TECHNICAL MANUAL
VERSION 1.1.0

Maintenance of Human Pluripotent Stem Cells in TeSR™-E8™

STEMCELL™ TECHNOLOGIES

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1.0 Introduction

The maintenance and propagation of human embryonic stem (ES) cells and human induced pluripotent stem (iPS) cells in feeder-free conditions requires the use of complex media formulations, in combination with careful handling techniques, to maintain high quality cultures at each passage. Recent efforts have focused on improving the general utility and reproducibility of human ES and iPS cell culture protocols by developing new and more straightforward strategies that increase consistency by the removal of undefined or unnecessary components from the culture system. A simplified version of the commercially available mTeSR™1 and TeSR™2 human ES and iPS cell maintenance media, referred to as E8, has been reported by Dr. James Thomson's lab[1-4]. E8 was developed via the pairwise removal of extraneous additives from the TeSR™ core media formulation, resulting in an albumin-free media with a minimum set of components.

TeSR™-E8™ is a low protein, feeder-free maintenance medium for human ES and iPS cells that is based on the published E8 formulation. When combined with the protocols described in this manual, TeSR™-E8™ is capable of maintaining high quality human ES and iPS cells similar to those cultured in mTeSR™1 and TeSR™2 media. This medium may be used with either BD Matrigel™, or alternatively with Vitronectin XF™ (developed and manufactured by Primorigen Biosciences Inc.) recombinant protein matrix if a fully defined culture system is desired.

2.0 Materials, Reagents and Equipment

2.1 TeSR™-E8™ Medium

The TeSR™-E8™ Medium Kit (Catalog #05940) includes:

COMPONENT	VOLUME	STORAGE CONDITIONS
TeSR™-E8™ Basal Medium (#05941)	474 mL	Product stable at 2 - 8°C for 1 year from date of manufacture as indicated on label
TeSR™-E8™ 20X Supplement (#05942)	25 mL	Product stable at -20°C for 1 year from date of manufacture as indicated on label
TeSR™-E8™ 500X Supplement (#05943)	1 mL	Product stable at -20°C for 1 year from date of manufacture as indicated on label
TeSR™-E8™ Complete Medium Bottle (#05945)	N/A	N/A

2.2 Additional Reagents Required for Human ES and iPS Cell Culture

PRODUCT	CATALOG #
Gentle Cell Dissociation Reagent	07174
Vitronectin XF™ Kit	07190
Vitronectin XF™	07180
CellAdhere™ Dilution Buffer	07183
Non-Tissue Culture Treated 6-well Plates*	27147
BD Matrigel™ hESC-qualified Matrix	BD, Catalog #354277
DMEM/F-12	36254
Tissue culture-treated cultureware**	e.g. 6-well plates, BD Catalog #353046
Cell scrapers	e.g. Corning, Catalog #3010 or Fisherbrand Catalog #08-100-240
Cryostor®CS10	07930

*Required for use with Vitronectin XF™; **Required for use with BD Matrigel™

For a complete list of products for human ES and iPS cell research available from STEMCELL Technologies Inc., please see our website www.stemcell.com

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) with a swinging bucket rotor
  Note: All centrifugation protocols described in this manual can be performed with the brake on
- Pipette-aid (e.g. Drummond Scientific) with appropriate serological pipettes
- Micropipette (e.g. Eppendorf, Gilson) with appropriate tips
- Inverted microscope with a total magnification of 20X to 100X (e.g. Olympus CKX31)
- Isopropanol freezing container (e.g. Nalgene, Fisher; Catalog #1535050)
- -150°C freezer or liquid nitrogen (LN_2) vapor tank
- -80°C freezer
- -20°C freezer
- Refrigerator (2 - 8°C)

3.0 Culturing Human ES and iPS Cells Using TeSR™-E8™

Culture of human ES and iPS cells in TeSR™-E8™ medium may require different techniques than culture in other media. The procedures described in this manual are general and may require optimization for use with specific cell lines.

3.1 Morphology of Cells Cultured in TeSR™-E8™

Undifferentiated human ES cells (Figure 1A and Figure 2A) and iPS cells (Figure 3A and Figure 4A) cultured in TeSR™-E8™ medium grow as compact, multicellular colonies characterized by distinct borders. The individual cells should be tightly packed and also exhibit a high nuclear-to-cytoplasm ratio and have prominent nucleoli. Healthy colonies will merge together seamlessly, and be multilayered in the center, resulting in dense clusters of cells when viewed under phase contrast. Colonies grown in TeSR™-E8™ have a more condensed and round morphology when grown on Vitronectin XF™ matrix compared to colonies grown on BD Matrigel™, which are more diffuse and irregularly shaped. On both matrices, differentiation is characterized by loss of colony border integrity, regions of irregular cell morphology within a colony, and/or the emergence of alternate cell types (Figure 1B, Figure 2B, Figure 3B and Figure 4B).

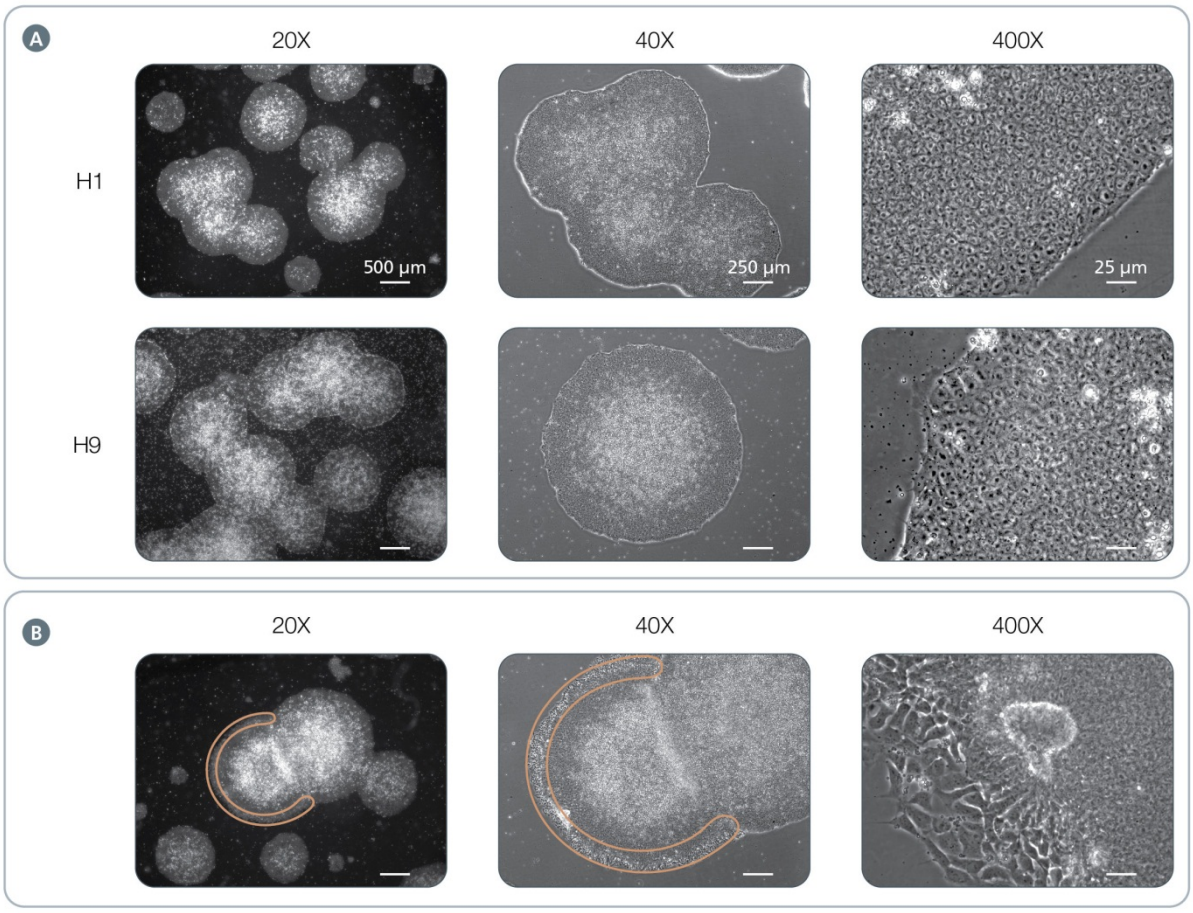


Figure 1. Morphology of Human ES Cells Cultured on Vitronectin XF™ Matrix in TeSR™-E8™ Medium. (A) Undifferentiated human ES cells (H1 and H9) at the optimal time of passaging. (B) Area of differentiation (orange outline) at the border of an undifferentiated H1 colony. Images taken using three magnifications: 20X, 40X and 400X.

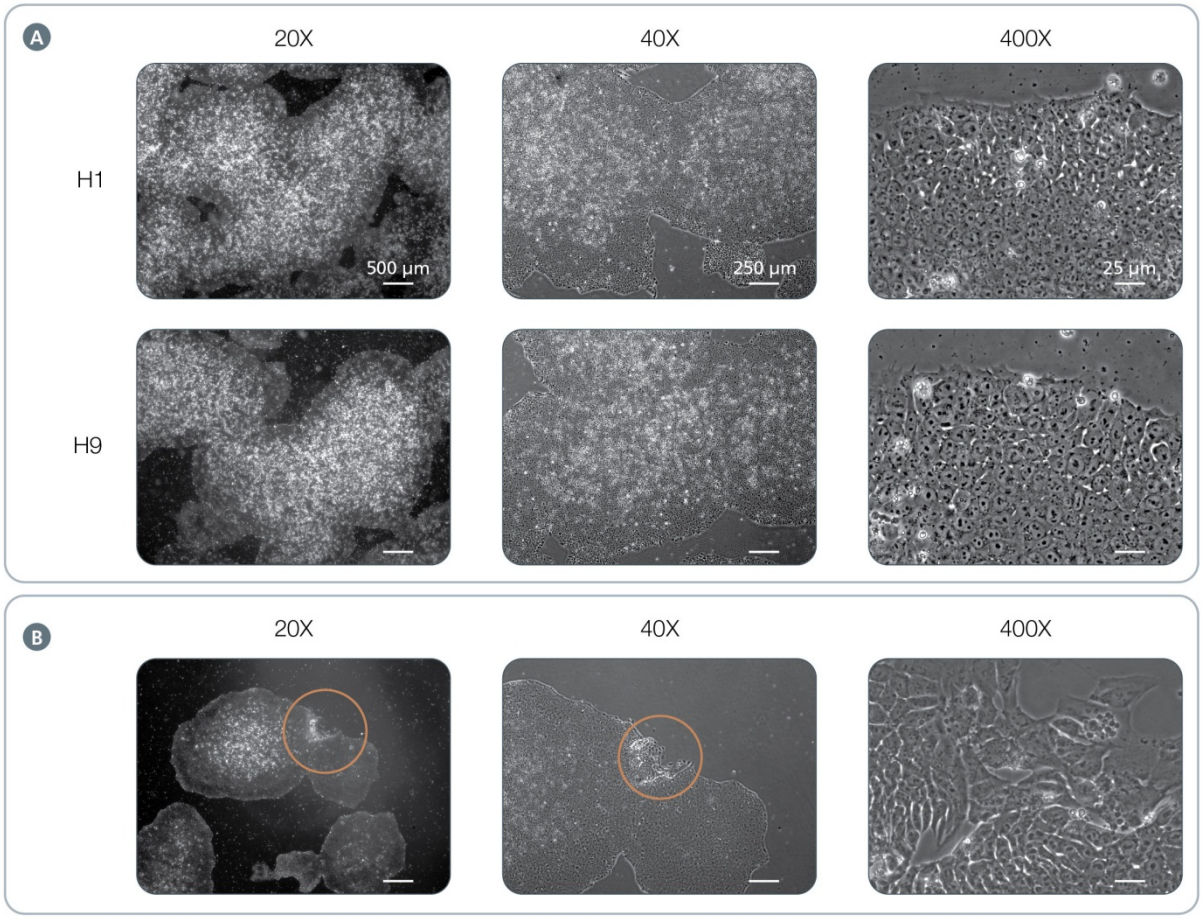


Figure 2. Morphology of Human ES Cells Cultured on BD Matrigel™ Matrix in TeSR™-E8™ Medium. (A) Undifferentiated human ES cells (H1 and H9) at the optimal time of passaging. (B) Area of differentiation (orange circle) between undifferentiated H1 colonies. Images taken using three magnifications: 20X, 40X and 400X.

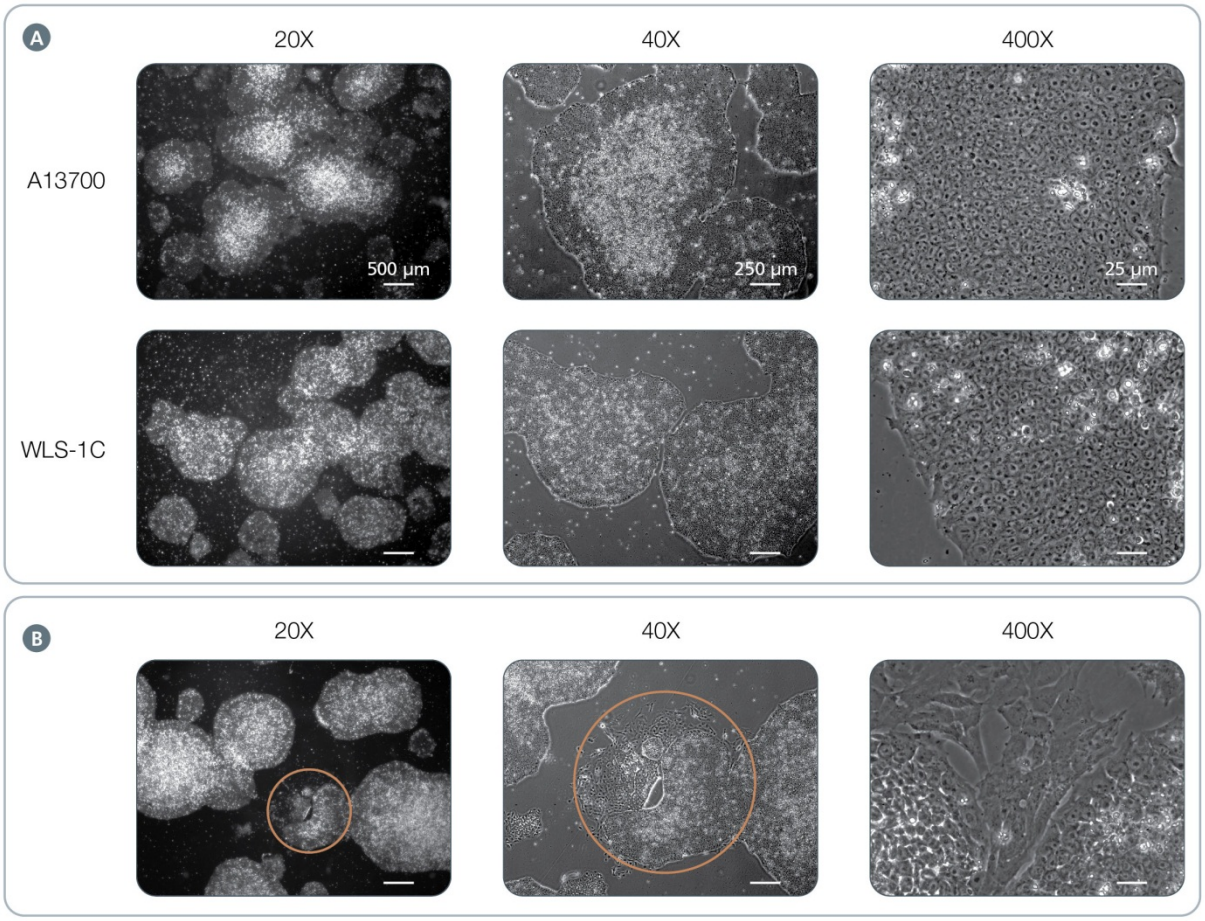


Figure 3. Morphology of Human iPS Cells Cultured on Vitronectin XF™ Matrix in TeSR™-E8™ Medium. (A) Undifferentiated human iPS cells (A13700 and WLS-1C) at the optimal time of passaging. (B) Area of differentiation (orange circle) within an undifferentiated WLS-1C colony. Images taken using three magnifications: 20X, 40X and 400X.

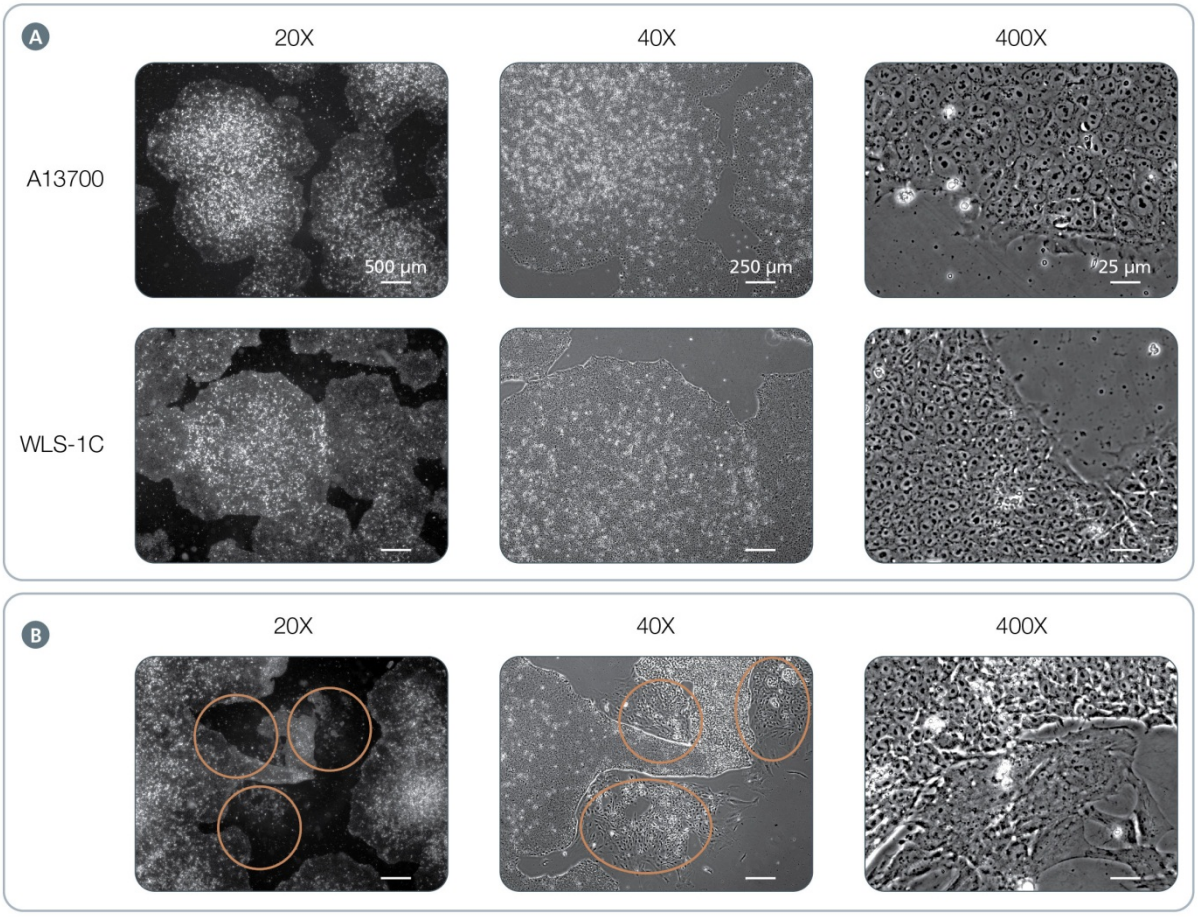


Figure 4. Morphology of Human iPS Cells Cultured on BD Matrigel™ Matrix in TeSR™-E8™ Medium. (A) Undifferentiated human iPS cells (A13700 and WLS-1C) at the optimal time of passaging. (B) Areas of differentiation (orange circles) between undifferentiated A13700 colonies. Images taken using three magnifications: 20X, 40X and 400X.

3.2 Critical Parameters for Successful Cell Culture with TeSR™-E8™

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 passaged at the appropriate time. The following tips will help ensure successful human ES and iPS cell culture with TeSR™-E8™ medium.

Preparation and Storage of Complete TeSR™-E8™ Medium

It is critical to prepare and store complete TeSR™-E8™ in the provided TeSR™-E8™ Complete Medium Bottle for optimal performance. Do not use other containers. For instructions on how to prepare complete TeSR™-E8™ medium, see section 5.1.

Choosing an Appropriate Matrix for Use with TeSR™-E8™

Cells may be cultured in TeSR™-E8™ medium using either BD Matrigel™ or Vitronectin XF™ as the surface coating matrix. For instructions on how to use either of these matrices, see section 5.2. BD Matrigel™ is a routinely used surface coating matrix for a variety of different applications, but this matrix is animal-derived and the composition is undefined. In contrast, Vitronectin XF™ consists of a single humanized protein matrix that is fully defined. Use of Vitronectin XF™ is recommended as the surface coating matrix for applications where a fully defined culture system and/or minimal exposure to animal derived components is desired.

Enzyme-Free Passaging

Ensure that you have optimized the exposure time to the enzyme-free dissociation reagent used; the appearance of colonies during dissociation should be as shown in Figure 9 and Figure 10. The incubation time may vary when using different cell lines or other non-enzymatic cell dissociation reagents, therefore dissociation should be monitored under the microscope until the optimal time is determined. Avoid over-exposure that leads to the generation of single cells, as these may put unwanted selective pressure on cell populations and could lead to genetic aberrations. If single cells are generated, refer to section 9.0 for troubleshooting suggestions.

Colony Density of Human ES and iPS Cell Cultures

Maintaining a proper colony density is a critical aspect of maintaining human ES and iPS cells in TeSR™-E8™ medium. Cultures that are either too sparsely or too densely populated can lead to undesired spontaneous differentiation of cells. A range of colony densities can be tolerated in TeSR™-E8™, as shown in Figure 12; it is recommended to adjust plating density to maintain the culture at the desired confluence (i.e. increase/decrease split ratio). By altering this parameter, the optimal day of passage may be influenced (see section 7.0 and Appendix 1).

Work Quickly

Colonies grown in TeSR™-E8™ that are dissociated with enzyme-free passaging protocols may be slightly more fragile than those cultured in other media or passaged by enzymatic methods. For this reason, it is important to ensure that cell aggregates are removed from the well after exposure to the dissociation reagent and replated as quickly as possible to minimize breakup and improve attachment. It is recommended to avoid centrifugation or other processing steps that may delay replating of cell aggregates.

Characterization of Human ES and iPS Cells

It is good practice to monitor your cultures frequently to ensure maintenance of pluripotency and a normal karyotype. Flow cytometry protocols for assessing pluripotency are described in detail in Appendix 2. Human ES and iPS cells should retain a normal genetic makeup during routine culture, expansion and manipulation. Nonetheless, chromosomal and genetic aberrations may appear during long-term passaging. Accordingly, it is important to periodically (approximately every 10 - 20 passages) check human ES and iPS cell cultures to exclude the possibility of an abnormal karyotype.

Transitioning Cells Grown in Other Feeder-free Media to TeSR™-E8™

Human ES and iPS cells cultured in mTeSR™1, TeSR™2, or other feeder-free media can be conveniently transferred to TeSR™-E8™ (see section 8.1). Cells should transition smoothly into TeSR™-E8™ with minimal differences in morphology, pluripotency and growth rate.

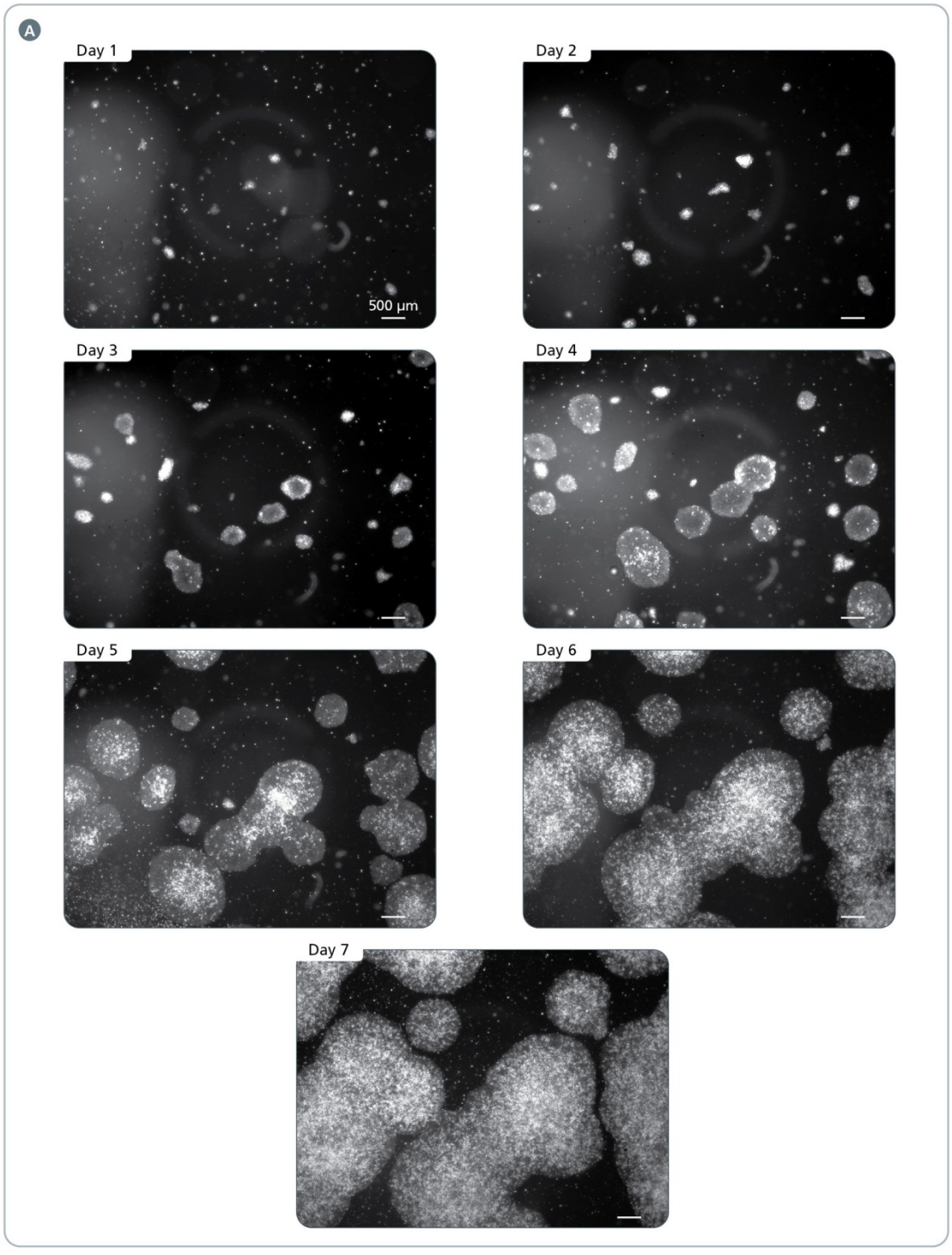
Transitioning Cells Grown on a Feeder Layer to TeSR™-E8™

Human ES and iPS cells cultured on a layer of feeder cells can be conveniently transferred to TeSR™-E8™ (see section 8.4). Cells should adapt to feeder-free culture within 1 - 2 passages and thereafter exhibit morphology consistent with feeder-free human pluripotent stem cells.

4.0 Assessing TeSR™-E8™ Cultures to Determine Day of Passage

Human ES and iPS cells grown in TeSR™-E8™ are ready to passage when the majority of colonies are large, compact, and have centers that are dense compared to their edges (see Figure 5, Figure 6, Figure 7 and Figure 8). 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 plating in TeSR™-E8™, 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 changes significantly in the last 1 - 2 days before passaging.

If colonies are passaged too early or too frequently, the cell aggregates may not attach well when replated, yields will be decreased and cells may start to differentiate (characterized by the emergence of cell types with different morphologies). If colonies are passaged too late, the culture may begin to show signs of differentiation. There is an approximate 24 - 48 hour window that is optimal for passaging. If there are large colonies, with dense centers, passage the cells within 24 hours (for further help, see section 9.0).



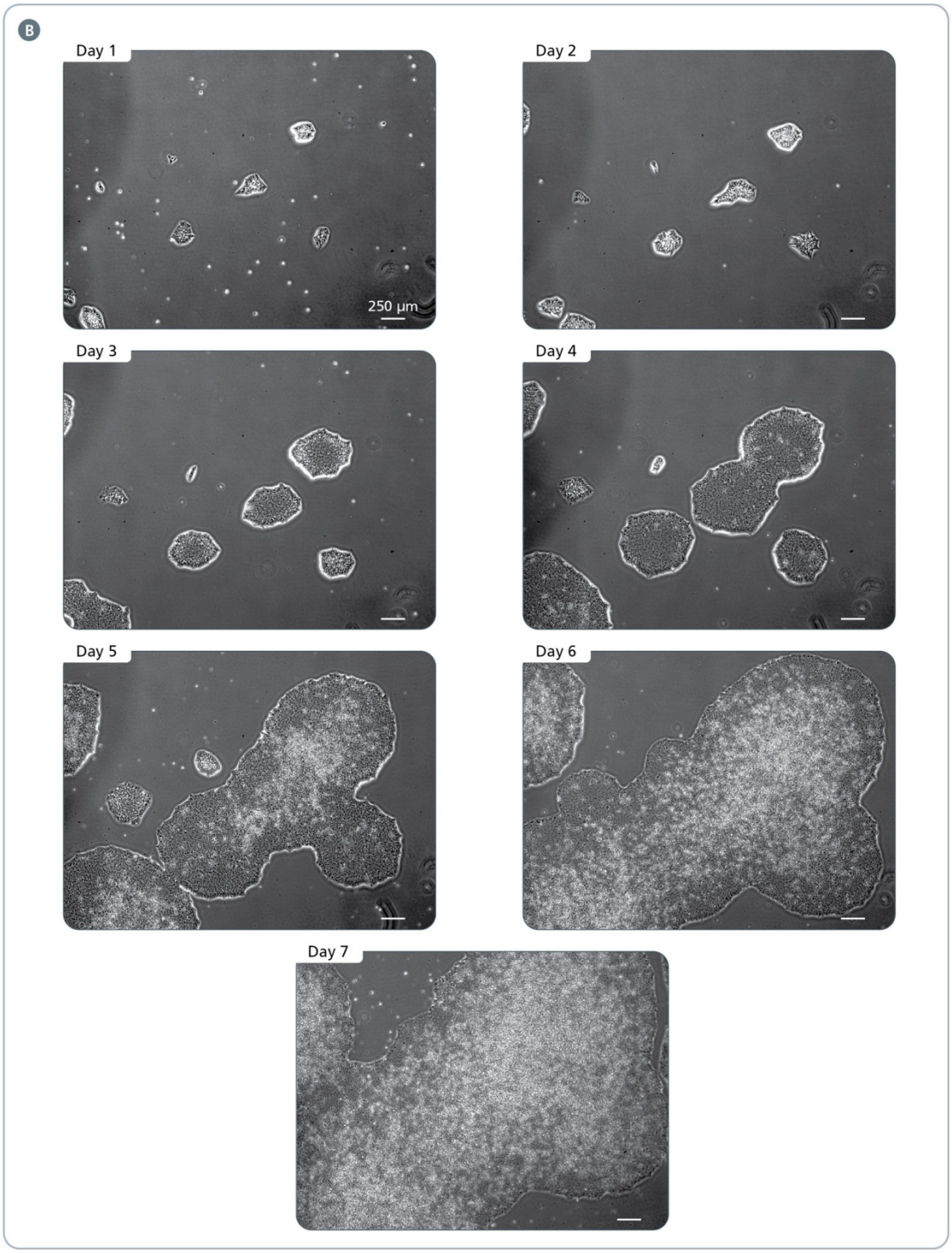
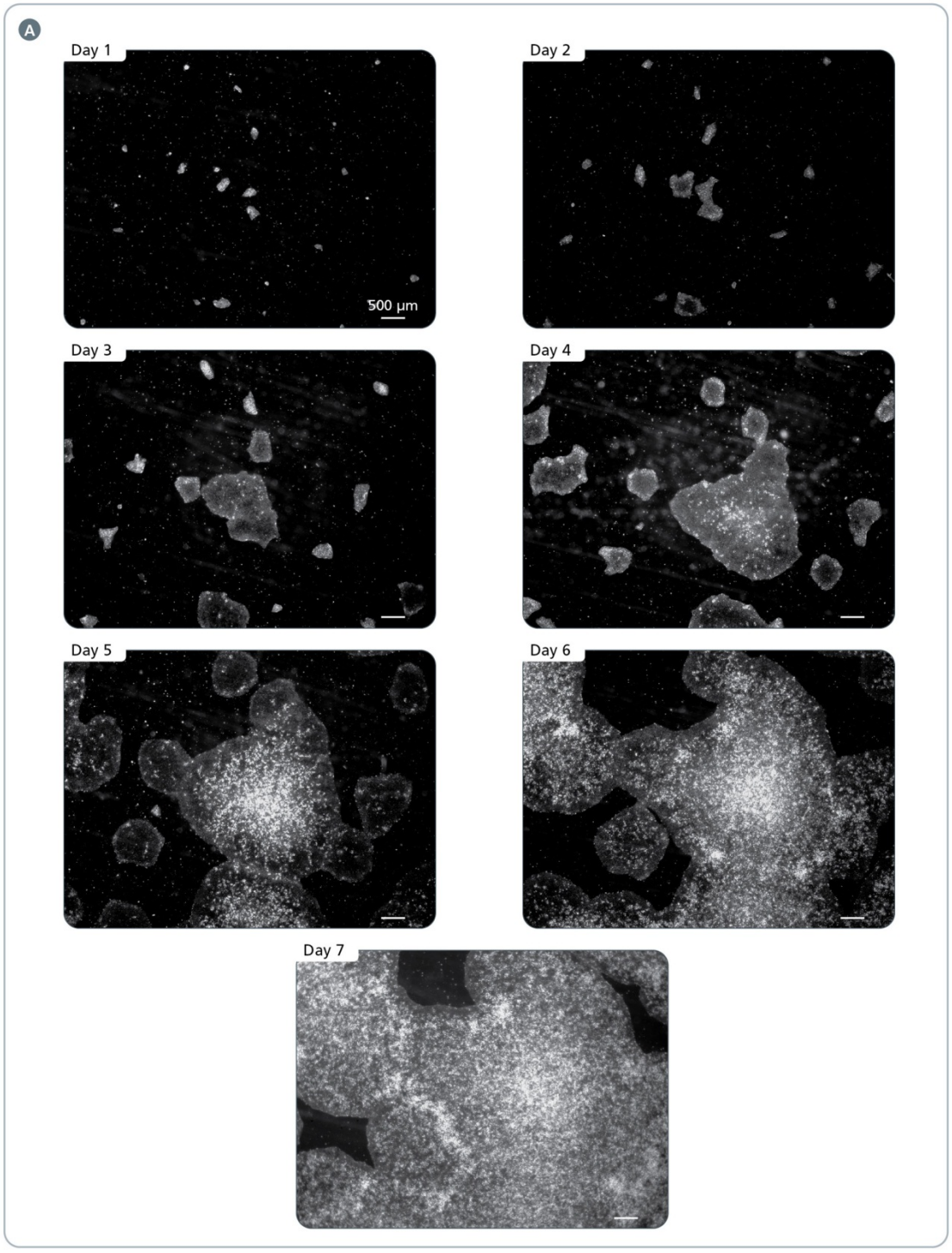


Figure 5. Human ES Cells Cultured on Vitronectin XF™ Matrix in TeSR™-E8™ Medium at Days 1 - 7 after Passaging. Human ES cells (H1) taken using a magnification of 20X (A) and 40X (B). For this culture, Day 6 or 7 would be the optimal window for passaging. Note that the optimal day of passage for each culture will depend on seeding density and aggregate size used (see section 7.0).



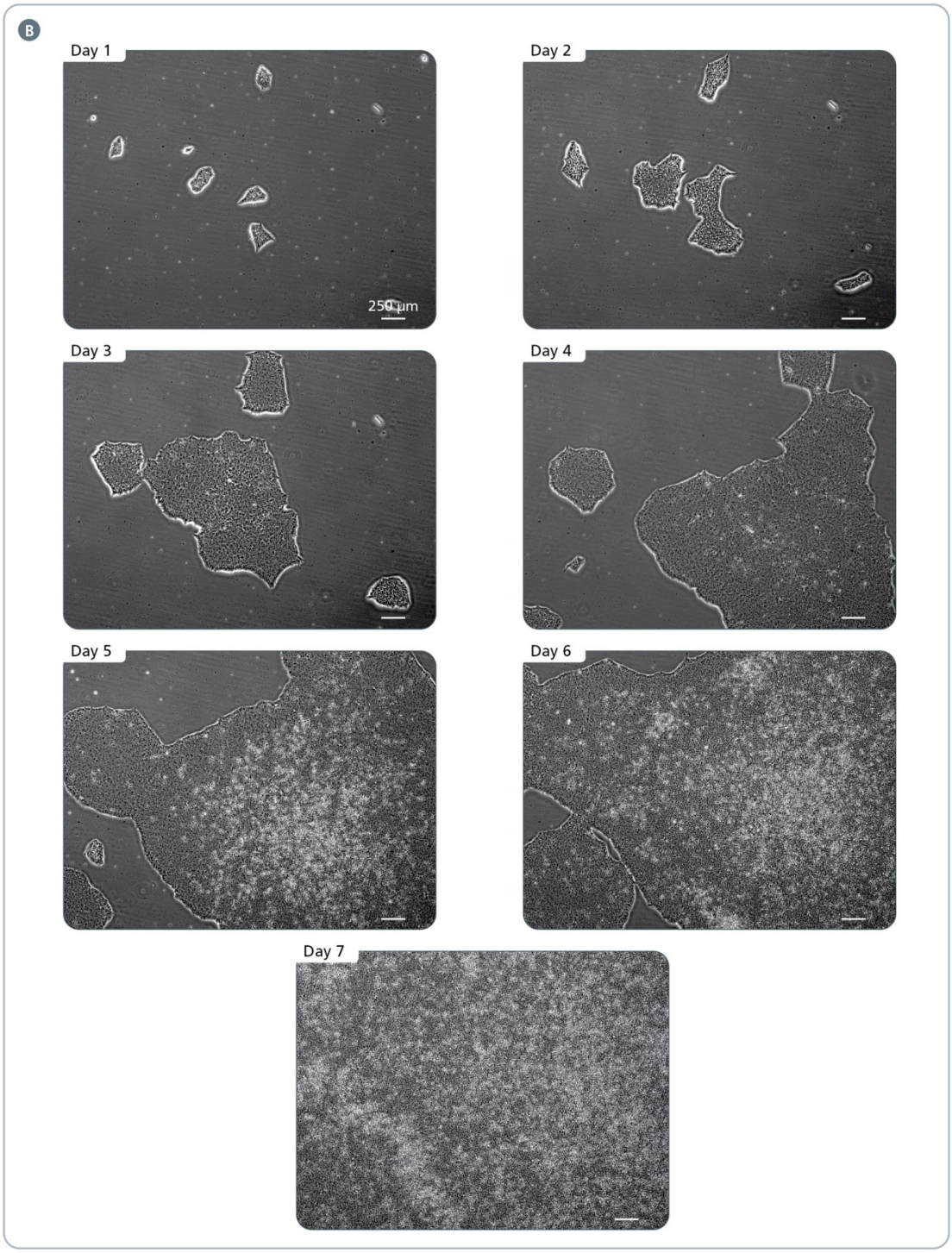
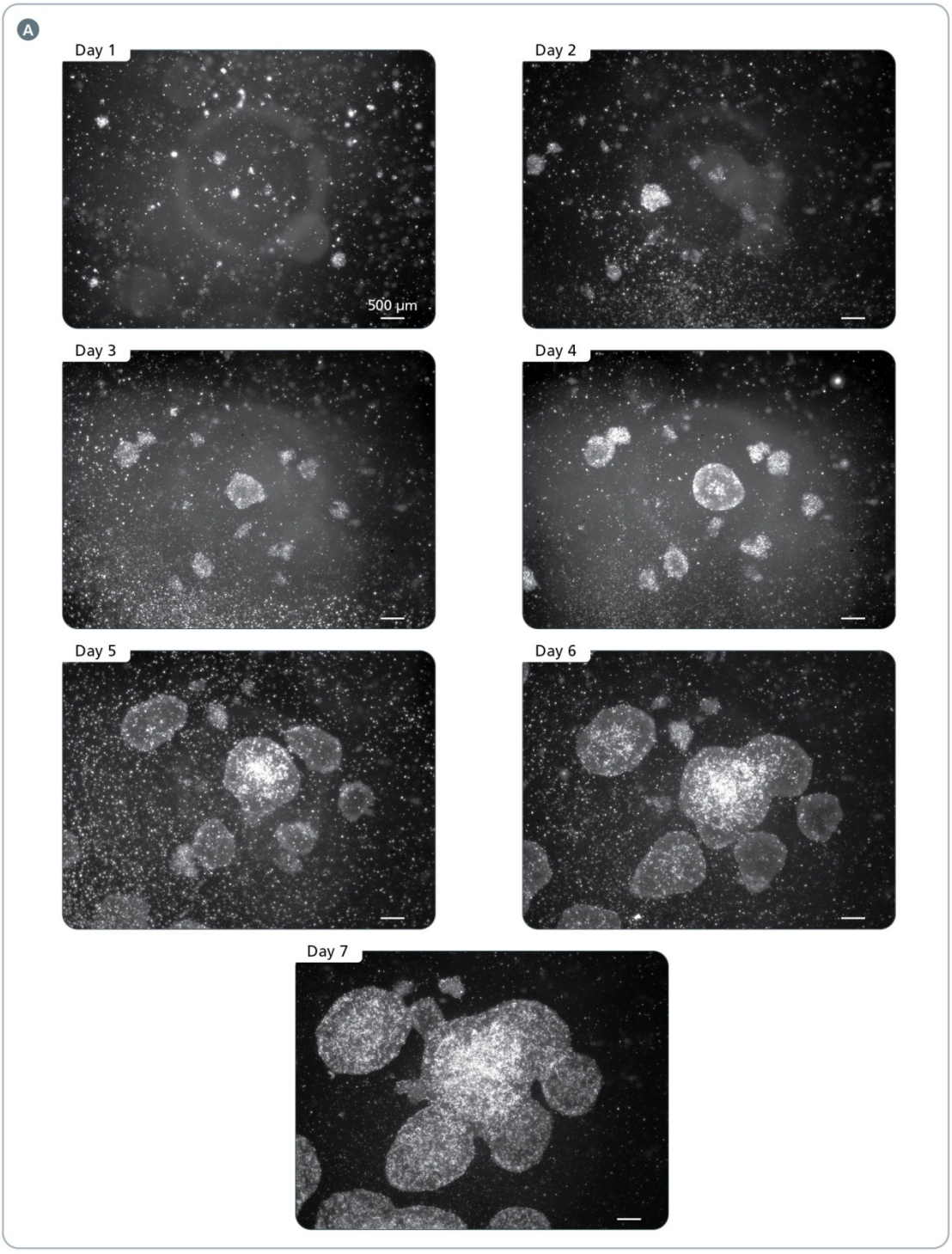


Figure 6. Human ES Cells Cultured on BD Matrigel™ Matrix in TeSR™-E8™ Medium at Days 1 - 7 after Passaging. Human ES cells (H1) taken using a magnification of 20X (A) and 40X (B). For this culture, Day 5 or 6 would be the optimal window for passaging. Note that the optimal day of passage for each culture will depend on seeding density and aggregate size used (see section 7.0).



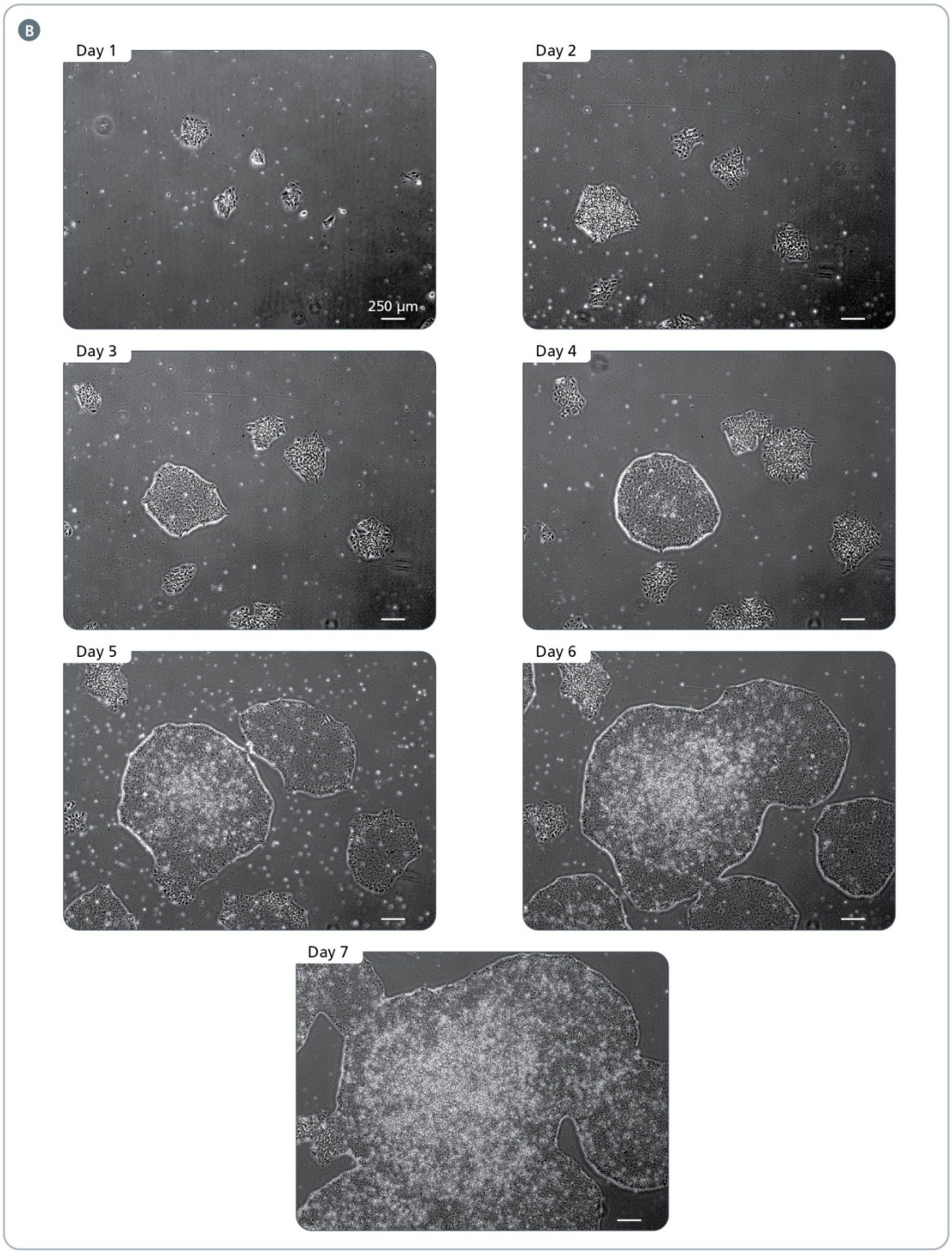
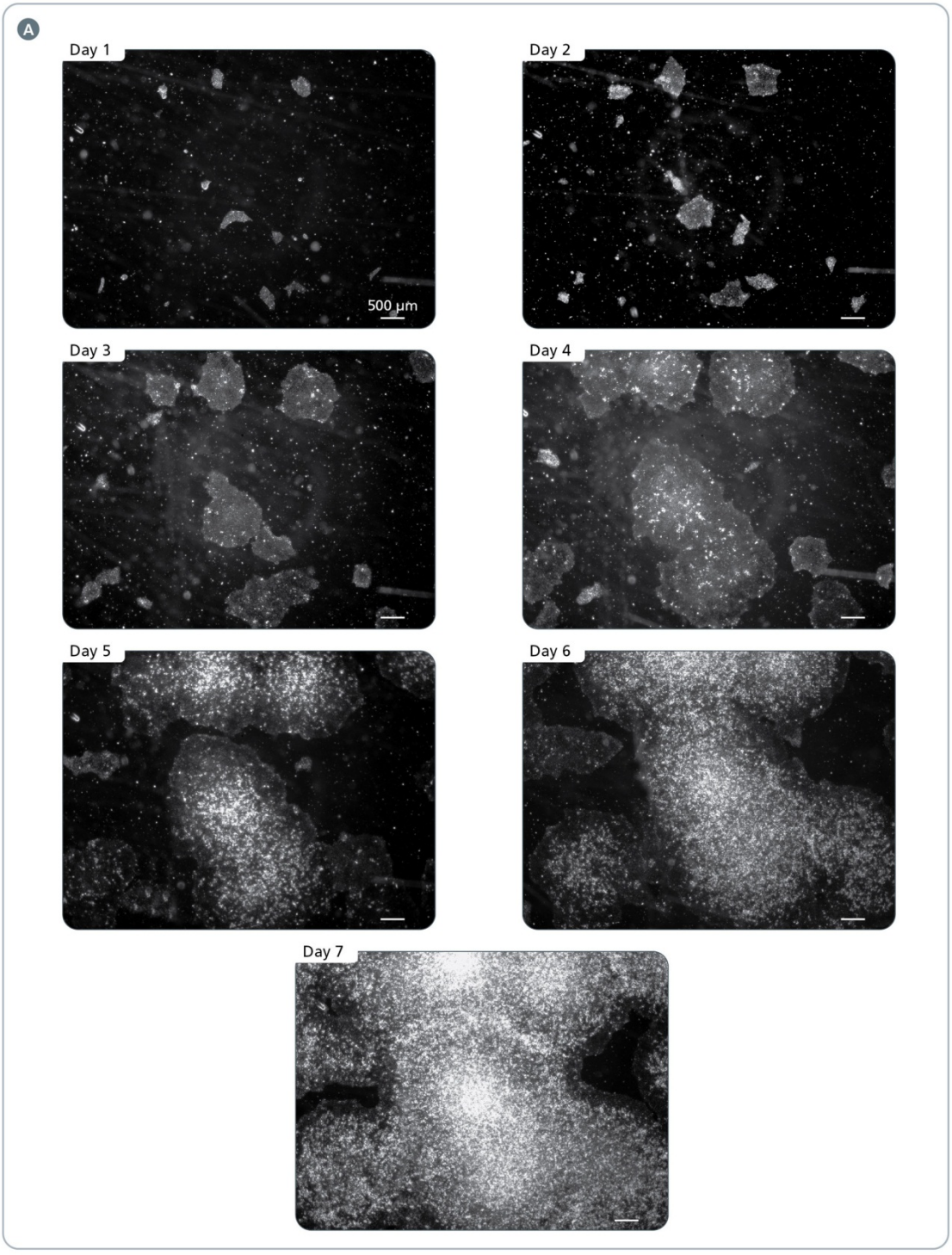


Figure 7. Human iPS Cells Cultured on Vitronectin XF™ Matrix in TeSR™-E8™ Medium at Days 1 - 7 after Passaging. Images of human iPS cells (WLS-1C) taken using a magnification of 20X (A) and 40X (B). For this culture, Day 6 or 7 would be the optimal window for passaging. Note that the optimal day of passage for each culture will depend on seeding density and aggregate size used (see section 7.0).



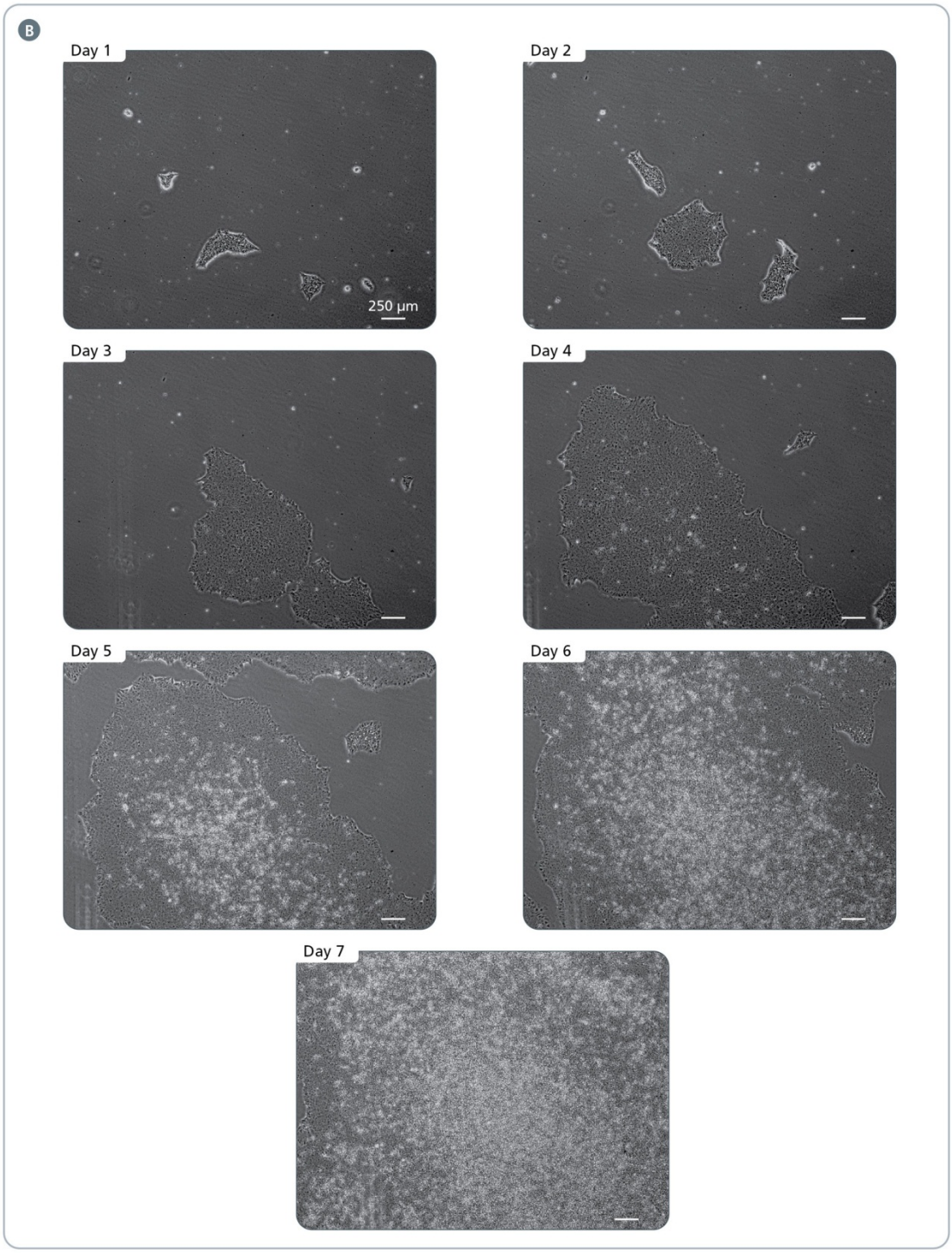


Figure 8. Human iPS Cells Cultured on BD Matrigel™ Matrix in TeSR™-E8™ Medium at Days 1 - 7 after Passaging. Images of human iPS cells (A13700) taken using a magnification of 20X (A) and 40X (B). For this culture, Day 6 would be the optimal time for passaging. Note that the optimal day of passage for each culture will depend on seeding density and aggregate size used (see section 7.0).

5.0 Preparation of Reagents and Materials

5.1 Preparing Complete TeSR™-E8™ Medium

Use sterile techniques when preparing complete TeSR™-E8™ medium. Instructions given in this section are for preparing 500 mL of TeSR™-E8™ medium.

Prepare complete TeSR™-E8™ medium in the TeSR™-E8™ Complete Medium Bottle. Do not use other containers. Store complete TeSR™-E8™ medium at 2 - 8°C and use within 2 weeks.

1. Thaw TeSR™-E8™ 20X Supplement and TeSR™-E8™ 500X Supplement at room temperature (15 - 25°C) or at 2 - 8°C just prior to use. Do not thaw supplements in a 37°C water bath.
2. Add the entire 474 mL of TeSR™-E8™ Basal Medium to the TeSR™-E8™ Complete Medium Bottle.
3. Pipette the entire 25 mL of thawed TeSR™-E8™ 20X Supplement and the entire 1 mL of thawed TeSR™-E8™ 500X Supplement to the TeSR™-E8™ Complete Medium Bottle, for a total volume of 500 mL. Mix well.

If prepared using sterile techniques, complete TeSR™-E8™ medium is ready for use and does not require filtering.

Complete TeSR™-E8™ medium (freshly prepared) may be stored at -20°C in the TeSR™-E8™ Complete Medium Bottle or alternatively aliquots can be stored in 50 mL polypropylene tubes (e.g. BD Catalog #352070) or Corning Square Polycarbonate Storage Bottles (Corning Catalog #431430 [125 mL]; #431431 [250 mL]). Store frozen medium for up to 1 month. Once thawed, use medium within 1 week. Do not refreeze medium.

5.2 Matrices for Coating Cultureware

Successful culture of human ES and iPS cells in TeSR™-E8™ requires the use of a suitable matrix to allow attachment of cell aggregates. Vitronectin XF™ (Catalog #07180) or BD Matrigel™ hESC-Qualified Matrix (BD, Catalog #354277) are recommended for use with TeSR™-E8™. Vitronectin XF™ recombinant protein matrix is recommended if a fully defined culture system is desired.

5.2.1 Vitronectin XF™

Use sterile techniques when coating cultureware with Vitronectin XF™.

Note: Use non-tissue culture-treated cultureware *(e.g. Non-Tissue Culture-Treated 6-well Plates; Catalog #27147).*

1. Thaw Vitronectin XF™ at room temperature (15 - 25°C).
2. Dilute Vitronectin XF™ in CellAdhere™ Dilution Buffer (Catalog #07183) to reach a final concentration of 10 µg/mL (i.e. use 40 µL of Vitronectin XF™ per mL of CellAdhere™ Dilution Buffer). Use a 50 mL polypropylene conical tube (e.g. BD Catalog #352070) to dilute the Vitronectin XF™.
3. Gently mix the diluted Vitronectin XF™. Do not vortex.
4. Immediately use the diluted Vitronectin XF™ solution to coat non-tissue culture-treated cultureware. See Table 1 for recommended coating volumes.

Table 1. Volumes Recommended for Coating Cultureware with Vitronectin XF™

NON-TISSUE CULTURE-TREATED CULTUREWARE	VOLUME OF DILUTED VITRONECTIN XF™
6-well plate	1 mL/well
100 mm dish	6 mL/dish
T-25cm^2 flask	3 mL/flask
T-75cm^2 flask	8 mL/flask

5. Gently rock the cultureware back and forth to spread the Vitronectin XF™ solution evenly across the surface.

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

   Note: If not used immediately, the cultureware must be sealed to prevent evaporation of the Vitronectin XF™ solution (e.g. with Parafilm®) and can be stored at 2 - 8°C for up to 1 week after coating. Allow stored coated cultureware to come to room temperature (15 - 25°C) for 30 minutes before moving on to the next step.
7. Gently tilt the cultureware onto one side and allow the excess Vitronectin XF™ solution to collect at the edge. Remove the excess solution using a serological pipette or by aspiration. Ensure that the coated surface is not scratched.
8. Wash the cultureware once using CellAdhere™ Dilution Buffer (e.g. use 2 mL/well if using a 6-well plate).
9. Aspirate wash solution and add TeSR™-E8™ medium (e.g. 2 mL/well if using a 6-well plate).

5.2.2 BD Matrigel™ hESC-Qualified Matrix Solution

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.

Note: Use tissue culture-treated cultureware *(e.g. 6-well plates, BD Catalog #353046).*

1. Thaw one aliquot of BD Matrigel™ on ice.
2. Dispense 25 mL of cold 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 2 for recommended coating volumes.

Table 2. Volumes Recommended for Coating Cultureware with BD Matrigel™

TISSUE CULTURE-TREATED CULTUREWARE	VOLUME OF DILUTED BD MATRIGEL™
6-well plate	1 mL/well
100 mm dish	6 mL/dish
T-25cm^2 flask	3 mL/flask
T-75cm^2 flask	8 mL/flask

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

   Note: If the cultureware's surface is not fully coated by the BD Matrigel™ solution, it should not be used for human ES or 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 at the edge. Remove the excess BD Matrigel™ solution using a serological pipette or by aspiration. Ensure that the coated surface is not scratched.
8. Immediately add TeSR™-E8™ medium (e.g. 2 mL/well if using a 6-well plate).

6.0 Passaging of Human ES and iPS Cells Grown in TeSR™-E8™

The following protocol is recommended when first culturing cells in TeSR™-E8™ medium. Once familiar with this protocol, it is possible to adjust the time at which cells are ready to be passaged by altering the cell aggregate size or plating density. For a description of how to customize the passaging protocol in this way, see section 7.0.

6.1 Passaging Protocol

This passaging protocol uses enzyme-free dissociation methods to remove colonies from the cultureware. The cell aggregates generated using this protocol may be fragile, and they should be replated as quickly as possible.

The following are instructions for use with 6-well plates. If using alternative cultureware, adjust volumes accordingly.

1. At least 1 hour before passaging, coat new plates with either Vitronectin XF™ (section 5.2.1) or BD Matrigel™ (section 5.2.2).
2. Aliquot sufficient complete TeSR™-E8™ medium and warm to room temperature (15 - 25°C).

   Note: Do not warm complete TeSR™-E8™ medium in a 37°C water bath.
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. Remove regions of differentiation by scraping with a pipette tip or by aspiration. Avoid having the culture plate out of the incubator for more than 15 minutes at a time.

   Note: Selection may not be required if differentiation is <5%. Selection should not exceed 20% of the well if the culture is of high quality. For representative pictures of regions of differentiation see Figure 1B, Figure 2B, Figure 3B and Figure 4B.
4. Aspirate medium from the well and add 1 mL/well of Gentle Cell Dissociation Reagent.
5. Incubate at room temperature (15 - 25°C). Refer to Table 3 for recommended incubation times.

Table 3. Gentle Cell Dissociation Reagent Incubation Times with Cultures Plated on Different Matrices

MATRIX	INCUBATION TIME WITH GENTLE CELL DISSOCIATION BUFFER	REPRESENTATIVE EXAMPLE
Vitronectin XF™	10 - 12 minutes	Figure 9
BD Matrigel™	6 - 8 minutes	Figure 10

Note: Incubation time is based on STEMCELL Technologies' Gentle Cell Dissociation Reagent. Ensure that you have optimized the exposure time to the enzyme-free dissociation reagent used; the appearance of colonies during dissociation should be as shown in the representative example. The incubation time may vary when using different cell lines or other non-enzymatic cell dissociation reagents, therefore dissociation should be monitored under the microscope until the optimal time is determined.

6. Aspirate the Gentle Cell Dissociation Reagent, and add 1 mL/well of TeSR™-E8™ medium.
7. Gently detach the colonies by scraping with a serological glass pipette or a cell scraper (e.g. Corning Catalog #3010 or Fisherbrand Catalog #08-100-240).

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

   Note: Centrifugation of cell aggregates is not required.

9. Carefully pipette the cell aggregate mixture up and down to break up the aggregates as needed. Refer to Table 4 for suggestions on how to break up the cell aggregates grown on different types of matrices. Do not create a single-cell suspension.

Table 4. Suggested Methods for Breaking Up Cell Aggregates

MATRIX	PIPETTE TYPE	TIMES TO PIPETTE UP AND DOWN*
Vitronectin XF™	1 mL Micropipette	1 - 2
BD Matrigel™	2 mL serological pipette	2 - 5

* Number can be adjusted to obtain desired cell aggregate size (see section 7.1). Refer to Figure 11 for examples of appropriate cell aggregate sizes and adjust procedure as necessary to achieve desired results.

10. Plate cell aggregate mixture onto pre-coated wells containing TeSR™-E8™ medium (2 mL/well) at the desired density. If the colonies are at an optimal density, the cultures can be split every 4 - 7 days using 1 in 10 to 1 in 40 splits (i.e. cell aggregates from 1 well can be plated in 10 to 40 wells). If the colonies are too dense or too sparse, at the next time of passaging adjust the split ratio accordingly (see section 7.0 for more details).

    Note: Work quickly to transfer cell aggregates into new cultureware to maximize viability and attachment.

11. Place the plate in a 37°C incubator, with 5% CO_2 and 95% humidity. Move the 6-well plate in several quick, short, back-and-forth and side-to-side motions to distribute the cell aggregates across the surface of the wells. Do not disturb the plate for 24 hours.

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

12. Perform daily medium changes and visually assess cultures to monitor growth and to determine timing of next passaging.

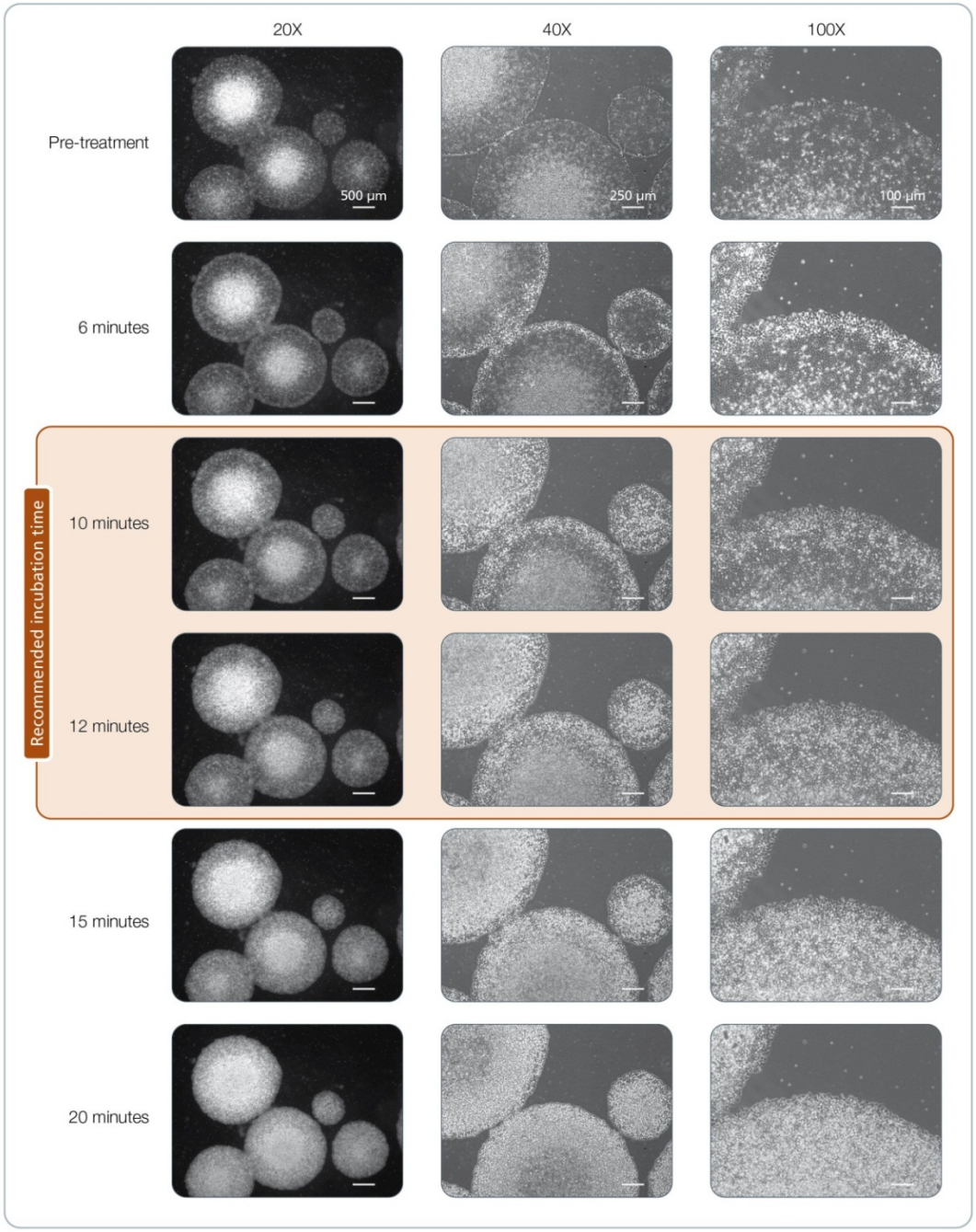


Figure 9. Effect of Gentle Cell Dissociation Reagent on Human ES Cells Cultured on Vitronectin XF™ Matrix in TeSR™-E8™ Medium. Human ES cells (H9) at different time points during incubation with the Gentle Cell Dissociation Reagent. Images were taken using three magnifications: 20X, 40X and 100X. Recommended incubation time (10 - 12 minutes) occurs when gaps appear between cells located on the edges of the colonies. At earlier time points the colonies are not sufficiently dissociated, whereas at later time points the colonies are excessively dissociated and may break up into unwanted single cells upon scraping. Note that the incubation time may vary when using different cell lines or other non-enzymatic cell dissociation reagents, therefore dissociation should be monitored under the microscope until the optimal time is determined.

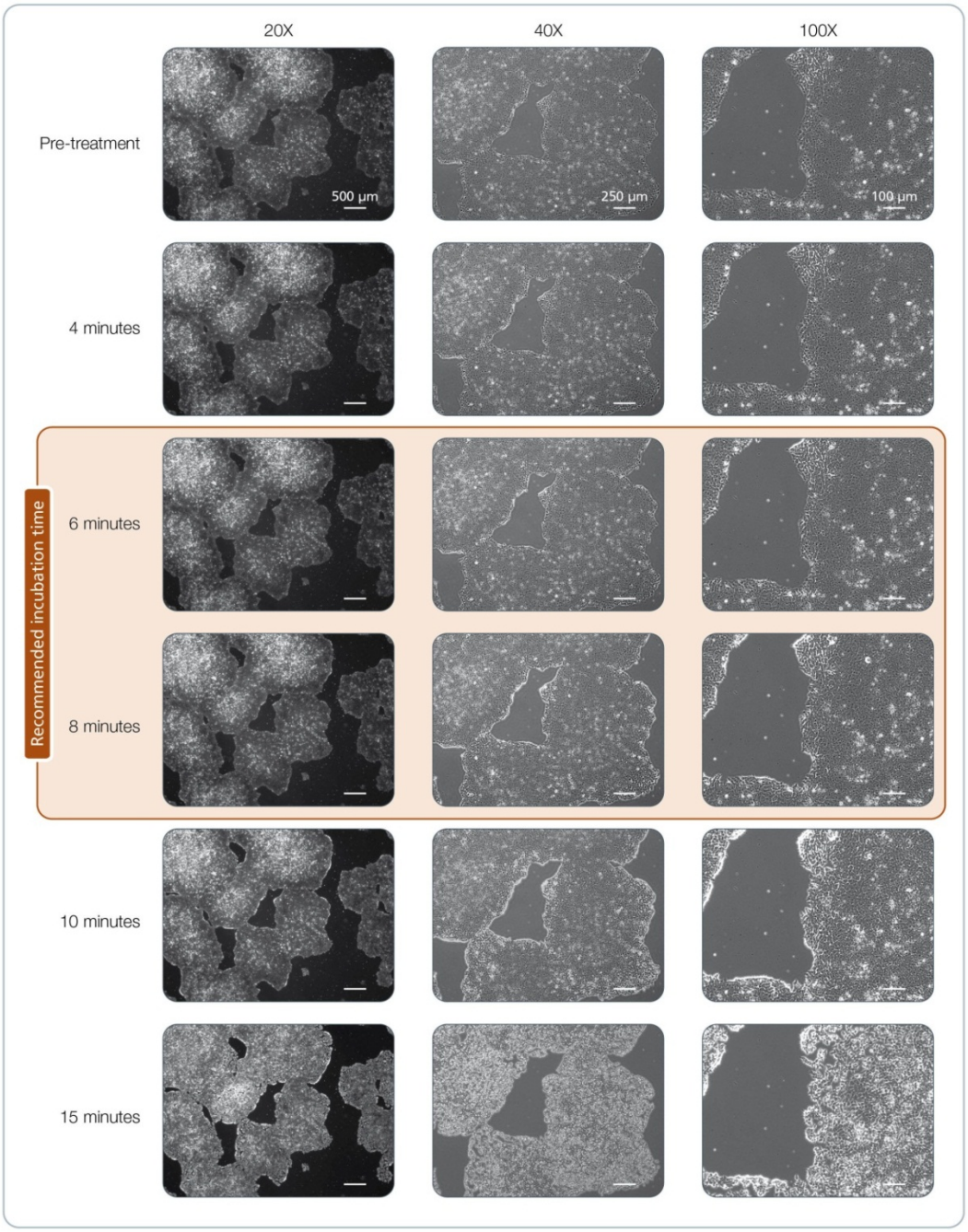


Figure 10. Effect of Gentle Cell Dissociation Reagent on Human iPS Cells Cultured on BD Matrigel™ Matrix in TeSR™-E8™ Medium. Human iPS cells (A13700) at different time points during incubation with the Gentle Cell Dissociation Reagent. Images were taken using three magnifications: 20X, 40X and 100X. Recommended incubation time (6 - 8 minutes) occurs when gaps are beginning to appear between cells located on the edges of the colonies. At earlier time points the colonies are not sufficiently dissociated, whereas at later time points the colonies are excessively dissociated and may break up into unwanted single cells upon scraping. Note that the incubation time may vary when using different cell lines or other non-enzymatic cell dissociation reagents, therefore dissociation should be monitored under the microscope until the optimal time is determined.

7.0 Customizing the Passaging Protocol

Culturing human ES and iPS cells in TeSR™-E8™ medium allows some flexibility in the passaging schedule, as cultures can be passaged between 4 and 7 days after plating in TeSR™-E8™. The next time the cells are ready for passaging depends on the size and density of the plated cell aggregates. For example, if large cell aggregates are plated at a high density, the next passaging time will most likely occur on day 4 or 5, whereas if small cell aggregates are plated at a low density, the next passaging will most likely occur on day 6 or 7 (Table 5). For representative images of large and small cell aggregates, see Figure 11. Regardless of the plating density and cell aggregate size used, the majority of colonies should be densely packed and multilayered in the center when ready for passage.

Table 5. Parameters that Affect Next Passaging Time

AT TIME OF PLATING		AT TIME OF PASSAGING	
CELL AGGREGATE SIZE*	PLATING DENSITY	COLONY DENSITY**	DAYS IN CULTURE
Large	High	High	4 - 5
Large	Low	Low	5 - 6
Medium	Medium	Medium	
Small	High	High	
Small	Low	Low	6 - 7

*For representative images see Figure 11
**For representative images see Figure 12

7.1 Cell Aggregate Size

At the time of plating, the desired cell aggregate size can be obtained by adjusting the number of times the cell aggregate suspension is pipetted up and down (Table 4). Refer to Figure 11 for a recommended range of cell aggregate sizes. Do not generate single cells.

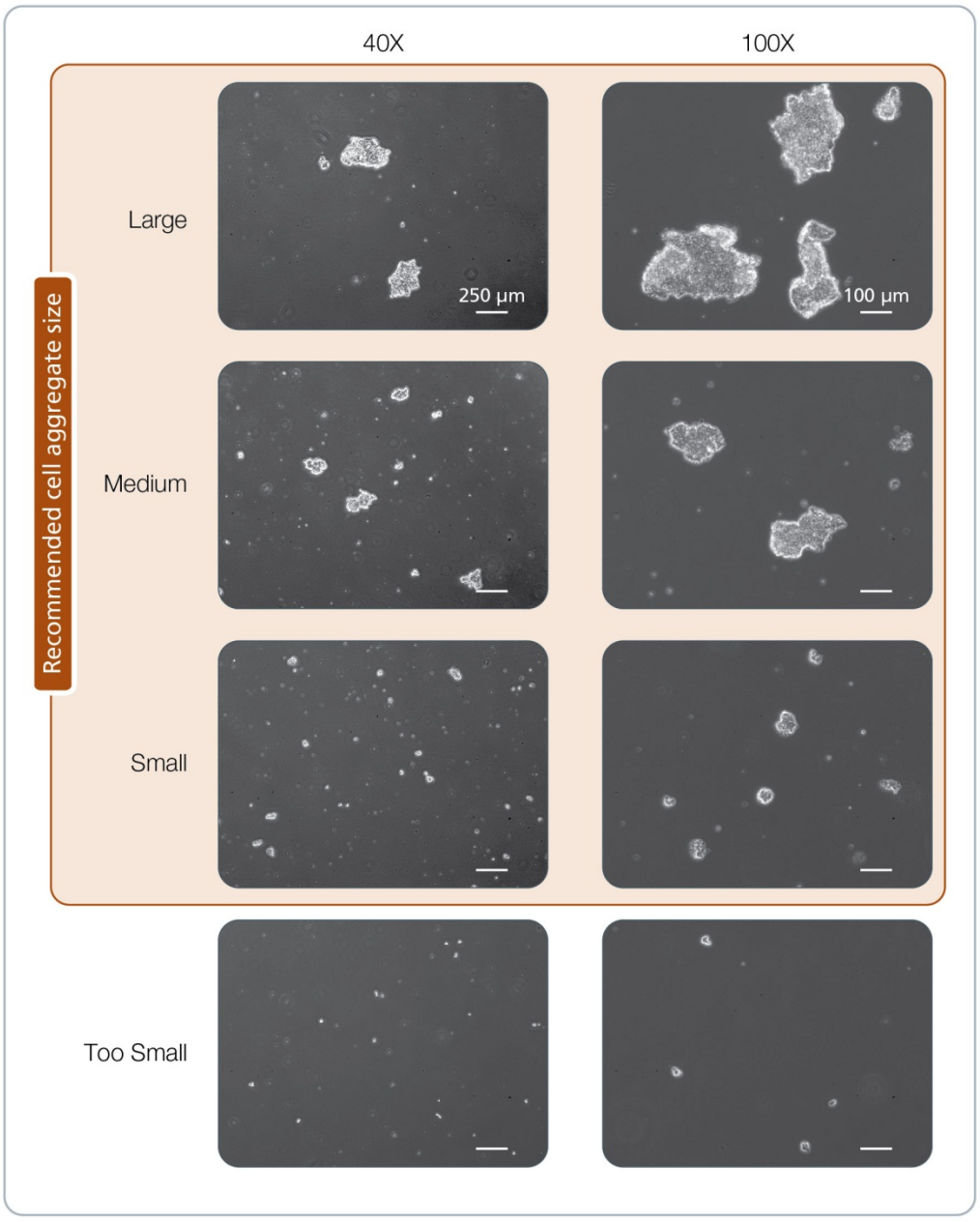


Figure 11. Acceptable Size Range for Cell Aggregates at the Time of Plating. Cell aggregate size can be adjusted by altering the number of times the cell aggregate mixture is pipetted up and down (see Table 4). Avoid generating single cells. Images taken using two magnifications: 40X and 100X.

7.2 Colony Density at Time of Passaging

The number of cell aggregates plated at the time of passaging is correlated to the colony density observed at the next time of passaging. By altering the split ratios at the time of plating you can increase or decrease the colony density as desired. For example, a lower split ratio at the time of plating will result in a higher colony density at the next passaging time. Typical split ratios are 1 in 10 to 1 in 40 however; this can vary depending on the cell line used and the individual operator. Refer to Figure 12 for a recommended range of colony densities that you should observe at the time of passage. Counting cell aggregates is an alternate way to determine and adjust plating densities (see Appendix 1).

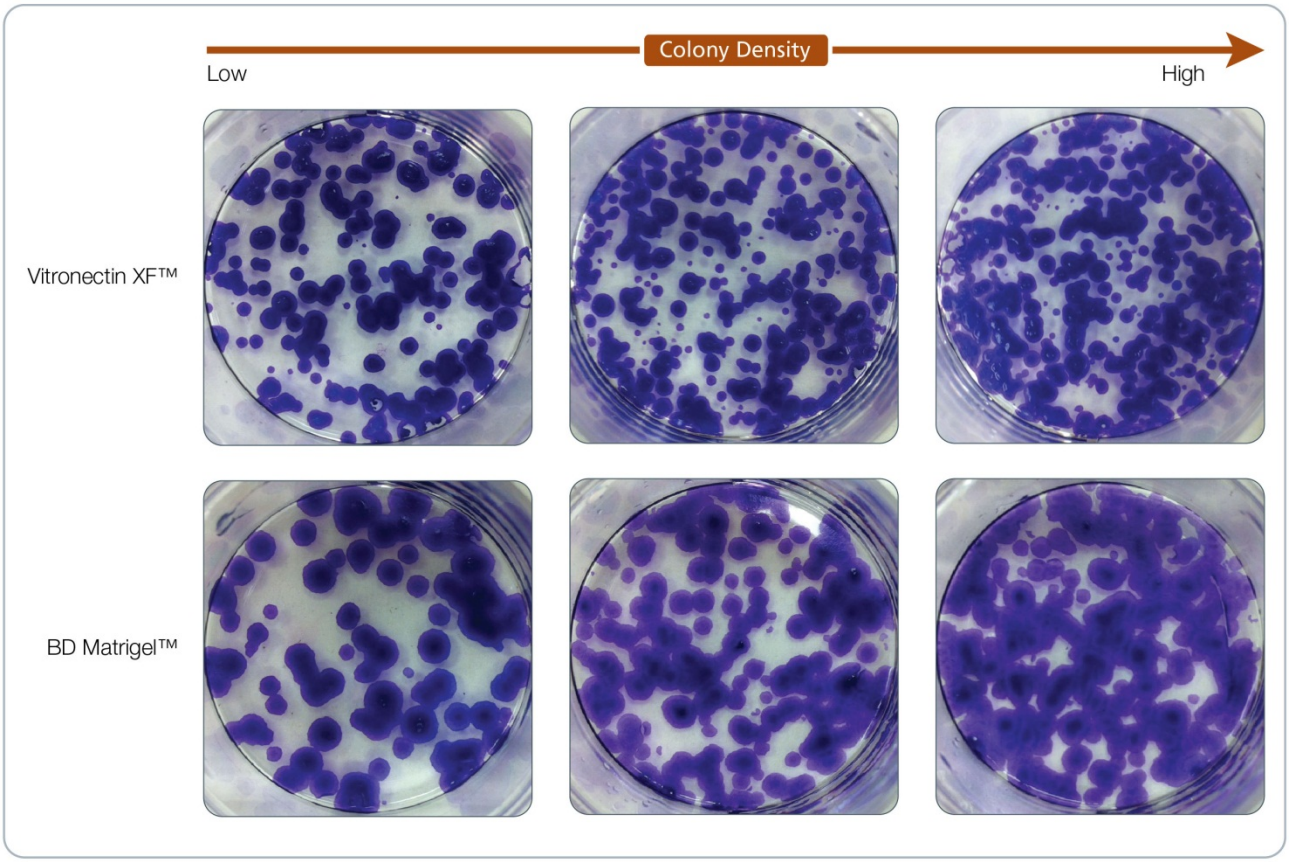


Figure 12. Acceptable Colony Densities for Cultures Grown on either Vitronectin XF™ or BD Matrigel™ in TeSR™-E8™ Medium. Human ES cells (H1) were cultured in TeSR™-E8™ medium and stained with Giemsa at the optimal time of passaging (note that the optimal time of passaging may be different for each colony density, see Table 5). Each image represents a single well of a 6-well plate.

8.0 Additional Protocols

8.1 Transitioning Cells from Feeder-Free Media to TeSR™-E8™

No adaptation step is required when plating human ES and iPS cells from mTeSR™1 (Catalog #05850), TeSR™2 (Catalog #05860), or other feeder-free maintenance medium to TeSR™-E8™ medium. Follow the enzyme-free passaging protocol from section 6.1 and replate cell aggregates on to pre-coated cultureware containing TeSR™-E8™ medium. Use of enzymatic passaging protocols is not recommended when transitioning cells from feeder-free media to TeSR™-E8™. It is recommended that a culture using the previous feeder-free medium and culture system is initially maintained in parallel to ensure that the chosen plating density in TeSR™-E8™ is appropriate.

8.2 Transitioning Cells from TeSR™-E8™ to mTeSR™1 or TeSR™2

No adaptation step is required when plating human ES and iPS cells from TeSR™-E8™ to mTeSR™ or TeSR™2 media. Follow the enzyme-free passaging protocol from section 6.1 and replate cell aggregates on to pre-coated cultureware containing mTeSR™1 or TeSR™2 media. Do not use enzymatic passaging protocols.

8.3 Transitioning Cells Plated on BD Matrigel™ to Vitronectin XF™

Human ES and iPS cells cultured on BD Matrigel™ in TeSR™-E8™ medium may be conveniently transitioned to Vitronectin XF™. When cells are ready for passage, follow the protocol in section 6.1 as though you were to plate cells back onto BD Matrigel™ (i.e. use incubation times recommended for BD Matrigel™ in Table 3), however, ensure that the cell aggregates are plated on Vitronectin XF™-coated cultureware (see section 5.2.1). The passaging protocol for cells grown on Vitronectin XF™ may then be used for subsequent passages.

Note that both the media and the matrix can be changed at the same time (e.g. cells cultured on BD Matrigel™ using mTeSR™1 medium may be transitioned onto Vitronectin XF™ with TeSR™-E8™ in one step, see section 8.1).

8.4 Transitioning Cells Cultured on a Feeder Layer to TeSR™-E8™

Human ES and iPS cells cultured on a layer of feeder cells may be conveniently transferred to feeder-free conditions with TeSR™-E8™. Cell aggregates can be harvested using protocols established in your institute for feeder-dependent cells and plated on the desired matrix in TeSR™-E8™ medium. Alternatively use the protocol below. Plating efficiency can be affected during the transition, therefore initial plating of cell aggregates at 2 - 3 times higher density than routinely used for passaging may improve adaptation of cells to feeder-free conditions.

The following are instructions for use with 6-well plates. If using alternative cultureware, adjust volumes accordingly.

1. At least 1 hour before passaging, coat new plates with either Vitronectin XF™ (section 5.2.1) or BD Matrigel™ (section 5.2.2).
2. Aliquot sufficient complete TeSR™-E8™ medium, DMEM/F-12 and Collagenase Type IV (Catalog #07909). Warm to room temperature (15 - 25°C).

   Note: Do not warm complete TeSR™-E8™ medium in a 37°C water bath.
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. Remove regions of differentiation by scraping with a pipette tip or by aspiration.

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

4. Aspirate medium from the well and add 1 mL/well of collagenase.
5. Incubate at 37°C for 20 minutes.

   Note: Incubation time is based on STEMCELL Technologies' Collagenase Type IV. If using another collagenase, incubation times may need to be adjusted.
6. Aspirate the collagenase, and wash twice with 1 mL of DMEM/F-12.
7. Aspirate wash medium and add 1 mL/well of TeSR™-E8™ medium.
8. Gently detach the colonies by scraping with a serological glass pipette or a cell scraper (e.g. Corning Catalog #3010 or Fisherbrand Catalog #08-100-240).
9. Transfer the detached cell aggregates to a 15 mL conical tube.

   Optional: Rinse the well with an additional 1 - 2 mL of TeSR™-E8™ to collect remaining cell aggregates. Add the rinse to the 15 mL tube.
10. Carefully pipette the cell aggregate mixture up and down 2 - 3 times with a 2 mL serological pipette to break up the cell aggregates. A uniform suspension of aggregates approximately 100 µm in size is optimal; do not create a single-cell suspension. For representative examples of cell aggregates refer to Figure 11 (medium and large).
11. Plate cell aggregate mixture onto pre-coated wells containing TeSR™-E8™ medium (2 mL/well) at appropriate density.

    Note: Feeder cells will continue to be present in the first 1 - 2 passages after transition, but should not persist beyond passage 2 following transfer. It is recommended that a culture grown on a layer of feeder cells is initially maintained in parallel to ensure that the chosen plating density in TeSR™-E8™ is appropriate.

8.5 Cryopreserving Cells Using Cryostor®CS10

CryoStor®CS10 (Catalog #07930) is an animal protein-free freezing medium. It is ready to use and contains cryoprotectant agents.

The following are instructions for cryopreserving cultures grown in TeSR™-E8™ medium in 6-well plates. Cultures should be frozen when ready for passaging. Each vial should contain the cell aggregates from one well of a 6-well plate. If using alternative cultureware, adjust volumes accordingly

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.
2. Remove regions of differentiation by scraping with a pipette tip or by aspiration.

   Note: This selection should not exceed 20% of the well if the culture is of high quality. For representative pictures of regions of differentiation see Figure 1B, Figure 2B, Figure 3B and Figure 4B.
3. Aspirate medium from the well and add 1 mL/well of Gentle Cell Dissociation Reagent.
4. Incubate at room temperature (15 - 25°C). Refer to Table 6 for recommended incubation times.

Table 6. Gentle Cell Dissociation Reagent Incubation Times with Cultures Plated on Different Matrices

MATRIX	INCUBATION TIME WITH GENTLE CELL DISSOCIATION BUFFER	REPRESENTATIVE EXAMPLE
Vitronectin XF™	10 - 12 minutes	Figure 9
BD Matrigel™	6 - 8 minutes	Figure 10

Note: Incubation time is based on STEMCELL Technologies' Gentle Cell Dissociation Reagent. Ensure that you have optimized the exposure time to the enzyme-free dissociation reagent used; the appearance of colonies during dissociation should be as shown in the representative example. The incubation time may vary when using different cell lines or other non-enzymatic cell dissociation reagents, therefore dissociation should be monitored under the microscope until the optimal time is determined.

5. Aspirate the Gentle Cell Dissociation Reagent, and add 1 mL/well of TeSR™-E8™ or DMEM/F12.
6. Gently detach the colonies by scraping with a cell scraper (e.g. Corning Catalog #3010 or Fisherbrand Catalog #08-100-240).

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

   Optional: Rinse each well with an additional 2 mL of TeSR™-E8™or DMEM/F12 to collect remaining cell aggregates. Add the rinse to the 15 mL tube.
8. Centrifuge at 300 x *g* for 5 minutes at room temperature (15 - 25°C).

   Note: Prepare and label cryovials while the tube is centrifuging.
9. Gently aspirate the supernatant taking care not to disrupt the cell pellet.
10. Gently resuspend the pellet with cold (2 - 8°C) CryoStor®CS10 using a serological pipette. Minimize the break-up of cell aggregates when dislodging the pellet.

    Note: 1 mL of CryoStor®CS10 should be used for each well of a 6-well plate being frozen.
11. Transfer 1 mL of cell aggregates in CryoStor®CS10 into each labeled cryovial using a 2 mL pipette.
12. Freeze cell aggregates using either:
    - a standard slow rate controlled cooling protocol that reduces temperatures at approximately -1°C/min, followed by long-term storage at -135°C (liquid nitrogen) or colder. Long-term storage at -80°C is not recommended.
    - a multi-step protocol where cells are kept at -20°C for 2 hours, followed by -80°C for 2 hours, followed by long-term storage at -135°C (liquid nitrogen) or colder.

8.6 Thawing Cryopreserved Cells

This protocol is for thawing human ES and iPS cells that were maintained in TeSR™-E8™ medium prior to cryopreservation. Cells cultured using other maintenance protocols (e.g. on a layer of feeders or their conditioned medium, or feeder-free media such as mTeSR™1 or TeSR™2) should be thawed into the same medium and conditions used prior to cryopreservation. Once they have recovered from the thaw, cells can be transitioned into TeSR™-E8™ (see section 8.1 and 8.4).

Human ES and iPS cells should be thawed into previously-coated cultureware (see section 5.2). Generally, one vial of cells cryopreserved as described in section 8.5 can be successfully thawed into 2 - 4 wells of a 6-well plate, as described below. If the cells have been cryopreserved using other methods, this may vary.

1. Have all tubes, warmed TeSR™-E8™ (15 - 25°C) and pre-coated plates (see section 5.2) ready before starting the protocol to ensure that the thawing procedure is done as quickly as possible.
2. Quickly thaw the cells in a 37°C water bath by gently shaking the cryovial continuously until only a small frozen cell pellet remains.
3. Remove the cryovial from the water bath and wipe it with 70% isopropanol to sterilize.
4. Use a 2 mL serological pipette to transfer the contents of the cryovial to a 15 mL conical tube.

   Note: Using a 2 mL serological pipette instead of a 1 mL micropipette will minimize breakage of cell aggregates.
5. Add 5 - 7 mL of warm TeSR™-E8™ dropwise to the 15 mL 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. Gently resuspend the cell pellet in 1 mL of TeSR™-E8™ using a 2 mL serological pipette. Take care to maintain the cells as aggregates.

8. Transfer 0.5 mL of TeSR™-E8™/cell mixture per well of a pre-coated 6-well plate.

   Note: Number of wells plated may need to be adjusted depending on how many cell aggregates were cryopreserved. Typically many more aggregates will need to be plated after thawing than during routine passaging.

9. Place the plate in a 37°C incubator, with 5% CO_2 and 95% humidity. Move the 6-well plate in several quick, short, back-and-forth and side-to-side motions to distribute the cell aggregates across the surface of the wells. Do not disturb the plate for 24 hours.

   Note: Ensure that cell aggregates are evenly distributed between wells. Uneven distribution may result in differentiation of human ES and iPS cells.

10. Perform daily medium changes. Check for undifferentiated colonies that are ready to be passaged (dense centered) approximately 6 - 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 (i.e. without splitting) on a newly coated cultureware plate.

8.7 Preparation of a Single-Cell Suspension for Downstream Applications

1. Warm medium (DMEM/F-12 or TeSR™-E8™) to room temperature (15 - 25°C) before use.
2. 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	6 mL/dish

3. 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. Optimal exposure time may also vary for different cell lines.

4. Harvest cells by pipetting up and down with either a serological pipette or a 1 mL micropipette 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 TeSR™-E8™) and add the rinse to the tube containing the cells.
5. Centrifuge cells at 300 x *g* for 5 minutes.
6. Resuspend cells in appropriate medium for desired downstream applications.

9.0 Troubleshooting

PROBLEM	SOLUTION
Excessive (>20%) differentiation in cultures	• Ensure the freshly prepared complete TeSR™-E8™ medium kept at 2 - 8°C is less than 2 weeks old and is stored in the provided TeSR™-E8™ Complete Medium Bottle. If frozen complete medium is thawed, use within 1 week. • Ensure areas of differentiation are removed prior to passaging. • Avoid having the culture plate out of the incubator for more than 15 minutes at a time. • Ensure that the cell aggregates generated after passaging are evenly sized. • Do not warm complete TeSR™-E8™ medium in a 37°C water bath.
Low attachment observed after plating	• Plate a higher number of cell aggregates initially (e.g. 2 - 3 time higher numbers) and maintain a more densely confluent culture. • Work quickly after cells are treated with Gentle Cell Dissociation Reagent to minimize the duration that cell aggregates are in suspension. • Reduce incubation time with the Gentle Cell Dissociation Reagent during passaging, as your cell line/culture may be more sensitive. This is particularly important if cells are passaged prior to cell multilayering within the colony. • Do not excessively pipette up and down to break up cell aggregates to reach the desired size. Instead, increase the incubation time with the Gentle Cell Dissociation Reagent by 1 - 2 minutes. This is particularly important if colonies are very dense and cell aggregates are difficult to break up. • Ensure that non-tissue culture-treated plates are used when coating with Vitronectin XF™. • Ensure that tissue culture-treated plates are used when coating with BD Matrigel™.
Single cells are generated during colony dissociation	• Work quickly after cells are treated with Gentle Cell Dissociation Reagent to minimize the duration that cell aggregates are in suspension. • Reduce incubation time with the Gentle Cell Dissociation Reagent during passaging, as your cell line/culture may be more sensitive. This is particularly important if cells are passaged prior to cell multilayering within the colony. • Minimize the manipulation of cell aggregates after dissociation.
Cells do not adhere to the coated cultureware	• Avoid using enzymatic dissociation reagents (e.g. dispase). • Ensure that non-tissue culture-treated plates are used when coating with Vitronectin XF™. • Ensure that tissue culture-treated plates are used when coating with BD Matrigel™.
Cells detach during Day 1 medium change	• Gently change medium during cell feeding. • Start changing medium on Day 2 after initial plating of cells to allow cell aggregates to fully attach.
Low cell expansion	• Allow cells to culture longer prior to passaging. The majority of cell expansion occurs just prior to optimal passage points.
Non-uniform cell aggregate attachment	• Ensure that the well is completely covered with the culture matrix during the coating step. • Ensure that cell aggregates are evenly distributed throughout the well when placing in the incubator and that the plate is not disturbed for 24 hours after plating.
Significant scraping is required to dislodge cells	• Ensure that the Gentle Cell Dissociation Reagent is being used as described in section 6.1. • Adjust incubation time with the Gentle Cell Dissociation Reagent, to determine optimal conditions for your cell line. • Wash cells with Gentle Cell Dissociation Reagent prior to dissociation step.
Cell aggregates are very large after the scraping step during the passaging protocol	• Increase the incubation time with the Gentle Cell Dissociation Reagent. • Increase pipetting up and down of the cell aggregates. • Add a wash step using calcium-free PBS before adding the Gentle Cell Dissociation Reagent.

10.0 References

1. Chen G, et al. Chemically defined conditions for human iPSC derivation and culture. Nat Methods 8(5): 424-429, 2011
2. Beers J, et al. Passaging and colony expansion of human pluripotent stem cells by enzyme-free dissociation in chemically defined culture conditions. Nat Protoc 7(11): 2029-40, 2012
3. Ludwig TE, et al. Derivation of human embryonic stem cells in defined conditions. Nat Biotechnol 24(2): 185-187, 2006
4. Ludwig TE, et al. Feeder-independent culture of human embryonic stem cells. Nat Methods 3(8): 637-646, 2006

Appendix 1: Plating Human ES and iPS Cells Using the Cell Aggregate Count Method

Counting cell aggregates is an alternate way to determine and adjust plating densities; it allows a more controlled way to plate an appropriate number of cell aggregates at the time of passaging. This can be a valuable learning tool for those new to human ES and iPS cell culture.

In the protocol below, count cell aggregates ≥60 µm in diameter, as these are the most likely to attach and grow into healthy colonies. An eyepiece micrometer can help to identify cell aggregates of this size. The following protocol should be carried out during the passaging at the time of plating cell aggregates (i.e. on step 10 in section 6.1).

1. Draw a "+" centered on the bottom of 2 wells of a 96-well flat-bottom plate to serve as a counting grid.
2. Aliquot 40 µL of DMEM/F-12 (Catalog #36254) into each well.
3. Add 5 µL of a freshly resuspended cell aggregate mixture to each well.
4. Count the cell aggregates in each well that are approximately ≥60 µm in diameter. Average the results from the two wells to obtain the average number of cell aggregates (N_A) in the 5µL sample.
5. Calculate the concentration of cell aggregates (C) and the total number of cell aggregates in the mixture (N_T) using the total volume of the mixture (V_T):

$$C = \frac{N_A}{5\ \mu L}$$

$$N_T = C \times V_T$$

6. Determine the target number of cell aggregates to plate (N_P, refer to Table 7). Ensure that the total target number of cell aggregates to plate for all conditions in your experiment (i.e. N_P x number of conditions) does not exceed N_T.

Table 7. Number of Cell Aggregates Recommended to Plate

CULTUREWARE	TARGET # OF CELL AGGREGATES TO PLATE (N_P, PLATING DENSITY)		
	LOW	MEDIUM	HIGH
1 well of a 6-well plate	350	700	1000
100 mm dish	2100	4200	6000
T-75cm^2 flask	2800	5600	8000

7. Calculate the volume of cell aggregate mixture to plate (V_P) for each condition in your experiment:

$$V_P = \frac{N_P}{C}$$

8. Gently mix the cell aggregate mixture prior to plating to ensure a uniform suspension.
9. Add calculated volume of cell aggregate mixture (V_P) on to pre-coated wells containing TeSR™-E8™ medium.
10. Continue with steps 11 and 12 of the passaging protocol (see section 6.1).

Appendix 2: Flow Cytometry Protocols

Reagents and Materials

Antibodies

Antibodies can be used to characterize human ES and iPS cells by flow cytometry. The tables below contain information about a selection of antibodies available from STEMCELL Technologies that can be used to characterize human ES and iPS cells. For a complete list of antibodies, visit our website at www.stemcell.com.

Indirect Surface Antigen Labeling

PRIMARY ANTIBODY*	CATALOG #	SHORT DESCRIPTION	RECOMMENDED SECONDARY ANTIBODY*	CATALOG #
Anti-Mouse SSEA-1 Antibody, Clone MC-480	60060	Mouse monoclonal IgM antibody against human, mouse SSEA-1, unconjugated	Anti-mouse IgM Antibody, FITC-conjugated	10211
Anti-Mouse SSEA-3 Antibody, Clone MC-631	60061	Rat monoclonal IgM antibody against human, mouse, rhesus SSEA-3, unconjugated	Anti-rat IgM Antibody, APC-conjugated	10215
Anti-Human SSEA-4 Antibody, Clone MC-813-70	60062	Mouse monoclonal IgG3 antibody against human, mouse, rhesus SSEA-4, unconjugated	Anti-mouse IgG (H+L) Antibody, FITC-conjugated	10210
Anti-Human TRA-1-60 Antibody, Clone TRA-1-60R	60064	Mouse monoclonal IgM antibody against human, rhesus, rabbit TRA-1-60, unconjugated	Anti-mouse IgM Antibody, FITC-conjugated	10211
Anti-Human TRA-1-81 Antibody, Clone TRA-1-81	60065	Mouse monoclonal IgM antibody against human, rhesus TRA-1-81, unconjugated	Anti-mouse IgM Antibody, FITC-conjugated	10211
Anti-Human TRA-2-49 Antibody, Clone TRA-2-49/6E	60066	Mouse monoclonal IgG1 antibody against human, chimpanzee, gibbon TRA-2-49, unconjugated	Anti-mouse IgG (H+L) Antibody, FITC-conjugated	10210
Anti-Human TRA-2-54 Antibody, Clone TRA-2-54/2J	60067	Mouse monoclonal IgG1 antibody against human, chimpanzee, gibbon TRA-2-54, unconjugated	Anti-mouse IgG (H+L) Antibody, FITC-conjugated	10210

*Optimal working dilutions of the primary and secondary antibodies should be determined by the end user.

Direct Surface Antigen Labeling

DIRECTLY CONJUGATED ANTIBODY*	CATALOG #	SHORT DESCRIPTION
Anti-Mouse SSEA-1 Antibody, Clone MC-480, Alexa Fluor® 488	60060AD 60060AD.1	Mouse monoclonal IgM antibody against human, mouse SSEA-1, Alexa Fluor® 488-conjugated
Anti-Mouse SSEA-1 Antibody, Clone MC-480, PE	60060PE 60060PE.1	Mouse monoclonal IgM antibody against human, mouse SSEA-1, PE-conjugated
Anti-Mouse SSEA-3 Antibody, Clone MC-631, Alexa Fluor® 488	60061AD 60061AD.1	Rat monoclonal IgM antibody against human, mouse, rhesus SSEA-3, Alexa Fluor® 488-conjugated
Anti-Mouse SSEA-3 Antibody, Clone MC-631, PE	60061PE 60061PE.1	Rat monoclonal IgM antibody against human, mouse, rhesus SSEA-3, PE-conjugated
Anti-Human SSEA-4 Antibody, Clone MC-813-70, Alexa Fluor® 488	60062AD 60062AD.1	Mouse monoclonal IgG3 antibody against human, mouse, rhesus SSEA-4, Alexa Fluor® 488-conjugated
Anti-Human SSEA-4 Antibody, Clone MC-813-70, PE	60062PE 60062PE.1	Mouse monoclonal IgG3 antibody against human, mouse, rhesus SSEA-4, PE-conjugated
Anti-Human TRA-1-60 Antibody, Clone TRA-1-60R, Alexa Fluor® 488	60064AD 60064AD.1	Mouse monoclonal IgM antibody against human, rhesus, rabbit TRA-1-60, Alexa Fluor® 488-conjugated
Anti-Human TRA-1-60 Antibody, Clone TRA-1-60R, PE	60064PE 60064PE.1	Mouse monoclonal IgM antibody against human, rhesus, rabbit TRA-1-60, PE-conjugated
Anti-Human TRA-1-81 Antibody, Clone TRA-1-81, PE	60065PE 60065PE.1	Mouse monoclonal IgM antibody against human, rhesus TRA-1-81, PE-conjugated

*Optimal working dilutions of the directly conjugated antibody should be determined by the end user.

Intracellular Antigen Labeling

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

General Reagents and Materials

REAGENTS AND MATERIALS	CATALOG #
D-PBS (without Mg++ and 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

Additional Reagents Required for Intracellular Antigen Labeling

Saponin Permeabilization Buffer (SPB)*

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

*Mix well and store at 2 - 8°C.

Preparation of a Single-Cell Suspension for Flow Cytometry

1. Warm medium (DMEM/F-12 or TeSR™-E8™) to room temperature (15 - 25°C) before use.
2. 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	6 mL/dish

3. 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. Optimal exposure time may also vary for different cell lines.
4. Harvest cells by pipetting up and down with either a serological pipette or a 1 mL micropipette 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 TeSR™-E8™) and add the rinse to the tube containing the cells.
5. Centrifuge cells at 300 x *g* for 5 minutes.
6. Resuspend cells in medium and perform a viable cell count using Trypan Blue.
7. The single-cell suspension may now be used for surface antigen and/or intracellular antigen labeling (see below for detailed protocols).

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 or the directly conjugated antibody mix (100 µL/sample) using the appropriate 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 to each tube.
    - If using a primary antibody, gently mix and centrifuge at 300 x *g* for 5 minutes. While the samples are centrifuging, make a sufficient quantity of the secondary antibody mix (100 µL/sample) using the appropriate secondary antibody at the predetermined optimal working dilution. Continue to step 7.
    - If using a directly conjugated antibody, gently mix and centrifuge at 300 x *g* for 5 minutes. Continue to step 9.
7. 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.
8. Add 1 mL of 2% FBS/PBS to each tube. Gently mix and centrifuge at 300 x *g* for 5 minutes.
9. Carefully remove the supernatant without disturbing the cell pellet and resuspend the cells in 200 - 300 µL of 2% FBS/PBS. Transfer to a 5 mL FACS tube if necessary.

    Optional: Propidium iodide (PI) can be added at a final concentration of 1 µg/mL to assess viability.
10. Place samples on ice and analyze by flow cytometry as soon as possible.

Intracellular Antigen Labeling Protocol for Oct-3/4

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 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 SPB as the diluent.

    Note: The suggested working dilution of the Oct-3/4 primary antibody (Catalog # 01550) 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 SPB as the diluent.

    Note: When using Oct-3/4 primary antibody (Catalog # 01550) 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 of 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 13.

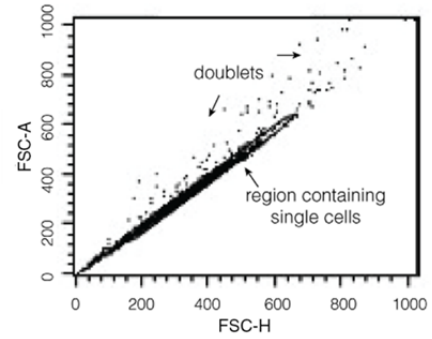


Figure 13. An Example of Doublet Discrimination by Flow Cytometry

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