Title:	**WiCell Feeder Based (MEF) Protocol 01 for Culture of MJFF iSCORE Lines**
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1. Preface

This booklet of protocols is intended to serve as a primer for culturing pluripotent stem cells with iSCORE 01 medium on mouse embryonic fibroblast (MEF) feeder cells. These protocols are representative of how the cells were cultured and banked. 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. 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 Characterization Laboratory; to learn more about WiCell Characterization's offerings and how to submit samples visit our website.

If you have any additional questions, please contact us through technical support on the WiCell website at www.wicell.org.

2. Protocols

Section 1: Culturing Conditions Recommendations

Stem Cells cultured with iSCORE 01 medium are cultured in "low" oxygen conditions. A humidified incubator capable of maintaining 5% CO_2 and 5% O_2 was used in the culture and banking of cells cultured with iSCORE 01 medium on MEFs.

Section 2: MEF Plating Density Recommendations

For MEF based culture systems cultured with iSCORE 01 medium, WiCell recommends a plating density for freshly inactivated MEFs is 150,000 cells/mL at 2.5mL per well in a 6 well plate (375,000 cells/well, which is equal to 39,060 cells/cm^2). **Note: This is twice the MEF density of WiCell's standard Feeder Dependent culture protocols.** If inactivated MEFs have been frozen and thawed before use, WiCell recommends plating at a density of 180,000 cells/mL at 2.5mL per well in a 6 well plate (450,000 cells/well, which is equal to 46,880 cells/cm^2) to compensate for the loss of viability following the freeze thaw process. There can be significant variation in MEF quality from lot to lot, and therefore WiCell recommends qualifying all MEFs used to assure that they will support pluripotent stem cell culture before use with critical cell lines.

For MEFs to adhere properly, vessels must be coated with a sterile 0.1% gelatin (Sigma G1890-100G) in water solution. It is recommended that gelatin be allowed to coat plates for 24 hours in

a 37°C incubator prior to use. In cases of extreme urgency, a minimum of one hour of coating time in a 37°C incubator is required. Use the MEFs plates within 7 days of plating, with a total life span of 14 days. The cells do not require medium exchange, they will maintain in the same plating medium until they are used.

Section 3: Thawing Pluripotent Cells: Feeder Based (MEF) Protocol 01 for iSCORE Lines

Note 1: 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).

Required Equipment

1. Biosafety cabinet
2. 37°C / 5% CO_2 / 5% O_2 incubator
3. 37°C water bath
4. Centrifuge
5. Microscope
6. 10µl, 200µl and 1000µl micropipettes

Required Supplies

1. Forceps
2. Cryogenic handling gloves and eye protection
3. Two 6-well plates with freshly inactivated MEF cells prepared at least the day before use (only 1or 2 wells of MEFs are needed on each plate)
4. 5mL sterile serological pipettes (Fisher, 13-678-27E)
5. 95% Ethanol
6. 1.5mL Microcentrifuge tubes, sterilized (Fisher, 05-408-129)
7. Microcentrifuge tube holders
8. Freezer storage boxes
9. Sterile 15mL or 50mL conical tubes (Corning, 352096, 430291)
10. Sterile 10µl, 200µl and 1000µl micropipette tips (Fisher, 21-236-35, 2069, 2079E)
11. Sterile Pasteur Pipettes (Fischer, 13-678-20D)

Required Reagents

1. DMEM/F-12 Medium (Thermo Fisher, 11330-032)

2. Fetal Bovine Serum (FBS) (R&D systems, S12450), heat-inactivated
3. Knockout Serum Replacement (KOSR) (Thermo Fisher, 10828-028)
4. L-glutamine, non-animal, cell culture tested (Sigma, G-8540)
5. MEM Non-Essential Amino Acid Solution (100X) (Thermo Fisher, 11140-050)
6. Basic Fibroblast Growth Factor (β-FGF) (Thermo Fisher, PHG0021)
7. 2-Mercaptoethanol (Sigma, M3148)
8. Rock inhibitor (Y-27632 dihydrochloride; BD Biosciences, 562822)
9. Sterile water (Cytiva, SH3052901)
10. 0.1% BSA in PBS with $CaCl_2$ and $MgCl_2$

iSCORE 01 medium (500mL)

1. To make iSCORE 01 medium, combine following components, filter sterilize, store at 4ºC for up to 14 days, or for up to one year at -20ºC. If frozen, use within 14 days after thaw.

iSCORE 01 medium	
Component	**Amount**
DMEM/F12	390mL
FBS	75mL
Knockout Serum Replacement (KOSR)	25mL
L-Glutamine (100X) Solution	5mL
MEM Non-Essential Amino Acids (100X)	5mL
Basic FGF (25µg/mL) Solution	80µL
2-Mercaptoethanol (10,000X) Solution	50µL

a. **L-Glutamine (100X) Solution (for iSCORE 01 Medium)**

Combine the following components just prior to making iSCORE 01 medium, ensure solution is well mixed. Discard any extra. May be scaled up or down based on need.

L-Glutamine Solution (100X)	
Component	**Amount**
L-Glutamine, non-animal, cell culture tested	14.6 g
Sterile water	500mL

b. **2-Mercaptoethanol (10,000X) Solution (for iSCORE 01 medium)**

Combine the following components just prior to making iSCORE 01 medium, ensure solution is well mixed. Discard any extra. May be scaled up or down based on need.

2-Mercaptoethanol (10,000X)	
Component	**Amount**
2-Mercaptoethanol	0.78mL
Sterile water	9.22mL

c. **Basic FGF (25µg/mL) Solution (for iSCORE 01 Medium)**

Combine the following components, sterile filter, and aliquot. Store aliquots in -20°C for up to 6 months. Thaw aliquot at room temperature (or at 4°C) just prior to making iSCORE 01 medium. Do not re-freeze aliquots.

Basic FGF Solution (25µg/mL)	
Component	**Amount**
Basic Fibroblast Growth Factor (β-FGF)	100µg
0.1% BSA in PBS with $CaCl_2$ and $MgCl_2$	4mL

Reconstitute ROCK Inhibitor and Aliquot Working Stock Solution

ROCK Inhibitor Working Solution (1000X)	
Component	**Amount**
Rock Inhibitor	1mg
Sterile water	295µL

Note: Perform work sterilely.

1. Make 10mM working stock solution by diluting 1mg ROCK inhibitor (Formula Weight 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.
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 to achieve a final concentration of 10µM (for example: Add 50µl of 10mM working stock solution to 50mL iSCORE 01 Medium). Aliquots can be stored long term at -80°C for up to 1 year and up to 2 months at 4°C.

3. Filtering the medium after addition of ROCK inhibitor is not necessary if working with sterile stock solution. However, filtering may be performed to ensure sterility of final product if desired.

Remove Vial and Thaw

1. Prior to removing vial from storage, 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.
2. Wear eye protection as vials stored in liquid nitrogen may accidentally explode when warmed.
3. Wear ultra-low temperature cryogenic gloves. Remove the cell vial from the liquid nitrogen storage tank using forceps.
4. Quickly remove the label or copy the information written on the tube in your notebook. The writing or printed information may come off the vial in the ethanol bath. This should take no longer than 10 seconds.
5. Ensure the vial cap is tightly closed, grasp vial with forceps
6. Immerse the vial in a 37°C water bath without submerging the cap. Swirl the vial gently.
7. When only a small ice crystal remains, remove the vial from the water bath.
8. 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 or 50mL conical tube using a 1mL or 5mL glass pipette. Do not allow cells to flow down the side of the tube, as this will cause shearing and reduce attachment.
2. Slowly, add 10mL of warmed iSCORE 01 medium drop-wise to cells in the 15mL conical tube. While adding the medium, gently move the tube back and forth to mix the cells. This reduces osmotic shock to the cells.
3. Centrifuge the cells at 200 x g for 5 minutes.
4. Aspirate and discard the supernatant with a sterilized Pasteur pipette.
5. Suspend the cell pellet in 2mL iSCORE 01 medium supplemented with ROCK Inhibitor 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 1 well, re-suspend the pellet in 2mL.
6. Gently pipette cells up and down in the tube a few times.
7. Set aside while preparing the MEF plate to receive cells.

Prepare MEF Plate and Transfer Stem Cells

1. Label a 6-well plate containing inactivated MEF cells with the passage number from the vial, the date and your initials.
2. Aspirate the MEF medium from well(s) that will receive cells (thaw recommendations vary among lots, refer to the Certificate of Analysis to know how many wells one vial should be seeded into). Rinse the well(s) with 1mL sterile DMEM/F-12.
3. Aspirate the DMEM/F-12 rinse medium.
4. Slowly add the 2mL stem cell suspension into the well (if plating into more than 1 well, gently mix the cell suspension prior to adding to plate).
5. Place plate into the incubator and gently move it back and forth and side to side to evenly distribute the cells—avoid circular motions to prevent pooling in the center of the well.

The Day After Thaw

1. The next day, add 2mL fresh iSCORE 01 medium to each well containing cells without aspirating spent media.
2. Return plate to incubator and gently move it back and forth and side to side to redistribute the remaining aggregates in suspension.
    a. Be careful with plates as they now will be very full of media.

Maintain Cells

1. The second day after thaw, aspirate spent medium
2. Gently add 2mL of iSCORE 01 medium to thaw well(s) and return to incubator.
3. Feed and monitor all cells daily until ready to passage or freeze. Cells are typically ready to passage for the first time out of freeze between day 6 and 14.

Section 4: Feeding Pluripotent Stem Cells: Feeder Based (MEF) Protocol 01 for iSCORE Lines

Required Equipment

1. Biosafety cabinet
2. 37°C / 5% CO_2 / 5% O_2 incubator
3. Microscope
4. Water bath

Required Supplies

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

2. Sterile Pasteur Pipettes (Fischer, 13-678-20D)

Required Reagents

1. iSCORE 01 medium as in section 2

Feeding Pluripotent Stem Cells

1. Observe the stem cells using a microscope. If they require passaging, follow the passaging protocol below.
2. Use a 37°C water bath to warm enough medium to feed 2-3mL for each well that will be fed.
3. Aspirate the spent medium with a sterilized Pasteur pipette. If feeding more than one plate, use a different pipette for each plate to reduce risk of contamination.
4. Add 2-3mL of warmed iSCORE 01 medium to each well. After pipettes are used once, they must be disposed to reduce the contamination potential. Do not reinsert a used pipette into sterile medium for any reason.
5. Return the cells to the 37°C incubator.
6. Repeat procedure daily until ready to passage or freeze.

Section 5: Passaging Pluripotent Stem Cells: Feeder Based (MEF) Protocol 01 for iSCORE Lines

Required Equipment

1. Biosafety cabinet
2. 37°C / 5% CO_2 / 5% O_2 incubator
3. 37°C water bath
4. Hood (biosafety or static) equipped with stereomicroscope

Required Supplies

1. 5mL sterile serological pipettes (Fisher, 13-678-27E)
2. Sterile Pasteur Pipettes (Fisher, 13-678-20D)
3. Sterile 50mL conical tubes (Fisher, 430291)

Required Reagents

1. iSCORE 01 medium as in section 2
2. Collagenase type IV (Invitrogen, 17104-019)
3. DMEM/F-12 Medium (Invitrogen, 11330-032)
4. Fetal Bovine Serum (FBS) (R&D systems, S12450), heat-inactivated

5. Knockout Serum Replacement (KOSR) (Invitrogen, 10828-028)
6. L-Glutamine Solution (100X) as in section 2
7. MEM Non-Essential Amino Acid Solution (100X) (Invitrogen, 11140-050)
8. DPBS without $CaCl_2$ or $MgCl_2$ (Invitrogen, 14190-250)

KSR Medium

To make KSR Medium, combine following components, filter sterilize, store at 4ºC for up to 14 days, or for up to one year at -20ºC. If frozen, use within 14 days after thaw.

Component	Amount
DMEM/F12 Medium	390mL
Knockout Serum Replacement	100mL
L-Glutamine Solution (100X)	5mL
MEM Non-Essential Amino Acid Solution	5mL

KSR Collagenase Solution

To make KSR Collagenase Solution, combine following components, filter sterilize, store at 4ºC for up to 14 days, or for up to one year at -20ºC. If frozen, use within 14 days after thaw.

Component	Amount
Collagenase type IV	10mg
KSR Medium	10mL

Wash Medium

To make Wash Medium, combine following components, filter sterilize, store at 4ºC for up to 14 days, or for up to one year at -20ºC. If frozen, use within 14 days after thaw.

Component	Amount
DMEM/F12 Medium	475mL
FBS	25mL

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

1. In general, split cells when the first of the following occur:
    a. Mouse Embryonic Fibroblasts (MEF) feeder layer is two weeks old.

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

c. Increased differentiation occurs.

2. The split ratio is variable, though generally between 1:2 and 1:4. Occasionally cells will grow at a different rate and the split ratio will need to be adjusted. A general rule is to observe the last split ratio and adjust the ratio according to the appearance of the pluripotent stem cell colonies. If the cells look healthy and colonies have enough space, split using the same ratio, if they are overly dense and crowding, increase the ratio, and if the cells are sparse, decrease the ratio.
3. Cells will need to be split every 4-6 days based upon appearance.
4. Warm enough iSCORE 01 medium in a 37ºC water bath.

Passaging Cells with KSR Collagenase

Note: Determine total number of wells you are passaging into. The volumes in this section may need to be adjusted based on culture conditions. The final plating volume should be 2.0mL/well.

1. Place plate in the biosafety cabinet.
2. Aspirate the spent medium from the wells to be passaged with a Pasteur pipette. If possible, at least one well of cells should be fed independently 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. Wash each well to be passaged with 1mL DPBS.
4. Add 1mL room temperature KSR Collagenase Solution to each well to be passaged.
5. Incubate for 30-45 minutes at 37°C. Colonies should be starting to lift.
6. Add 2mL wash medium to each well.
7. Pipette repeatedly with a 5mL serological pipette to lift colonies, but do not scrape the plate.

    a. Be careful no to carry over too many MEFs.

8. Collect into a 50mL conical tube.
9. Use 5mL Wash Medium to rinse plate and add to conical tube.
10. Gravity precipitate the cell aggregates for 5-10 minutes.
11. Aspirate the Wash Medium, leaving 0.5mL.

    a. Do not disturb the aggregates that are loosely pelleted.

12. Add 5-10mL Wash Medium to the cell aggregates.
13. Gravity precipitate the cell aggregates for 5-10 minutes.

14. Aspirate the Wash Medium, leaving 0.5mL.
    a. Do not disturb the aggregates that are loosely pelleted.
15. Add 5mL iSCORE 01 medium to the aggregates.
16. Gravity precipitate the cell aggregates for 5-10 minutes.
17. Aspirate the iSCORE 01 medium, leaving 0.5-1mL.
    a. Do not disturb the aggregates that are loosely pelleted.
18. Using a p1000 pipette, triturate 4-10 times to break up the aggregates into smaller clusters.
    a. Do not completely dissociate into single cells.
    b. Avoid introducing air bubbles.

Prepare MEF Plate to Receive Cells

1. While cells are incubating, ready the prepared MEF plate for culture by aspirating the MEF medium from the wells, and rinsing with 1mL/well sterile DMEM/F-12.
2. Aspirate rinse medium and add 1mL of warmed iSCORE 01 medium to each well.
3. Label plate appropriately (WiCell recommends at minimum the cell line name, passage number on the vial, date and technician initials), and place in incubator until ready to plate passaged cells.

Plate Cells

1. Add enough iSCORE 01 medium to the triturated aggregate suspension so that each well will receive 1mL.
2. Gently re-suspend the cells using a 5mL pipette.
3. Add 1mL of cell suspension to each well of the prepared MEF plate.
4. 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 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.
5. Incubate cells overnight to allow colonies to attach.
6. If passage was successful and free of contamination, the backup well can be discarded the following day.
7. The next day, add 2mL fresh iSCORE 01 medium to each well containing cells <u>without</u> aspirating spent media.

8. Return plate to incubator and gently move it back and forth and side to side to redistribute the remaining aggregates in suspension.
    a. Be careful with plates as they now will be very full of media.
9. From the second day after passage, feed culture as previously described until ready to passage or freeze.

Section 6: Freezing Stem Cells: Feeder Based (MEF) Protocol 01 for iSCORE Lines

Required Equipment

1. Biosafety cabinet
2. 37°C / 5% CO_2 / 5% O_2 incubator
3. Microscope
4. 37°C Water bath
5. P1000 Micropipette

Required Supplies

1. 5mL and 10mL sterile serological pipettes (Fisher: 13-678-27E, 13-67827F)
2. 1000uL pipette tips (Fisher, 2079E)
3. Sterile Pasteur Pipettes (Fischer, 13-678-20D)
4. Isopropanol freezing containers (Fisher, 15-350-50)

Required Reagents

1. iSCORE 01 medium as in section 2
2. KSR Collagenase as in section 4
3. Wash Medium as in section 4
4. DPBS without $CaCl_2$ or $MgCl_2$ (Invitrogen, 14190-250)
5. Dimethyl Sulfoxide (DMSO) 10mL ampoules (Sigma Aldrich, D2438)
6. Fetal Bovine Serum (FBS) (R&D systems, S12450), **do not heat-inactivate**

Freezing Medium I

To make Freezing Medium I, combine the following components sterilely and keep on ice until ready to use. May be scaled up or down based on need.

Component	Amount
iSCORE 01 medium	5mL
FBS (non-heat inactivated)	5mL

Freezing Medium II

To make Freezing Medium II, combine the following components sterilely and keep on ice until ready to use. Do not filter DMSO, it will degrade the filter membrane. May be scaled up or down based on need.

Component	Amount
FBS (non-heat inactivated)	8mL
DMSO	2mL

Prepare for Freeze

1. Label cryovials with the cell line, passage number (increase the passage number on the vial label by 1 from what was on the culture plate at time of harvest), 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 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.
3. Obtain a recharged, room-temperature isopropanol freezing container. Each container can hold 18 vials. The isopropanol must be replaced every 5 uses.
4. View all cells under the microscope. Discard any contaminated plates.

Harvest Cells

1. Place plate in the biosafety cabinet.
2. Wash each well to be passaged with 1mL DPBS.
3. Add 1mL room temperature KSR Collagenase Solution to each well to be passaged.
4. Incubate for 30-45 minutes at 37°C. Colonies should be starting to lift.
5. Add 3mL iSCORE 01 medium to each well.
6. Pipette repeatedly with a 5mL or 10mL serological pipette to lift colonies, but do not scrape the plate.

a. Be careful no to carry over too many MEFs.

7. Collect into a 50mL conical tube.
8. Use 5mL Wash Medium to rinse plate, and add to conical tube.
9. Gravity precipitate the cell aggregates for 5-10 minutes.
10. Aspirate Wash Medium to 1mL.
11. Add 10mL Wash Medium.
12. Gravity precipitate cells for 10 minutes.
13. Aspirate Wash Medium to 1mL or less.
14. Using a P1000 pipet, triturate the aggregates 5-10 times against the bottom of the tube to break up the cell clusters.
    a. Do not completely dissociate into single cells.
    b. Avoid introducing air bubbles.
15. Resuspend cells in 10mL iSCORE 01 medium.
16. Centrifuge cells at 200 x g for 5 minutes at room temperature.

Resuspend Pellet and Vial Cells

1. Aspirate the supernatant being careful not to disturb the cell pellet.
2. Carefully resuspend the cell pellet in 0.5mL Freezing Medium I per vials to be frozen.
3. Add 0.5mL Freezing Medium II per vial to be frozen dropwise to cell suspension while gently tapping the tube to mix the suspension.
    a. **Note:** At this point, cells are in contact with DMSO, and work must be performed efficiently. Once cells are in contact with DMSO, they should be aliquoted and frozen within 2-3 minutes.
4. Pipette the cells very gently to evenly mix suspension.
5. With the same pipette, distribute 1mL of cell suspension to each of the prepared cryovials. Mix the cell pool every 6-10 vials for even distribution.
6. Quickly, tighten caps and place cryovials into an isopropanol containing freezing container. Place the freezing container in the -80ºC freezer overnight.
7. The next day, transfer cell vials to liquid nitrogen storage.

3. Version History

Version	Version History	Effective Date

1.0	CC-3357, Document Initiation	See Qualio or Controlled Copy

4. Attachments

1. No attachments.