

TECHNICAL MANUAL

Maintenance of Human Pluripotent Stem Cells in TeSR™-AOF

Critical Parameters for Successful Cell Culture with TeSR™-AOF

What is TeSR™-AOF?

TeSR™-AOF is an animal origin-free (AOF) human pluripotent stem cell (hPSC) maintenance medium to support researchers as an increasing number of approved cell therapies move towards the clinic. To address traceability and viral safety concerns, TeSR™-AOF is manufactured using animal origin-free raw materials with traceability to the secondary level of manufacturing. Critical medium components in TeSR™-AOF have been stabilized to allow for the maintenance of hPSCs with versatile every-other-day or weekend-free feeding schedules. Furthermore, TeSR™-AOF has been optimized to improve robustness and consistency of the medium, resulting in improved hPSC attachment and expansion.

What is Animal Origin-Free (AOF)?

AOF products do not contain any raw materials derived from animal tissues, cells, or fluids. The components used to manufacture the raw material ingredients are also not derived from an animal tissue, cell, or body fluid. Ingredients and components may be produced by or extracted from plants, bacteria or yeast, or cell-free systems, or they may be produced by organic synthesis.

This differs from animal component-free (ACF) claims on some other STEMCELL products, such as TeSR™-E8™, that do not contain ingredients derived from animal tissues or fluids but may contain recombinant animal or non-animal proteins derived from animal cell lines or fermentation processes.

Choosing an Appropriate Matrix for Use with TeSR™-AOF

Cells may be cultured in TeSR™-AOF using Corning® Matrigel®, Vitronectin XF™, or CellAdhere™ Laminin-521 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™ and CellAdhere™ Laminin-521 consist of single humanized proteins and can be used if 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™-AOF

Enzyme-free passaging methods are recommended for cells cultured in TeSR™-AOF (section 5.0) because of 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 embryonic stem cells (hESCs) and induced pluripotent stem cells (hiPSCs) as cell aggregates (section 5.1). With any passaging protocol, ensure to optimize the exposure time to the passaging reagent being used; the appearance of colonies after dissociation should be as shown in Figure 3 (ReLeSR™) or Figure 4 (Gentle Cell Dissociation Reagent). 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.

Medium Changes

Typically, hESCs and hiPSCs require daily medium changes for optimal growth; however, TeSR™-AOF enables flexible feeding schedules without affecting culture quality through the use of stabilized components (including fibroblast growth factor 2; FGF2).

Cells can be fed daily, every other day, or up to two consecutive days of feeding can be skipped when using TeSR™-AOF. To design a convenient schedule that suits your lab's routine, refer to the table below to determine the volume of medium required at each feed. Any combination of feeding intervals can be used throughout a passage when following these guidelines. It is important to note that if higher density cultures are being maintained, either late in the passage or on a shortened passaging schedule (4 - 5 days), it is recommended to feed cells when they reach a confluency of > 50%.

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FEEDING INTERVAL		
DAILY FEEDING	SKIP ONE DAY	SKIP TWO CONSECUTIVE DAYS
Standard feed volume (e.g. 2 mL per well of a 6-well plate)	Standard feed volume (e.g. 2 mL per well of a 6-well plate)	Double feed volume (e.g. 4 mL per well of a 6-well plate)

Aggregate vs. Single-Cell Passaging

The protocols in this manual for the routine expansion of hESCs and hiPSCs are for passaging cells as small aggregates of approximately 50 - 200 μm in diameter. These established methods have been shown to allow the long-term expansion of many different cell lines while maintaining a normal karyotype. It is possible to passage hESCs and hiPSCs 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 (Buzzard et al.; Draper et al.). Therefore, when performing single-cell passaging of hESCs or hiPSCs in any culture medium, check the karyotype frequently to ensure that the culture has retained a normal karyotype.

Characterization of hESCs and hiPSCs

It is good practice to monitor 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. hESCs and hiPSCs should retain a normal genetic makeup during routine culture, expansion, and manipulation. Nonetheless, chromosomal and genetic aberrations may appear during long-term passaging (Buzzard et al.; Draper et al.; Mitalipova et al.). Accordingly, it is important to periodically (approximately every 10 - 20 passages) check hESC and hiPSC cultures to exclude the possibility of an abnormal karyotype. More frequent screening for recurrent abnormalities commonly associated with hESC/hiPSC culture can be performed using tools such as the hPSC Genetic Analysis Kit (Catalog #07550).

Colony Density of hESC and hiPSC Cultures

Maintaining a proper colony density is a critical aspect of maintaining hESCs and hiPSCs in TeSR™-AOF. Cultures that are densely populated (> 50%) may require more frequent feed days nearing the end of the passage. A range of colony densities, as shown in Figure 6, can be tolerated in TeSR™-AOF; 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 6.4 and Appendix 1).

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

hESCs and hiPSCs cultured in mTeSR™1, mTeSR™ Plus, or other feeder-free media, can also be conveniently transferred to TeSR™-AOF with no adaptation step required (section 7.1.1), and a similar split ratio can be maintained when adapting from these media. An enhanced growth rate may be observed for hESCs and hiPSCs cultured in TeSR™-E8™, resulting in higher confluence cultures sooner after passaging. Cultures should be monitored carefully after the transition to ensure that the optimal day of passaging is not missed, as this may be one day sooner than for TeSR™-E8™ cultures plated at the same density. Alternatively, the split ratio can be adjusted to compensate for enhanced growth, for example, by seeding 20 - 25% fewer aggregates in TeSR™-AOF if any overgrowth is observed after transitioning.

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1.0 Introduction

The maintenance and expansion of human pluripotent stem cells (hPSCs) (human embryonic stem cells [hESCs] and induced pluripotent stem cells [hiPSCs]) 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. Use of feeder-free conditions eliminates the inherent biological variability of feeder cells, an undefined component of the culture system. Historically, efforts have focused on improving the reproducibility of hESC and hiPSC culture protocols by simplifying and removing undefined components from all aspects of the culture system: media, matrices, and passaging reagents. Recently, there have been increased efforts to improve the traceability of components used in hPSC maintenance media.

TeSR™-AOF is an animal origin-free (AOF), stabilized, serum-free cell culture medium for the feeder-free maintenance and expansion of hESCs and hiPSCs. It aims to improve component traceability and viral safety for users.

Similar to mTeSR™ Plus, TeSR™-AOF contains formulation-stabilized critical medium components such as fibroblast growth factor 2 (FGF2; also known as basic FGF [bFGF]). These modifications enhance the flexibility of the medium, allowing for greater hESC and hiPSC expansion with daily feeding, while also maintaining cell quality during restricted feeding schedules such as every-other-day or weekend-free schedules.

TeSR™-AOF is capable of maintaining high-quality hESCs and hiPSCs when used with Corning® Matrigel® as the matrix, or if a fully defined culture system is desired, Vitronectin XF™ (developed and manufactured by Nucleus Biologics), or CellAdhere™ Laminin-521. Any of these combinations provide highly robust cell culture systems that are compatible with many novel applications that are rapidly accelerating stem cell research.

hESCs and hiPSCs maintained in TeSR™-AOF 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, TRA-1-60, and NANOG)
- Have a high cloning efficiency when plated at a clonal density in TeSR™-AOF with CloneR™ (Catalog #05888)
- 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 TeSR™-E8™, mTeSR™ 1, mTeSR™ Plus, or other feeder-free maintenance media

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2.0 Materials, Reagents, and Equipment

2.1 TeSR™-AOF (Catalog #100-0401)

For component storage and stability information, refer to the Product Information Sheet (PIS) for TeSR™-AOF, available at www.stemcell.com, or contact us to request a copy.

The following components are sold as a complete kit and are not available for individual sale:

COMPONENT #	COMPONENT NAME	SIZE
100-0402	TeSR™-AOF Basal Medium	475 mL
100-0403	TeSR™-AOF 20X Supplement	25 mL

2.2 Additional Materials for hESC and hiPSC Culture

CATEGORY	PRODUCT	CATALOG #
Culture Media	mTeSR™1	85850
	mTeSR™ Plus	100-0276
	TeSR™-E8™	05990
Passaging Reagents	Gentle Cell Dissociation Reagent	100-0485
	ReLeSR™	100-0484
Matrices and Related Materials	CellAdhere™ Dilution Buffer	07183
	CellAdhere™ Laminin-521	77003
	Corning® Matrigel® hESC-Qualified Matrix	Corning 354277
	Non-tissue culture-treated 6-well plates*	e.g. Corning 351146
	Tissue culture-treated cultureware**	e.g. 38016 (6-well plates)
	Vitronectin XF™	07180
Cryopreservation Media	CryoStor® CS10	07930
	FreSR™-S	05859
	mFreSR™	05855
Other Materials	15 mL and 50 mL conical tubes	e.g. 38009 and 38010
	Cell scrapers	e.g. 200-0598
	CloneR™	05888
	DMEM/F-12 with 15 mM HEPES	36254
	D-PBS (Without Ca++ and Mg++)	37350
	hPSC Genetic Analysis Kit	07550
	Serological pipettes	e.g. 38002
	Trypan Blue	07050
	Y-27632 (Dihydrochloride)	72302

*Required for use with Vitronectin XF™

**Required for use with Corning® Matrigel® or CellAdhere™ Laminin-521

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2.3 Equipment Required for hESC and hiPSC 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% CO₂ 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 (LN₂) vapor tank
- -80°C freezer
- -20°C freezer
- Refrigerator (2 - 8°C)

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3.0 Culturing hESCs and hiPSCs Using TeSR™-AOF

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™-AOF

Undifferentiated hESCs and hiPSCs (Figure 1A) cultured in TeSR™-AOF 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 will be multi-layered in the center, resulting in dense clusters of cells when viewed under phase contrast. Halo-like multi-layering around the outer edges is also typical for cells grown in TeSR™-AOF. Colonies cultured in TeSR™-AOF have a more condensed and round morphology when cultured on Vitronectin XF™ compared to colonies cultured on Corning® Matrigel®, 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).

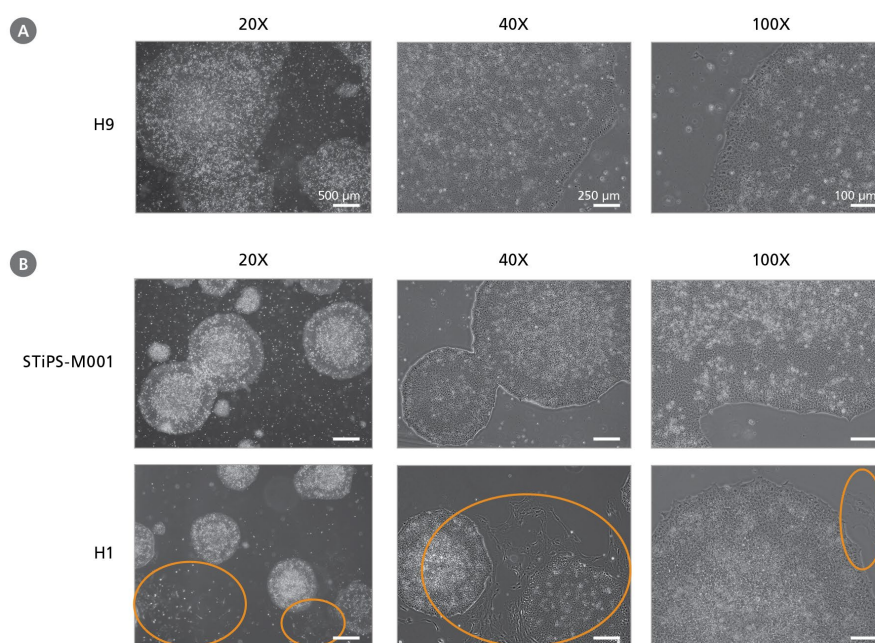


Figure 1. Morphology of hESCs and hiPSCs Cultured in TeSR™-AOF

(A) Undifferentiated hESCs (H9) on Corning® Matrigel® and hiPSCs (STiPS-M001) on Vitronectin XF™ at the optimal time of passaging. Cells were fed using a flexible feed schedule (section 6.1).

(B) Area of spontaneous differentiation (orange circle) between undifferentiated H1 colonies on Vitronectin XF™. Magnifications: 20X, 40X, and 100X.

3.2 Assessing TeSR™-AOF Cultures to Determine Day of Passage

hESCs and hiPSCs cultured in TeSR™-AOF are ready to passage when the majority of colonies are large, compact, and have centers that are dense compared to their edges (Figure 2). For up to 4 days after plating in TeSR™-AOF, colonies may not be very densely packed with cells. The density and robustness of the

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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, and yields will be decreased. If colonies are passaged too late, the culture may begin to show signs of differentiation or loose packing morphology. There is an approximate 24-hour window that is optimal for passaging. If there are large colonies with dense centers, passage the cells within 24 hours (for additional information, see section 8.0).

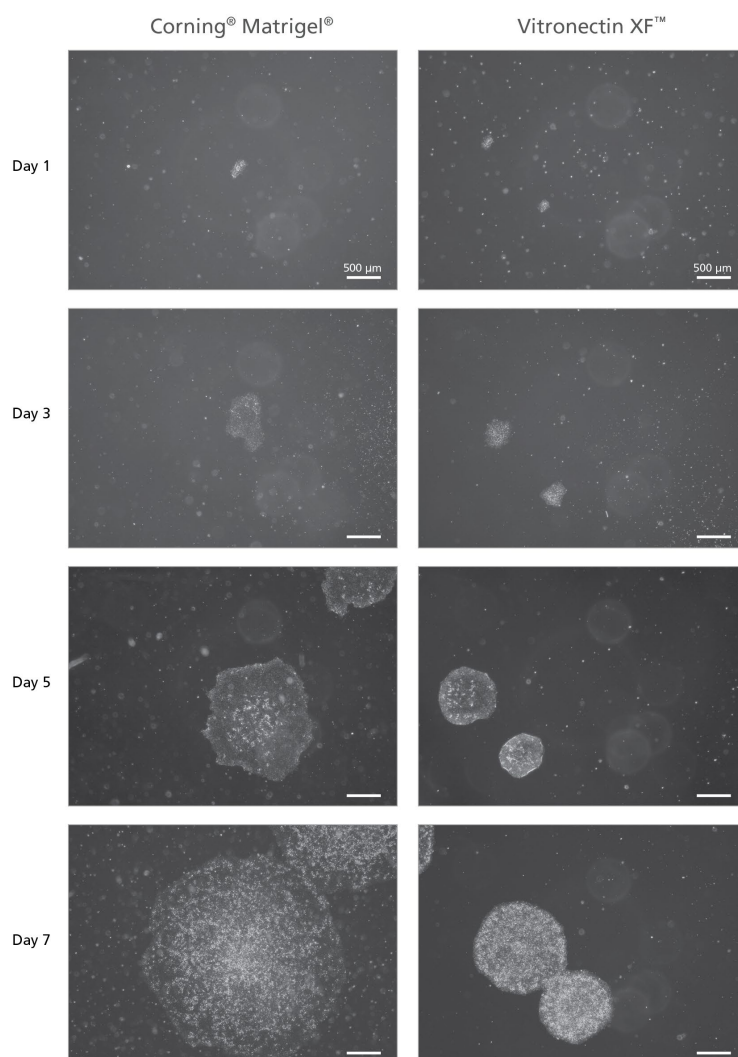


Figure 2. hESCs Cultured on Corning® Matrigel® and Vitronectin XF™ in TeSR™-AOF at Days 1 - 7 After Passaging

hESCs (H9) cultured in TeSR™-AOF on Matrigel® (left) and Vitronectin XF™ (right) at a magnification of 20X. 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 6.4) and aggregate size (section 6.3). Cells were fed on a flexible feeding schedule (section 6.1). Cells in TeSR™-AOF expand more rapidly than cells in TeSR™-E8™ when plated at the same density.

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4.0 Preparation of Reagents and Materials

4.1 Complete TeSR™-AOF Medium

Use sterile technique to prepare complete TeSR™-AOF medium (Basal Medium + 20X 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™-AOF 20X Supplement at room temperature (15 - 25°C) or overnight at 2 - 8°C.

Warm to room temperature. Mix 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 25 mL of TeSR™-AOF 20X Supplement to 475 mL of TeSR™-AOF Basal Medium. Mix thoroughly.

Note: If not used immediately, store complete TeSR™-AOF medium at 2 - 8°C for up to 2 weeks. Alternatively, aliquot and store at -20°C for up to 3 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™-AOF 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 hESCs and hiPSCs in TeSR™-AOF requires the use of a suitable matrix to allow attachment of cell aggregates. Corning® Matrigel®, Vitronectin XF™, or CellAdhere™ Laminin-521 can be used as a surface coating matrix with TeSR™-AOF. Corning® Matrigel® is a routinely used surface coating matrix for a variety of different applications, although the composition is undefined. In contrast, Vitronectin XF™ or CellAdhere™ Laminin-521 can be used 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®), 4.2.2 (Vitronectin XF™), or 4.2.3 (CellAdhere™ Laminin-521). Refer to Table 1 for recommended volumes of diluted matrix for coating various cultureware.

Table 1. Recommended Volumes for Coating Cultureware

CULTUREWARE*	VOLUME OF DILUTED MATRIGEL® OR VITRONECTIN XF™	VOLUME OF DILUTED CELLADHERE™ LAMININ-521
6-well plate	1 mL/well	1.5 mL/well
100 mm dish	6 mL/dish	8 mL/well
T-25 cm ² flask	3 mL/flask	4 mL/flask
T-75 cm ² flask	8 mL/flask	10.5 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 with 15 mM HEPES into a 50 mL conical tube 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 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 hESC or hiPSC 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™-AOF (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; Corning Catalog #351146) 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. add 40 µL of Vitronectin XF™ to 960 µL of CellAdhere™ Dilution Buffer). Use a 50 mL polypropylene conical tube 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.

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 hESC or hiPSC 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 proceeding to step 7.*

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

4.2.3 CellAdhere™ Laminin-521

Note: Use tissue culture-treated cultureware (e.g. Falcon® 6-Well Flat-Bottom Plate, Tissue Culture-Treated, Catalog #38016) with CellAdhere™ Laminin-521.

1. Thaw CellAdhere™ Laminin-521 at 2 - 8°C.
Note: If not using immediately, store at 2 - 8°C for up to 3 months.
2. Dilute CellAdhere™ Laminin-521 in Dulbecco's phosphate-buffered saline with Ca++ and Mg++ to a final concentration of 10 - 20 µg/well.
Note: When adapting to the new culture matrix, cells may benefit from a higher concentration of CellAdhere™ Laminin-521 for the first few passages.
3. Gently mix the diluted CellAdhere™ Laminin-521. Do not vortex.
4. Immediately add diluted CellAdhere™ Laminin-521 to cultureware. See Table 1 for recommended coating volumes.
5. Gently rock the cultureware back and forth to spread the solution evenly across the entire surface.
6. Seal the cultureware to prevent evaporation of the CellAdhere™ Laminin-521 solution (e.g. with Parafilm®). Incubate at 2 - 8°C overnight. If a more rapid coating is required, incubate at 37°C for at least 2 hours before use.
Note: If not used immediately, the cultureware can be stored at 2 - 8°C for up to 4 weeks after coating. Do not allow the culture surface to dry, as the matrix will become inactivated.
7. Aspirate the CellAdhere™ Laminin-521 solution when cells are ready to be plated.
Note: The coating does not require washing before use.
8. Immediately add TeSR™-AOF (e.g. 2 mL/well if using a 6-well plate).

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5.0 Enzyme-Free Passaging of hESCs and hiPSCs Cultured in TeSR™-AOF

hESCs and hiPSCs maintained in TeSR™-AOF on Corning® Matrigel® or Vitronectin XF™ are recommended to be passaged using non-enzymatic methods as described in this section. If CellAdhere™ Laminin-521 is used as the matrix, passaging protocols must be optimized by the user.

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 ReLeSR™

ReLeSR™ is an enzyme-free reagent for dissociation and passaging of hESCs and hiPSCs as aggregates **without** manual selection of differentiated areas or scraping to remove 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 plates with Corning® Matrigel® (section 4.2.1) or Vitronectin XF™ (section 4.2.2).
2. Aliquot sufficient TeSR™-AOF and warm to room temperature (15 - 25°C).
Note: Do not warm TeSR™-AOF 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 to completely remove the ReLeSR™ immediately or within 1 minute (if working with multiple wells), so that colonies are exposed to only the residual liquid.
5. Incubate at room temperature for 4 - 8 minutes. With some cell lines (e.g. Healthy Control Human iPSC Line, Female, SCTi003-A; Catalog #200-0511), a shorter incubation at room temperature is preferred (e.g. 4 - 6 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 (see Figure 3 and section 8.0). For more information, refer to the product information sheet (PIS) of the respective cell line.
6. Add 1 mL of TeSR™-AOF.
7. Detach the colonies by placing the plate on a plate vortexer (e.g. Multi-MicroPlate Genie, 120 V, 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. Using a 5 mL serological pipette, transfer the detached cell aggregates to a 15 mL conical tube. Cell aggregates should be appropriately sized for plating (mean aggregate size of approximately 50 - 200 µm; see Figure 3).
Note: To plate cell aggregates directly from the passaged well (i.e. without transferring into a tube), pipette the aggregate mixture up and down once using a 5 mL serological 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™-AOF. 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 split ratios (i.e. cell aggregates from 1 well can be plated in 10 to 50 wells). If the colonies are too dense or too sparse, adjust the split ratio accordingly at the next passage (see section 6.4).

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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 hESCs and hiPSCs.

11. Perform medium changes as needed using TeSR™-AOF, and visually assess cultures to monitor growth until the next passaging time. Medium can be changed daily or every other day. To skip two consecutive days of feeding, add twice the volume of medium.

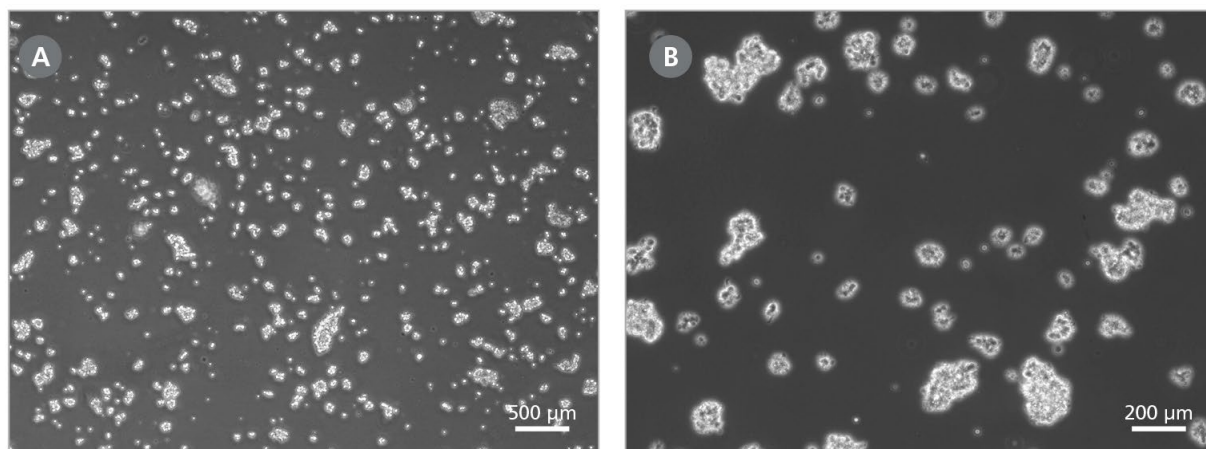


Figure 3. Aggregate Size of hPSCs Following Passaging With ReLeSR™

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).

5.2 Gentle Cell Dissociation Reagent (GCDR)

Gentle Cell Dissociation Reagent (GCDR) is an enzyme-free reagent for passaging hESCs and hiPSCs 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 6.3) or plating density (section 6.4).

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 Corning® Matrigel® (section 4.2.1) or Vitronectin XF™ (section 4.2.2).
2. Aliquot sufficient complete TeSR™-AOF and warm to room temperature (15 - 25°C).
Note: Do not warm TeSR™-AOF 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 1.

5. Aspirate medium from the well and add 1 mL of GCDR.

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6. Incubate at room temperature for 6 - 8 minutes. Refer to Figure 4 for a representative example of the desired appearance following incubation.

Note: 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 GCDR, then add 1 mL of TeSR™-AOF. 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™-AOF 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 2 for suggestions on how to break up 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 2. 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
CellAdhere™ Laminin-521	2 mL serological pipette	2 - 5

*Number can be adjusted to obtain desired cell aggregate size (section 6.3). Refer to Figure 5 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™-AOF. 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 split ratios (i.e. cell aggregates from 1 well can be plated in 10 - 50 wells). If the colonies are too dense or too sparse, at the next passage adjust the split ratio accordingly (see section 6.4).

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 hESCs and hiPSCs.

12. Perform medium changes as needed using TeSR™-AOF and visually assess cultures to monitor growth until the next passaging time. Medium can be changed daily or every other day. To skip two consecutive days of feeding, add twice the volume of medium.

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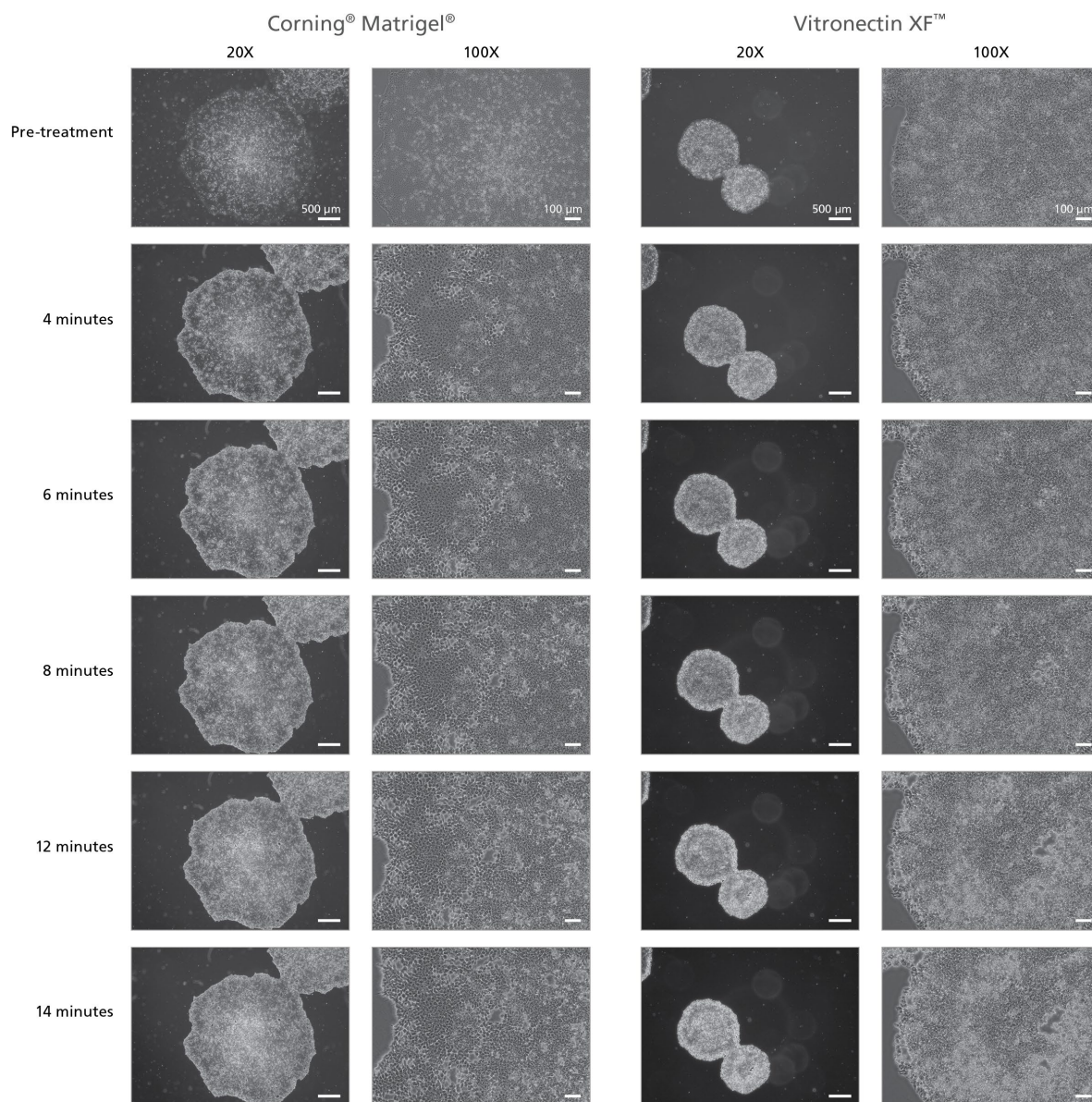


Figure 4. Effect of GCDR on hESCs Cultured on Corning® Matrigel® or Vitronectin XF™ in TeSR™-AOF

hESCs (H9) at different timepoints during incubation with GCDR. After the recommended incubation time (in this case, 6 - 10 minutes), 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. Magnifications: 20X and 100X.

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.

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6.0 Using Flexible Feeding and Passaging Schedules

Culturing hESCs and hiPSCs in TeSR™-AOF allows great flexibility in feeding and passaging schedules; cultures can be fed daily, or on every-other-day, or weekend-free schedules, and can be passaged between 4 and 7 days after plating in TeSR™-AOF.

6.1 Feeding Schedule

Typically, hESCs and hiPSCs require daily medium changes for optimal growth; however, TeSR™-AOF enables more flexible feeding schedules without affecting culture quality through the use of stabilized components (including FGF2).

Cells can be fed daily, every other day, or up to two consecutive days of feeding can be skipped when using TeSR™-AOF. To design a convenient schedule, refer to Table 3 to determine the volume of medium required at each feed. Any combination of feeding intervals can be used throughout a passage when following these guidelines. However, if cultures increase in confluency to > 50%, it is recommended that cultures be fed daily.

Table 3. Medium Volumes for Different Feeding Intervals

FEEDING INTERVAL		
DAILY FEEDING	SKIP ONE DAY	SKIP TWO CONSECUTIVE DAYS
Standard feed volume (e.g. 2 mL per well of a 6-well plate)	Standard feed volume (e.g. 2 mL per well of a 6-well plate)	Double feed volume (e.g. 4 mL per well of a 6-well plate)

Rapidly growing or very confluent cultures may still benefit from daily medium changes, or an increase in medium feed volumes.

6.2 Passaging Schedule

The passaging schedule 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; if small cell aggregates are plated at a low density, the next passage will most likely occur on day 6 or 7 (see Table 4). For representative images of large and small cell aggregates, see section 6.3.

For representative images of colony densities that should be observed at the time of passage, see section 6.4. 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 passaging.

Table 4. Parameters that Affect Passaging Schedule

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	6 - 7
Small	Low	Low	

*For representative images see Figure 5.

**For representative images see Figure 6.

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6.3 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 2). Refer to Figure 5 for a recommended range of cell aggregate sizes. Do not generate single cells.

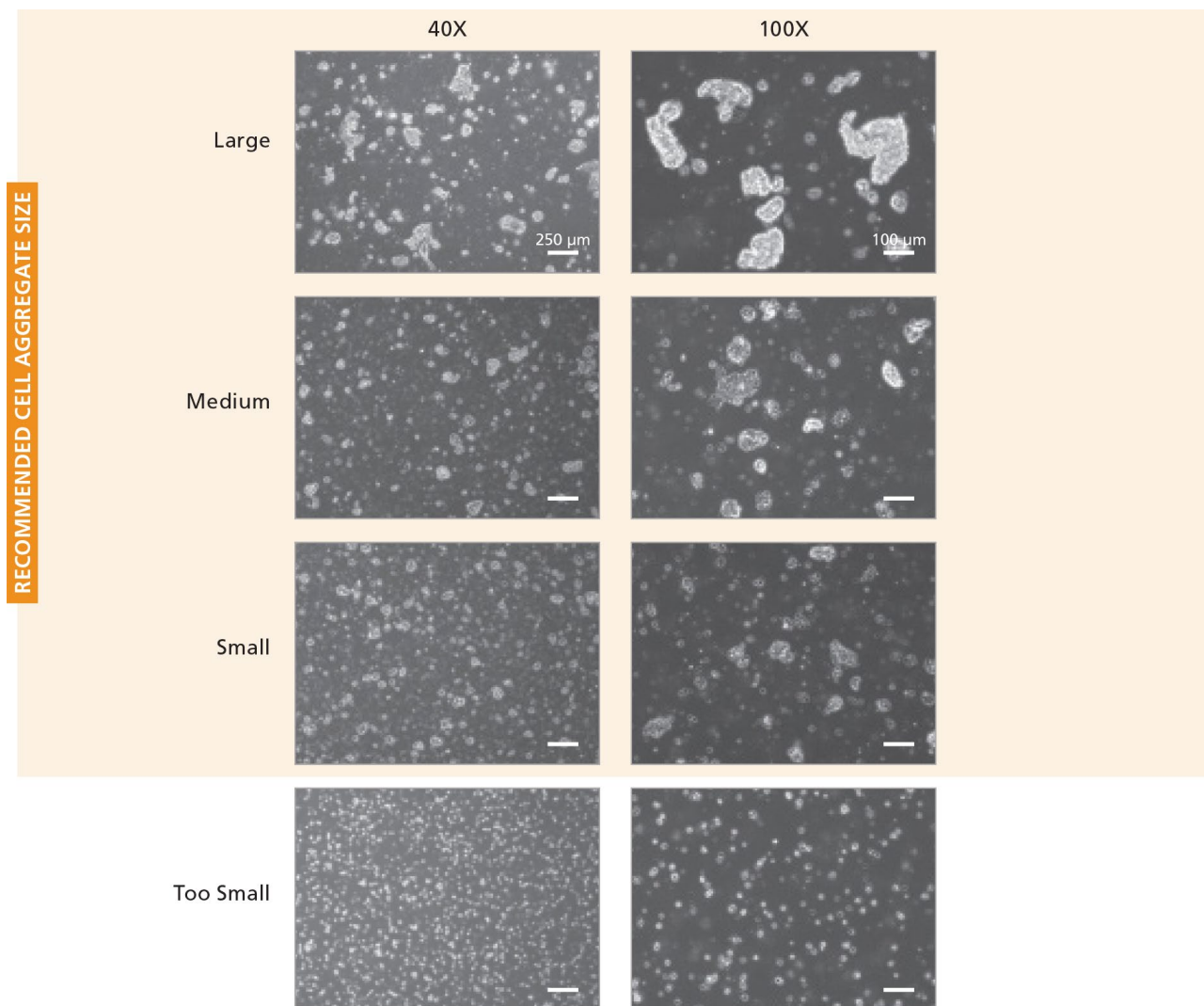


Figure 5. Acceptable Size Range for Cell Aggregates at the Time of Plating

Cell aggregate size can be adjusted by altering the number of times the cell aggregate mixture is pipetted up and down. Do not generate single cells. Magnifications: 40X and 100X.

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6.4 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 passaging schedule, the cell line used, and the individual operator. Refer to Figure 6 for a recommended range of colony densities that should be observed at the time of passage. Counting cell aggregates is an alternate way to determine and adjust plating densities (see Appendix 1).

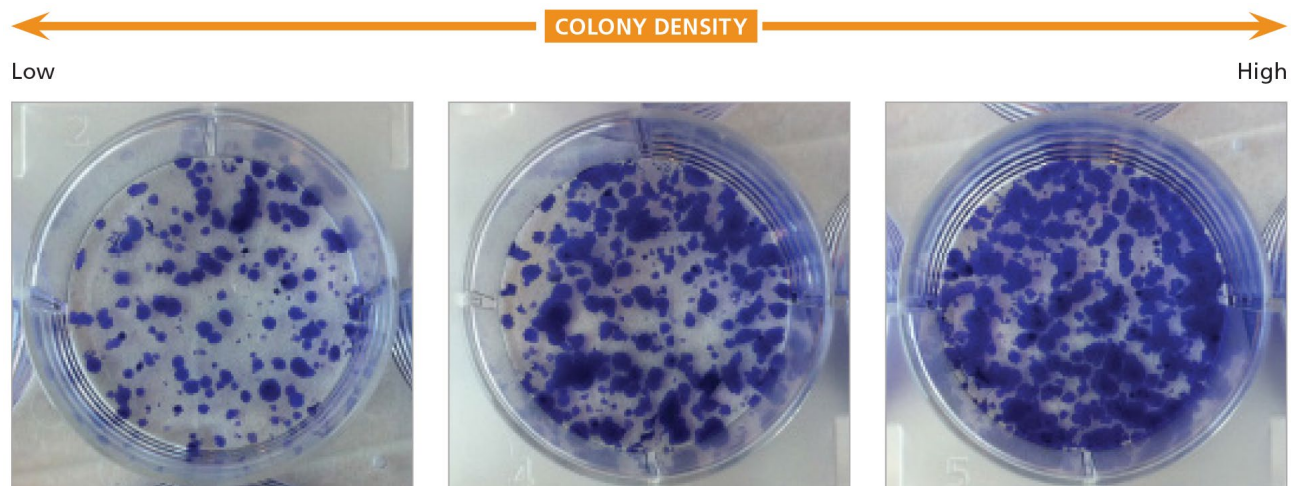


Figure 6. Example Colony Densities for hESC or hiPSC Cultures

hESCs (H1) stained with Giemsa at the optimal time of passaging (the optimal time of passaging may be different for each colony density; see Table 4). Each image represents a single well of a 6-well plate.

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7.0 Additional Protocols

7.1 Transitioning Cells

7.1.1 From Feeder-Free Media to TeSR™-AOF

Typically, no adaptation step is required when plating hESCs and hiPSCs from mTeSR™ Plus, mTeSR™ 1, TeSR™-E8™, or other feeder-free maintenance medium to TeSR™-AOF. When transitioning from these media, enzyme-free passaging protocols are recommended.

After enzyme-free passaging (section 5.0), plate cell aggregates onto coated cultureware containing TeSR™-AOF. 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™-AOF is appropriate.

For hPSCs maintained in low-protein medium (e.g. TeSR™-E8), an enhanced growth rate may also be observed when transitioning cultures to TeSR™-AOF, resulting in higher confluence cultures sooner after passaging. Cultures should be monitored carefully after the transition to ensure that the optimal day of passaging is not missed, as this may be one day sooner than for cultures plated at the same density in non-stabilized media. Alternatively, the split ratio can be adjusted to compensate for enhanced growth, for example, by seeding 20 - 25% fewer aggregates in TeSR™-AOF if any overgrowth is observed after transitioning.

7.1.2 Cultured on Corning® Matrigel® to Vitronectin XF™ or CellAdhere™ Laminin-521

hESCs and hiPSCs cultured on Corning® Matrigel® in TeSR™-AOF may be conveniently transitioned to Vitronectin XF™ or CellAdhere™ Laminin-521 without an adaptation step.

Note: Both the medium and the matrix can be changed at the same time (e.g. cells cultured on Corning® Matrigel® using TeSR™-E8™ may be transitioned onto Vitronectin XF™ with TeSR™-AOF in one step; see section 7.1.1).

7.2 Preparing a Single-Cell Suspension for Downstream Applications

The following are instructions for preparing a single-cell suspension using either Gentle Cell Dissociation Reagent (GCDR) or ACCUTASE™, from cultures grown in TeSR™-AOF in 6-well plates. If using other cultureware, adjust volumes accordingly. Cultures should be harvested and used for downstream applications at the time they would normally be ready for passaging.

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

Note: Do not warm TeSR™-AOF in a 37°C water bath.

2. Wash the well to be passaged with 1 mL of D-PBS (Without Ca++ and Mg++).
3. If using GCDR: Add 1 mL of GCDR. Incubate at 37°C for 8 - 10 minutes. Proceed to step 4.

If using ACCUTASE™:

- a) Add 1 mL of ACCUTASE™. Incubate at 37°C for ~5 minutes.
- b) Add 2 mL of TeSR™-AOF to the well.
- c) Using a 1 mL pipettor, gently wash the cells from the surface of the plate by spraying the solution directly onto the colonies.
- d) Proceed to step 4.

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™-AOF) 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 (Dihydrochloride) is recommended when plating single-cell suspensions of hESCs or hiPSCs, 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

hPSCs maintained in TeSR™-AOF can be cryopreserved as aggregates using CryoStor® CS10, or as single cells using FreSR™-S. Either cryopreservation method is appropriate for use with cultures routinely passaged as aggregates using the methods described in this manual.

The thawing protocols are for hESCs and hiPSCs that were maintained in TeSR™-AOF prior to cryopreservation. Cells cultured using other maintenance protocols (e.g. feeder-free media such as mTeSR™ Plus, mTeSR™1, or TeSR™-E8™) 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™-AOF (section 7.1.1).

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™-AOF in 6-well plates using CryoStor® CS10. 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 an enzyme-free passaging protocol until step 8 (section 5.1 or 5.2).
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. Gently resuspend the pellet with 1 mL per well harvested of cold (2 - 8°C) CryoStor® CS10 using a serological pipette. Minimize the break-up of cell aggregates when dislodging the pellet.
5. Transfer 1 mL of cell aggregates mixture into each labeled cryovial using a 2 mL serological pipette.
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

hESCs and hiPSCs 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, warm TeSR™-AOF (15 - 25°C), 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™-AOF 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 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 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™-AOF 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™-AOF using a 2 mL serological pipette. Take care to maintain the cells as aggregates.

10. Transfer 0.5 mL of the cell mixture into one well of a coated 6-well plate containing TeSR™-AOF (i.e. two wells can be plated from each cryovial).

Note: The 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 hESCs and hiPSCs.

12. Perform medium changes as needed using TeSR™-AOF, and visually assess cultures daily 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. Medium can be changed daily or every other day. To skip two consecutive days of feeding, add twice the volume of medium.

Note: For some cell lines (e.g. Healthy Control Human iPSC Line, Female, SCTi003-A; Catalog #200-0511), spontaneous differentiation must be removed manually at the first passage after thaw, independent of which passaging reagent is used (including ReLeSR™). If only a few undifferentiated colonies are observed after thawing, it may be necessary to select only these colonies for passaging and replat 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 hESCs/hiPSCs 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™-AOF 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 (TeSR™-AOF or DMEM/F-12 with 15 mM HEPES), Gentle Cell Dissociation Reagent, and D-PBS (Without Ca++ and Mg++) to room temperature (15 - 25°C) before use.

Note: Do not warm TeSR™-AOF 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 a serological pipette or a 1 mL pipettor to ensure a single-cell suspension, then transfer cells to a 15 mL conical tube. Rinse wells with an additional 2 - 4 mL of medium (TeSR™-AOF or DMEM/F-12 with 15 mM HEPES) and add the rinse to the tube containing the cells.
5. Perform a viable cell count using Trypan Blue.
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 10⁶ cells/mL and mix thoroughly.
9. Transfer 1 mL of the single-cell suspension to each cryovial.

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10. Cryopreserve cells using either:

- A standard slow rate-controlled cooling protocol that reduces temperatures at approximately $-1^{\circ}\text{C}/\text{min}$, 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

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

1. Have all tubes, warm TeSR™-AOF ($15 - 25^{\circ}\text{C}$) and 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™-AOF in a 37°C water bath.

2. Add Y-27632 (Dihydrochloride) to TeSR™-AOF to reach a final concentration of $10 \mu\text{M}$.
3. Wipe the outside of the vial of cells 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.
8. Centrifuge cells at $300 \times g$ for 5 minutes at room temperature ($15 - 25^{\circ}\text{C}$).
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™-AOF containing $10 \mu\text{M}$ Y-27632 (Dihydrochloride) to the tube. Mix gently.
11. Plate cells onto coated cultureware.

Note: In general, one frozen cryovial containing 1×10^6 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 medium changes as needed using TeSR™-AOF (without Y-27632 [Dihydrochloride]) and visually assess cultures daily to monitor growth until the next passaging time (i.e. 80 - 90% confluent). This takes approximately 2 - 5 days after thawing. Medium can be changed daily or every other day. To skip two consecutive days of feeding, add twice the volume of medium.

Note: The time to reach 80 - 90% confluency 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. using ReLeSR™ [section 5.1])

Note: It is not recommended to perform serial single-cell passaging due to the increased risk of karyotype abnormalities (Buzzard et al.; Draper et al.).

8.0 Troubleshooting

PROBLEM	SOLUTION
Excessive differentiation (> 20%) in cultures	<ul style="list-style-type: none"> • Ensure that complete TeSR™-AOF 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. • Ensure cultures are passaged when the majority of colonies are large, compact, and have centers that are dense compared to their edges, before centers overgrow. • Decrease the colony density by plating fewer cell aggregates during passaging. • Reduce incubation time with ReLeSR™ during passaging, as your cell line/culture may be more sensitive.
Cell aggregate size obtained with ReLeSR™ (section 5.1) is not ideal (i.e. aggregates do not look like the example in Figure 3)	<ul style="list-style-type: none"> • Larger aggregates are obtained (i.e. mean aggregate size is > 200 µm) <ul style="list-style-type: none"> ○ Pipette the cell aggregate mixture up and down. Avoid generating a single-cell suspension. ○ Increase the incubation time by 1 - 2 minutes. • Smaller aggregates are obtained (i.e. mean aggregate size is < 50 µm) <ul style="list-style-type: none"> ○ 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	<ul style="list-style-type: none"> • 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™	<ul style="list-style-type: none"> • Decrease the incubation time with ReLeSR™ by 1 - 2 minutes.
Colonies remain attached and/or significant scraping is required to dislodge cells	<ul style="list-style-type: none"> • Ensure that the passaging reagents are used as described in section 5.0. • Increase the incubation time by 1 - 2 minutes.
Low attachment observed after plating	<ul style="list-style-type: none"> • 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™ or Gentle Cell Dissociation Reagent) to minimize the duration 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. • 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® and CellAdhere™ Laminin-521.

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PROBLEM	SOLUTION
Single cells are generated during colony dissociation	<ul style="list-style-type: none"> • 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	<ul style="list-style-type: none"> • Ensure that non-tissue culture-treated plates are used when coating with Vitronectin XF™.
Cells detach during day 1 medium change	<ul style="list-style-type: none"> • 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	<ul style="list-style-type: none"> • 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	<ul style="list-style-type: none"> • 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.
Morphology is poor when cells are highly confluent	<ul style="list-style-type: none"> • Increase feed volumes when cells are highly confluent. • Feed daily if using restricted feeding schedules.

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Appendix 1: Plating hESCs and hiPSCs 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 hESC and hiPSC culture.

In the protocol below, count cell aggregates $\geq 50 \mu\text{m}$ 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 $\geq 50 \mu\text{m}$ 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):

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

$$N_T = C \times V_T$$

6. Determine the target number of cell aggregates to plate (N_P , refer to Table 5). Ensure that the total target number of cell aggregates to plate for all conditions in your experiment (i.e. $N_P \times$ number of conditions) does not exceed N_T .

Table 5. 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	300	600	850
100 mm dish	1800	3600	5000
T-75 cm^2 flask	2400	4800	7000

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 the calculated volume of cell aggregate mixture (V_P) to coated wells containing TeSR™-AOF.
10. Continue with the appropriate passaging protocol (section 5.0).

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Appendix 2: Flow Cytometry Protocols

Reagents and Materials

Antibodies

Antibodies can be used to characterize hESCs and hiPSCs by flow cytometry. The tables below contain information about a selection of antibodies available from STEMCELL Technologies that can be used to characterize hESCs and hiPSCs. 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 (CD15) 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.

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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	e.g. 38038
5 mL round-bottom tubes	e.g. 38007
15 mL conical tubes	e.g. 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	e.g. Sigma A1595	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 (e.g. Catalog #100-1181). 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 1×10^5 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.

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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 $4 - 8 \times 10^5$ 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 to each 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 analysis by flow cytometry.

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.

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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 μ L/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 μ L 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 that 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 (see Figure 7).

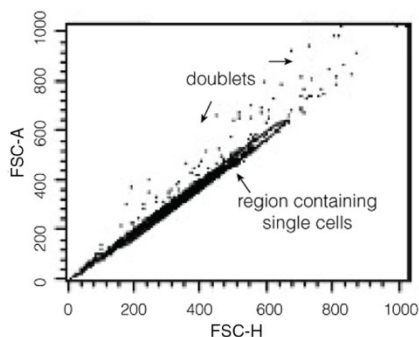


Figure 7. Example of Doublet Discrimination by Flow Cytometry

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