



# WiCell Feeder–Independent Pluripotent Stem Cell Protocols

## Supplement: Culturing with Zeocin

## STANDARD OPERATING PROCEDURE



Feeder Independent Stem Cell Protocols Supplement: Culturing with Zeocin

**SOP Number: SOP-SH-008**

**Version: A**

### 1. PURPOSE

1.1. This Standard Operating Procedure (SOP) provides additional information culturing with a Zeocin additive. If you have any additional questions, please contact us through technical support on the WiCell website at [www.wicell.org](http://www.wicell.org).

### 2. REFERENCES

2.1. WiCell SOP-SH-002

### 3. DEFINITIONS

#### 4. Required Material

4.1. Zeocin (Invitrogen, R250-1)

### 5. PROCEDURE

5.1. Thaw

**Note: Before thawing, check the certificate of analysis included in the shipping packet insert to determine the recommended number thaw ratio (number of wells into which a single vials of cells should be thawed).**

5.1.1. Thaw according to WiCell Feeder Independent Pluripotent Stem Cell Protocol (SOP-SH-002). Zeocin or any other antibiotic is not used in the thaw medium.

5.1.2. The next day, feed as per the WiCell Feeder Independent Pluripotent Stem Cell Protocol, using mTeSR1+2ug/ml of Zeocin.

5.2. Culture

5.2.1. Continue to observe cells and feed with mTeSR1 containing 2ug/ml Zeocin daily until cells require passaging. Expect to see increased cell debris from unselected differentiated cells killed by the antibiotic. Cell morphology should remain normal and consistent with the addition of zeocin.


5.3. Passage

5.3.1. Passage according to the WiCell Feeder Independent Pluripotent Stem Cell Protocol, plating cells into mTeSR1+2ug/ml of Zeocin. During all passages, the Zeocin may remain in the medium.

5.4. Cryopreservation

5.4.1. Cryopreserve cells by removing Zeocin containing medium from the wells, and follow the WiCell Feeder Independent Pluripotent Stem Cell Protocols.

## STANDARD OPERATING PROCEDURE

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	<b>SOP Number: SOP-SH-008</b>	<b>Version: A</b>

### 6. REVISION HISTORY

Version	Change Description	Effective Date
A	CC00402, Document Initiation.	See SPDC

### 7. APPROVALS

5/8/2015	5/12/2015
<b>X</b> JKG	<b>X</b> TEL
JKG Senior Quality Assurance Specialist Signed by: Gay, Jenna	TEL Director - WiCell Stem Cell Bank Signed by: Ludwig, Tenneille



# WiCell Feeder–Independent Pluripotent Stem Cell Protocols

## mTeSR™1 Medium



## Preface

This booklet of protocols is intended to serve as a primer for culturing pluripotent stem cells in a Feeder-Independent system using mTeSR™1 . Please note that mTeSR™1 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 that we have sent to 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](http://www.wicell.org/cytogenetics).

If you have any additional questions, please contact us through technical support on the WiCell website at [www.wicell.org](http://www.wicell.org).

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## Section 1: Aliquoting and Plating Matrigel™

### 1.1. Required Equipment

#### 1.1.1. For aliquoting

- 1.1.1.1. Sterile biosafety cabinet
- 1.1.1.2. -20°C freezer
- 1.1.1.3. 4°C refrigerator
- 1.1.1.4. 200µl or 1000µl Pipetman

#### 1.1.2. For plating

- 1.1.2.1. Sterile biosafety cabinet
- 1.1.2.2. Pipet-Aid

### 1.2. Required Supplies

#### 1.2.1. For aliquoting

- 1.2.1.1. 1.5ml Microcentrifuge tubes, sterilized (Fisher, 05-408-129), labeled
- 1.2.1.2. Microfuge tube holders
- 1.2.1.3. Ice Bucket with lid filled with ice
- 1.2.1.4. 2 small containers filled with ice
- 1.2.1.5. Freezer Boxes
- 1.2.1.6. Sterile 200µl or 1000µl Pipette tips (Molecular Bio, 2770)

#### 1.2.2. For plating

- 1.2.2.1. 15ml conical tube (Corning, 430052)
- 1.2.2.2. 10ml sterile serological pipettes (Fisher, 13-678-27F) or equivalent
- 1.2.2.3. 6-well tissue culture plates (Nunc, 140675)
- 1.2.2.4. Parafilm

### 1.3. Required Reagents

#### 1.3.1. For aliquoting

- 1.3.1.1. Matrigel™, Growth Factor Reduced (BD Biosciences®, 354230) or equivalent

#### 1.3.2. For plating

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



1.3.2.2. Matrigel™, Growth Factor Reduced aliquot (as prepared in following section)

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.)

$\frac{1\text{ml}}{X\text{ml}} = \frac{\text{given concentration}}{1\text{mg}}$ <p>X = ml of Matrigel required per tube</p> <p><i>Example:</i> Concentration of lot: 8.6mg/ml</p> $\frac{1\text{ml}}{X\text{ml}} = \frac{8.6\text{mg}}{1\text{mg}}$ <p>X=0.1163ml = <b>116µl per tube for 1mg per tube</b></p>
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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

**Note:** As an alternative to Matrigel™, mTeSRTM1 medium can also be used with rhVitronectin as a supporting matrix.

### 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<sub>2</sub> and MgCl<sub>2</sub> (Invitrogen, 14190-250) or equivalent

## 2.4. Aliquoting rhVitronectin

- 2.4.1. Reconstitute 50µg rhVitronectin in 500µl sterile PBS without CaCl<sub>2</sub> and MgCl<sub>2</sub>.
- 2.4.2. Aliquot 60µl per tube into sterile microcentrifuge tubes. This will be 6µg of rhVitronectin, or enough to coat 3 wells at 2µg/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

**Note:** In general, ROCK inhibitor is not required for appropriate attachment and expansion in mTeSR™1 medium for most cell lines. Please consult your Certificate of Analysis to determine if ROCK inhibitor is recommended for use with a specific cell line.

### 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 – mTeSR™1 Protocol

**Note:** Before thawing, check the certificate of analysis to acquire the recommended number of wells one vial should be thawed into (this can vary across lots).

### 4.1. Required Equipment

4.1.1. Biosafety cabinet

4.1.2. 37°C / 5% CO<sub>2</sub> 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. mTeSR™1 Medium (Stem Cell Technologies, 05850)
- 4.3.3. 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 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.
- 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 mTeSR™1 Medium 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 a small 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 mTeSR™1 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 mTeSR™1 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.
- 4.6.6. To increase attachment add 1µl per ml ROCK inhibitor working stock solution (as previously described) for every well that will receive cells.
- 4.6.7. Very gently pipette cells up and down in the tube a few times.

#### 4.7. Plate Pluripotent Stem Cells

- 4.7.1. Slowly add 0.5ml of the cell suspension drop-wise into each well.
- 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 cells from 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 across the well.
- 4.7.3. The next day, remove the spent medium and 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 backup should be maintained with separate medium and in a separate incubator.
- 4.7.4. Add 2.0ml of mTeSR™1 medium to the first original well/plate. Place both plates gently into an incubator overnight.
- 4.7.5. Feed cells daily as described below until ready to be passaged or harvested.

## Section 5: Feeding Pluripotent Stem Cells: Feeder Independent – mTeSR™1 Protocol

### 5.1. Required Equipment:

- 5.1.1. Biosafety cabinet
- 5.1.2. 37°C / 5% CO<sub>2</sub> incubator
- 5.1.3. Light microscope (a camera is recommended)

### 5.2. Required Supplies:

- 5.2.1. 5ml and 10ml sterile serological pipettes (Fisher, 13-678-27E, 13-67827F) or equivalent
- 5.2.2. Sterilized Pasteur pipettes (Fisher, 13-678-20D)



**5.3. Required Reagents:**

- 5.3.1. mTeSR™1 Medium (Stem Cell Technologies, 05850)

**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. If they do not require passaging, aspirate the spent medium with a sterilized Pasteur pipette. If feeding more than one plate, use a different pipette for each plate in order to reduce risk of contamination.
- 5.4.3. Add 2.0ml of mTeSR™1 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.
- 5.4.4. Repeat procedure daily until cells require passaging.

**Section 6: Passaging Pluripotent Stem Cells: Feeder Independent - mTeSR™1 Protocols**

Note: There are two methods for passaging. A “no-spin” method of passaging is recommended for thaws with greater than 10-20 colonies and is suitable for standard passaging. A manual passaging method is recommended for sparse (fewer than 10-20 colonies) thaws or splits. The manual passaging method is also used when there is significant differentiation present and the culture must be maintained. Either Dispase or EDTA (Versene®) can be used to passage cells using the “no-spin” method. While WiCell currently recommends passaging with EDTA (Versene®) for best results, Dispase based passaging protocols have been included for convenience for those laboratories choosing to use this method.

**6.1. Required Equipment:**

- 6.1.1. Biosafety cabinet
- 6.1.2. Hood (biosafety or static) equipped with stereomicroscope
- 6.1.3. Colony marker
- 6.1.4. 37°C / 5% CO<sub>2</sub> incubator
- 6.1.5. 37°C water bath

**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. mTeSR™1 Medium (Stem Cell Technologies, 05850)
- 6.3.3. DMEM/-F-12 Medium (Invitrogen, 11330-057)

6.3.4. Dispase or Versene® Solution for passaging

**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 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 4-10 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.

**6.4.3. Prepare Culture for Passaging**

- 6.4.3.1. 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

- 6.4.3.2. **Pick-To-Remove:** If removing differentiation by the pick-to-remove method, transfer the plate into a hood equipped with a stereomicroscope. Remove all areas of differentiation with a modified pipette or micropipette tip.

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

- 6.4.3.3. **Removal by Suction:** If removing differentiation by suction, on the last aspiration, confirm the Pasteur pipette tip is intact and not chipped. Carefully press the pipette tip to the circled area and suction off the differentiated cells. Confirm the complete removal under the microscope. Passage immediately after removal of differentiation.

**6.5. Passaging Cells Using No-Spin Method**

### 6.5.1. Passaging Cells Using Versene®

#### 6.5.1.1. Harvest Cells

6.5.1.1.1. Remove pluripotent stem cell plate to be passaged from incubator and place it in the biosafety cabinet.

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

6.5.1.1.3. Rinse each well with 1ml room temperature Versene®. Aspirate.

6.5.1.1.4. Add 1ml room temperature Versene® to each well.

6.5.1.1.5. Treat with Versene® for 6-9 minutes at room temperature.

6.5.1.1.6. While treating with Versene®, determine volume of cells to add to each new well by dividing total volume of cell suspension by the split ratio.

EXAMPLE 1: 3 wells collected in a total of 3ml at a split ratio of 1:20  
→  $3\text{ml} \div 20 = 0.150\text{ml} = 150\text{ul}$  per well.

EXAMPLE 2: 4 wells collected in a total of 6ml at a split ratio of 1:15  
→  $6\text{ml} \div 15 = 0.4\text{ml} = 400\text{ul}$  per well.

6.5.1.1.7. Aspirate the Versene® with a Pasteur pipette carefully without disturbing the attached cell layer.

6.5.1.1.7.1. 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  $\mu\text{l}$  of cells/well to add.

6.5.1.1.8. Using 1-3ml per well of mTeSR™1 Medium, hold a 5ml pipette perpendicular to the plate and gently dispense the medium over the surface of the plate. 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)

**Note:** Minimize bubbles by pipetting gently. Cells should wash off without touching the pipette to the bottom of the plate.

6.5.1.1.9. 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.

#### 6.5.1.2. Plate Cells

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

6.5.1.2.2. Add determined volume of cell suspension to each well of the new plate.

6.5.1.2.3. Return the plate to the incubator after plating the cells. Move the plate in several quick, short, back and-forth and side-to-side motions to further disperse cells across the surface of the wells.

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

6.5.1.2.4. Incubate cells overnight to allow colonies to attach.

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

## 6.5.2. Passaging Using Dispase

### 6.5.2.1. Prepare Dispase solution

#### 6.5.2.1.1. 2mg/ml Dispase Solution Recipe

6.5.2.1.1.1. To make 2mg/ml Dispase Solution Recipe Combine the following components. Filter sterilize, store at 4°C for up to 14 days.

6.5.2.1.1.2. 100mg Dispase

6.5.2.1.1.3. 50ml DMEM-F12

6.5.2.2. Remove pluripotent stem cell plate from incubator and place it in the biosafety cabinet.

### 6.5.2.3. Harvest Cells

6.5.2.3.1. 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 problems with the split that would otherwise jeopardize the culture (contamination, etc.).

6.5.2.3.2. Add 1ml room temperature Dispase Solution to each well to be passaged.

6.5.2.3.3. Incubate for 7 minutes at 37°C.

6.5.2.3.4. Aspirate the Dispase Solution with a Pasteur pipette. Remove the Dispase carefully without disturbing the attached cell layer.

6.5.2.3.5. Gently wash the attached cells three times with 1ml of DMEM/F-12 per wash.

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

6.5.2.3.6. Add 1-2ml of mTeSR™1 Medium to each well (depending on determined passage ratio: If 1:4 or greater use 2ml/well. If <1:4 use less medium).

6.5.2.3.7. Hold a 5ml pipette perpendicular to the plate and gently scrape the surface of the plate while simultaneously dispensing medium. Repeat if necessary.

**Note:** Minimize bubbles by scraping and pipetting gently.

6.5.2.3.8. Pipette the medium slowly up and down to wash the cells off the surface. Be careful not to create bubbles.



- 6.5.2.3.9. Leave the contents in the wells until all wells are scraped.
- 6.5.2.3.10. 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.
- 6.5.2.3.11. 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.
- 6.5.2.3.12. Determine how much additional medium is required to 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)

#### 6.5.2.4. Plate Cells

- 6.5.2.4.1. Gently re-suspend the cells using a 5ml pipette.
- 6.5.2.4.2. Add 1ml of cell suspension to each well of the new plate.
- 6.5.2.4.3. Return the plate to the incubator after plating the cells. Move the plate in several quick, short, back and-forth and side-to-side motions to further disperse cells across the surface of the wells.  
**Note:** While cells are attaching, try to limit opening and closing the incubator doors, and if you need to access the incubator, open and close the doors carefully. This will prevent disturbing the even distribution of cells to the surface of the well.
- 6.5.2.4.4. Incubate cells overnight to allow colonies to attach.
- 6.5.2.4.5. Feed culture as previously described until ready to passage or freeze.

### 6.6. 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.

- 6.6.1. Ready prepared Matrigel™ or rh Vitronectin plates as described previously.
  - 6.6.1.1. 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 left and 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.
  - 6.6.1.2. Add 1.0ml mTeSR™1 Medium to each well containing pluripotent stem cells.
  - 6.6.1.3. Transfer the plate of pluripotent stem cells to be passaged into a hood or static chamber equipped with a stereomicroscope.

- 6.6.1.4. Remove the undifferentiated colonies from the pluripotent stem cell plate by cutting each colony into several (4-5) pieces and nudge them so they are floating in the medium. This can be done with a modified pipette, pipetman tip, needle or SweMed™ instrument.
- 6.6.1.5. Transfer the pieces into the prepared plate. Transfer up to 50 colony pieces into each prepared well.
- 6.6.1.6. Return the plate to the incubator. Move the plate in several quick, short, back-and-forth and side-to-side motions to further disperse cells across the surface of the wells.  
**Note:** While cells are attaching, open and close the incubator doors carefully. This will prevent disturbing the even distribution of cells to the surface of the well.
- 6.6.1.7. 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.
- 6.6.1.8. Feed culture as previously described until ready to passage or freeze.

## Section 7: Freezing Pluripotent Stem Cells: Feeder Independent - mTeSR™1 Protocol

### 7.1. Required Equipment

- 7.1.1. Biosafety cabinet
- 7.1.2. 37°C / 5% CO<sub>2</sub> Incubator
- 7.1.3. 37°C water bath
- 7.1.4. Centrifuge
- 7.1.5. Light microscope
- 7.1.6. Liquid Nitrogen storage tank, and liquid nitrogen

### 7.2. Required Supplies

- 7.2.1. Metal forceps
- 7.2.2. Cryogenic handling gloves and eye protection
- 7.2.3. Isopropanol freezing containers, and isopropanol
- 7.2.4. 1.5ml Cryovials, (Nunc, 5000-1020)
- 7.2.5. Plastic cryovial holders
- 7.2.6. 5ml and 10ml sterile serological pipettes (Fisher,13-678-27E, 13-67827F) or equivalent
- 7.2.7. 95% Ethanol

### 7.3. Required Reagents



- 7.3.1. mTeSR™1 Medium (Stem Cell Technologies, 05850)
- 7.3.2. mFreSR™ Cryopreservation Medium (Stem Cell Technologies, 05855)
- 7.3.3. DMEM-F12 Medium (Invitrogen, 11330-057)
- 7.3.4. Versene® or Dispase Solution (2mg/ml) as required.

**7.4. Prepare for Freeze**

- 7.4.1. 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. Placed in biosafety cabinet.
- 7.4.2. Sterilize 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.
- 7.4.3. Obtain a recharged isopropanol freezing container. The isopropanol must be replaced every 5 uses.
- 7.4.4. View all cells under the microscope. Discard any contaminated plates.
- 7.4.5. Identify and remove differentiation as described above if necessary.

**7.4.6. Freezing Using Versene® Harvest**

**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 detaching from the plate.

- 7.4.6.1. 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.
- 7.4.6.2. Treat pluripotent stem cells with Versene® for 6-8 minutes at room temperature.
- 7.4.6.3. **Harvest and Wash Cells**
  - 7.4.6.3.1. Aspirate Versene® from each well, taking care not to remove any floating colonies.
  - 7.4.6.3.2. Gently wash the cells off with 3ml of mFreSR™1 Medium for each plate, transferring the medium from well to well and add the medium to the 50ml conical tube.
  - 7.4.6.3.3. Pool the cells in a sterile 50ml centrifuge tube.
  - 7.4.6.3.4. Wash each plate again with 3ml of mFreSR™1 Medium to collect any remaining cells, transferring the medium from well to well and add the medium to the 50ml conical tube.
  - 7.4.6.3.5. Repeat harvest for any remaining plates. Continue to pool all cells to create a uniform lot.
  - 7.4.6.3.6. Add mFreSR™1 Medium to reach desired freezing density. Vials frozen using Versene® can be less dense than other standard methods. Freezing 1 plate at 2 cryovials/well will require 12ml of mFreSR™1 Medium



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

**7.4.7. Freeze Using Dispase Harvest**

- 7.4.7.1. Remove spent medium and add 1ml of Dispase Solution to each well of each 6-well plate.
- 7.4.7.2. Incubate pluripotent stem cells for 5 minutes at 37°C.
- 7.4.7.3. Confirm colony separation by viewing the plate under the microscope. Look for the perimeter of the colony to appear folded back.

**7.4.7.4. Harvest and Wash Cells**

- 7.4.7.4.1. Aspirate Dispase Solution from each well, taking care not to remove any floating colonies.
- 7.4.7.4.2. 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.
- 7.4.7.4.3. Add 1ml of mTeSR™1 medium to each well.
- 7.4.7.4.4. Take up 1ml of medium from the well in the 5ml pipette and scrape the surface of the plate. At the same time, slowly pipette the mTeSR™1 Medium up and down to wash the cells off the surface. Be careful to break clumps as little as possible. Cells will recover from the thaw more efficiently if frozen in large aggregates.
- 7.4.7.4.5. Pool the cells in a sterile 50ml centrifuge tube per plate.
- 7.4.7.4.6. Wash each plate with 3ml of mTeSR™1 Medium, transferring the medium from well to well and add the medium to the 50ml conical tube.
- 7.4.7.4.7. Centrifuge cells at 200xg for 5 minutes
- 7.4.7.4.8. Aspirate the supernatant being careful not to disturb the cell pellet. Gently re-suspend each cell pellet by adding 1ml mFreSR™1 Medium per well very slowly drop wise. Freezing 1 plate at 1 well/cryovial will require 6ml of mFreSR™1 Medium. If there is more than one conical tube, you may want to pool them at this point for a uniform lot.
- 7.4.7.4.9. Pipette pooled cells very gently to evenly mix suspension.
- 7.4.7.4.10. With the same pipette, distribute 1ml of cell suspension to each prepared vial. Mix cell pool every 6-10 vials for even distribution.



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

7.4.7.4.12. 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](http://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	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	08-April-2010
C	Added attachment 2: MSDS to be included with WiCell produced cells only	13-August-2010
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.	24-August-2011
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	09-July-2013
F	CC00389. Added use of Rock Inhibitor note to section 3, and amended E8 reference to mTeSR™1.	09-October-2013
G	CC00392, Corrected spelling and grammatical errors.	30-January-2014
H	CC00445, reordered sections to allow for better flow and added copyright.	See SPDC

#### 9.2. Approvals

5/8/2015	5/12/2015
 <hr/> JKG Senior Quality Assurance Specialist Signed by: Gay, Jenna	 <hr/> TEL Director - WiCell Stem Cell Bank Signed by: Ludwig, Tenneille

### Attachment 1: Photographs of Pluripotent Stem cells grown on Matrigel™ in mTeSR™ 1

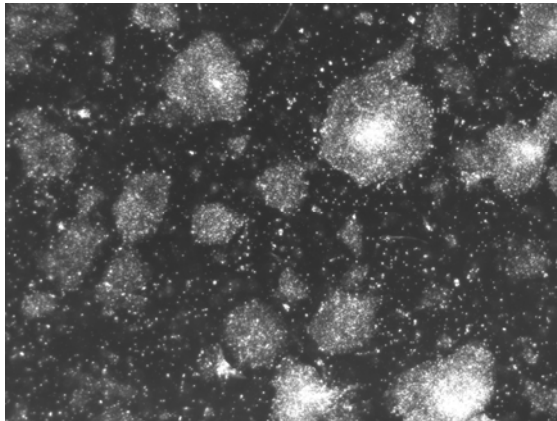


Fig 1: Pluripotent Stem cells mid split cycle (2.5x)

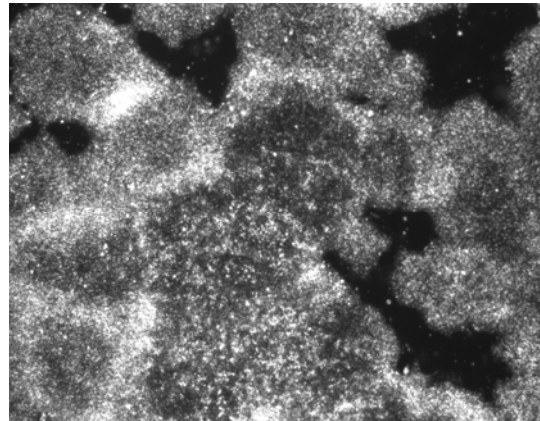


Fig 2: Pluripotent Stem cells, minimal differentiation (2.5x)

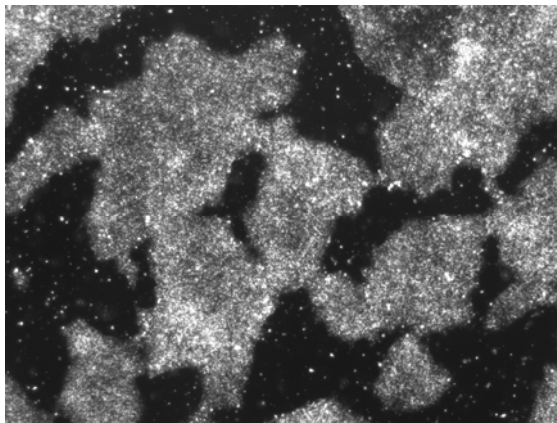


Fig 3: Pluripotent Stem cells pre-split (2.5x)

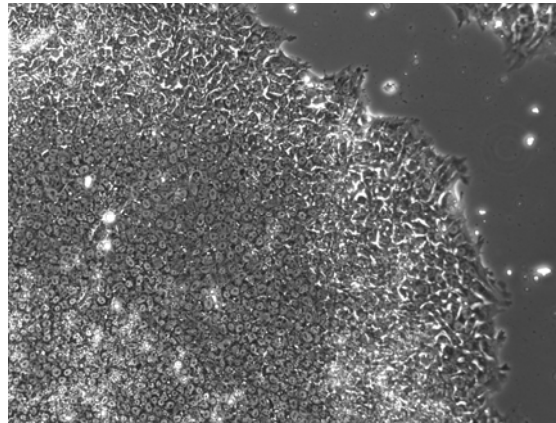


Fig 4: Pluripotent Stem cell colony pre-split (10x)

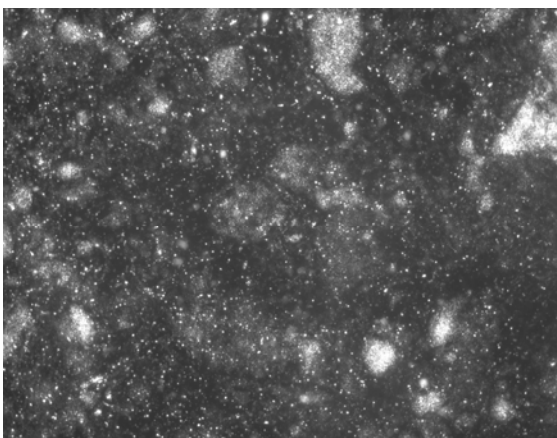


Fig 5: Pluripotent Stem differentiation (2.5x)

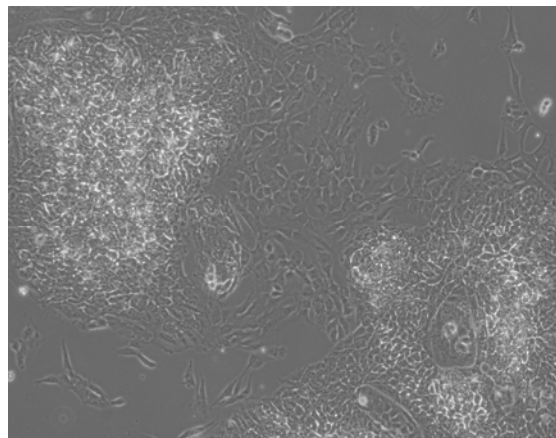


Fig 6: Pluripotent Stem differentiation (10x) between colonies