetman

3.2. Required Supplies

3.2.1. 1.5ml Microcentrifuge tubes, sterilized (Fisher, 05-408-129)

3.2.2. Microcentrifuge tube holders

3.2.3. Freezer Boxes

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

3.3. Required Reagents

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

3.3.2. Sterile water (Sigma, W3500)

3.4. Dilute and Aliquot ROCK Inhibitor Working Stock Solution

Note: Perform work sterilely.

3.4.1. Make 10mM working stock solution by diluting 1mg ROCK inhibitor (FW 320.26) into 295µl sterile water to achieve a 10mM solution. Note: if FW of material is not 320.26, dilute appropriately to achieve a 10mM solution.

3.4.2. Aliquot into appropriate working volumes (recommended at 20-50µl). ROCK inhibitor working stock solution will be used at 1µl to 1ml final medium volume. Aliquots can be stored long term at -80°C for up to 1 year and up to 2 months at 4°C.

Section 4: Thawing Pluripotent Stem Cells: Feeder Independent - E8 Protocol

Note: Before thawing, check the certificate of analysis included in the shipping packet insert to acquire the recommended number of wells one vial should be thawed into (this can vary among different lots). Also, check the certificate of analysis to see if ROCK inhibitor is recommended. If ROCK inhibitor is not recommended, it will not be referenced on the certificate of analysis.

4.1. Required Equipment

4.1.1. Sterile biosafety cabinet

4.1.2. 37˚C / 5% CO_2 incubator

4.1.3. 37˚C water bath

4.1.4. Centrifuge

4.2. Required Supplies

4.2.1. Forceps

4.2.2. Cryogenic handling gloves and eye protection

4.2.3. 5ml sterile serological pipettes (Fisher,13-678-27E) or equivalent

4.2.4. 95% Ethanol

4.2.5. Sterilized Pasteur pipettes (Fisher, 13-678-20D)

4.3. Required Reagents

4.3.1. Matrigel™, Growth Factor Reduced (BD Biosciences®, 354230) or rhVitronectin (R&D Systems, 2308-VN) coated 6-well plate (Nunc, 140675), prepared as indicated previously

4.3.2. E8 medium (per Chen et al., Nature Methods 2011)

4.3.3. ROCK inhibitor working stock solution as previously described

4.3.4. DMEM/F-12 medium (Invitrogen, 11330-057)

4.4. Prepare Plate to Receive Cells

4.4.1. If prepared Matrigel™ or rhVitronectin 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.

4.4.2. 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.

4.4.3. Remove excess plating medium from prepared Matrigel™ or rhVitronectin coated plate from wells which will receive cells, and add 1.5 ml E8 medium plus 1µl per ml sterile ROCK inhibitor working stock solution to every well that will receive cells.

4.4.4. Label plate appropriately (WiCell recommends at minimum the cell line name, passage number on the vial, date and technician initials).

4.5. 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.

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

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

4.5.3. Using long forceps immerse the vial in a 37˚C water bath without submerging the cap. Swirl the vial gently.

4.5.4. When only an ice crystal remains, remove the vial from the water bath.

4.5.5. 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.

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

4.6.1. Transfer the cells gently into a sterile 15ml conical tube using a 1ml or 5ml glass pipette.

4.6.2. Slowly, add 11ml of E8 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.

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

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

4.6.5. Re-suspend the cell pellet in 0.5ml E8 medium plus 1µl per ml ROCK inhibitor working stock solution (as previously described) for every well that will receive cells (number of wells receiving cells is based on the thaw recommendation found in the certificate of analysis which is included in the shipping packet insert). *For example*: When the thaw recommendation is to thaw 1 vial into 4 wells, re-suspend the pellet in 2ml, 0.5ml will be plated into each well.

4.6.6. Very gently pipette cells up and down in the tube a few times, be careful to keep the cell in small clumps.

4.7. Plate Pluripotent Stem Cells

4.7.1. Slowly add 0.5ml of the cell suspension drop-wise into prepared well of the warmed plate.

4.7.2. Place plate gently 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.

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

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

4.7.4. Add 2.0ml of standard E8 medium to the first original well/plate. Place both plates gently into an incubator overnight.

4.7.5. Feed daily as outlined below.

Section 5: Feeding Pluripotent Stem Cells: Feeder Independent - E8 Protocol

5.1. Required Equipment

5.1.1. Sterile biosafety cabinet

5.1.2. 37˚C / 5% CO_2 incubator

5.2. Required Supplies

5.2.1. 5ml and 10ml sterile serological pipettes (Fisher, 13-678-27E, 13-67827F) or equivalent

5.2.2. Light microscope (a camera is recommended)

5.2.3. Sterilized Pasteur pipettes (Fisher, 13-678-20D)

5.3. Required Reagents

5.3.1. E8 medium (per Chen et al., Nature Methods 2011)

5.4. Feeding Pluripotent Stem Cells

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

5.4.2. 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.

5.4.3. Add 2.0ml of E8 medium to each well. Return the 6-well plate to the 37°C incubator.

5.4.4. Repeat procedure daily until cells are ready to be passaged or harvested.

Section 6: Passaging Pluripotent Stem Cells: Feeder Independent - E8 Protocol

There are two methods for passaging: A "no-spin" method is recommended for thaws with greater than 10-20 colonies and is suitable for standard passaging. The manual passaging method is used when there is significant differentiation present and the culture must be maintained.

6.1. Required Equipment

6.1.1. For Versene® passaging

6.1.1.1. Sterile biosafety cabinet

6.1.1.2. Hood (biosafety or static) equipped with stereomicroscope

6.1.1.3. Light microscope (a camera is recommended)

6.1.1.4. Colony marker

6.1.1.5. 37˚C / 5% CO_2 incubator

6.1.1.6. 37˚C water bath

6.1.2. For manual passaging

6.1.2.1. Hood (biosafety or static) equipped with stereomicroscope

6.1.2.2. Microscope

6.2. Required Supplies

6.2.1. 5ml sterile serological pipettes (Fisher, 13-678-27E) or equivalent

6.2.2. Sterilized Pasteur pipettes (Fisher, 13-678-20D)

6.3. Required Reagents

6.3.1. Matrigel™, Growth Factor Reduced (BD Biosciences, 354230) or rhVitronectin (R&D Systems, 2308-VN) coated 6-well plate (Nunc, 140675), prepared as indicated previously

6.3.2. E8 medium (per Chen et al., Nature Methods 2011)

6.3.3. ROCK inhibitor Working Stock Solution as previously described

6.3.4. DMEM/F-12 medium (Invitrogen, 11330-057)

6.3.5. Versene® (0.02% EDTA solution; Lonza, 17-711E) or equivalent (not required for manual passaging)

6.3.6. Cutting tool (for manual passaging only: this can be a needle, modified pipette, pipetman tip, SweMed™ instrument, etc.)

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

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

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

6.4.2. Increased differentiation occurs.

Note: The split ratio is variable, though when using Versene® you can generally expect to passage between 1:2 and 1:6 for the first passage from thaw, and between 1:8 and 1:20 for subsequent passages. 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 4-7 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.

6.5. Prepare Plate to Receive Cells

6.5.1. If prepared Matrigel™ or rhVitronectin 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.

6.5.2. Remove plating medium from prepared Matrigel™ or rhVitronectin coated plate, and add 1.5 ml E8 medium plus 1µl per ml ROCK inhibitor working stock solution to every well that will receive cells.

6.5.3. Label plate appropriately (WiCell recommends at minimum the cell line name, passage number on the vial, date and technician initials).

6.6. Prepare Culture for Passaging Using Versene®

6.6.1. Examine the cultures to be passaged and remove differentiation if necessary. Use the colony marker on the microscope to mark all areas of differentiation on the plate of pluripotent stem cells. Determine which method of differentiation removal 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

6.6.2. **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 sterile 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.

6.6.3. **Removal by Suction**: If removing differentiation by suction, become familiar with the passaging protocol detailed below as this occurs during the procedure. Just prior to removing the Versene® 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 and any remaining Versene®. Add 1ml/well of Versene® and begin treatment time. Continue the passage procedure as detailed below.

6.6.4. Determine appropriate split ratio for the culture.

6.6.5. Use split ratio to determine appropriate volume of cell suspension to add to each new well at passage. Each well will be collected in 3ml total of medium. 3ml divided by the split ratio = the volume of cell suspension to add to each new well. EXAMPLE: Split ratio is determined to be 1:10. 3ml/10 wells = 0.3ml/well or 300µl/well.

6.7. Passaging Cells Using Versene®

6.7.1. Remove plate to be passaged from incubator and place it in the sterile biosafety cabinet.

6.7.1.1. 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 issues that may occur as a result of the split that would otherwise jeopardize the culture (contamination, etc.).

6.7.1.2. Rinse each well to be passaged with 1ml room temperature Versene®. Aspirate, removing marked areas of differentiation as previously described (removal by suction).

6.7.1.3. Add 1ml room temperature Versene® to each well to be passaged.

6.7.1.4. Incubate for 2-7 minutes at room temperature depending on cell line.

6.7.1.5. While incubating, for every well that will be passaged, place 3ml E8 medium plus 1µL per ml ROCK inhibitor working stock solution into a conical tube.

6.7.1.6. Once incubation is complete, aspirate the Versene® from the culture to be passaged with a Pasteur pipette.

6.7.1.6.1. 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 µl of cells/well to add to each new well.

6.7.1.7. Using 1-2ml per well of medium from the conical tube (from Step 6.7.1.5.), hold a 5ml pipette perpendicular to the plate and gently dispense the medium over the surface of the plate to wash cell off of plate. Repeat if necessary. Transfer cells to a new sterile conical tube. 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).

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.

6.7.1.8. Rinse plate with remaining medium to collect any residual cells, add to conical tube containing previously collected cells to create pooled the cell suspension.

6.7.1.9. Check final volume of cell suspension, and correct to approximately 3ml/well passaged.

6.7.2. **Plate Cells**

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

6.7.2.2. Add determined volume of cell suspension to each well of the newly prepared plate.

6.7.2.3. Place plate gently 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.

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.

6.7.2.4. Incubate cells overnight to allow colonies to attach.

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

6.8. Manual Passaging

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

6.8.1. Prepare plate to receive cell as described in a previous section, adding 2ml E8 medium plus 1µl/ml ROCK inhibitor working stock solution to each well that will be used.

6.8.2. Remove pluripotent stem cell plate from incubator and place it in the sterile biosafety cabinet. Aspirate the spent medium from the wells to be passaged with a Pasteur pipette. If possible, one well of cells should be left and used as a backup to protect

against issues arising from 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.

6.8.3. Add 1.0ml E8 medium plus 1µl/ml ROCK inhibitor working stock solution to each well containing pluripotent stem cells.

6.8.4. 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).

6.8.5. Remove the undifferentiated colonies from the pluripotent stem cell plate by cutting each colony into several (4-5) pieces and nudging them so they are floating in the medium. This can be done with a sterile modified pipette, pipetman tip, needle or SweMed™ instrument, etc.

6.8.6. Transfer the pieces into a new Matrigel™ or rhVitronectin coated plate prepared to receive cells as previously described. Transfer up to 50 colony pieces into each prepared well.

6.8.7. Return the plate with the freshly plated colony pieces 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.

6.8.8. Rinse and feed the plate that cells were harvested from, and return it to the incubator as well.

6.8.9. 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.

6.8.10. Once the new plate is determined to be free of contamination with appropriately attached colonies, discard the initial culture plate.

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

Section 7: Freezing Pluripotent Stem Cells: Feeder Independent - E8 Protocol

7.1. Freezing Using Versene® Harvest

7.1.1. Required Equipment

7.1.1.1. Sterile biosafety cabinet

7.1.1.2. 37˚C / 5% CO_2 incubator

7.1.1.3. 37˚C water bath

7.1.1.4. Centrifuge

7.1.1.5. Light microscope

7.1.2. Required Supplies

7.1.2.1. Metal forceps

7.1.2.2. Cryogenic handling gloves and eye protection

7.1.2.3. Liquid Nitrogen storage tank, and liquid nitrogen

7.1.2.4. Isopropanol freezing containers, and isopropanol

7.1.2.5. 1.5ml Cryovials (Nunc, 5000-1020)

7.1.2.6. Plastic cryovial holders

7.1.2.7. 5ml and/or 10ml sterile serological pipettes (Fisher,13-678-27E, 13-67827F) or equivalent

7.1.2.8. 95% Ethanol

7.1.3. **Required Reagents**

7.1.3.1. E8 medium (as per Chen et al., Nature Methods, 2011)

7.1.3.2. DMSO (Sigma, D2438)

7.1.3.3. DMEM-F12 medium (Invitrogen, 11330-057)

7.1.3.4. Versene® (0.02% EDTA solution; Lonza, 17-711E) or equivalent

7.1.4. **Prepare for Freeze**

7.1.4.1. Label cryovials appropriately using ethanol and liquid nitrogen resistant methods. 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 in biosafety cabinet.

7.1.4.2. Prepare E8 cryopreservation medium.

Note: Prepare sterilely and do not filter after addition of DMSO**.** Keep on ice until ready to use.

7.1.4.2.1. 80% E8 medium

7.1.4.2.2. 20% Sterile DMSO

7.1.4.3. 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.

7.1.4.4. Obtain a recharged room temperature isopropanol freezing container (Mr. Frosty). Each container can hold 18 vials. The isopropanol must be replaced every 5 uses.

7.1.4.5. View all cultures under the microscope. Discard any contaminated plates.

7.1.4.6. Identify and remove differentiation as described above if necessary.

Note: If cells from more than one plate are being frozen, stagger the Versene® wash and treatment by 3-4 minutes between plates to avoid overexposure. Overexposure to Versene® will lead to the cells individualizing and/or detaching from the plate.

7.1.4.7. Remove spent medium and add 1ml of Versene® to each well of each 6-well plate. Aspirate. Again, add 1ml of Versene® to each well and incubate for 2 to 7 minutes at room temperature depending on cell line.

7.1.5. **Harvest and Wash Cells**

7.1.5.1. Aspirate Versene® from each well taking care not to remove any floating colonies.

7.1.5.2. Gently wash the cells off with 3ml of E8 medium per plate, transferring the medium from well to well and add the medium to the 50ml conical tube.

7.1.5.3. Pool the cells in a sterile vessel (e.g. a sterile 50ml centrifuge tube).

7.1.5.4. Wash each plate again with 3ml of E8 medium to collect any remaining cells, transferring the medium from well to well and add the medium to the vessel of pooled cells.

7.1.5.5. Repeat harvest for any remaining plates. Continue to pool all cells to create a uniform lot.

7.1.5.6. Add E8 medium cell pool so that the final volume is equal to the total number of wells harvested (some medium will have been lost in the harvest).

7.1.5.7. Very slowly and drop-wise, add an equal volume of E8 cryopreservation medium. For example, is you have harvested 6 wells and now have 6ml of pooled cell suspension, add 6ml cryopreservation medium. Gently pipette up and down two times to mix, being careful not to break up the colonies further.

Note: At this point, cells are in contact with DMSO, and work should be performed efficiently. Once cells are in contact with DMSO, they should be aliquoted to vials and frozen within 2-3 minutes. For large scale freezing, it is best to do this step in small batches.

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

7.1.5.9. Quickly, place cryovials into an isopropanol containing freezing container. Place the freezing containers in the -80˚C freezer overnight.

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

Section 8: 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-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 described in WiCell's Feeder-Free protocols using Dispase (available online at www.WiCell.org), 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.

Section 9: Revision History and Protocol Approvals

9.1. Revision History

Version	Change Description	Effective Date
A	CC00211, Document Initiation	07-September-2012
B	CC00283, Corrected typos and minor changes for clarity.	18-September-2012
C	CC00327, updated preface, directed to plate 5µg/well instead of 2µg/well	11-March-2014
D	CC00545, added copyright	28-May-2015
E	CC00635, Added details about Rock Inhibitor use in thawing section note. Added detail on cell clumps in resuspension of PSC section.	See SPDC

9.2. Approvals

1/31/2017 X JKG JKG Quality Assurance Signed by: Gay, Jenna	X TEL Director - WiCell Stem Cell Bank