TECHNICAL MANUAL
VERSION 3.0.0

Maintenance of Human Pluripotent Stem Cells in mTeSR™1 and TeSR™2

STEMCELL™ TECHNOLOGIES

Table of Contents

1.0 Introduction

Undifferentiated human pluripotent stem cells have the potential for unlimited expansion with the retention of normal karyotype and the ability to generate cells of all three germ layers - endoderm, mesoderm and ectoderm[1,5,19 - 20]. These germ layer cells can then further differentiate into many specific cell lineages[11]. Because of this ability, their use has been proposed in a variety of clinical applications and as a tool for the study of human cellular and developmental systems. Human cells with pluripotent characteristics were initially derived from the inner cell mass of pre-implantation blastocysts and termed human embryonic stem (ES) cells [1]. The discovery that human fibroblasts[15,19 - 20] can be reprogrammed by the transient overexpression of a small number of genes into human induced pluripotent stem (iPS) cells, which functionally and phenotypically resemble human ES cells, raises the possibility that cellular therapies using patient-specific input cells may be a reality in the future. Collectively, human ES cells and human iPS cells are known as human pluripotent stem cells.

Basic techniques to culture human ES and iPS cells are well established[1,12,20], although limitations remain in many of these procedures. In particular, many existing lines have been cultured using mouse embryonic fibroblast (MEF) feeder cells and serum or other animal-sourced medium components. MEFs support self-renewal either by providing necessary factors or by removing inhibitory factors. MEF-conditioned medium, produced by harvesting spent culture medium from MEF cell cultures, also supports self-renewal. Despite this, the continued use of feeders and animal-derived components will hinder the development of clinical applications due to: a) the presence of immunogenic material; b) the risk of transmitting animal virus or prion material; and c) difficulty with quality control of these undefined components.

Therefore, an important advancement in the field is the definition of culture conditions that support the proliferation and culture of human ES and iPS cells without the need for feeders or animal-derived components. An optimal culture system would include an appropriately buffered medium that contains all metabolites, cytokines and growth factors required for self-renewal and survival of pluripotent cells as well as a culture matrix that supports cell growth.

1.1 Development of Serum-Free and Feeder-Free Culture Systems for Human ES and iPS Cells

Several groups have developed culture conditions for human ES cells that are, to various degrees, serum- and feeder-free. Xu et al reported a culture system that utilized BD Matrigel™ as a culture matrix and MEF conditioned medium (consisting of animal component-containing serum replacement and basic fibroblast growth factor, bFGF) that allowed human ES cells to be cultured without direct contact with feeders[2]. True feeder-free culture has been reported using extracellular matrix and a combination of transforming growth factor β (TGFβ) and bFGF or high levels of bFGF alone[3,4], although these studies relied on an animal component-containing serum replacement. Richards et al reported the first xeno-free culture system for human ES cells, which replaced traditionally used MEFs with human feeder cells of fetal or adult fallopian tube-epithelial cell origin. However, the culture medium used was supplemented with 20% human serum[5] and, thus, has issues with batch-to-batch variability due to its undefined nature.

A number of subsequent publications have described defined xeno- or feeder-free media formulations for the maintenance of human ES cells[6-10]. “TeSR” is a serum-free, xeno-free medium that was shown to support derivation and long-term feeder-free culture of human ES cells, and was developed by Tenneille Ludwig and colleagues at the WiCell Research Institute (Madison, WI)[9]. The formulation of “TeSR” included high levels of bFGF, together with TGFβ, gamma-aminobutyric acid (GABA), pipecolic acid, and lithium chloride. The original publication described the use of cell support matrix composed of four human components (collagen IV, fibronectin, laminin, and vitronectin). In an effort to reduce the cost of this defined system for everyday research, Ludwig and colleagues continued their development and formulated “mTeSR”, which does include some animal-sourced proteins yet retains the advantages of being fully-defined and serum-free and supports the self-renewal of human ES cells without requiring feeder cells[10].

1.2 mTeSR™1 and TeSR™2: STEMCELL Technologies' Serum-Free and Feeder-Free Media for Human ES and iPS Cells

STEMCELL Technologies Inc. has developed mTeSR™1 (Catalog #05850/05857/05870/05875) and TeSR™2 (Catalog #05860/05880) as standardized media for feeder-free maintenance of human ES and iPS cells in culture. They are both complete, serum-free, defined formulations based on the publications by Ludwig et al [9,10] made under license from the WiCell Research Institute. mTeSR™1 contains a bovine albumin source that supports the long-term, feeder-free culture of human ES and iPS cells as well as the derivation of human iPS cells in feeder-free conditions[15,16]. TeSR™2 is an animal protein-free formulation that similarly supports the long-term, feeder-free culture of human ES and iPS cells. Neither media require any further addition of growth factors. They have been designed to maintain and expand undifferentiated human ES and iPS cells and can be used with BD Matrigel™ hESC-qualified Matrix (BD, Catalog #354277) as a substrate. STEMCELL Technologies has pre-qualified each batch of BD Matrigel™ to ensure consistency, reproducibility, and reliability in performance. To obtain a complete animal protein-free culture system, TeSR™2 can be used with alternate matrices as described in Section 5.4.

Human ES and iPS cells maintained in mTeSR™1 and TeSR™2:

- are phenotypically homogeneous and karyotypically normal
- express high levels of multiple antigens associated with pluripotency: Oct-3/4, SSEA-3, SSEA-4, TRA-1-60, TRA-1-81
- express genes required for pluripotency: Oct-3/4, nanog
- form teratomas containing derivatives of endo-, meso-, and ectodermal lineages
- can be differentiated into functional mature cell types in vitro (e.g. hematopoietic cells)
- require no adaptation period (i.e. no period of low cell yield) when transferred from feeder-based culture

Advantages of using mTeSR™1 and TeSR™2 include:

- consistent conditions for human ES and iPS cells culture due to the elimination of undefined medium components and removal of the inherent variability associated with feeder cells and conditioned media
- standardization in culture methods leading to increased reproducibility of data
- time savings due to elimination of the labor involved in preparing feeder cells or conditioned media
- complete medium formulation with no additional growth factors or other supplements necessary

2.0 Materials and Reagents

2.1 mTeSR™1 and TeSR™2 for Maintenance of Human ES and iPS Cells

The mTeSR™1 Medium Kit (Catalog #05850) includes:

COMPONENT	VOLUME	STORAGE CONDITIONS
mTeSR™1 Basal Medium (#05851)	400 mL	2 - 8°C
mTeSR™1 5X Supplement (t #05852)	100 mL	-20°C

The TeSR™2 Medium Kit (Catalog #05860) includes:

COMPONENT	VOLUME	STORAGE CONDITIONS
TeSR™2 Basal Medium (#05861)	400 mL	2 - 8°C
TeSR™2 5X Supplement (#05862)	100 mL	-20°C
TeSR™2 250X Supplement (#05863)	2 mL	-20°C

2.2 Additional Reagents Required for Human ES and iPS Cell Culture

PRODUCT	CATALOG #
Gentle Cell Dissociation Reagent	07174
mFreSR™	05854/05855
Cryostor®CS10	07930
Dispase (1 mg/mL)	07923
Dispase (5 mg/mL)	07913
D-PBS (without Mg^{++} and Ca^{++})	37350
BD Matrigel™ hESC-qualified Matrix	BD, Catalog #354277
Trypan Blue	07050
70% Ethanol or Isopropanol	-
Conical tubes (15 mL)	e.g. BD, Catalog #352196
Conical tubes (50 mL)	e.g. BD, Catalog #352070
4-well tissue culture-treated plates	e.g. BD, Catalog #353654
6-well tissue culture-treated plates	e.g. BD, Catalog #353046
10 cm tissue culture-treated plates	e.g. BD, Catalog #353003
Cell scrapers	e.g. Corning, Catalog #3010
Serological pipettes (2 mL, 5 mL, 10 mL)	e.g. BD, Catalog #357507, 357543, 357551

2.3 Equipment Required for Human ES and iPS Cell Culture

- Vertical laminar flow hood certified for Level II handling of biological materials
- Incubator with humidity and gas control to maintain 37°C and >95% humidity in an atmosphere of 5% CO_2 in air
- Low speed centrifuge (e.g. Beckman GS-6)

  Note: All protocols described in this technical manual can be performed with the brake on.
- Tabletop centrifuge (e.g. Eppendorf 5417R)
- Pipette-aid (e.g. Drummond Scientific)
- Hemacytometer (e.g. Nebauer, Reichert)
- Inverted microscope with 2X, 4X, and 10X phase objectives (e.g. Olympus CKX31)
- Isopropanol freezing container (e.g. Nalgene, Fisher; Catalog #1535050)
- -150°C freezer or liquid nitrogen vapor tank

2.4 Preparation of Reagents and Materials

Use sterile techniques when preparing reagents and materials.

2.4.1 Preparation of mTeSR™1

1. Thaw mTeSR™1 5X Supplement at room temperature (15 - 25°C) or overnight at 2 - 8°C.

   If desired, 5X Supplement can be dispensed into working aliquots and stored at -20°C. Use frozen aliquots within 3 months. Thawed aliquots should be used within 1 day to prepare complete mTeSR™1 medium. Do not refreeze aliquots after thawing.
2. Add the entire 100 mL of thawed mTeSR™1 5X Supplement to the entire 400 mL mTeSR™1 Basal Medium for a total volume of 500 mL. Mix well. Complete mTeSR™1 is stable when stored at 2 - 8°C for up to 2 weeks or is stable when frozen at -20°C for up to 6 months. Thaw frozen complete medium at room temperature (15 - 25°C) or overnight at 2 - 8°C before use.

   Complete mTeSR™1 is ready for use and does not require filtering. However, the medium can be filtered using a 0.2 µm, low-protein binding filter, if desired.

2.4.2 Preparation of TeSR™2

1. Thaw TeSR™2 5X Supplement and TeSR™2 250X Supplement at room temperature (15 - 25°C) or overnight at 2 - 8°C before use.

   If desired, the 5X Supplement and 250X Supplement can be dispensed into working aliquots and stored at -20°C. Use frozen aliquots within 6 months. Thawed aliquots should be used within 1 day to prepare complete TeSR™2 medium. Do not refreeze aliquots after thawing.
2. Add the entire 100 mL of thawed 5X Supplement and 2 mL of thawed 250X Supplement to the entire 400 mL TeSR™2 Basal Medium for a total volume of 502 mL. Mix well. Complete TeSR™2 is stable when stored at 2 - 8°C for up to 2 weeks or is stable when frozen at -20°C for up to 6 months. Thaw frozen complete medium at room temperature (15 - 25°C) or overnight at 2 - 8°C.

   Complete TeSR™2 is ready for use and does not require filtering. However, the medium can be filtered using a 0.2 µm, low-protein binding filter, if desired.

2.4.3 Preparation of Dispase

Dispase can be used for passaging human ES and iPS cells grown in mTeSR™1 or TeSR™2. STEMCELL Technologies sells dispase at two concentrations: 1 mg/mL (Catalog #07923) and 5 mg/mL (Catalog #07913). Dispase at a concentration of 1 mg/mL is ready to use but it is highly recommended that dispase be aliquoted into smaller working volumes and stored at -20°C. Dispase can be stored at 2 - 8°C for up to 2 weeks.

If starting with the 5 mg/mL concentration, dispase must be prepared as follows:

1. Aliquot a 100 mL bottle of stock dispase solution (5 mg/mL) by thawing and dispensing into smaller volumes (5 or 10 mL).
2. Store aliquots at -20°C and thaw as required.
3. Aliquots should only be thawed once; repeated freeze/thaw is not recommended.
4. Prepare a working solution of dispase at 1 mg/mL by diluting 1 in 5 with DMEM/F-12 (e.g. 10 mL of dispase plus 40 mL of DMEM/F-12). Diluted dispase can be stored at 2 - 8°C for up to 2 weeks.

   It is highly recommended that the diluted dispase solution is aliquoted into smaller working volumes to avoid repeated warming.

2.4.4 Coating Plates with BD Matrigel™ hESC-qualified Matrix

BD Matrigel™ hESC-qualified Matrix should be aliquoted and frozen. Consult the Certificate of Analysis supplied with the BD Matrigel™ for the recommended aliquot size (Dilution Factor) to make up 25 mL of diluted matrix. Make sure to always keep BD Matrigel™ on ice when thawing and handling to prevent it from gelling.

1. Thaw one aliquot of BD Matrigel™ on ice.
2. Dispense 25 mL of dilution medium (DMEM/F-12; Catalog #36254) into a 50 mL conical tube and keep on ice.
3. Add thawed BD Matrigel™ to the cold dilution medium (in the 50 mL tube) and mix well. The vial may be washed with cold medium if desired.
4. Immediately use the diluted BD Matrigel™ solution to coat tissue culture-treated cultureware. See Table 1 for recommended coating volumes.

Table 1: Volumes Recommended for Coating Cultureware with BD Matrigel™

CULTUREWARE	VOLUME OF DILUTED BD MATRIGEL™
6-well plate	1 mL/well
100 mm dish	8 mL/dish
T-25cm^2 flask	2 mL/flask
T-75cm^2 flask	5 mL/flask

5. Swirl the cultureware to spread the BD Matrigel™ solution evenly across the surface.

   Note: Tissue culture-treated cultureware should be used for coating with BD Matrigel™. If the cultureware's surface is not fully coated by the BD Matrigel™ solution, it should not be used for human ES and iPS cell culture.
6. Incubate at room temperature (15 - 25°C) for at least 1 hour before use. Do not let the BD Matrigel™ solution evaporate.

   Note: If not used immediately, the cultureware must be sealed to prevent evaporation of the BD Matrigel™ solution (e.g. with Parafilm®) and can be stored at 2 - 8°C for up to 7 days after coating. Allow stored coated cultureware to come to room temperature (15 - 25°C) for 30 minutes before moving onto the next step.

7. Gently tilt the cultureware onto one side and allow the excess BD Matrigel™ solution to collect in that corner.
8. Remove the excess BD Matrigel™ solution using a serological pipette or by aspiration. Ensure that the coated surface is not scratched.
9. Immediately add an appropriate volume of medium and use to plate pluripotent cells (e.g. total 2 mL/well if using a 6-well plate).

3.0 Culture of Human ES and iPS Cells Using mTeSR™1 or TeSR™2 on BD Matrigel™

Culture of human ES and iPS cells in mTeSR™1 or TeSR™2 may require different techniques than culture in other media formulations. The procedures described in this technical manual are general and may require optimization for use with specific cell lines.

3.1 Thawing Cryopreserved Human ES and iPS Cells

Human ES and iPS cells should be thawed into either 4- or 6-well plates coated with BD Matrigel™. For instructions on how to coat tissue culture-treated plates with BD Matrigel™ see Section 2.4.4. If unsure of the number of cell aggregates frozen down, a 4-well plate is recommended. Generally, human ES and iPS cells from 1 well of a 6-well plate cryopreserved in STEMCELL Technologies' cryopreservation medium, mFreSR™ (Catalog #05854/05855) or Cryostor®CS10 (Catalog #07930) can be successfully thawed into 1 well of a 6-well plate. If the cells have been cryopreserved using other methods, this may vary.

Human ES and iPS cells cultured using other maintenance protocols (e.g. with mouse embryonic feeders or their conditioned medium) should be thawed under the same media and conditions used prior to cryopreservation. Once they have recovered from the thaw, cells can be transitioned into mTeSR™1 or TeSR™2 culture.

The procedure described below uses mTeSR™1 medium; TeSR™2 can be substituted without further changes.

1. Have all tubes, warmed mTeSR™1 medium (15 - 25°C) and pre-coated plates (see Section 2.4.4) ready before starting the protocol to ensure that the thawing procedure is done as quickly as possible.
2. Quickly thaw cells in a 37°C water bath by gently shaking the cryovial continuously until only a small frozen pellet remains.
3. Remove the cryovial from the water bath and wipe with 70% ethanol to sterilize.
4. Use a 2 mL pipette to transfer the contents of the cryovial to a 15 mL conical tube.

   Using a 2 mL pipette will minimize breakage of cell aggregates.
5. Add 5 - 7 mL of warm mTeSR™1 dropwise to the tube, gently mixing as the medium is added.
6. Centrifuge cells at 300 *x g* for 5 minutes at room temperature (15 - 25°C).
7. Aspirate the medium, leaving the cell pellet intact and using a 2 mL pipette, gently resuspend the cell pellet in 0.5 - 2 mL of mTeSR™1. Take care to maintain the cells as aggregates.
8. Transfer the appropriate amount of medium containing the cell aggregates to a pre-coated 4-well or 6-well plate.

   Transfer 0.5 mL/well if using a 4-well plate. Transfer 2 mL/well if using a 6-well plate. If using more than one well, ensure that cell aggregates are evenly distributed between wells.
9. Place the plate into the 37°C incubator and move the plate in quick side to side, forward to back motions to evenly distribute the cell aggregates within the wells. Culture the cells at 37°C, with 5% CO_2 and 95% humidity.
10. Perform daily medium changes. Check for undifferentiated colonies that are ready to be passaged (dense centered) approximately 5 - 7 days after thawing.

    Note: If only a few undifferentiated colonies are observed after thawing, it may be necessary to select only these colonies for passaging and replate them in the same size well on a newly-coated plate.

3.2 Passaging Human ES and iPS Cells Grown in mTeSR™1 or TeSR™2

Human ES and iPS cells grown in mTeSR™1 or TeSR™2 are ready to passage when the colonies are large, beginning to merge, and have centers that are dense and phase-bright compared to their edges (see Figure 1). Depending on the size and density of seeded aggregates, cultures are usually passaged 5 - 7 days after seeding in mTeSR™1 and passaged 4 - 6 days after seeding in TeSR™2. **Thus, cells grown in TeSR™2 may need to be passaged approximately 1 day earlier than the same cells grown in mTeSR™1**.

It is to be expected that colony morphology will look different when compared to cells grown using other culture conditions. For up to 4 days after seeding in mTeSR™1, colonies may appear transparent and not very densely packed with cells. The density and robustness of the colonies increases rapidly after this time point and the morphology will change significantly in the last few days before passaging. Colonies that form in TeSR™2 medium will be more densely packed with cells earlier than colonies grown in mTeSR™1. For representative pictures of colonies passaged in mTeSR™1, see Appendix 1. For representative pictures of colonies passaged in TeSR™2, see Appendix 2.

In both media formulations, if colonies are passaged too early or too frequently, the cells may not attach well, yields will be decreased and cells may start to differentiate. If colonies are passaged too late, the culture will begin to show signs of differentiation (characterized by the emergence of cell types with different morphologies). There is an approximate 24 hour window that is optimal for passaging. If there are large colonies, with dense centers, and the colonies are beginning to merge, passage the cells within 24 hours (for further help, see Section 5.1).

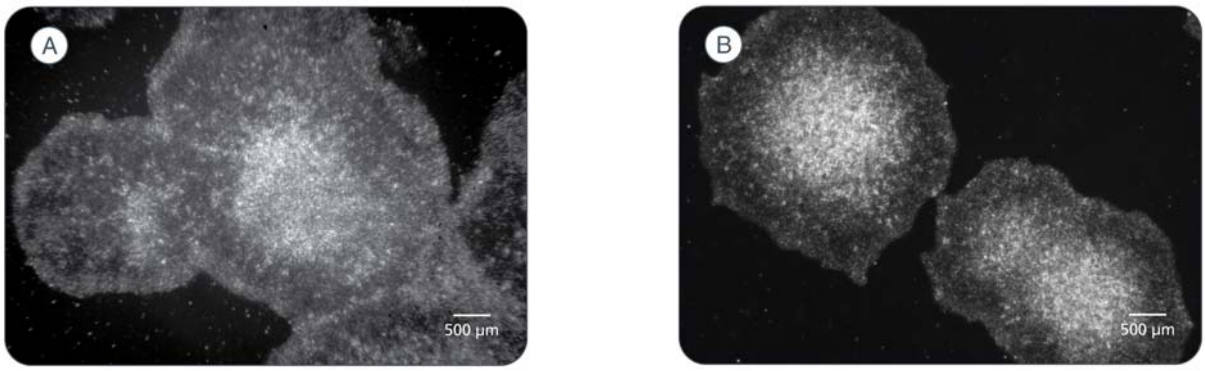


Photo courtesy of Dr. T. Ludwig, WiCell Research Institute

Figure 1: Morphology of (A) human ES H9 colonies cultured in mTeSR™1 and (B) human iPS (IMR90)-1 colonies cultured in TeSR™2 that are ready to be passaged. Note the dense, bright center when viewed with phase contrast or dark field under low magnification.

3.2.1 Enzymatic Passaging Using Dispase

Cells cultured in TeSR™2 are more sensitive to enzymatic dissociation (see step 7 below). If using TeSR™2, the dispase incubation period should be decreased to 3 - 4 minutes. More care should also be taken when clumps are in suspension as they are more fragile than those cultured in mTeSR™1.

The procedure described below uses mTeSR™1 medium. If TeSR™2 is used instead, adjust the incubation time as indicated in step 7. Volumes are listed for 6-well plates; if using alternate sizes of cultureware, adjust volumes according to surface area.

1. At least 1 hour before passaging, coat new dishes with BD Matrigel™ (see Section 2.4.4).
2. Aliquot sufficient mTeSR™1, Dispase (1 mg/mL) (Catalog #07923), and DMEM/F-12 (Catalog #36254) to passage cells and warm to room temperature (15 - 25°C).
3. Use a microscope to visually identify regions of differentiation. Mark these using a felt tip or lens marker on the bottom of the plate.

   Note: This selection should not exceed 20% of the well if the culture is of high quality.
4. Remove regions of differentiation by scraping with a pipette tip or by aspiration.
5. Aspirate medium from the well and rinse with DMEM/F-12 (2 mL/well).
6. Add 1 mL/well of dispase at a concentration of 1 mg/mL.
7. Incubate at 37°C for 7 minutes if using mTeSR™1 or for 3 - 4 minutes if using TeSR™2.

   Note: This incubation time is based on STEMCELL Technologies' dispase. If using dispase from another supplier, incubation times may need to be adjusted due to differences in activity of the enzyme. After incubation the colony edges will appear slightly folded back but the colonies should remain attached to the plate.
8. Aspirate dispase, and gently rinse each well 2 - 3 times with 2 mL/well of DMEM/F-12 to dilute away any remaining dispase.
9. Add 2 mL/well of DMEM/F-12 or mTeSR™1. Gently detach colonies by scraping with a glass pipette or a cell scraper (e.g. Corning, Catalog #3010).

   Note: Take care to minimize the breakup of colonies.
10. Transfer the detached cell aggregates to a 15 mL conical tube.

    Optional: Rinse the well with an additional 2 mL of DMEM/F-12 or mTeSR™1 to collect any remaining aggregates. Add the rinse to the 15 mL tube.

    Note: If cells are scraped in mTeSR™1, Steps 11 and 12 are not necessary. Adjust volume of medium for an appropriate split and proceed to Step 13.
11. Centrifuge the 15 mL tube containing the cell aggregates at 300 x *g* for 5 minutes at room temperature (15 - 25°C).
12. Aspirate the supernatant. For each well of cell aggregates collected in the 15 mL tube, add 1 - 2 mL of mTeSR™1.
13. Carefully pipette the cell aggregate mixture up and down 2 - 3 times with a 2 mL serological pipette to break up the aggregates. A uniform suspension of aggregates approximately 100 µm in size is optimal; do not create a single-cell suspension (for more information, see Section 5.1).
14. Plate the cell aggregates with mTeSR™1 onto a new pre-coated plate.

    If the colonies are at an optimal density, the cells can be split every 4 - 7 days using 1 in 6 - 10 splits (i.e. aggregates from 1 well can be plated in 6 - 10 new wells). If the colonies are too dense or too sparse, adjust the split ratio accordingly. For an alternative method of determining the amount of cell aggregates to plate, see Section 5.3. Please note that these guidelines are based on the growth characteristics of the H1 and H9 human ES cell lines, and may vary between different lines and laboratories.

15. Place the plate in a 37°C incubator. Move the plate in several quick, short, back-and-forth and side-to-side motions to disperse cells across the surface of the well.

    Note: Ensure that newly seeded cell aggregates are evenly dispersed across the entire surface. Uneven distribution may result in differentiation of human ES/iPS cells.

3.2.2 Enzyme-Free Passaging Protocol Using Gentle Cell Dissociation Reagent

The procedure described below uses mTeSR™1 medium; TeSR™2 can be substituted without further changes. Volumes are listed for 6-well plates; if using alternate sizes of cultureware, adjust volumes according to surface area.

1. At least 1 hour before passaging, coat new passaging dishes with BD Matrigel™ (see Section 2.4.4).
2. Aliquot sufficient mTeSR™1 and warm to room temperature (15 - 25°C).
3. Use a microscope to visually identify regions of differentiation. Mark these using a felt tip or lens marker on the bottom of the plate.
4. Remove regions of differentiation by scraping marked areas of the plate with a pipette tip or aspirator.

   Note: This selection should not exceed 20% of the well if the culture is of high quality.
5. Aspirate medium from the well and add 1 mL/well of Gentle Cell Dissociation Reagent.
6. Incubate at room temperature (15 - 25°C) for 6 - 8 minutes.

   Note: This incubation time is based on STEMCELL Technologies' Gentle Cell Dissociation Reagent. If using a non-enzymatic cell dissociation reagent from another supplier, incubation times may need to be adjusted.
7. Aspirate the Gentle Cell Dissociation Reagent and add 1 mL/well of mTeSR™1 medium.
8. Gently detach colonies by scraping with a glass pipette or a cell scraper.

   Note: Take care to minimize the breakup of colonies.
9. Transfer the detached cell aggregates to a 15 mL conical tube.

   Optional: Rinse the well with an additional 1 mL of mTeSR™1 to collect remaining cell aggregates.

   Note: Centrifugation of aggregates is not required.
10. Carefully pipette the cell aggregate mixture up and down 2 - 3 times with a 2 mL serological pipette to break up the aggregates. A uniform suspension of aggregates approximately 100 µm in size is optimal; do not create a single cell suspension.
11. Plate the cell aggregate mixture onto a fresh matrix-coated dish. Add mTeSR™ as required to bring the volume up to a total of 2 mL per well. If the colonies are at an optimal density, the cultures can be split every 5 - 7 days using 1 in 10 - 40 splits (i.e. aggregates from 1 well can be plated in 10 - 40 new wells).

    Note: Work quickly to transfer cell aggregates into new cultureware to maximize viability and attachment.
12. Place the plate in a 37°C incubator. Move the 6-well plate in several quick, short, back-and-forth and side-to-side motions to disperse cell aggregates across the surface of the well.

    Note: Ensure that newly seeded cell aggregates are evenly dispersed across the entire surface. Uneven distribution may result in differentiation of human ES/iPS cells.

3.3 Cryopreservation of Human ES and iPS Cells

Before cryopreservation, human ES and iPS cell cultures should be of high quality (primarily undifferentiated with less than 20% of the cells being differentiated). Cryopreservation should be done when cells are ready to passage or up to 1 day earlier. The following protocols are based on cultures in 6-well plates where wells are 60 - 75% confluent at the time of cryopreservation; if wells are more or less confluent, or alternate cultureware is used, the number of vials cryopreserved should be adjusted accordingly. Human ES and iPS cells will have improved survival following thawing if cryopreserved as large clumps.

3.3.1 Cryopreserving cells Using mFreSR™

mFreSR™ (Catalog #05854/05855) is a defined, serum-free cryopreservation medium designed specifically for human ES and iPS cells. It is ready to use and contains cryoprotectant agents*. Note: Wipe down outside of bottle with 70% ethanol before opening.*

1. Thaw the required amount of mFreSR™ and keep on ice.
2. Use a microscope to visually identify regions of differentiation. Mark these using a felt tip or lens marker on the bottom of the plate.

   Note: This selection should not exceed 20% of the well if the culture is of high quality.
3. Remove regions of differentiation by scraping with a pipette tip or by aspiration.
4. Aspirate medium from wells and rinse with DMEM/F-12 (2 mL/well).
5. Add 1 mL/well of dispase at a concentration of 1 mg/mL.
6. Incubate at 37°C for 7 minutes if using mTeSR™1 or for 3 - 4 minutes if using TeSR™2.

   Note: This incubation time is based on STEMCELL Technologies' dispase. If using dispase from another supplier, incubation times may need to be adjusted due to differences in activity of the enzyme. After incubation the colony edges will appear slightly folded back but the colonies should remain attached to the plate.
7. Aspirate dispase, and gently rinse each well 2 - 3 times with 2 mL/well of DMEM/F12 to dilute away any remaining dispase.
8. Add 2 mL/well of DMEM/F12 or mTeSR™1 or TeSR™2 and scrape colonies off using a cell scraper or a 5 mL serological pipette.

   Note: Take care to keep the cell aggregates as big as possible.
9. Transfer the detached cell aggregates into a 15 mL conical tube and rinse the wells with additional 2 mL/well medium to collect any remaining aggregates. Add the rinse to the 15 mL tube containing the cell aggregates.
10. Centrifuge the 15 mL tube containing the aggregates at 300 x *g* for 5 minutes at room temperature (15 - 25°C).

    Note: Prepare and label cryovials while cells are centrifuging.
11. Gently aspirate the supernatant taking care to keep the cell pellet intact.
12. Gently resuspend the pellet in cold (2 - 8°C) mFreSR™, using a 2 mL pipette. Take care to leave the cell aggregates larger than would normally be done for passaging.

    Note: 1 mL of mFreSR™ should be used for every well of a 6-well plate being frozen. However, if the wells are at low density (less than 50% confluent), 1 mL of mFreSR™ may be used for every 2 wells.
13. Gently flick the tube to mix the suspension and mFreSR™. Then transfer 1 mL of cell aggregates in mFreSR™ into each labeled cryovial using a 2 mL pipette. To ensure even distribution of cell aggregates between the vials, draw up 1 mL at a time and mix gently before taking each aliquot.
14. Place vials into an isopropanol freezing container and place the container at -80°C to -150°C overnight.
15. Transfer to a liquid nitrogen vapor tank or liquid nitrogen the next day.

3.3.2 Cryopreserving Human ES and iPS Cells Using Cryostor®CS10

CryoStor®CS10 (Catalog #07930) is an animal-component free cryopreservation medium. It is ready to use and contains cryoprotectant agents. *Note: Wipe down outside of bottle with 70% ethanol before opening.*

1. Use a microscope to visually identify regions of differentiation. Mark these using a felt tip or lens marker on the bottom of the plate.

   Note: This selection should not exceed 20% of the well if the culture is of high quality.

2. Remove regions of differentiation by scraping with a pipette tip or by aspiration.
3. Aspirate medium from wells and rinse with DMEM/F-12 (2 mL/well).
4. Add 1 mL/well of dispase at a concentration of 1 mg/mL.
5. Incubate at 37°C for 7 minutes if using mTeSR™1 or for 3 - 4 minutes if using TeSR™2.

   Note: This incubation time is based on STEMCELL Technologies' dispase. If using dispase from another supplier, incubation times may need to be adjusted due to differences in activity of the enzyme. After incubation the colony edges will appear slightly folded back but the colonies should remain attached to the plate.

6. Aspirate dispase, and gently rinse each well 2 - 3 times with 2 mL/well of DMEM/F12 to dilute away any remaining dispase.
7. Add 2 mL/well of DMEM/F12 or mTeSR™1 or TeSR™2 and scrape colonies off using a cell scraper or a 5 mL serological pipette.

   Note: Take care to keep the cell aggregates as big as possible.

8. Transfer the detached cell aggregates into a 15 mL conical tube and rinse the wells with additional 2 mL/well of medium to collect any remaining aggregates. Add the rinse to the 15 mL tube containing the cell aggregates.
9. Centrifuge the 15 mL tube containing the aggregates at 300 x *g* for 5 minutes at room temperature (15 - 25°C).

   Note: Prepare and label cryovials while cells are centrifuging.

10. Gently aspirate the supernatant taking care to keep the cell pellet intact.
11. Gently resuspend pellets with cold (2 - 8°C) CryoStor®CS10, using a 2 mL pipette. Take care to leave the cell aggregates larger than would normally be done for passaging.

    Note: 1 mL of CryoStor®CS10 should be used for every well of a 6-well plate being frozen. However, if the wells are at low density (less than 50% confluent), 1 mL of CryoStor®CS10 may be used for every 2 wells.

12. Transfer 1 mL of cell aggregates in CryoStor®CS10 into each labeled cryovial using a 2 mL pipette.
13. Freeze cells using a standard slow rate controlled cooling protocol (approximately -1°C/min) and store at LN_2 temperature (-135°C). Long-term storage at -80°C is not recommended.

    Alternatively, cells can be frozen using an isopropanol freezing vessel and a multi-step protocol: -20°C for 2 hours, followed by -80°C for 2 hours, followed by storage at LN_2 temperature (-135°C).

4.0 Characterization of Undifferentiated Human ES and iPS Cells

4.1 Morphology

Undifferentiated human ES and iPS cells grow as compact, multicellular colonies, as shown in Figure 2. They should also exhibit a high nuclear-to-cytoplasm ratio and prominent nucleoli. These colonies are characterized by a distinct border (this may be less pronounced in colonies cultured on BD Matrigel™ in mTeSR™1 or TeSR™2 compared to those grown on feeder cells). Healthy human ES and iPS cell colonies will be multilayered in the center, resulting in clusters of phase-bright cells. Differentiation is characterized by loss of border integrity, gross non-uniformity of cell morphology within a colony, and the emergence of obvious alternate cell types.

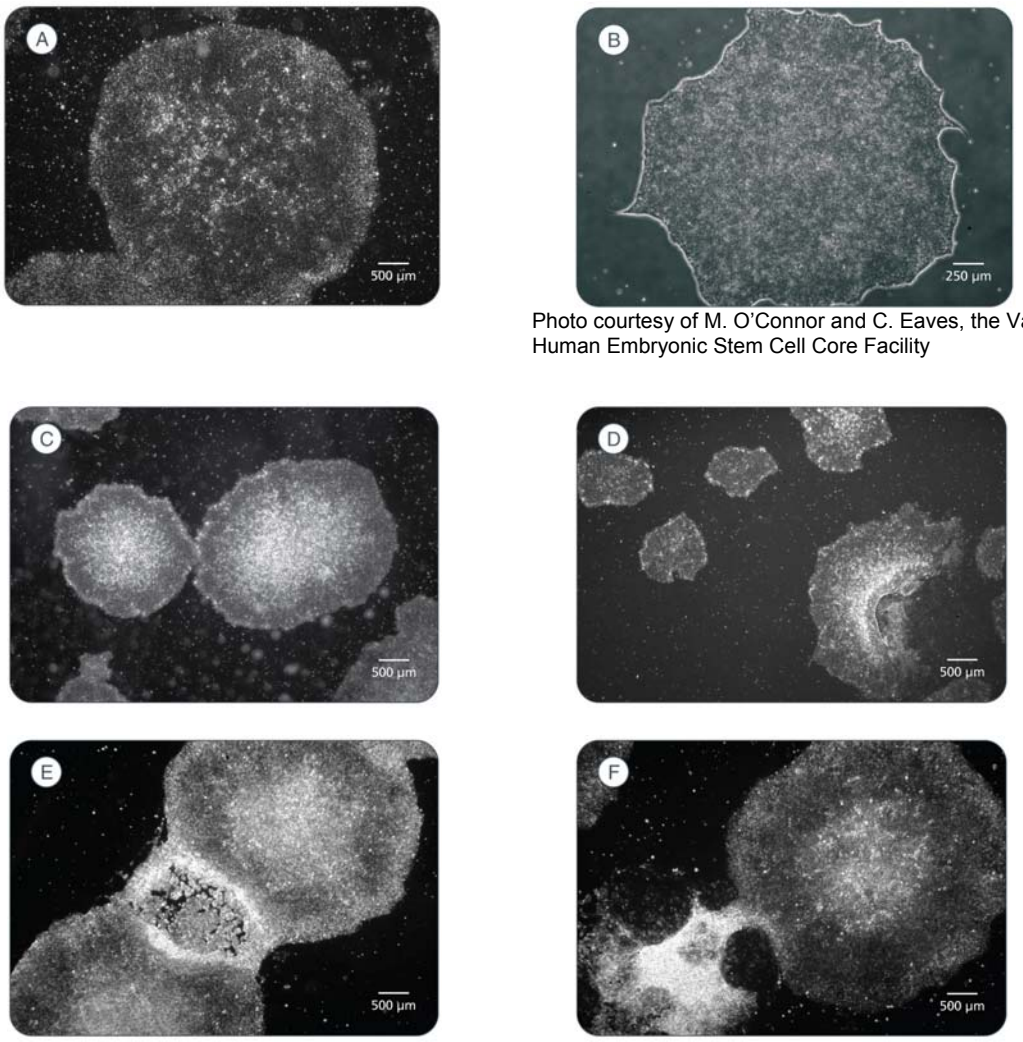


Photo courtesy of M. O'Connor and C. Eaves, the Vancouver Human Embryonic Stem Cell Core Facility

Figure 2: Morphology of Cultured Human ES and iPS Cells. (A) An undifferentiated human ES H9 colony and (B) an undifferentiated human iPS (IMR90)-3 colony cultured in mTeSR™1. Both cultures will be ready to passage in approximately 1 - 2 days. (C) H9 cell line cultured in TeSR™2 ready to be passaged. (D) H9 cell line in TeSR™2 showing an area of differentiation. (E) Area of differentiation between 2 undifferentiated human ES H1 colonies cultured in mTeSR™1. (F) H1 cell line in mTeSR™1 showing a differentiated colony adjacent to an undifferentiated colony.

4.2 Flow Cytometry Protocols

4.2.1 Reagents and Materials

Antibodies

Antibodies can be used to characterize cells by flow cytometry. The table below contains information about primary and secondary antibodies available from STEMCELL Technologies that can be used to characterize human ES and iPS cells.

FOR INTRACELLULAR ANTIGEN LABELING				
PRIMARY ANTIBODY	CATALOG #	ISOTYPE	RECOMMENDED SECONDARY ANTIBODY	CATALOG #
Oct-3/4 Antibody	01550/01551	IgG1 (Mouse)	FITC-conjugated goat anti-mouse IgG	10210

FOR SURFACE ANTIGEN LABELING				
PRIMARY ANTIBODY	CATALOG #	ISOTYPE	RECOMMENDED SECONDARY ANTIBODY	CATALOG #
SSEA-1 Antibody	01552	IgM (Mouse)	FITC-conjugated goat anti-mouse IgM	10211
SSEA-3 Antibody	01553	IgM (Rat)	APC-conjugated goat anti-rat IgM	10215
SSEA-4 Antibody	01554	IgG3 (Mouse)	FITC-conjugated goat anti-mouse IgG	10210
TRA-1-60 Antibody	01555	IgM (Mouse)	FITC-conjugated goat anti-mouse IgM	10211
TRA-1-81 Antibody	01556	IgM (Mouse)	FITC-conjugated goat anti-mouse IgM	10211
TRA-2-49 Antibody	01557	IgG1 (Mouse)	FITC-conjugated goat anti-mouse IgG	10210
TRA-2-54 Antibody	01558	IgG1 (Mouse)	FITC-conjugated goat anti-mouse IgG	10210

General Reagents and Materials

REAGENTS AND MATERIALS	CATALOG #
D-PBS (without Mg++ or Ca++)	37350
DMEM/F-12	36254
Trypan Blue	07050
Gentle Cell Dissociation Reagent	07174
D-PBS with 2% FBS (2%FBS/PBS)	07905
1.5 mL tubes	e.g. Eppendorf, Catalog #022364111
5 mL FACS tubes	e.g. BD, Catalog #352058
Conical tubes (15 mL)	e.g. BD, Catalog #352196
Nuclear stain (optional: e.g. 1 mg/mL propidium iodide diluted 1 in 1000 in 2% FBS/PBS)	e.g. Sigma, #81845

4.2.2 Preparation of a Single Cell Suspension for Flow Cytometry

1. Warm medium (DMEM/F-12 or mTeSR™1) to room temperature (15 - 25°C) before use. The Gentle Cell Dissociation Reagent should already be at room temperature. Calculate the total volume of Gentle Cell Dissociation Reagent required based on the number and size of the cultureware, using the table below:

CULTUREWARE	GENTLE CELL DISSOCIATION REAGENT
6-well plate	1 mL/well
100 mm dish	4 mL/dish

2. Aspirate the culture medium and add the Gentle Cell Dissociation Reagent. Incubate at 37°C for 15 minutes.

   Note: This incubation time is based on STEMCELL Technologies' Gentle Cell Dissociation Reagent. If using a non-enzymatic cell dissociation reagent from another supplier, incubation times may need to be adjusted.

3. Harvest cells by pipetting up and down with either a serological pipette or a P1000 micropipetter to ensure a single-cell suspension and transfer cells to a 15 mL conical tube. Rinse wells with an additional 2 - 4 mL of medium (DMEM/F-12 or mTeSR™1) and add the rinse to the tube containing the cells.
4. Centrifuge cells at 300 x *g* for 5 minutes.
5. Resuspend cells in medium and perform a viable cell count using Trypan Blue.
6. The single cell suspension may now be used for surface antigen and/or intracellular antigen labeling (see below for detailed protocols).

4.2.3 Surface Antigen Labeling Protocol

Note: Optimal concentrations of primary and secondary antibodies need to be predetermined by titration for each antibody.

1. Determine the number of samples required to perform flow cytometry including necessary labeling controls.
2. Aliquot approximately 1 x 10^5 cells per sample into a 5 mL FACS tube or a 1.5 mL tube and place on ice.
3. Centrifuge cells at 300 x *g* for 5 minutes.
4. While the samples are centrifuging, make a sufficient quantity of the primary antibody mix (100 µL/sample) using the appropriate primary antibody at the predetermined optimal working dilution.
5. Carefully remove the supernatant without disrupting the cell pellet and resuspend cells in the primary antibody mix. Gently mix and incubate on ice for 15 - 60 minutes.
6. Add 1 mL of 2% FBS/PBS per tube. Gently mix and centrifuge at 300 x *g* for 5 minutes.
7. While the samples are centrifuging, make a sufficient quantity of the secondary antibody mix (100 µL/sample) using the appropriate secondary fluorochrome-conjugated antibody at the predetermined optimal working dilution.
8. Carefully remove the supernatant without disturbing the cell pellet and resuspend the cells in the secondary antibody mix. Gently mix and incubate on ice for 15 - 60 minutes. Protect samples from exposure to direct light.
9. Add 1 mL of 2% FBS/PBS to each tube. Gently mix and centrifuge at 300 x *g* for 5 minutes.
10. Carefully remove the supernatant without disturbing the cell pellet and resuspend the cells in 200 - 300 µL 2% FBS/PBS. Transfer to a 5 mL FACS tube if necessary.
11. Place samples on ice and analyze by flow cytometry as soon as possible.

    Optional: Propidium iodide (PI) can be added at a final concentration of 1 µg/mL to assess viability by flow cytometry.

4.2.4 Intracellular Antigen Labeling Protocol for Oct-3/4

Additional Reagents Required

Saponin Permeabilization Buffer (SPB)*

COMPONENT	CATALOG #	FINAL CONCENTRATION
Saponin	e.g. Fluka Biochemika, Catalog #47036	1 mg/mL
10% BSA Solution	04915	1%
PBS (without Mg^{++} or Ca^{++})	37350	to final volume

*Mix well and store at 2 - 8°C for up to 1 month.

2% Paraformaldehyde Solution

COMPONENT	CATALOG #	FINAL CONCENTRATION
Paraformaldehyde	e.g. Affymetrix, Catalog #19943 1 LT	2%
PBS (without Mg^{++} or Ca^{++})	37350	to final volume

*Mix well and store at 2 - 8°C.

Protocol

Note: Optimal concentrations of primary and secondary antibodies need to be predetermined by titration for each antibody.

1. Determine the number of samples required to perform flow cytometry including necessary labeling controls.
2. Aliquot approximately 4 - 8 x 10^5 cells per sample into a 5 mL FACS tube or a 1.5 mL tube.
3. Centrifuge cells at 300 x *g* for 5 minutes.
4. Carefully remove the supernatant without disrupting the cell pellet and resuspend cells in 250 µL of 2% Paraformaldehyde Solution/tube. Gently mix and incubate on ice for 15 - 30 minutes.
5. Add 1 mL of 2% FBS/PBS/tube. Gently mix and centrifuge at 300 x *g* for 5 minutes.
6. Carefully remove the supernatant without disrupting the cell pellet and resuspend cells in 500 µL of Saponin Permeabilization Buffer (SPB)/tube. Gently mix and incubate at room temperature (15 - 25°C) for 15 minutes.

   Note: Cells should remain in SPB until the final resuspension step, prior to flow cytometric analysis.
7. While the samples are incubating, make a sufficient quantity of the primary antibody mix (100 µL/sample) using SBP as the diluent.

   Note: The suggested working dilution of the Oct-3/4 primary antibody (Catalog # 01550/01551) is 1 in 100.
8. Centrifuge cells at 300 x *g* for 5 minutes.
9. Carefully remove the supernatant without disrupting the cell pellet and resuspend cells in the primary antibody mix (100 µL/sample). Gently mix and incubate on ice for 15 - 60 minutes.
10. Add 1 mL of SPB/tube. Gently mix and centrifuge at 300 x *g* for 5 minutes.
11. While the tubes are centrifuging, make a sufficient quantity of the secondary antibody mix (100 µL/sample) using SBP as the diluent.

    Note: When using Oct-3/4 primary antibody (Catalog # 01550/01551) the suggested secondary antibody is FITC-conjugated goat anti-mouse IgG (Catalog #10210) diluted 1 in 100 from the reconstituted stock.

12. Carefully remove the supernatant without disturbing the cell pellet and resuspend cells in the secondary antibody mix (100 µL/sample). Gently mix and incubate on ice for 15 - 60 minutes. Protect samples from exposure to direct light.
13. Add 1 mL of SPB/tube. Gently mix and centrifuge at 300 x *g* for 5 minutes.
14. Carefully remove the supernatant without disturbing the cell pellet and resuspend the cells in 300 µL 2% FBS/PBS. Transfer to a 5 mL FACS tube if necessary.
15. Place samples on ice and analyze by flow cytometry as soon as possible.

    Optional: In order to ensure only single cells are assessed, examine a plot of FSC area versus FSC height in the linear range and gate out events that deviate from diagonal as in Figure 3.

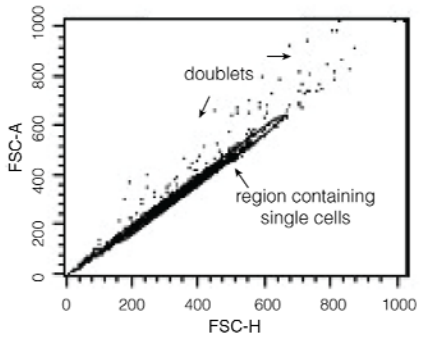


Figure 3: An Example of Doublet Discrimination by Flow Cytometry

5.0 Helpful Hints

5.1 Successful Culture of Human ES and iPS Cells

Culturing human ES and iPS cells is a very time-consuming process. It requires daily medium changes, use of the highest quality reagents and frequent morphologic observations of the cultures to ensure that they are maintained at the optimum density. The following tips will help ensure success with human ES and iPS cell culture.

A) Preparation of Human ES and iPS Cell Clumps for Passaging

Preparation of a uniform suspension of suitable sized human ES and iPS cell clumps for passaging is very important for the successful culture of human ES and iPS cells. If the clumps are too large, an increased rate of differentiation within the colonies may occur. If the clumps are too small with many single cells present, cell survival (and resulting number and health of colonies) will be compromised. Following the scraping step, one or two gentle draws with a micropipettor and P1000 tip or a 2 mL serological pipette should be sufficient to generate appropriately-sized clumps for passaging (approximately 100 µm). If the clumps are the correct size, the majority will remain in suspension after this step. If large clumps are present that rapidly sink to the bottom of the tube, perform one or two more gentle draws with the micropipettor or pipette.

B) Density

Culture density is a critical aspect of maintaining human ES and iPS cells in mTeSR™1 or TeSR™2. Cultures that are either too sparsely or too densely populated can lead to differentiation. Many colonies in the dish should be just beginning to touch each other at the time of passaging (i.e. plate is approximately 60 - 75% confluent). Adjust plating and/or split ratios to achieve a balance between having too much space between colonies and having a confluent culture (see Figure 1, Section 3.2, Appendix 1 and Appendix 2 for examples of human ES and iPS cell cultures ready for passaging). As a general guideline, an acceptable density would be approximately 150 colonies per well of a 6-well plate.

C) Differentiation in a Maintenance Culture

It is important that the starting culture is of high quality and is primarily undifferentiated. Cultures that are compromised or have large amounts of differentiated cells will continue to show these symptoms after transition into mTeSR™1 or TeSR™2. If necessary, areas of differentiation can be selectively removed by scraping off or aspirating before passaging the cultures. The morphologic hallmarks of differentiation should appear in less than 20% of colonies in a healthy culture.

D) Feeding Regime

Human ES and iPS cells generally require daily medium change for optimal growth. Some human ES and iPS cell lines will tolerate occasional double feeding (adding twice the required volume of medium). For instance, it is possible to perform a double feed on a Friday, with the next medium change on Sunday. However, it is not recommended to either go longer than 1 day without a medium change, or to feed the cultures every other day continuously.

5.2 Transitioning Human ES and iPS Cells to mTeSR™1 or TeSR™2

No adaptation step is required when seeding cells from feeder or conditioned medium cultures to mTeSR™1 or TeSR™2. Simply replate human ES and iPS cell aggregates into mTeSR™1 or TeSR™2 at the time of passaging. It is, however, recommended that a culture using the previous medium and culture system is initially maintained in parallel to ensure that the chosen plating density in mTeSR™1 or TeSR™2 is appropriate.

5.2.1 Transitioning from Feeder Culture

The following describes a collagenase method for passaging human ES or iPS cell from feeders on 6-well plates. It is also possible to use mechanical passaging or alternate methods to generate clumps for seeding cells in mTeSR™1 or TeSR™2.

1. Under a low power microscope (e.g. using a 4X or lower objective), mark any differentiated colonies on the maintenance plate of cells to be removed prior to passaging (use a felt-tip or objective marker to indicate the regions that contain differentiated colonies on the bottom of the plate).
2. Remove the differentiated colonies by gently scraping marked regions with either a 2 mL plastic pipette, or a 1 mL plastic pipette tip attached to a P1000 micropipette. Aspirate the medium and scraped colonies. After aspiration, check under the microscope to ensure that the cells in the marked regions are completely removed.
3. Wash the wells with 2 mL/well DMEM/F-12 to remove any scraped colonies that remain loosely attached.
4. Add 1 mL/well of room temperature (15 - 25°C) collagenase solution (1 mg/mL; Catalog #07909). Incubate at 37°C for 20 minutes.
5. Using a 2 mL serological pipette or a cell scraper, scrape cells off the surface of the well. While scraping, gently pipette the collagenase up and down to wash the cells off the surface.
6. After the cell aggregates are detached from the surface of the plate, pool the suspension into a sterile 15 mL conical tube.
7. Rinse each well with 1 mL mTeSR™1 or TeSR™2 and transfer the rinse to the 15 mL conical tube containing the colonies removed from the plate.
8. Pellet cell aggregates by centrifuging at 300 x *g* for 5 minutes at room temperature (15 - 25°C).
9. Aspirate or pour off the supernatant from the pellet. Resuspend the pellet with 1 - 2 mL mTeSR™1 or TeSR™2 using a P1000 micropipetter or a 2 mL pipette by gently pipetting up and down 1 - 2 times to break up large clumps.

   Note: Be careful not to generate a single cell suspension with excessive pipetting.
10. Seed an appropriate volume of clumps on BD Matrigel™-coated plates with mTeSR™1 or TeSR™2.

    Note: No adaptation is required when switching to mTeSR™1 or TeSR™2 from medium containing Knockout™ Serum Replacement. It is recommended that cultures are seeded at the same density as on feeders.

5.2.2 Transitioning from Cultures in Feeder-free Conditioned Media

Transitioning from conditioned media does not require any adaptation; cells can be plated in mTeSR™1 or TeSR™2 on BD Matrigel™-coated plates at the time of passage.

5.3 Plating Human ES and iPS Cells by the Clump Count Method

An alternative to splitting clump suspensions into defined volumes is to perform clump counts on the human ES and iPS cell suspension and to always plate a defined number of clumps according to the size of the well or dish that is being seeded. This can be a valuable learning tool for those new to human ES and iPS cell culture because it aids in defining how much a suspension should be pipetted to achieve optimally-sized clumps. An eyepiece micrometer placed in the microscope eyepiece is required to enumerate clumps of appropriate size (≥60 µm in diameter) that are likely to attach and grow. Eyepiece micrometers are available from most microscope manufacturers.

Performing a Clump Count*

1. Aliquot 40 µL of DMEM/F-12 (Catalog #36254) into 2 wells of a 96-well flat-bottom plate.
2. Draw a "+" centered on the bottom of these wells to serve as a counting grid.
3. Add 5 µL of a freshly mixed clump suspension to each well. Count clumps that are approximately 60 µm or greater in diameter (using a calibrated eyepiece micrometer). This corresponds to clumps with an area of approximately 3500 μm^2
4. Perform duplicate counts, then average the results and calculate the total number (x) of clumps.

$$\frac{\text{\# of clumps counted}}{5\ \mu L} = \frac{\text{x clumps}}{\text{total volume of suspension (µL)}}$$

* Protocol kindly provided by the Vancouver Human Embryonic Stem Cell Core Facility.

5. Calculate the volume of clump suspension (y) required to seed new dishes using the following guide for appropriate seeding densities:

CULTUREWARE	TARGET # OF CLUMPS
100 mm dish	2400 clumps
60 mm dish	1000 clumps
Wells in a 6-well dish	300 clumps

For example, to seed 1 well of a 6-well dish, the volume of clump suspension required for 300 clumps is calculated as follows:

$$\frac{\text{\# of clumps counted}}{5\ \mu L} = \frac{300\ \text{clumps}}{y\ \mu L}$$

6. Completely remove excess BD Matrigel™ from a pre-coated 6-well plate (performed as described in Section 2.4.4). Add the appropriate volume of mTeSR™1 or TeSR™2 to each well.
7. Gently mix the clump suspension prior to plating to ensure a uniform suspension.
8. Add the required seeding volume of clumps to each well.
9. Move the plate in several quick, short, back-and-forth and side-to-side motions to disperse cells across the surface of the wells. Return the plate to the incubator.

   Ensure that clumps are evenly dispersed across the entire surface of the plate. Uneven distribution of clumps may result in differentiation of human ES and iPS cells.

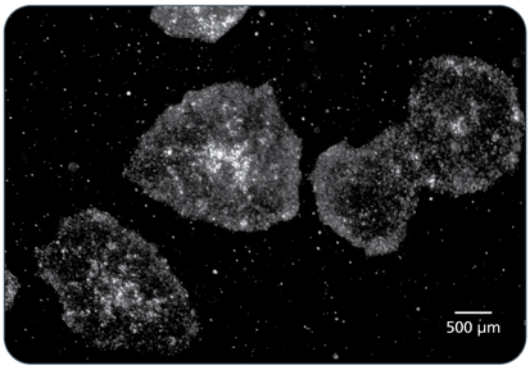
5.4 Alternative Matrices for an Animal Protein-Free Culture System

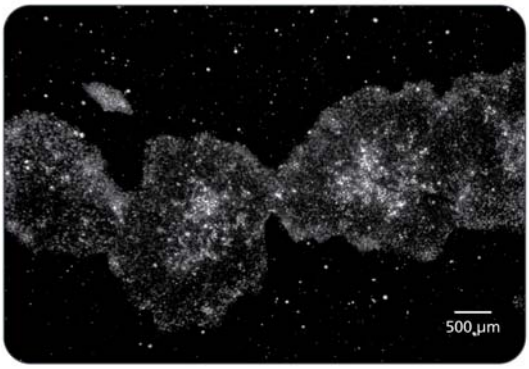
It is a major focus in the field of pluripotent cell culture to develop animal protein-free matrices for use with animal protein-free defined medium. To date, the most robust matrix for the growth of human ES and iPS cell is BD Matrigel™. However, as it is isolated from the mouse EHS tumor, it introduces animal proteins to the system. For those who are striving towards an animal protein-free culture system, the use of BD Matrigel™ is not desirable. Furthermore, understanding the specific requirements needed to create defined and animal component-free surfaces that are capable of supporting human ES and iPS cell attachment and growth has proven to be technically challenging[9]. Defined matrices often suffer from poor cell attachment, inability to support long-term passaging, or prohibitive production costs.

StemAdhere™ Defined Matrix for hPSC (Catalog #07160), developed and manufactured by Primorigen Biosciences®, is a defined, humanized matrix that supports the long-term culture of human ES and iPS cells with cells that consistently maintain puripotency[17]. It is an affordable and effective alternative to Matrigel™ and may be used with mTeSR™1 or TeSR™2 medium for a fully-defined and feeder-free culture system.[18] This system allows for complete control over the culture environment, resulting in more consistent cell populations and more reproducible results in downstream applications. Cells easily transition from Matrigel™ to StemAdhere™ and are maintained and passaged, thereafter, using a simple, enzyme-free protocol. For complete instructions on culturing cells on StemAdhere™ Defined Matrix for hPSC, please refer to the manual "Maintenance of hPSCs on StemAdhere™ Defined Matrix for hPSC" available at www.stemcell.com.

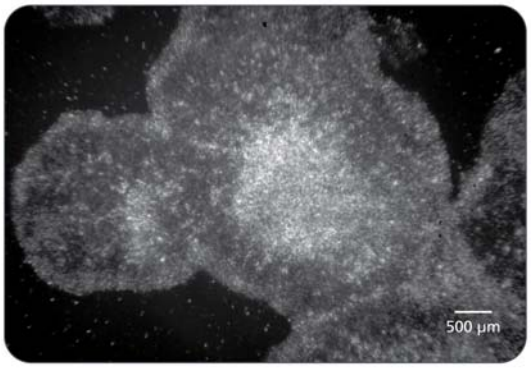
Appendix 1: Representative mTeSR™1 Cultures at Various Days After Passaging

Day 2: Colonies are small, transparent and not very densely packed with cells.





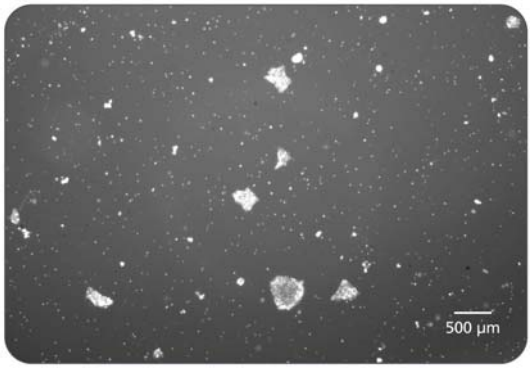
Day 4: Colonies rapidly increase in size and start to develop phase-bright centers when viewed under a phase contrast microscope. However, these colonies are not yet ready to be passaged.

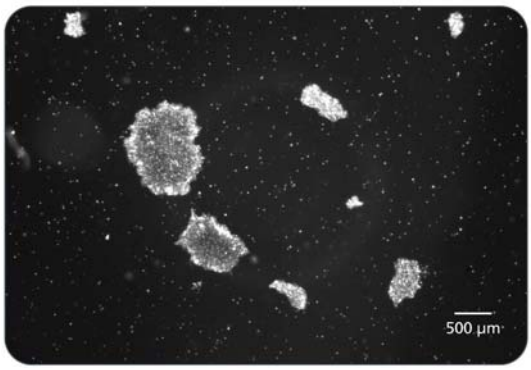


Day 6: Colonies begin to merge and have phase-bright centers that are densely packed with cells. These colonies are ready to passage.

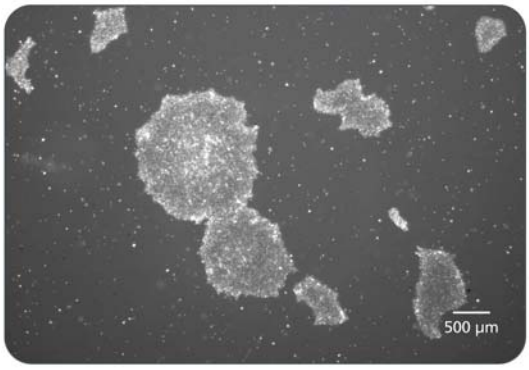
Appendix 2: Representative TeSR™2 Cultures at Various Days After Passaging

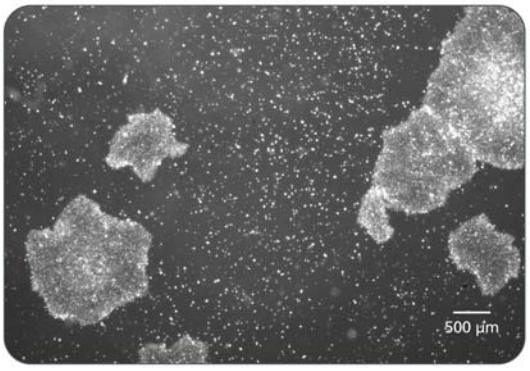
Note: cells grown in TeSR™2 may need to be passaged approximately 1 day earlier than the same cells grown in mTeSR™1. Please refer to Appendix 2 for representative mTeSR™1 cultures at various days after passaging.



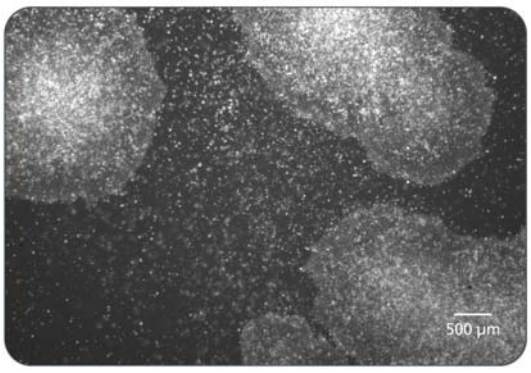


Days 1 and 2: Colonies are small, transparent and not very densely packed with cells.





Days 3 and 4: Colonies rapidly increase in size and start to develop phase-bright centers when viewed under a phase contrast microscope. However, these colonies are not yet ready to be passaged. Colonies at Day 4 should be passaged within 24 hours.



Day 5: Colonies begin to merge and have phase-bright centers that are densely packed with cells. These colonies are ready to passage.

6.0 References

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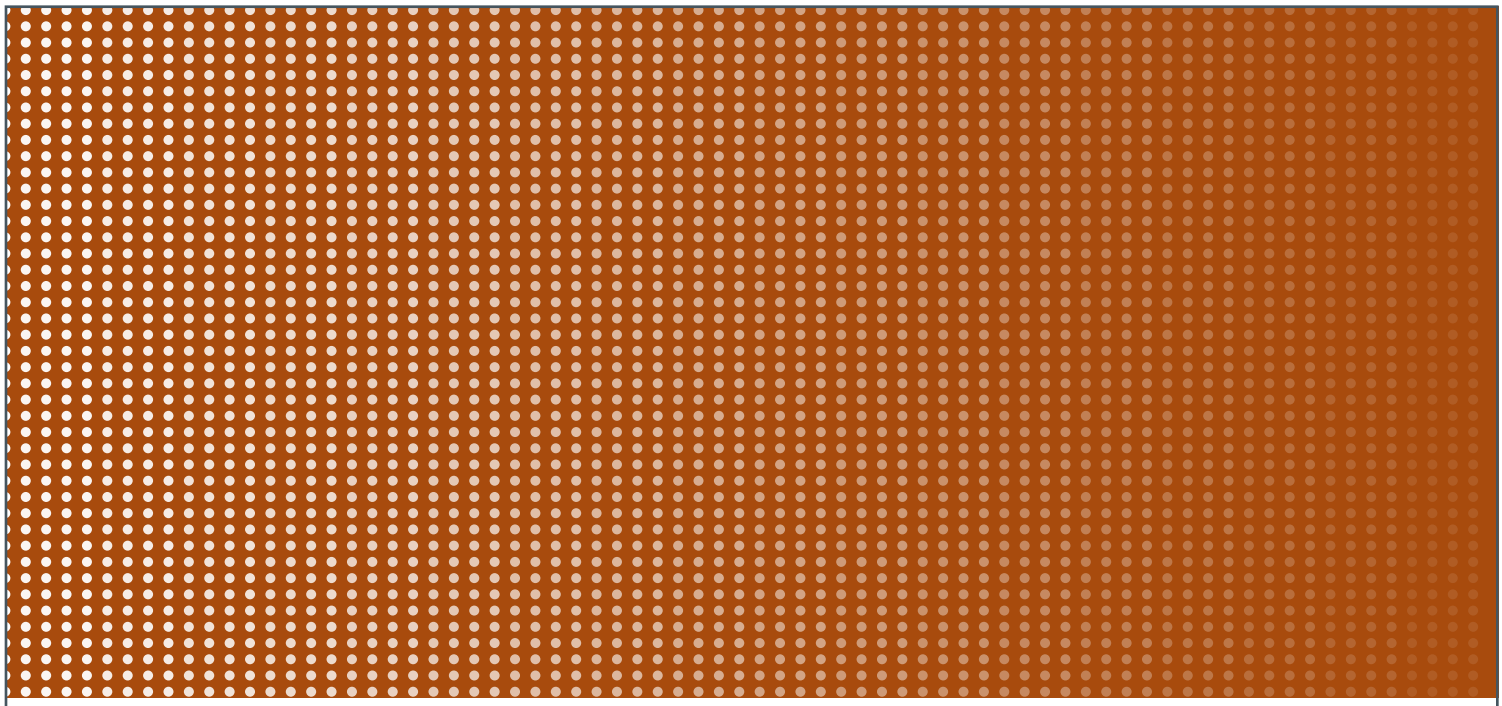

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