TECHNICAL MANUAL

Maintenance of Human Pluripotent Stem Cells in TeSR™2

Critical Parameters for Successful Cell Culture with TeSR™2

Choosing an Appropriate Matrix for Use With TeSR™2

Cells may be cultured in TeSR™2 using either Vitronectin XF™ or Corning® Matrigel® as the surface coating matrix. Corning® Matrigel® is a routinely used surface coating matrix for a variety of applications, although the composition is undefined. In contrast, Vitronectin XF™ consists of a single humanized protein that is fully defined. Vitronectin XF™ is recommended for applications where a fully defined culture system is desired. For coating cultureware with these matrices, see section 4.2.

Choosing an Appropriate Passaging Reagent for Use With TeSR™2

Cells cultured in TeSR™2 can be passaged using either enzyme-free (section 5.1) or enzymatic (section 5.2) passaging protocols depending on the matrix used (Table 2). Enzyme-free methods are recommended for their ease of use, high cell recovery, and for preserving the integrity of cell surface proteins that aid in the reattachment of cells to the matrix.

Among the enzyme-free passaging reagents, ReLeSR™ allows the quickest and most straightforward protocol for routine passaging of human ES and iPS cells as cell aggregates (section 5.1.1). With any passaging protocol, ensure that you have optimized the exposure time to the passaging reagent used; the appearance of colonies after dissociation should be as shown in Figure 9 (ReLeSR™), Figure 10 & 11 (Gentle Cell Dissociation Reagent), or Figure 12 (Dispase). The incubation time may vary when using different cell lines or other cell passaging reagents; dissociation should be monitored under the microscope until the optimal time is determined.

Aggregate vs. Single-Cell Passaging

The protocols in this manual for the routine expansion of human ES and iPS cells are for passaging cells as small aggregates of approximately 50 - 200 µm in diameter. These established methods have been shown to allow the expansion of many different cell lines for > 20 passages while maintaining a normal karyotype. It is possible to passage human ES and iPS cells as single cells; however, it has been demonstrated that this practice can place unwanted selective pressure on cell populations and could lead to genetic aberrations in the culture.[5,6] Therefore, if you perform single-cell passaging of human ES or iPS cells in any culture medium, check the karyotype frequently to ensure that the culture has retained a normal karyotype.

Characterization of Human ES and iPS Cells

It is good practice to monitor your cultures frequently to ensure maintenance of undifferentiated cells and a normal karyotype. Flow cytometry protocols for assessing the proportion of undifferentiated cells are described 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.[5,6,8] 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. More frequent screening for recurrent abnormalities commonly associated with ES/iPS cell culture can be performed using tools such as the hPSC Genetic Analysis Kit (Catalog #07550).

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™2. Cultures that are too densely populated can lead to undesired spontaneous differentiation of cells. A range of colony densities can be tolerated in TeSR™2, as shown in Figure 14; it is recommended to adjust plating density to maintain the culture at the desired confluence (i.e. increase/decrease split ratio). By altering the colony density parameter, the optimal day of passage may be influenced (section 6.0 and Appendix 1). Some cell lines may show increased spontaneous differentiation when cultured at higher densities; if increased differentiation is observed, the colony density may be decreased in the next passage by plating fewer cell aggregates.

Medium Changes

Human ES and iPS cells generally require daily medium changes for optimal growth; however, occasional double feeding (adding twice the required volume of medium during one feed) is possible without affecting culture quality. For instance, it is possible to perform a double feed on a Friday, with the next medium change on Sunday. It is not recommended to double feed more than once per passage. It is also possible to maintain high-quality cultures without feeding on weekends (i.e. skipping two days of feeding) using a specific protocol where feeds are reduced soon after passaging when the cell colonies are small and require fewer nutrients. For details of this protocol, see the Technical Bulletin: Weekend-Free Culture of Human Pluripotent Stem Cells in mTeSR™1 or TeSR™-E8™ (Document #28071), available at www.stemcell.com or contact us to request a copy. This document describes comprehensive results with related TeSR™ media.

Transitioning Cells Cultured in TeSR™-E8™ to TeSR™2

Human ES and iPS cells cultured in TeSR™-E8™ can be conveniently transferred to TeSR™2 (section 7.1.1). However, cells may experience an increase in spontaneous differentiation in the first 1 - 2 passages after transfer. Removal of differentiated regions either manually or using specialized dissociation reagents such as ReLeSR™ (section 5.1.1) will help ensure that the culture quickly adapts to the new environment without affecting the long-term health of the culture.

Transitioning Cells Cultured in Other Feeder-Free Media to TeSR™2

Human ES and iPS cells cultured in mTeSR™1, mTeSR™ Plus, or other feeder-free media can be conveniently transferred to TeSR™2 (section 7.1.1). Cells should transition smoothly into TeSR™2 with minimal differences in morphology, spontaneous differentiation, or growth rate. Adjust the split ratio as required if small changes in growth rate or confluency are observed.

Transitioning Cells Cultured on a Feeder Layer to TeSR™2

Human ES and iPS cells cultured on a layer of feeder cells can be conveniently transferred to TeSR™2 (section 7.1.4). Cells should adapt to feeder-free culture within 1 - 2 passages and thereafter exhibit morphology consistent with feeder-free human pluripotent stem cells.

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1.0 Introduction

The maintenance and expansion of human pluripotent stem cells (human embryonic stem [ES] cells and human induced pluripotent stem [iPS] cells) in feeder-free conditions requires the use of highly specialized media formulations, in combination with careful handling techniques, to maintain high-quality cultures at each passage. Use of feeder-free conditions eliminates the inherent biological variability of feeder cells, an undefined component of the culture system. Recent efforts have focused on improving the reproducibility of human ES and iPS cell culture protocols by simplifying and removing undefined components from all aspects of the culture system: media, matrices, and passaging reagents.

TeSR™2 is a defined, xeno- and feeder-free maintenance medium for human ES and iPS cells that is based on publications by Dr. James Thomson's lab.[1-4] This medium is capable of maintaining high-quality human ES and iPS cells when used with Corning® Matrigel® as the matrix, or alternatively with Vitronectin XF™ (developed and manufactured by Nucleus Biologics) if a fully defined culture system is desired.[7] Additionally, both enzyme-free and enzymatic passaging protocols are compatible with TeSR™2 cultures.

STEMCELL Technologies Inc. has developed both mTeSR™1 and TeSR™2 based on publications by Dr. James Thomson's lab.[3,4] mTeSR™1 is the most widely used feeder-free medium with more than 1500 publications and has been used to successfully maintain hundreds of different human ES and iPS cell lines. Protocols have been published for numerous applications including bioreactor expansion, single-cell cloning, and lineage-specific differentiation of cells maintained in mTeSR™1. For a complete list of publications, visit www.stemcell.com/mTeSR1publications. Closely related to mTeSR™1, TeSR™2 combines the advantages of mTeSR™1 with the added value of being free of xenogenic components.

Human ES and iPS cells maintained in TeSR™2 have been shown to:

- Be phenotypically homogeneous and karyotypically normal
- Express high levels of multiple genes associated with undifferentiated pluripotent stem cells (e.g. OCT4, SSEA-3, SSEA-4, TRA-1-60, TRA-1-81, and NANOG)
- Form teratomas containing derivatives of endo-, meso-, and ectodermal lineages
- Undergo directed differentiation to mature cell types from all three germ layers (i.e. mesoderm, endoderm, and ectoderm)
- Require no adaptation period (i.e. no period of low cell yield) when transferred from feeder-based culture
- Be compatible with bioreactor and suspension cultures

2.0 Materials, Reagents, and Equipment

2.1 TeSR™2

PRODUCT	COMPONENT NAME	COMPONENT #	SIZE	STORAGE	SHELF LIFE
TeSR™2 Complete Kit (Catalog #05860)	TeSR™2 Basal Medium	05861	400 mL	Store at 2 - 8°C.	Stable until expiry date (EXP) on label.
	TeSR™2 5X Supplement	05862	100 mL	Store at -20°C.	Stable until expiry date (EXP) on label.
	TeSR™2 250X Supplement	05863	2 mL	Store at -20°C.	Stable until expiry date (EXP) on label.
TeSR™2 Complete Kit, 10 Pack (Catalog #05880)	TeSR™2 Basal Medium	05861	10 x 400 mL	Store at 2 - 8°C.	Stable until expiry date (EXP) on label.
	TeSR™2 5X Supplement	05862	10 x 100 mL	Store at -20°C.	Stable until expiry date (EXP) on label.
	TeSR™2 250X Supplement	05863	10 x 2 mL	Store at -20°C.	Stable until expiry date (EXP) on label.

2.2 Additional Materials for Human ES and iPS Cell Culture

CATEGORY	PRODUCT	CATALOG #
Culture Media	mTeSR™1	85850
	mTeSR™ Plus	05825
	TeSR™-E8™	05990
Passaging Reagents	ReLeSR™	05872
	Gentle Cell Dissociation Reagent	07174
	Dispase (1 U/mL)	07923
Matrices and Related Materials	Vitronectin XF™ Kit with GCDR	07190
	Vitronectin XF™ Kit with ReLeSR™	07191
	Vitronectin XF™	07180
	CellAdhere™ Dilution Buffer	07183
	Non-tissue culture-treated 6-well plates*	27147 or 38040
	Corning® Matrigel® hESC-Qualified Matrix	07181
	Tissue culture-treated cultureware**	e.g. 38016 (6-well plates)
Cryopreservation Media	Cryostor® CS10	07930
	FreSR™-S	05859
Other Materials	DMEM/F-12 with 15 mM HEPES	36254
	Cell scrapers	e.g. 38065
	15 mL conical tubes	e.g. 38009
	D-PBS (Without Ca++ and Mg++)	37350
	Y-27632 (ROCK inhibitor)	72302
	Trypan Blue	07050
	CloneR™	05888
	hPSC Genetic Analysis Kit	07550

*Required for use with Vitronectin XF™.
**Required for use with Corning® Matrigel®.

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

2.3 Equipment Required for Human ES and iPS Cell Culture

- Biohazard safety cabinet 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 with a swinging bucket rotor
  Note: All centrifugation protocols described in this manual can be performed with the brake on.
- Pipette-Aid with appropriate serological pipettes
- Pipettor (e.g. Catalog #38058) with appropriate tips
- Inverted microscope with a total magnification of 20X to 100X
- Isopropanol freezing container (e.g. Fisher Catalog #1535050)
- -150°C freezer or liquid nitrogen (LN_2) vapor tank
- -80°C freezer and -20°C freezer
- Refrigerator (2 - 8°C)

3.0 Culturing Human ES and iPS Cells Using TeSR™2

Culture of human ES and iPS cells in TeSR™2 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™2

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

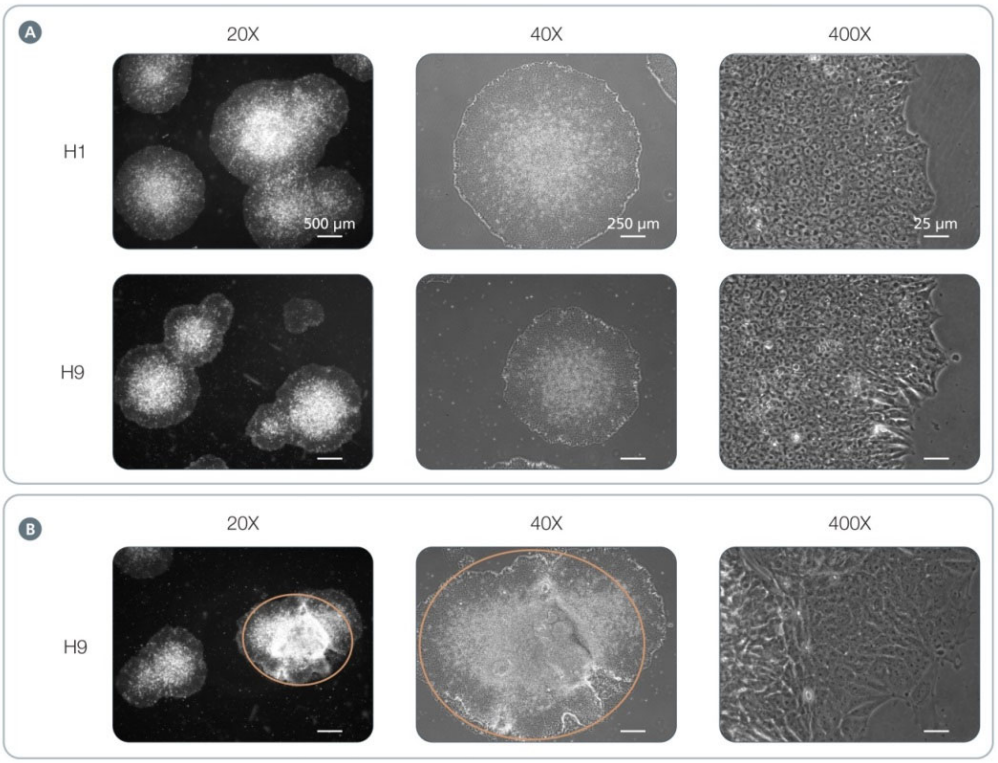


Figure 1. Morphology of Human ES Cells Cultured on Vitronectin XF™ in TeSR™2

(A) Undifferentiated human ES cells (H1 and H9) at the optimal time of passaging. (B) Area of spontaneous differentiation (orange circle) in H9 colonies. Magnifications: 20X, 40X, and 400X.

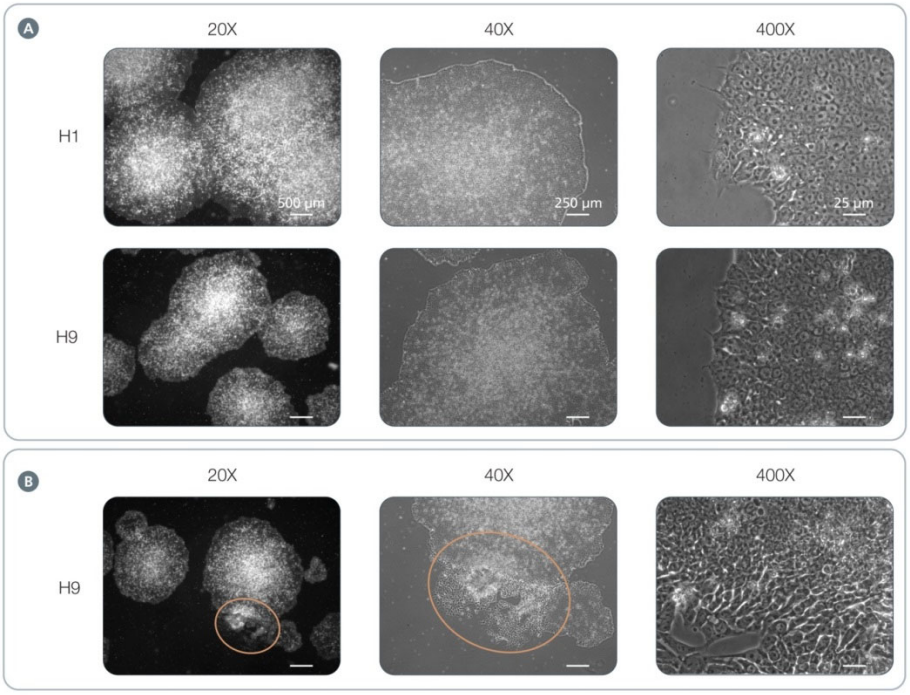


Figure 2. Morphology of Human ES Cells Cultured on Corning® Matrigel® in TeSR™2
(A) Undifferentiated human ES cells (H1 and H9) at the optimal time of passaging. (B) Area of spontaneous differentiation (orange circle) at the border of an undifferentiated H9 colony. Magnifications: 20X, 40X, and 400X.

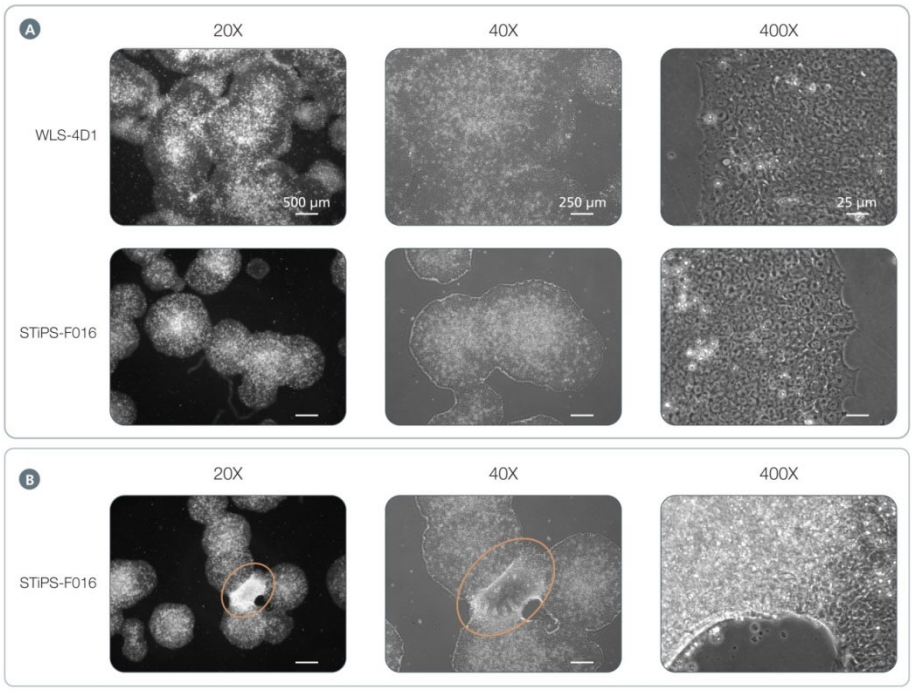


Figure 3. Morphology of Human iPS Cells Cultured on Vitronectin XF™ in TeSR™2
(A) Undifferentiated human iPS cells (WLS-4D1 and STiPS-F016) at the optimal time of passaging.
(B) Area of spontaneous differentiation (orange circle) between undifferentiated STiPS-F016 colonies.
Magnifications: 20X, 40X, and 400X.

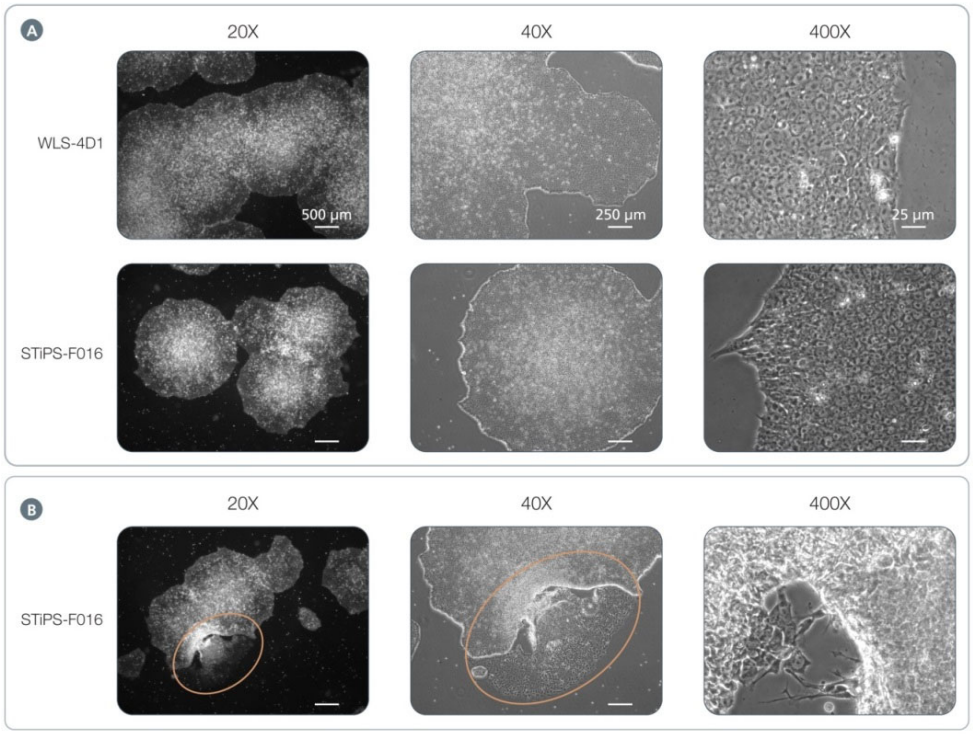
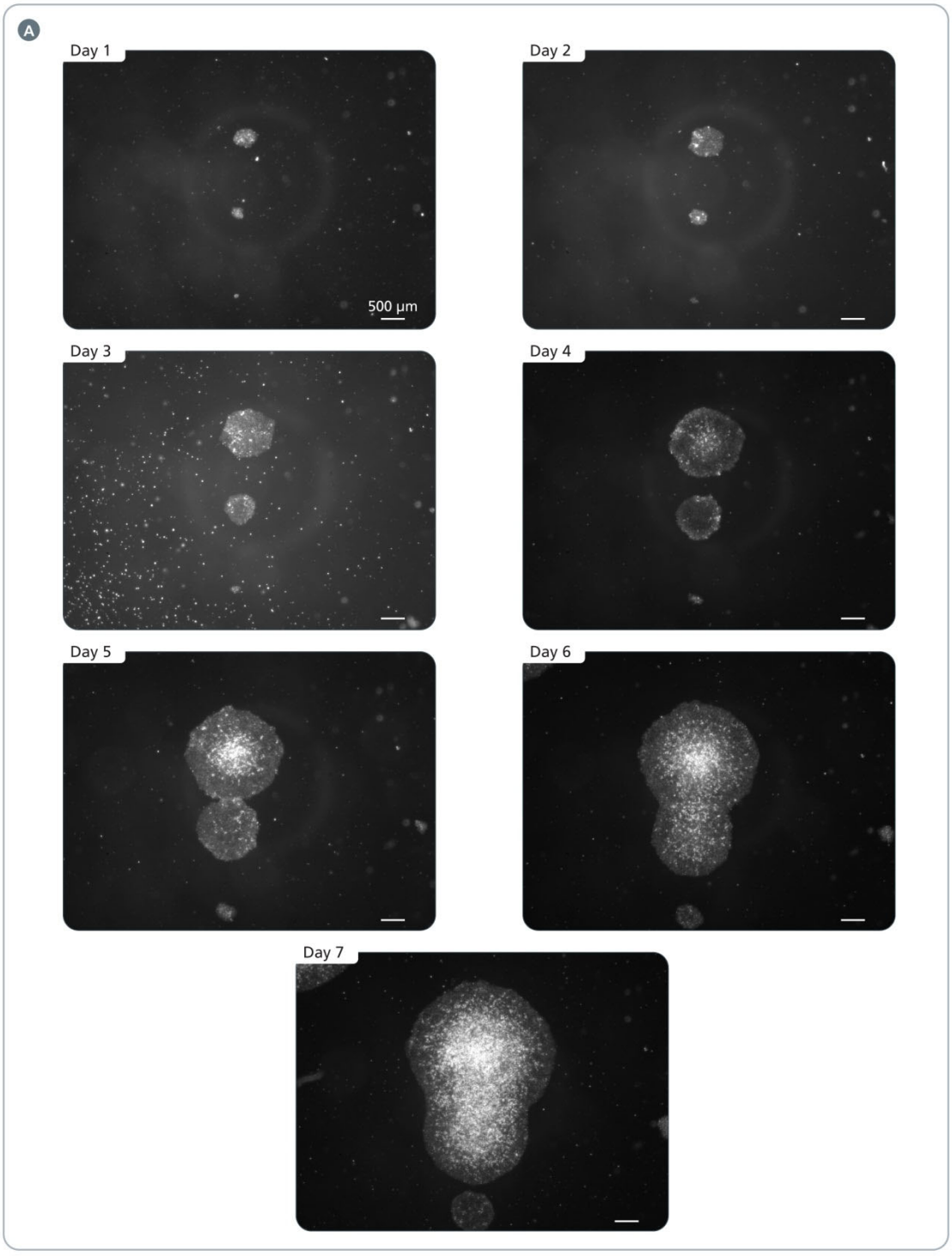


Figure 4. Morphology of Human iPS Cells Cultured on Corning® Matrigel® in TeSR™2
(A) Undifferentiated human iPS cells (WLS-4D1 and STiPS-F016) at the optimal time of passaging.
(B) Areas of spontaneous differentiation (orange circle) at the border of an undifferentiated STiPS-F016 colony.
Magnifications: 20X, 40X, and 400X.

3.2 Assessing TeSR™2 Cultures to Determine Day of Passage

Human ES and iPS cells cultured in TeSR™2 are ready to passage when the majority of colonies are large, compact, and have centers that are dense compared to their edges (Figures 5 - 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™2, colonies may not be very densely packed with cells. The density and robustness of the colonies increases rapidly after this timepoint 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- to 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 8.0).



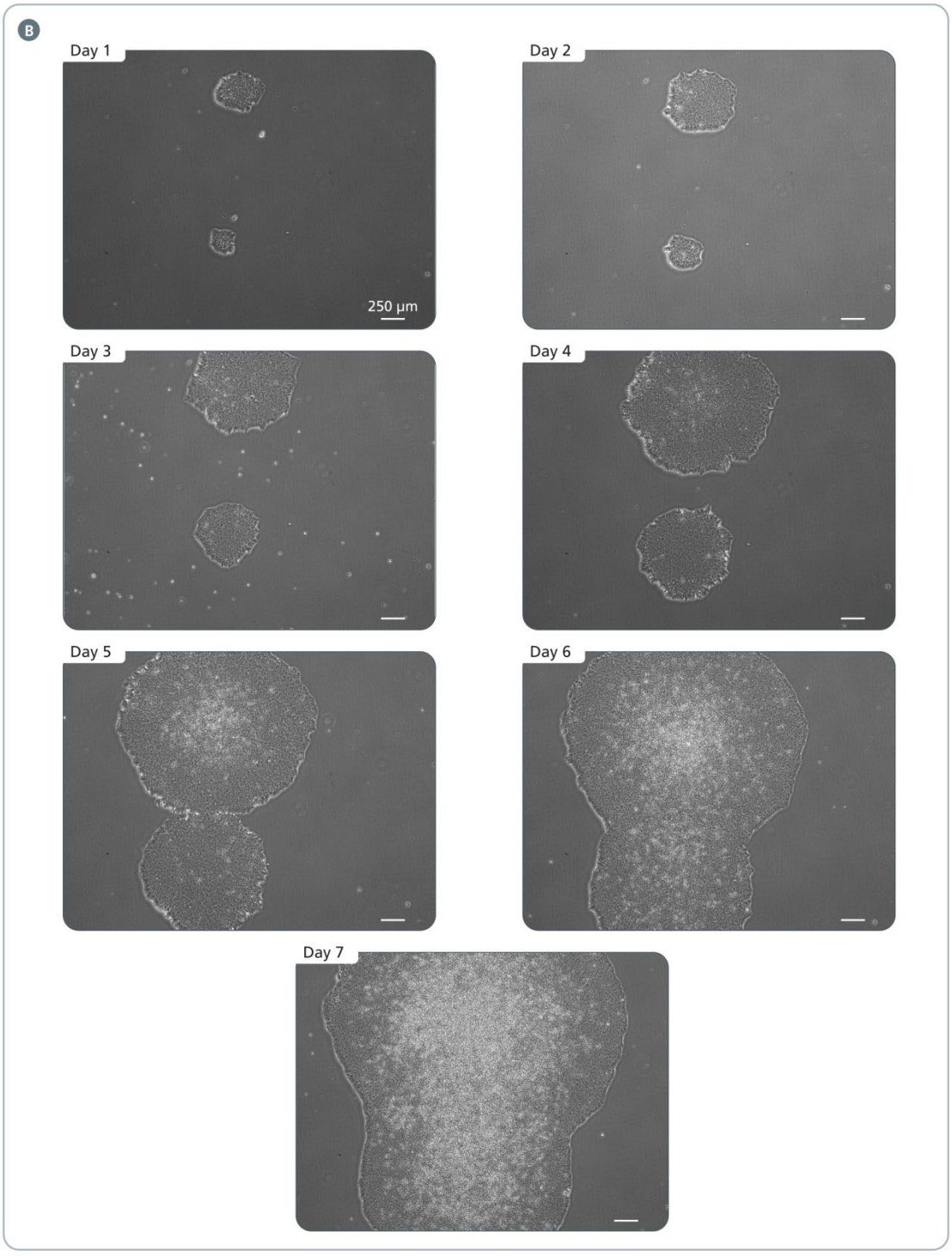
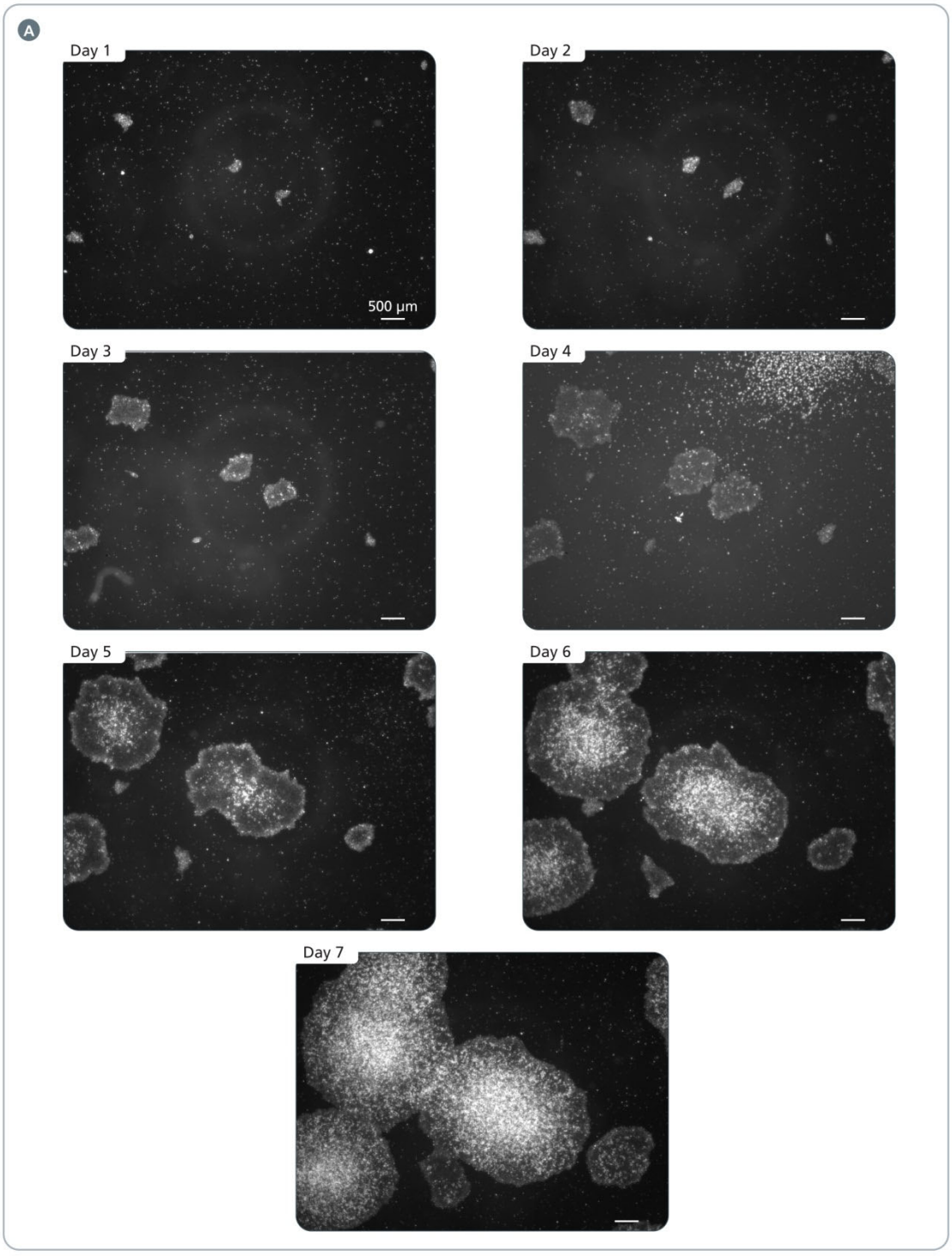


Figure 5. Human ES Cells Cultured on Vitronectin XF™ in TeSR™2 at Days 1 - 7 After Passaging
Human ES cells (H1) at a magnification of (A) 20X and (B) 40X. For this culture, Day 7 would be the optimal time for passaging. The optimal day of passage for each culture will depend on plating density (section 6.2) and aggregate size used (section 6.1).



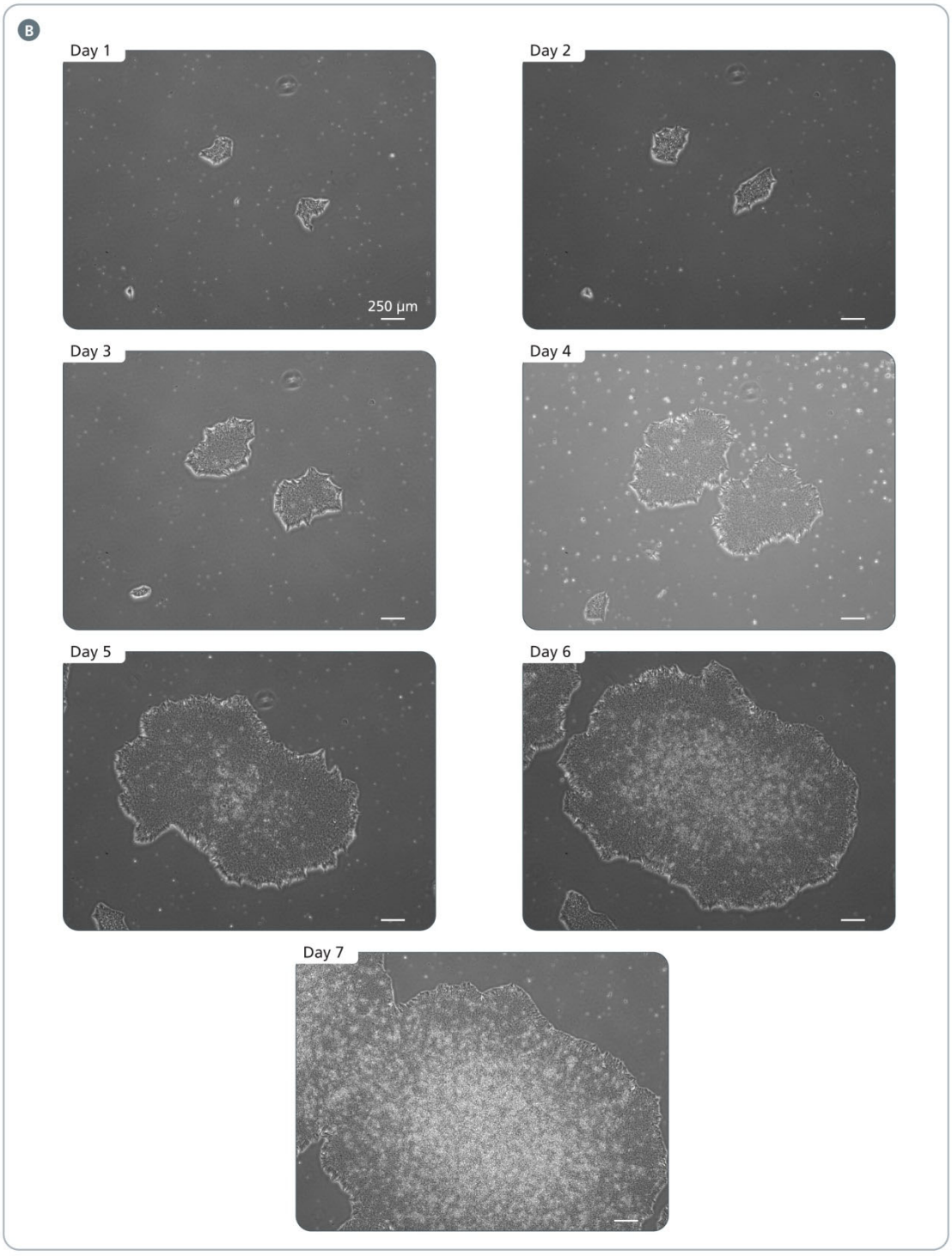
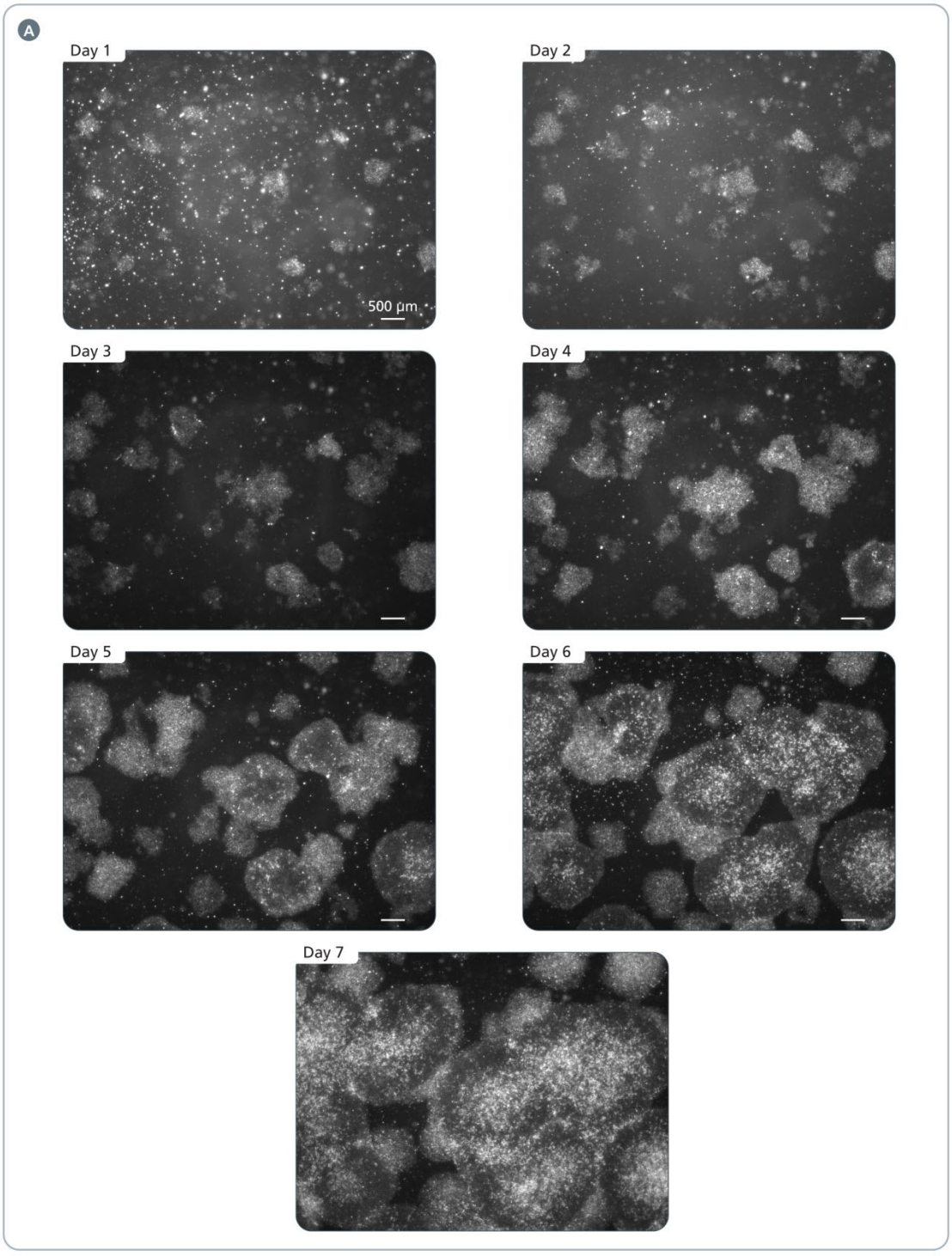


Figure 6. Human ES Cells Cultured on Corning® Matrigel® in TeSR™2 at Days 1 - 7 After Passaging
Human ES cells (H9) at a magnification of (A) 20X and (B) 40X. For this culture, Day 7 would be the optimal time for passaging. The optimal day of passage for each culture will depend on plating density (section 6.2) and aggregate size used (section 6.1).



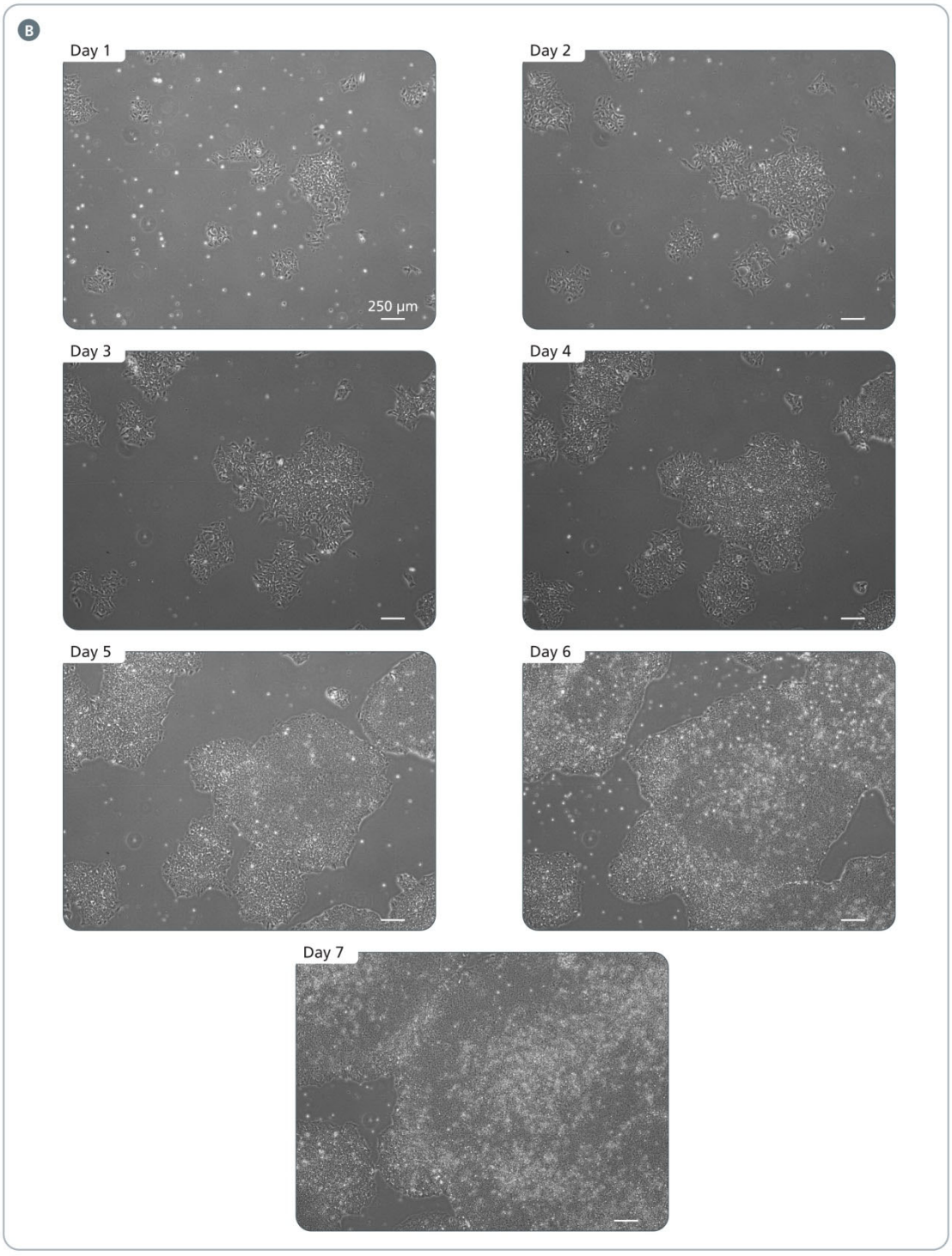
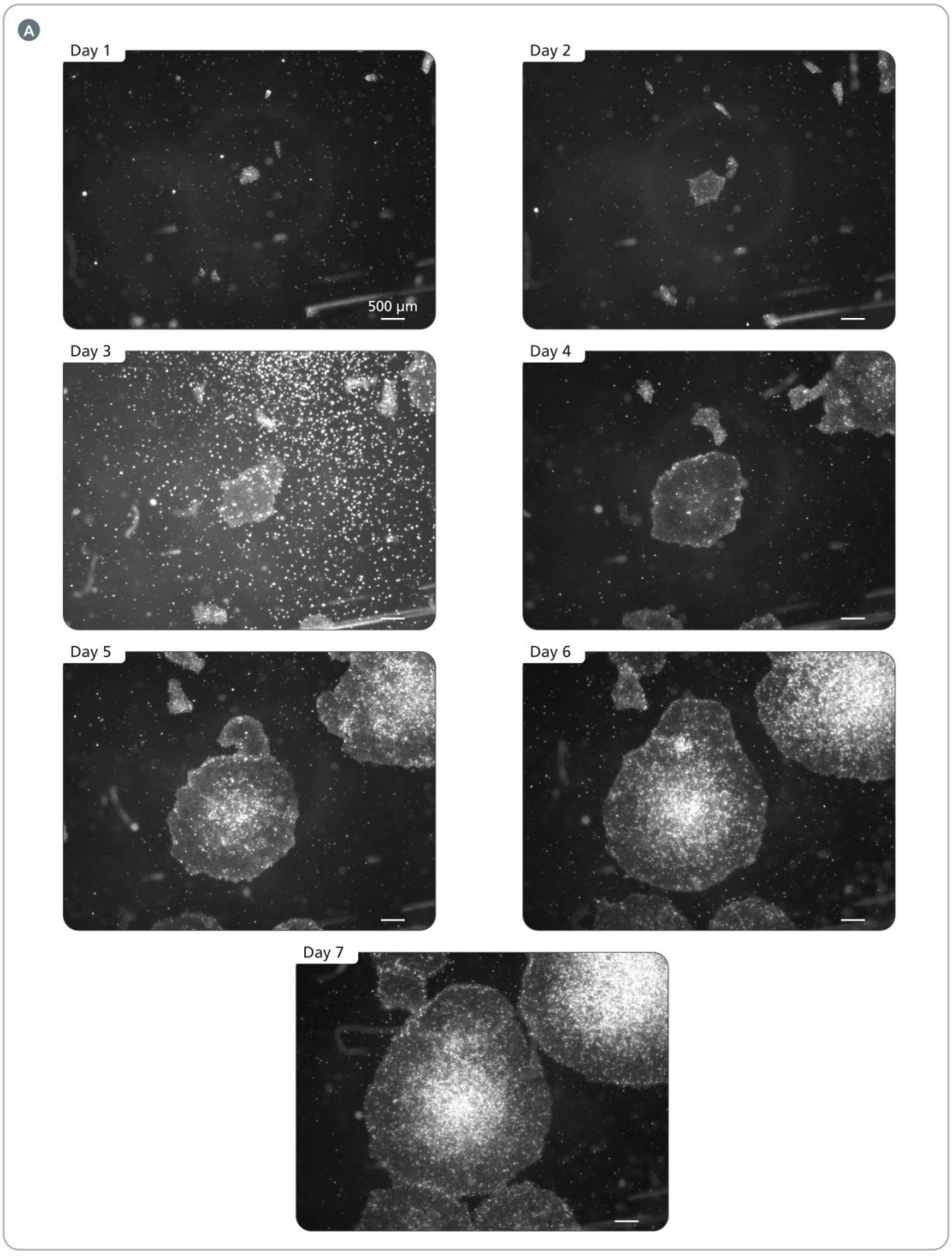


Figure 7. Human iPS Cells Cultured on Vitronectin XF™ in TeSR™2 at Days 1 - 7 After Passaging
Human iPS cells (WLS-4D1) at a magnification of (A) 20X and (B) 40X. For this culture, Day 7 would be the optimal time for passaging. The optimal day of passage for each culture will depend on plating density (section 6.2) and aggregate size used (section 6.1).



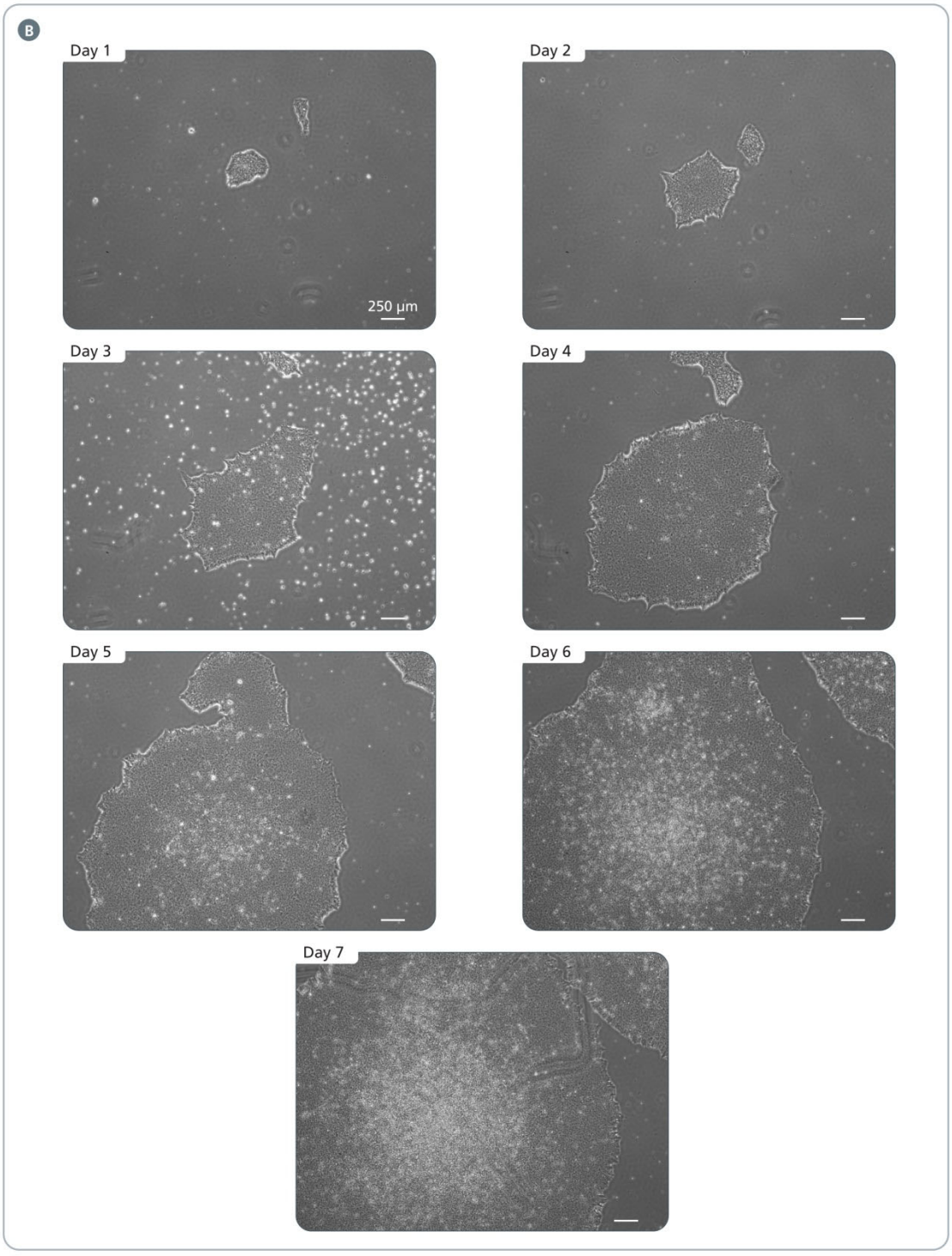


Figure 8. Human iPS Cells Cultured on Corning® Matrigel® in TeSR™2 at Days 1 - 7 After Passaging
Human iPS cells (STiPS-M001) at a magnification of (A) 20X and (B) 40X. For this culture, Day 7 would be the optimal time for passaging. The optimal day of passage for each culture will depend on plating density (section 6.2) and aggregate size used (section 6.1).

4.0 Preparation of Reagents and Materials

4.1 Complete TeSR™2 Medium

Use sterile technique to prepare complete TeSR™2 medium (Basal Medium + 5X Supplement + 250X Supplement). The following example is for preparing 500 mL of complete medium. If preparing other volumes, adjust accordingly.

Note: Thaw supplements or complete medium at room temperature (15 - 25°C) or overnight at 2 - 8°C. ***Do not thaw in a 37°C water bath.***

1. Thaw TeSR™2 5X Supplement and TeSR™2 250X Supplement. Mix each supplement thoroughly.

   Note: Once thawed, use supplement immediately or aliquot and store at -20°C for up to 3 months. Do not exceed the shelf life of the supplement. After thawing the aliquoted supplement, use immediately. Do not re-freeze.

2. Add 100 mL of TeSR™2 5X Supplement and 2 mL of TeSR™2 250X Supplement to 400 mL of TeSR™2 Basal Medium. Mix thoroughly.

   Note: If not used immediately, store complete TeSR™2 medium at 2 - 8°C for up to 2 weeks. Alternatively, aliquot and store at -20°C for up to 6 months. Do not exceed the shelf life of the individual components. After thawing the aliquoted complete medium, use immediately or store at 2 - 8°C for up to 2 weeks. Do not re-freeze.

If prepared aseptically, complete TeSR™2 medium is ready for use. If desired, the medium can be filtered using a 0.2 - 0.22 µm low protein binding polyethersulfone (PES) filter unit (e.g. Fisher 09-741-04 [0.2 µm, 250 mL]; Fisher SCGP00525 [0.22 µm, 50 mL]).

4.2 Matrices for Coating Cultureware

Successful culture of human ES and iPS cells in TeSR™2 requires the use of a suitable matrix to allow attachment of cell aggregates. Vitronectin XF™ or Corning® Matrigel® hESC-Qualified Matrix are recommended for use with TeSR™2. Vitronectin XF™ recombinant protein matrix is recommended if a fully defined culture system is desired.

Use sterile technique when coating cultureware. For specific instructions for each matrix, refer to section 4.2.1 (Corning® Matrigel®) or 4.2.1 (Vitronectin XF™). Refer to Table 1 **Error! Reference source not found.**for recommended volumes of diluted matrix for coating various cultureware.

Table 1. Recommended Volumes for Coating Cultureware

CULTUREWARE*	VOLUME OF DILUTED MATRIX
6-well plate	1 mL/well
100 mm dish	6 mL/dish
T-25 cm^2 flask	3 mL/flask
T-75 cm^2 flask	8 mL/flask

*Non-tissue culture-treated cultureware is required for use with Vitronectin XF™.

4.2.1 Corning® Matrigel®

Corning® Matrigel® hESC-Qualified Matrix should be aliquoted and frozen. Consult the Matrigel® Certificate of Analysis for the recommended aliquot size ("Dilution Factor") to prepare 25 mL of diluted matrix. Always keep Matrigel® on ice when thawing and handling to prevent it from gelling.

*Note: **Use tissue culture-treated cultureware** (e.g. Falcon® 6-Well Flat-Bottom Plate, Tissue Culture-Treated, Catalog #38016) with Corning® Matrigel®.*

1. Thaw one aliquot of Matrigel® on ice.
2. Dispense 25 mL of cold DMEM/F-12 into a 50 mL conical tube (e.g. Catalog #38010) and keep on ice.
3. Add thawed Matrigel® to the cold DMEM/F-12 (in the 50 mL tube) and mix well. The vial may be washed with cold medium if desired.
4. Immediately use the diluted Matrigel® solution to coat tissue culture-treated cultureware. See Table 1 **Error! Reference source not found.**for recommended coating volumes.
5. Swirl the cultureware to spread the Matrigel® solution evenly across the surface.

   Note: If the cultureware surface is not fully coated by the 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 Matrigel® solution evaporate.

   Note: If not used immediately, the cultureware must be sealed to prevent evaporation of the Matrigel® 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 proceeding to step 7.
7. Gently tilt the cultureware onto one side and allow the excess Matrigel® 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. Immediately add TeSR™2 (e.g. 2 mL/well if using a 6-well plate).

4.2.2 Vitronectin XF™

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

1. Thaw Vitronectin XF™ at room temperature (15 - 25°C).
2. Dilute Vitronectin XF™ in CellAdhere™ Dilution Buffer 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. Catalog #38010) 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 **Error! Reference source not found.**for recommended coating volumes.
5. Gently rock the cultureware back and forth to spread the solution evenly across the surface.

   Note: If the cultureware 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 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 proceeding to step 7.

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™2 (e.g. 2 mL/well if using a 6-well plate).

5.0 Passaging Human ES and iPS Cells Cultured in TeSR™2

Human ES and iPS cells maintained in TeSR™2 can be passaged using a number of different methods, both enzymatic and non-enzymatic, that are described in this section. For optimal performance, it is important to select an appropriate passaging reagent that is compatible with the matrix that is used (Table 2).

Table 2. Compatibility of Matrices and Passaging Reagents

MATRIX	COMPATIBLE PASSAGING REAGENTS
Vitronectin XF™	• ReLeSR™ • Gentle Cell Dissociation Reagent
Corning® Matrigel®	• ReLeSR™ • Gentle Cell Dissociation Reagent • Dispase

5.1 Enzyme-Free Passaging Protocols

These passaging protocols use enzyme-free dissociation methods to remove colonies from the cultureware. The cell aggregates generated using these protocols may be fragile, and they should be replated as quickly as possible.

5.1.1 ReLeSR™

ReLeSR™ is an enzyme-free reagent for dissociation and passaging of human ES and iPS cells as aggregates without manual selection of differentiated areas or scraping to remove cell aggregates. The following protocol is recommended when first culturing cells in TeSR™2.

The following instructions are for passaging cells from one well of a 6-well plate. If using other cultureware, adjust volumes accordingly.

1. At least 1 hour before passaging, coat new plates with either Corning® Matrigel® (section 4.2.1) or Vitronectin XF™ (section 4.2.1).
2. Aliquot sufficient TeSR™2 and warm to room temperature (15 - 25°C).

   Note: Do not warm TeSR™2 in a 37°C water bath.
3. Wash cells with 1 mL of D-PBS (Without Ca++ and Mg++) and aspirate.

   Note: There is no need to remove regions of differentiated cells.
4. Add 1 mL of ReLeSR™ and aspirate ReLeSR™ within 1 minute, so that colonies are exposed to a thin film of liquid.
5. Incubate at room temperature (15 - 25°C) for 5 - 8 minutes.

   Note: Optimal dissociation time may vary depending on the cell line used; when passaging a cell line with ReLeSR™ for the first time, the optimal dissociation time should be determined (for more information see Figure 9 and section 8.0).
6. Add 1 mL of TeSR™2.
7. Detach the colonies by placing the plate on a plate vortexer (e.g. Multi-MicroPlate Genie, 120V, Scientific Industries Model SI-4000, at 1200 rpm) for 2 - 3 minutes at room temperature. Alternatively, hold the plate with one hand and use the other hand to firmly tap the side of the plate for approximately 30 - 60 seconds.

8. Transfer the detached cell aggregates to a 15 mL conical tube using a 5 mL serological pipette (e.g. Catalog #38003). Cell aggregates should be appropriately sized for plating (mean aggregate size of approximately 50 - 200 µm; see Figure 9).

   Note: If you wish to plate cell aggregates directly from your passaged well (i.e. without transferring into a tube), pipette the aggregate mixture up and down once using a 5 mL pipette. This will ensure breakup of any large aggregates that may still be present.

9. Plate the cell aggregate mixture at the desired density onto coated wells containing TeSR™2. If the colonies are at an optimal density, the cultures can be split every 4 - 7 days using 1 in 10 to 1 in 50 splits (i.e. cell aggregates from 1 well can be plated in 10 - 50 wells). If the colonies are too dense or too sparse, adjust the split ratio accordingly at the next time of passaging (see section 6.2).
10. Place the plate in a 37°C incubator. Move the plate in several quick, short, back-and-forth and side-to-side motions to evenly distribute the cell aggregates. Do not disturb the plate for 24 hours.

    Note: Uneven distribution of aggregates may result in increased differentiation of human ES and iPS cells.

11. Perform daily medium changes using TeSR™2 and visually assess cultures to monitor growth until the next passaging time.

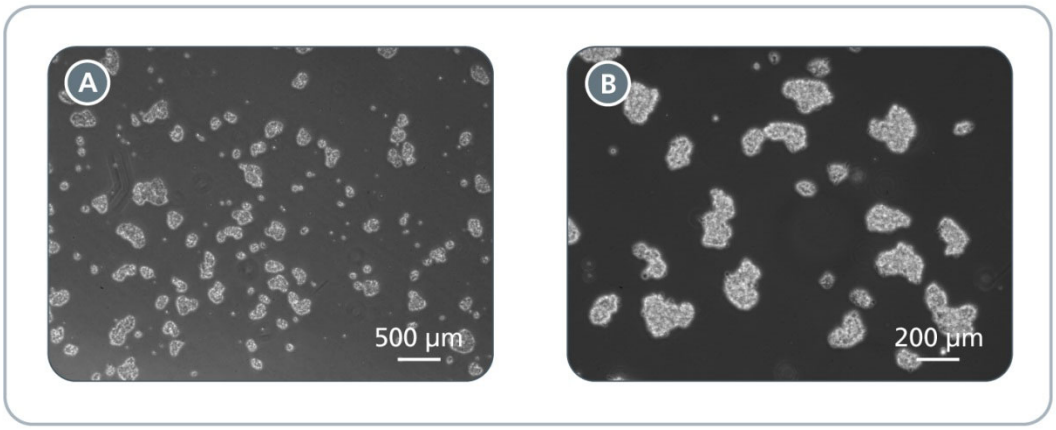


Figure 9. Effect of ReLeSR™ on Human Pluripotent Cells Cultured in TeSR™2
Examples of ideal cell aggregates (mean size of approximately 50 - 200 µm) obtained after step 8 of the ReLeSR™ protocol. Magnifications: (A) 40X and (B) 100X. If cell aggregates do not resemble these examples, the passaging protocol may require further optimization (for more information, refer to "Cell aggregate size obtained with ReLeSR™ is not ideal" in section 8.0).

5.1.2 Gentle Cell Dissociation Reagent (GCDR)

Gentle Cell Dissociation Reagent (GCDR) is an enzyme-free reagent for passaging of human ES and iPS cells as aggregates with manual scraping to generate cell aggregates. Once familiar with the following protocol, it is possible to adjust the time at which cells are ready to be passaged by altering the cell aggregate size (section 6.1) or plating density (section 6.2).

The following are instructions for passaging cells from one well of a 6-well plate. If using other cultureware, adjust volumes accordingly.

1. At least 1 hour before passaging, coat new plates with either Corning® Matrigel® (section 4.2.1) or Vitronectin XF™ (section 4.2.1).
2. Aliquot sufficient complete TeSR™2 and warm to room temperature (15 - 25°C).

   Note: Do not warm complete TeSR™2 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.
4. 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.
5. Aspirate medium from the well and add 1 mL of GCDR.
6. Incubate at room temperature. Refer to Table 3 for recommended incubation times.

Table 3. GCDR Incubation Times with Cultures Plated on Different Matrices

MATRIX	INCUBATION TIME WITH GCDR*	REPRESENTATIVE EXAMPLE
Vitronectin XF™	6 - 12 minutes	Figure 10
Corning® Matrigel®	6 - 8 minutes	Figure 11

*Incubation times may vary when using different cell lines or other non-enzymatic cell passaging reagents; dissociation should be monitored under the microscope until the optimal time is determined.

7. Aspirate the GCDR, and add 1 mL of TeSR™2. Gently detach the colonies by scraping with a serological glass pipette or a cell scraper.

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

   Optional: Rinse the well with an additional 1 mL of TeSR™2 to collect remaining cell aggregates.

   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. A uniform suspension of aggregates approximately 50 - 200 µm in size is optimal; do not create a single-cell suspension (for more information, see section 8.0).

Table 4. Suggested Methods for Breaking Up Cell Aggregates

MATRIX	PIPETTE TYPE	NUMBER OF TIMES TO PIPETTE UP AND DOWN*
Vitronectin XF™	1 mL pipettor	1 - 2
Corning® Matrigel®	2 mL serological pipette	2 - 5

*Number can be adjusted to obtain desired cell aggregate size (section 6.1). Refer to Figure 13 for examples of appropriate cell aggregate sizes and adjust procedure as necessary to achieve desired results.

10. Plate the cell aggregate mixture at the desired density onto coated wells containing TeSR™2. If the colonies are at an optimal density, the cultures can be split every 4 - 7 days using 1 in 10 to 1 in 50 splits (i.e. cell aggregates from 1 well can be plated in 10 to 50 wells). If the colonies are too dense or too sparse, at the next time of passaging adjust the split ratio accordingly (for more details see section 6.2).

    Note: Work quickly to transfer cell aggregates into new cultureware to maximize viability and attachment.
11. Place the plate in a 37°C incubator. Move the plate in several quick, short, back-and-forth and side-to-side motions to distribute the cell aggregates. Do not disturb the plate for 24 hours.

    Note: Uneven distribution of aggregates may result in increased differentiation of human ES and iPS cells.
12. Perform daily medium changes using TeSR™2 and visually assess cultures to monitor growth until the next passaging time.

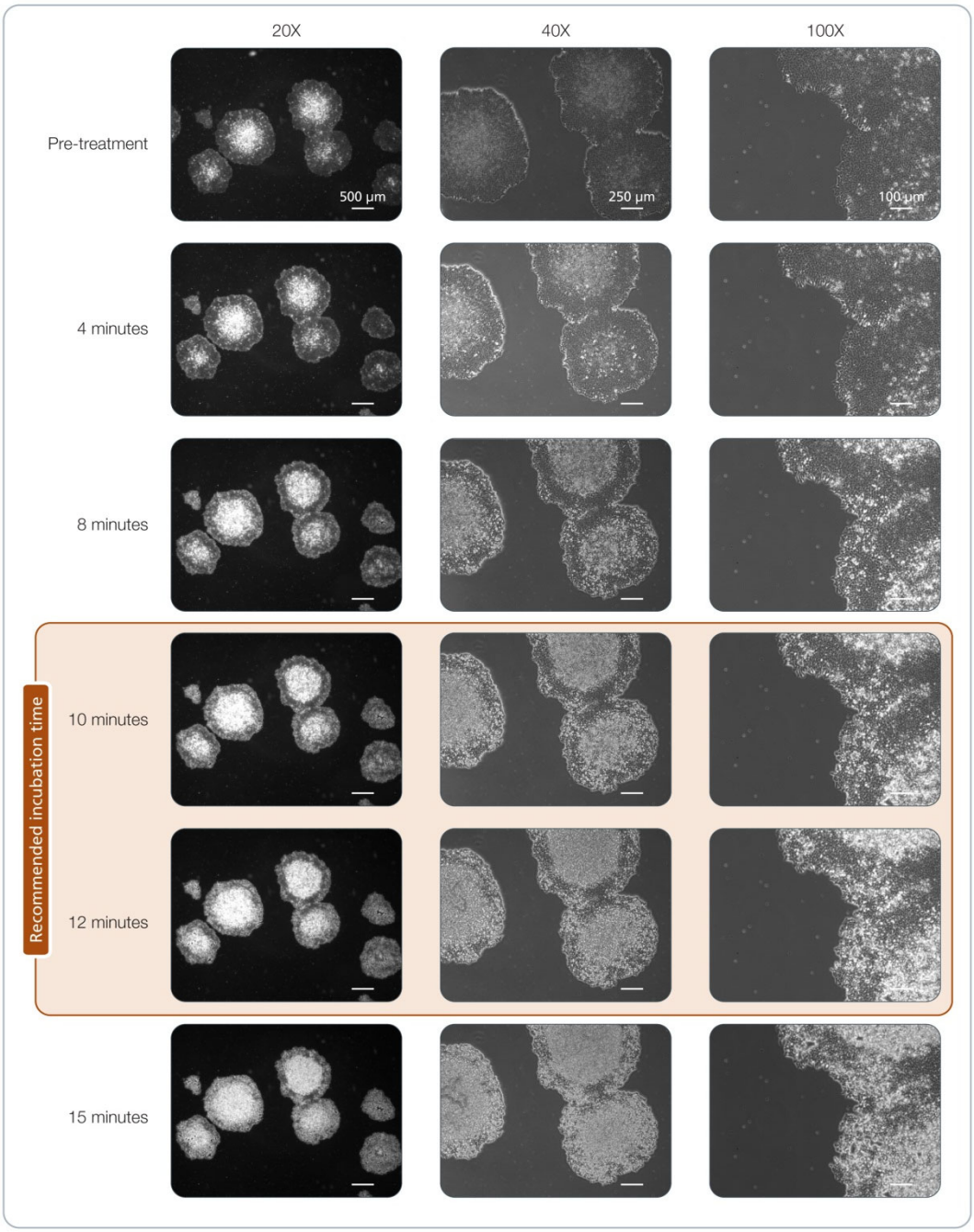


Figure 10. Effect of Gentle Cell Dissociation Reagent on Human ES Cells Cultured on Vitronectin XF™ in TeSR™2. Human ES cells (H9) at different timepoints during incubation with Gentle Cell Dissociation Reagent. Magnifications: 20X, 40X, and 100X. Recommended incubation time (10 - 12 minutes) occurs when gaps appear between cells located on the edges of the colonies. At earlier timepoints the colonies are not sufficiently dissociated, whereas at later timepoints the colonies are excessively dissociated and may break up into unwanted single cells upon scraping.

Note: The incubation time may vary when using different cell lines or other non-enzymatic cell passaging reagents; dissociation should be monitored under the microscope until the optimal time is determined based on appearance.

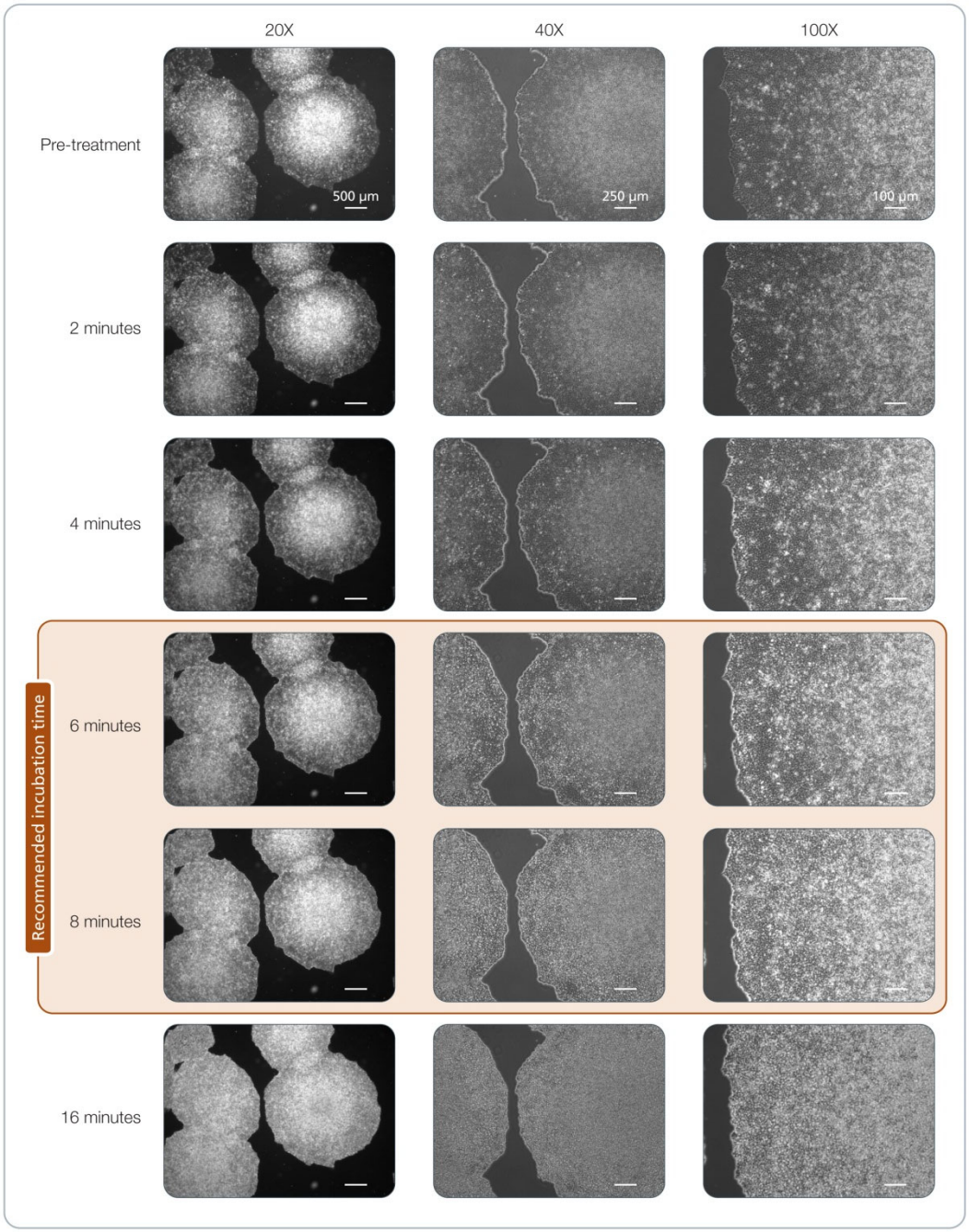


Figure 11. Effect of Gentle Cell Dissociation Reagent on Human iPS Cells Cultured on Corning® Matrigel® in TeSR™2. Human iPS cells (STiPS-F016) at different timepoints during incubation with Gentle Cell Dissociation Reagent. Magnifications: 20X, 40X, and 100X. Recommended incubation time (6 - 8 minutes) occurs when gaps are beginning to appear between cells located on the edges of the colonies. At earlier timepoints the colonies are not sufficiently dissociated, whereas at later timepoints the colonies are excessively dissociated and may break up into unwanted single cells upon scraping.

Note: The incubation time may vary when using different cell lines or other non-enzymatic cell passaging reagents; dissociation should be monitored under the microscope until the optimal time is determined based on appearance.

5.2 Enzymatic Passaging Protocol

This passaging protocol uses an enzymatic dissociation method to remove colonies from the cultureware.

Note: Enzyme-free methods (section 5.1) are recommended for their ease of use, high cell recovery, and for preserving the integrity of cell surface proteins that aid in the reattachment of cells to the matrix.

5.2.1 Dispase

Dispase is an enzymatic reagent for passaging of human ES and iPS cells as aggregates with manual scraping to generate cell aggregates.

The following instructions are for passaging cells from one well of a 6-well plate. If using other cultureware, adjust volumes accordingly.

1. At least 1 hour before passaging, coat new dishes with Corning® Matrigel® (section 4.2.1).
2. Aliquot sufficient TeSR™2, Dispase (1 U/mL), and DMEM/F-12 with 15 mM HEPES and warm to room temperature (15 - 25°C).

   Note: Do not warm TeSR™2 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.
4. 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.
5. Aspirate medium from the well and wash with 2 mL of DMEM/F-12 with 15 mM HEPES.
6. Add 1 mL of Dispase (1 U/mL).
7. Incubate at 37°C for 3 - 4 minutes, until the colony edges begin to loosen and lift from the dish. Refer to Figure 12 for additional information.

   Note: The incubation time may vary when using different cell lines or other enzymatic cell passaging reagents, therefore dissociation should be monitored under the microscope until the optimal time is determined.
8. Aspirate the Dispase, and gently wash each well 2 - 3 times with 2 mL of DMEM/F-12 with 15 mM HEPES.
9. Add 2 mL of DMEM/F-12 with 15 mM HEPES or TeSR™2. Gently detach colonies by scraping with a serological glass pipette or a cell scraper.

   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 with 15 mM HEPES or TeSR™2 to collect any remaining aggregates. Add the rinse to the 15 mL tube.

    Note: If cells are scraped in TeSR™2, 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.
12. Aspirate the supernatant. For each well of cell aggregates collected in the 15 mL tube, add 1 - 2 mL of TeSR™2.
13. Carefully pipette the cell aggregate mixture up and down 2 - 3 times using a 2 mL serological pipette to break up the aggregates. A uniform suspension of aggregates approximately 50 - 200 µm in size is optimal; do not create a single-cell suspension (see section 8.0).

14. Plate the cell aggregate mixture at the desired density onto coated wells containing TeSR™2. If the colonies are at an optimal density, the cultures can be split every 4 - 7 days using 1 in 6 to 1 in 10 splits (i.e. cell aggregates from 1 well can be plated in 6 - 10 wells). If the colonies are too dense or too sparse, at the next time of passaging adjust the split ratio accordingly (see section 6.2).
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 distribute the cell aggregates. Do not disturb the plate for 24 hours.

    Note: Uneven distribution of aggregates may result in increased differentiation of human ES and iPS cells.
16. Perform daily medium changes using TeSR™2 and visually assess cultures to monitor growth until the next passaging time.

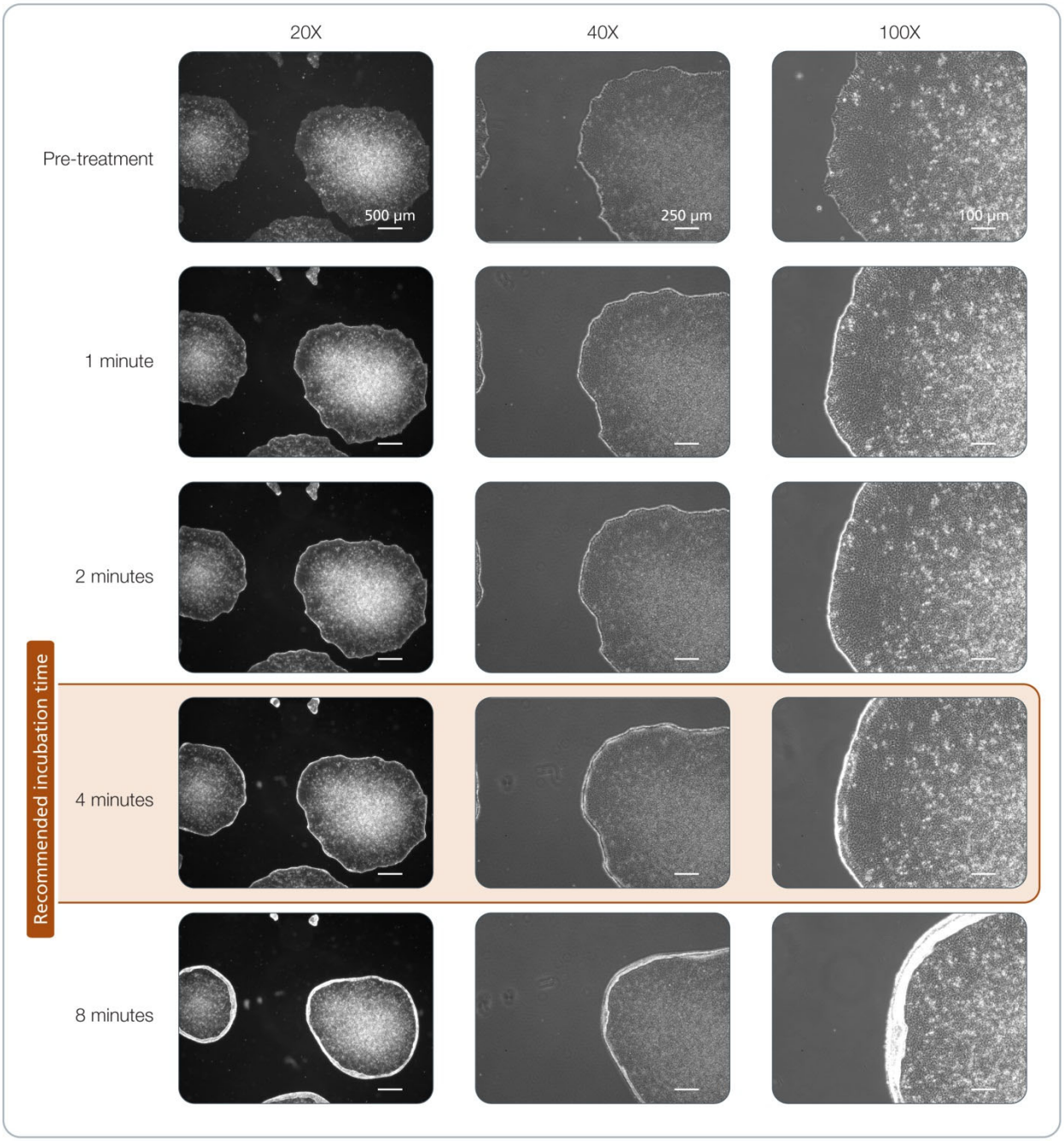


Figure 12. Effect of Dispase on Human iPS Cells Cultured on Corning® Matrigel® in TeSR™2
Human iPS cells (STiPS-F016) at different timepoints during incubation with Dispase. Magnifications: 20X, 40X, and 100X. Recommended incubation time (4 minutes) occurs when the edges of the colonies loosen and begin to lift from the dish. At earlier timepoints the colonies are not sufficiently dissociated, whereas at later timepoints the colonies are excessively dissociated and may break up into unwanted single cells upon scraping.

Note: The incubation time may vary when using different cell lines or other enzymatic cell passaging reagents; dissociation should be monitored under the microscope until the optimal time is determined based on appearance.

6.0 Customizing the Passaging Protocol

Culturing human ES and iPS cells in TeSR™2 allows some flexibility in the passaging schedule, as cultures can be passaged between 4 and 7 days after plating in TeSR™2. 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 13. Regardless of the plating density and cell aggregate size used, the majority of colonies should be densely packed and multi-layered 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 13.

**For representative images see Figure 14.

6.1 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 13 for a recommended range of cell aggregate sizes. Do not generate single cells.

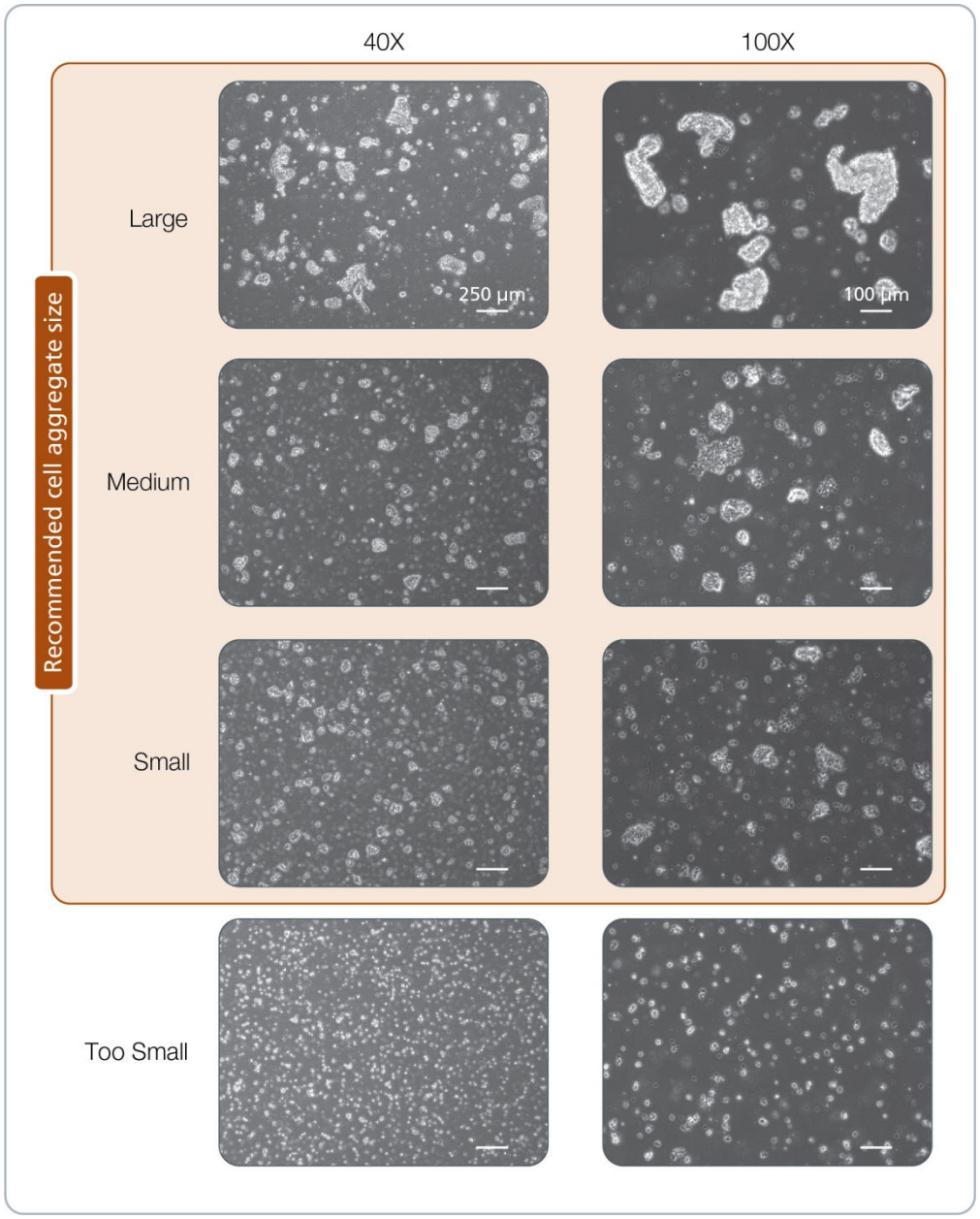


Figure 13. Acceptable Size Range for Cell Aggregates at the Time of Plating
Cell aggregate size can be adjusted by altering the number of times the cell aggregate mixture is pipetted up and down. Avoid generating single cells. Magnifications: 40X and 100X.

6.2 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 (i.e. plating more cell aggregates) will result in a higher colony density at the next passaging time. Typical split ratios are 1 in 10 to 1 in 50; however, this can vary depending on the cell line used and the individual operator. Refer to Figure 14 for a recommended range of colony densities that you should observe at the time of passage. Some cell lines may show increased spontaneous differentiation when cultured at higher densities; if increased differentiation is observed, the colony density may be decreased in the next passage by plating fewer cell aggregates. Counting cell aggregates is an alternate way to determine and adjust plating densities (see Appendix 1).

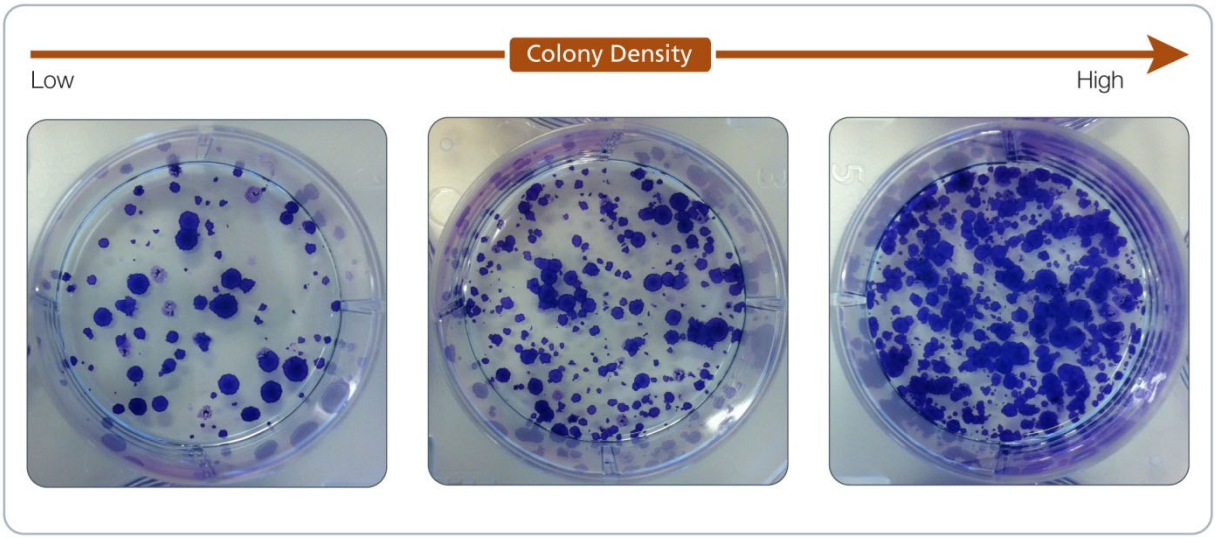


Figure 14. Example Colony Densities for Cultures Grown in TeSR™2
Human iPS cells (STiPS-F016) were cultured in TeSR™2 and stained with Giemsa at the optimal time of passaging (the optimal time of passaging may be different for each colony density; see Table 5). Each image represents a single well of a 6-well plate.

7.0 Additional Protocols

7.1 Transitioning Cells

7.1.1 From Feeder-Free Media to TeSR™2

No adaptation step is required when plating human ES and iPS cells from TeSR™-E8™, mTeSR™1, mTeSR™ Plus, or other feeder-free maintenance medium to TeSR™2.

When transitioning from:

- TeSR™-E8™ or mTeSR™ Plus to TeSR™2: Follow an enzyme-free passaging protocol (section 5.1)
- mTeSR™1 to TeSR™2: Follow either an enzyme-free (section 5.1) or enzymatic (section 5.2) passaging protocol

After passaging, plate cell aggregates onto coated cultureware containing TeSR™2. 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™2 is appropriate.

Cells may experience an increase in spontaneous differentiation in the first 1 - 2 passages after transfer. Removal of differentiated regions manually or using a specialized dissociation reagent such as ReLeSR™ (section 5.1.1) will help ensure that the culture quickly adapts to the new environment without affecting the long-term health of the culture.

7.1.2 From TeSR™2 to TeSR™-E8™, mTeSR™1, or mTeSR™ Plus

No adaptation step is required when plating human ES and iPS cells from TeSR™2 to TeSR™-E8™, mTeSR™1, or mTeSR™ Plus. Follow either an enzyme-free passaging protocol (section 5.1) or an enzymatic passaging protocol (section 5.2) and plate cell aggregates onto coated cultureware containing TeSR™-E8™, mTeSR™1, or mTeSR™ Plus.

7.1.3 Cultured on Corning® Matrigel® to Vitronectin XF™

Human ES and iPS cells cultured on Corning® Matrigel® in TeSR™2 may be conveniently transitioned to Vitronectin XF™ without an adaptation step. When cells are ready for passage, follow the protocol in section 5.1 as though you were to plate cells back onto Corning® Matrigel® (i.e. use incubation times recommended for Corning® Matrigel® in Table 3); however, ensure that the cell aggregates are plated on Vitronectin XF™-coated cultureware (section 4.2.2). For subsequent passages, an enzyme-free passaging protocol (section 5.1) for cells cultured on Vitronectin XF™ may then be used. Do not use an enzymatic passaging protocol when transitioning cells to Vitronectin XF™.

Note: Both the medium and the matrix can be changed at the same time (e.g. cells cultured on Corning® Matrigel® using mTeSR™1 may be transitioned onto Vitronectin XF™ with TeSR™2 in one step; see section 7.1.1).

7.1.4 Cultured on a Feeder Layer to TeSR™2

Human ES and iPS cells cultured on a layer of feeder cells may be conveniently transferred to feeder-free conditions with TeSR™2. Cell aggregates can be harvested using protocols established in your institute for feeder-dependent cells and plated on the desired matrix in TeSR™2. 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 passaging cells from one well of a 6-well plate. If using other cultureware, adjust volumes accordingly.

1. At least 1 hour before passaging, coat new plates with either Corning® Matrigel® (section 4.2.1) or Vitronectin XF™ (section 4.2.1).
2. Aliquot sufficient complete TeSR™2, DMEM/F-12 with 15 mM HEPES, and Collagenase Type IV (Catalog #07909). Warm to room temperature (15 - 25°C).

   Note: Do not warm complete TeSR™2 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 of Collagenase Type IV.
5. Incubate at 37°C for 20 minutes.

   Note: The incubation time may vary when using different cell lines or other enzymatic cell passaging reagents, therefore dissociation should be monitored under the microscope until the optimal time is determined.
6. Aspirate the collagenase, and wash the well twice with 1 mL of DMEM/F-12 with 15 mM HEPES.
7. Add 1 mL of TeSR™2. Gently detach the colonies by scraping with a serological glass pipette or a cell scraper.
8. Transfer the detached cell aggregates to a 15 mL conical tube.

   Optional: Rinse the well with an additional 1 - 2 mL of TeSR™2 to collect remaining cell aggregates. Add the rinse to the 15 mL tube.
9. Carefully pipette the cell aggregate mixture up and down 2 - 3 times using a 2 mL serological pipette to break up the cell aggregates. A uniform suspension of aggregates approximately 50 - 200 µm in size is optimal; do not create a single-cell suspension (see section 8.0).
10. Plate cell aggregate mixture at the desired density onto coated wells containing TeSR™2.

    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™2 is appropriate.
11. Place the plate in a 37°C incubator. Move the plate in several quick, short, back-and-forth and side-to-side motions to evenly distribute the cell aggregates. Do not disturb the plate for 24 hours.

    Note: Uneven distribution of aggregates may result in increased differentiation of human ES and iPS cells.
12. Perform daily medium changes using TeSR™2 and visually assess cultures to monitor growth until the next passaging time.

7.2 Preparing a Single-Cell Suspension for Downstream Applications

The following are instructions for preparing a single-cell suspension from cultures grown in TeSR™2 in 6-well plates. If using other cultureware, adjust volumes accordingly. Cultures should be harvested and frozen at the time they would normally be ready for passaging.

1. Warm medium (DMEM/F-12 with 15 mM HEPES or TeSR™2), Gentle Cell Dissociation Reagent, and D-PBS (Without Ca++ and Mg++) to room temperature (15 - 25°C) before use.

   Note: Do not warm TeSR™2 in a 37°C water bath.
2. Wash the well to be passaged with 1 mL of D-PBS (Without Ca++ and Mg++).
3. Aspirate the wash medium and add 1 mL of Gentle Cell Dissociation Reagent. Incubate at 37°C for 8 - 10 minutes.

   Note: The incubation time may vary when using different cell lines or other cell passaging reagents.
4. Harvest cells by pipetting up and down with either a serological pipette or a 1 mL pipettor 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 with 15 mM HEPES or TeSR™2) 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.

   Note: Addition of 10 µM Y-27632 (ROCK inhibitor) is recommended when plating single-cell suspensions of human ES or iPS cells, as it has been reported to increase cell survival. Alternatively, addition of CloneR™ is recommended when plating single cells at low or clonal densities to improve survival and cloning efficiency.

7.3 Cryopreserving and Thawing Cells

Human pluripotent stem cells maintained in TeSR™2 can be cryopreserved as aggregates using Cryostor® CS10 or as single cells using FreSR™-S. Either cryopreservation method is appropriate for use with cultures passaged as aggregates using the methods described in this manual.

The thawing protocols are for human ES and iPS cells that were maintained in TeSR™2 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 TeSR™-E8™, mTeSR™1, or mTeSR™ Plus) 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™2 (sections 7.1.1 and 7.1.4).

7.3.1 Cryostor® CS10 (Cell Aggregates)

CryoStor® CS10 is an animal component-free cryopreservation medium. It is ready to use and contains cryoprotectant agents.

Cryopreserving Cells

Note: Wipe down the outside of the bottle with 70% ethanol or isopropanol before opening.

The following are instructions for cryopreserving cultures grown in TeSR™2 in 6-well plates. Cultures should be harvested and cryopreserved at the time they would normally be ready for passaging. Each vial should contain the cell aggregates from one well of a 6-well plate. If using other cultureware, adjust volumes accordingly.

1. Passage cells using enzyme-free passaging protocols (section 5.1) until step 8 or the enzymatic passaging protocol (section 5.2) until step 10.
2. Centrifuge at 300 x *g* for 5 minutes at room temperature (15 - 25°C).

3. Gently aspirate the supernatant, taking care not to disrupt the cell pellet.
4. Using a serological pipette, gently resuspend the pellet with 1 mL per well harvested of cold (2 - 8°C) CryoStor® CS10. Minimize the breakup of cell aggregates when dislodging the pellet.
5. Using a 2 mL serological pipette, transfer 1 mL of cell aggregates mixture into each labeled cryovial.
6. Cryopreserve 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.

Thawing Cells

Human ES and iPS cells should be thawed into coated cultureware (section 4.2). In general, one vial of cells cryopreserved as described above can be successfully thawed into 1 - 2 wells of a 6-well plate.

1. Have all tubes, warmed TeSR™2 (15 - 25°C), and coated plates ready before starting the protocol to ensure that the thawing procedure is done as quickly as possible.

   Note: Do not warm TeSR™2 in a 37°C water bath.
2. Wipe the outside of the vial of cells with 70% ethanol or isopropanol.
3. In a biosafety cabinet, twist the cap a quarter-turn to relieve internal pressure, then retighten.
4. Quickly thaw the cells in a 37°C water bath by gently shaking the vial. Remove the vial when a small frozen cell pellet remains. Do not vortex cells.
5. Wipe the outside of the vial with 70% ethanol or isopropanol.
6. Use a 2 mL serological pipette (e.g. Catalog #38002) 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 pipettor will minimize breakage of cell aggregates.
7. Add 5 - 7 mL of warm TeSR™2 dropwise to the 15 mL tube, gently mixing as the medium is added.
8. Centrifuge cells at 300 x *g* for 5 minutes at room temperature (15 - 25°C).
9. Aspirate the medium, leaving the cell pellet intact. Gently resuspend the cell pellet in 1 mL of TeSR™2 using a 2 mL serological pipette. Take care to maintain the cells as aggregates.
10. Transfer 0.5 mL of the cell mixture onto a well of a coated 6-well plate containing TeSR™2 (i.e. two wells can be plated from each cryovial).

    Note: Number of wells plated may need to be adjusted depending on how many cell aggregates were cryopreserved. Typically more aggregates will need to be plated after thawing than during routine passaging.
11. Place the plate in a 37°C incubator. Move the plate in several quick, short, back-and-forth and side-to-side motions to distribute the cell aggregates. Do not disturb the plate for 24 hours.

    Note: Uneven distribution of aggregates may result in increased differentiation of human ES and iPS cells.
12. Perform daily medium changes using TeSR™2 and visually assess cultures to monitor growth until the next passaging time. 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 plate.

7.3.2 FreSR™-S (Single Cells)

FreSR™-S is a defined, serum-free and animal component-free medium for cryopreserving human ES/iPS cells as single cells. It is ready to use and contains cryoprotectant agents.

Cryopreserving Cells

Note: Wipe down the outside of the bottle with 70% ethanol or isopropanol before opening.

The following are instructions for cryopreserving cultures grown in TeSR™2 in 6-well plates. If using other cultureware, adjust volumes accordingly. Cultures should be harvested and cryopreserved at the time they would normally be ready for passaging.

1. Warm medium (DMEM/F-12 with 15 mM HEPES or TeSR™2), Gentle Cell Dissociation Reagent, and D-PBS (Without Ca++ and Mg++) to room temperature (15 - 25°C) before use.

   Note: Do not warm TeSR™2 in a 37°C water bath.
2. Wash the well to be passaged with 1 mL of D-PBS (Without Ca++ and Mg++).
3. Aspirate the wash medium and add 1 mL of Gentle Cell Dissociation Reagent. Incubate at 37°C for 8 - 10 minutes.

   Note: The incubation time may vary when using different cell lines or other cell passaging reagents.
4. Harvest cells by pipetting up and down with either a serological pipette or a 1 mL pipettor to ensure a single-cell suspension. Transfer cells to a 15 mL conical tube. Rinse wells with an additional 2 - 4 mL of medium (DMEM/F-12 with 15 mM HEPES or TeSR™2) and add the rinse to the tube containing the cells.
5. Perform a viable cell count using Trypan Blue and a hemocytometer.
6. Centrifuge cells at 300 x *g* for 5 minutes at room temperature (15 - 25°C).
7. Carefully remove the supernatant with a pipette, leaving a small amount of medium to ensure the cell pellet is not disturbed. Resuspend the cell pellet by gently flicking the tube.
8. Add cold (2 - 8°C) FreSR™-S to obtain a cell suspension of 1 x 10^6 cells/mL and mix thoroughly.
9. Transfer 1 mL of the single-cell suspension into each cryovial.
10. Cryopreserve cells 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.

Thawing Cells

Human ES and iPS cells should be thawed into coated cultureware (section 4.2). In general, one vial of 1 x 10^6 cells cryopreserved as described above can be successfully thawed into 1 - 2 wells of a 6-well plate.

1. Have all tubes, warmed TeSR™2 (15 - 25°C), DMEM/F-12 with 15 mM HEPES, and coated cultureware ready before starting the protocol to ensure that the thawing procedure is done as quickly as possible.

   Note: Do not warm TeSR™2 in a 37°C water bath.
2. Add Y-27632 to TeSR™2 to reach a final concentration of 10 µM.
3. Wipe the outside of the vial with 70% ethanol or isopropanol.
4. In a biosafety cabinet, twist the cap a quarter-turn to relieve internal pressure, then retighten.
5. Quickly thaw cells in a 37°C water bath by gently shaking the vial. Remove the vial when a small frozen cell pellet remains. Do not vortex cells.
6. Wipe the outside of the vial with 70% ethanol or isopropanol.
7. Use a 1 mL pipettor to slowly transfer the contents of the cryovial to a 15 mL conical tube containing 5 - 7 mL of DMEM/F-12 with 15 mM HEPES.
8. Centrifuge cells at 300 x *g* for 5 minutes at room temperature (15 - 25°C).
9. Carefully remove the supernatant with a pipette, leaving a small amount of medium to ensure the cell pellet is not disturbed.
10. Add 1 mL of TeSR™2 containing 10 µM Y-27632 to the tube. Mix gently.
11. Plate cells onto coated cultureware.

    Note: In general, one frozen cryovial containing 1 x 10^6 cells can be thawed and plated into 1 - 2 wells of a 6-well plate.
12. Place the cultureware in a 37°C incubator. Move the cultureware in several quick, short, back-and-forth and side-to-side motions to evenly distribute the cells across the surface.
13. Perform daily medium changes using TeSR™2 (without Y-27632) and visually assess cultures to monitor growth until the next passaging time (i.e. 80 - 90% confluent). This takes approximately 2 - 5 days after thawing.

    Note: This time may vary when using different cell lines; cultures should be monitored under the microscope until the optimal passaging time is determined.
14. Passage cultures using standard techniques to generate cell aggregates (e.g. ReLeSR™ [section 5.1.1])

    Note: It is not recommended to perform serial single-cell passaging due to the increased risk of karyotype abnormalities.[5,6]

8.0 Troubleshooting

PROBLEM	SOLUTION
Excessive (> 20%) differentiation in cultures	• Ensure the freshly prepared complete TeSR™2 medium kept at 2 - 8°C is less than 2 weeks old. • 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. • Decrease the colony density by plating less cell aggregates during passaging. • Reduce incubation time with the ReLeSR™ during passaging, as your cell line/culture may be more sensitive.

Cell aggregate size obtained with ReLeSR™ (section 5.1.1) is not ideal (i.e. aggregates do not look like the example in Figure 9)	• Larger aggregates are obtained (i.e. mean aggregates size is > 200 µm)
	  ○ Pipette the cell aggregate mixture up and down. Avoid generating a single-cell suspension.
	  ○ Increase the incubation time by 1 - 2 minutes.
	  ○ Increase the incubation temperature to 37°C.
	• Smaller aggregates are obtained (i.e. mean aggregates size is < 50 µm)
	  ○ Minimize the manipulation of cell aggregates after dissociation.
	  ○ Decrease the incubation time by 1 - 2 minutes.

Cell aggregates obtained during the passaging protocol are too large	• Increase the incubation time with Gentle Cell Dissociation Reagent. • Increase pipetting up and down of the cell aggregates. • Add a wash step using D-PBS (Without Ca++ and Mg++) before adding the non-enzymatic passaging reagents.
Differentiated cells are also detaching with the colonies when using ReLeSR™	• Decrease the incubation time with ReLeSR™ by 1 - 2 minutes.
Colonies remain attached and/or significant scraping is required to dislodge cells	• Ensure that the passaging reagents are being used as described in section 5.0. • Increase the incubation time by 1 - 2 minutes. • Increase the incubation temperature to 37°C when using ReLeSR™.
Low attachment observed after plating	• Plate a higher number of cell aggregates initially (e.g. 2 - 3 times higher) and maintain a more densely confluent culture. • Work quickly after cells are treated with passaging reagents (ReLeSR™, Gentle Cell Dissociation Reagent, or Dispase) to minimize the time that cell aggregates are in suspension. • Reduce incubation time with the passaging reagent during passaging, as your cell line/culture may be more sensitive. This is particularly important if cells are passaged prior to cell multi-layering 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 passaging 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 Corning® Matrigel®.

PROBLEM	SOLUTION
Single cells are generated during colony dissociation	• Work quickly after cells are treated with passaging reagents to minimize the time that cell aggregates are in suspension. • Reduce incubation time with the passaging reagents during passaging, as your cell line/culture may be more sensitive. This is particularly important if cells are passaged prior to cell multi-layering within the colony. • Minimize the manipulation of cell aggregates after dissociation.
Cells do not adhere to the coated cultureware	• Avoid using enzymatic passaging reagents (e.g. Dispase) when using Vitronectin XF™. • Ensure that non-tissue culture-treated plates are used when coating with Vitronectin XF™.
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 and has not evaporated prior to use. • 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.

9.0 References

1. Thomson JA et al. (1998) Embryonic stem cell lines derived from human blastocysts. Science 282(5391): 1145–7.
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7. Braam SR et al. (2008) Recombinant vitronectin is a functionally defined substrate that supports human embryonic stem cell self-renewal via alphavbeta5 integrin. Stem Cells 26(9): 2257–65.
8. Mitalipova MM et al. (2005) Preserving the genetic integrity of human embryonic stem cells. Nat Biotechnol 23(1): 19–20.

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 ≥ 50 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 passaging at the time of plating cell aggregates. Refer to section 5.0 for detailed passaging protocols.

1. Draw a "+" centered on the bottom of 2 wells of a 96-well flat-bottom plate (e.g. Catalog #38044) to serve as a counting grid.
2. Aliquot 40 µL of DMEM/F-12 with 15 mM HEPES 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 ≥ 50 µm in diameter. Average the results from the two wells to obtain the average number of cell aggregates (N_A) in the 5 µL 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 6). 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 6. Recommended Number of Cell Aggregates 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-75 cm^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) to coated wells containing TeSR™2.
10. Continue with the appropriate passaging protocol (section 5.0).

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, including other conjugates, sizes and clones, visit www.stemcell.com/antibodies.

Surface Antigen Labeling

PRIMARY ANTIBODY*	SPECIES REACTIVITY	ISOTYPE	CATALOG #
Anti-Mouse SSEA-1 Antibody, Clone MC-480	Human, Mouse, Rat	IgM, kappa (Mouse)	60060
Anti-Mouse SSEA-3 Antibody, Clone MC-631	Human, Mouse, Rat, Rhesus	IgM, kappa (Rat)	60061
Anti-Human SSEA-4 Antibody, Clone MC-813-70	Human, Mouse, Rat, Rhesus, Cat, Chicken, Dog, Rabbit	IgG3, kappa (Mouse)	60062
Anti-Human SSEA-5 Antibody, Clone 8e11	Human	IgG1, kappa (Mouse)	60063
Anti-Human TRA-1-60 Antibody, Clone TRA-1-60R	Human, Rhesus, Rabbit	IgM, kappa (Mouse)	60064
Anti-Human TRA-1-81 Antibody, Clone TRA-1-81	Human, Rat, Rhesus	IgM, kappa (Mouse)	60065
Anti-Human TRA-2-49 Antibody, Clone TRA-2-49/6E	Human, Chimpanzee, Gibbon, Gorilla, Orangutan, Owl Monkey, Squirrel Monkey, Cat, Pig, Rabbit, Tiger	IgG1, kappa (Mouse)	60066
Anti-Human TRA-2-54 Antibody, Clone TRA-2-54/2J	Human, Chimpanzee, Gibbon, Gorilla, Orangutan, Owl Monkey, Squirrel Monkey, Cat, Pig, Rabbit, Tiger	IgG1, kappa (Mouse)	60067

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

Intracellular Antigen Labeling

PRIMARY ANTIBODY*	SPECIES REACTIVITY	ISOTYPE	CATALOG #
Anti-Human OCT4 (OCT3) Antibody, Clone 3A2A20	Human	IgG2b, kappa (Mouse)	60093

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

General Reagents and Materials

REAGENTS AND MATERIALS	CATALOG #
D-PBS (Without Ca++ and Mg++)	37350
DMEM/F-12 with 15 mM HEPES	36254
Trypan Blue	07050
Gentle Cell Dissociation Reagent	07174
Dulbecco's Phosphate Buffered Saline with 2% Fetal Bovine Serum (2% FBS/PBS)	07905
1.7 mL microcentrifuge tubes	38038
5 mL round-bottom tubes	38007
15 mL conical tubes	38009
Propidium Iodide (optional for nuclear stain)	75002

Additional Reagents Required for Intracellular Antigen Labeling

Saponin Permeabilization Buffer (SPB)*

COMPONENT	CATALOG #	FINAL CONCENTRATION
Saponin	e.g. Fluka Biochemika 47036	1 mg/mL
10% BSA Solution	04915	1%
D-PBS (Without Ca++ and Mg++)	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 19943 1 LT	2%
D-PBS (Without Ca++ and Mg++)	37350	to final volume

*Mix well and store at 2 - 8°C.

Preparation of a Single-Cell Suspension for Flow Cytometry

Prepare a single-cell suspension as indicated in section 7.2. Perform a viable cell count using Trypan Blue and a hemocytometer. 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 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 round-bottom tube or a 1.7 mL microcentrifuge tube and place on ice.
3. Centrifuge cells at 300 x *g* for 5 minutes.

4. While the samples are centrifuging, prepare 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. If using a directly conjugated antibody, protect samples from exposure to direct light.
6. Add 1 mL of 2% FBS/PBS to each tube, gently mix and centrifuge at 300 x *g* for 5 minutes.
   - If using an unconjugated primary antibody: While the samples are centrifuging, prepare a sufficient quantity of the secondary antibody mix (100 µL/sample) using the appropriate secondary antibody at the predetermined optimal working dilution. Proceed to step 7.
   - If using a directly conjugated antibody: Proceed 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 round-bottom tube if necessary.

   Optional nuclear stain: Propidium Iodide (PI) can be added at a final concentration of 1 µg/mL to assess viability (e.g. add 1 mg/mL PI at a 1 in 1000 dilution).
10. Place samples on ice, while avoiding exposure to direct light, and analyze by flow cytometry as soon as possible.

Intracellular Antigen Labeling Protocol for OCT4

Note: Optimal concentrations of 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 round-bottom tube or a 1.7 mL microcentrifuge 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 per 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, prepare a sufficient quantity of the primary antibody mix (100 µL/sample) at the predetermined optimal working dilution, using SPB as the diluent.
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. If using a directly conjugated antibody, protect samples from exposure to direct light.

10. Add 1 mL of SPB to each tube, gently mix, and centrifuge at 300 x *g* for 5 minutes.
    - If using an unconjugated primary antibody: While the samples are centrifuging, prepare a sufficient quantity of the secondary antibody mix (100 µL/sample) using the appropriate secondary antibody at the predetermined optimal working dilution. Proceed to step 11.
    - If using a directly conjugated antibody: Proceed to step 13.
11. 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.
12. Add 1 mL of SPB to each tube. Gently mix and centrifuge at 300 x g for 5 minutes.
13. 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 round-bottom tube if necessary.
14. Place samples on ice, while avoiding exposure to direct light, and analyze by flow cytometry as soon as possible.

    Optional: 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 15.

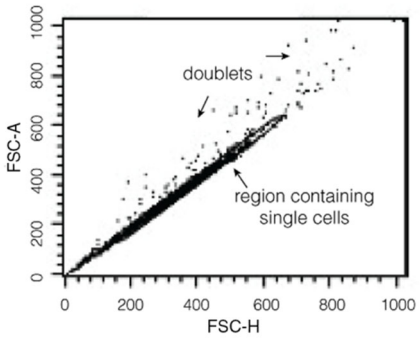

Figure 15. Example of Doublet Discrimination by Flow Cytometry

TECHNICAL MANUAL

Maintenance of Human Pluripotent Stem Cells in TeSR™2

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