TECHNICAL MANUAL

Expansion of Human Pluripotent Stem Cells in eTeSR™

Critical Parameters for Successful Cell Culture with eTeSR™

What is eTeSR™?

eTeSR™ is an enhanced feeder-free cell culture medium that is stabilized and optimized to support the maintenance and expansion of human pluripotent stem cells (hPSCs) when passaged as single cells. Whether for routine maintenance or application-specific single-cell culture, eTeSR™ is formulated to reduce cellular stress associated with single-cell passaging and maintain long-term genetic stability of hPSCs. eTeSR™ builds upon previous TeSR™ formulations,[1,2] the most widely published feeder-free cell culture media for hPSCs.

eTeSR™ has been specifically developed to support single-cell passaging which typically involves shorter passaging schedules at high cell density. To cope with the increased metabolic demand and increased cell stress associated with this technique, eTeSR™ contains stabilized components, improved buffering capacity, and optimized metabolites to produce high quality hPSCs with improved genetic stability.

Genomic Stability: Aggregate versus Single-Cell Passaging

Historically, hPSCs have been passaged as aggregates to support hPSC culture quality. STEMCELL provides many optimized protocols to support researchers during the routine maintenance of hPSCs as aggregates using mTeSR™1, mTeSR™ Plus, TeSR™-AOF, or TeSR™-E8™. However, the rising development of workflows requiring single-cell dissociation (e.g. CRISPR gene editing and hPSC scale-up) has resulted in the increased use of routine single-cell passaging to maintain hPSCs. As single-cell hPSC passaging is linked to increased genetic instability, there is a need for robust culture systems that alleviate the appearance of genetic variants in hPSC cultures.

eTeSR™ has been shown to significantly improve the long-term genetic stability of hPSCs when performing routine single-cell passaging compared to other hPSC maintenance media. Despite this, the risk of cultures acquiring cytogenetic changes still remains.[3,4] When performing long-term maintenance of hPSCs using any passaging method or cell culture medium, it is important to frequently verify the genetic stability of the cells with an appropriate method or combination of methods, including G-band karyotyping, microarray (SNP array or array CGH), qPCR (e.g. hPSC Genetic Analysis Kit [Catalog #07550]), or whole genome sequencing.

What is Single-Cell Passaging?

Routine maintenance of hPSCs using single-cell passaging requires enzymatic dissociation to generate a single-cell suspension of cells prior to re-plating. A single-cell suspension can be achieved by incubation at 37°C with enzymatic reagents such as TrypLE™ Express or ACCUTASE™, which may be quenched using a high-protein inhibitor solution or through dilution with protein-containing culture medium (e.g. eTeSR™). Cells may be centrifuged to remove residual dissociation reagent or sufficiently diluted to reduce the concentration of passaging reagent transferred during passaging. hPSCs are then seeded in culture medium containing ROCK inhibitors (e.g. Y-27632, thiazovivan) or seeding supplements (CloneR™, CloneR™2) to enhance cell survival and attachment. A full-medium change is performed 24 hours later to remove the additives and allow hPSCs to expand.

Choosing an Appropriate Matrix for Use with eTeSR™

eTeSR™ has been tested in combination with hESC-qualified Corning® Matrigel® and CellAdhere™ Laminin-521 surface coating matrices. hESC-qualified Corning® Matrigel® is a routinely used surface coating matrix for a variety of applications, although the composition is undefined. In contrast, CellAdhere™ Laminin-521 consist of single humanized proteins and can be used if a defined culture system is desired. For directions for coating cultureware with these matrices, see section 4.3. eTeSR™ may be compatible with additional hPSC culture matrices; user optimization may be required.

Choosing an Appropriate Dissociation Reagent for Use with eTeSR™

TrypLE™ Express or ACCUTASE™ can be used as dissociation reagents for passaging hPSCs as single cells. The optimal incubation time can vary when using different dissociation reagents, cell lines, cell culture matrices, and incubation temperatures. Therefore, dissociation should be monitored under the microscope until the optimal incubation time is determined for each application. Refer to section 4.0 for single-cell dissociation of hPSCs using TrypLE™ Express or ACCUTASE™.

Cell Culture Medium Changes

hPSC cultures require frequent medium changes for maintaining optimal growth and culture quality. eTeSR™ allows for daily and restricted schedules without affecting culture quality through the stabilization of key components, including fibroblast growth factor 2 (FGF2; also known as basic FGF [bFGF]). With eTeSR™, hPSC cultures can be fed daily, every other day, or every third day. Refer to section 5.0 for flexible feeding schedules.

Characterization of hPSCs

It is good practice to monitor hPSC cultures frequently to ensure maintenance of undifferentiated cells. Routine assessment of cell morphology and periodic assessment by flow cytometry for expression of undifferentiated markers is recommended. Flow cytometry protocols for assessing the proportion of undifferentiated cells are described in Appendix 1. When maintained as single cells in eTeSR™, hPSCs typically do not exhibit areas of spontaneous differentiation.

Transitioning hPSCs Routinely Passaged as Single Cells to eTeSR™

There is typically no adaptation step required when transitioning single-cell passaged hPSC cultures maintained in mTeSR™ Plus, mTeSR™1, or other feeder-free media to eTeSR™. An enhanced growth rate may be observed, resulting in higher confluence cultures sooner after passaging. Cultures should be monitored carefully after the transition to ensure that the optimal passaging day is not missed. Alternatively, hPSCs may be seeded at lower densities after transitioning to eTeSR™ to compensate for enhanced growth. It is important to plate a range of cell densities to ensure that at least one seeded well will reach optimal passaging confluence on the desired day. Additionally, a parallel culture may be maintained for 1 - 2 passages in the original feeder-free maintenance medium as a source of backup cells in the event that eTeSR™-transitioned cultures become over-confluent.

Transitioning hPSCs Routinely Passaged as Aggregates to eTeSR™

An adaptation step may be required when transitioning hPSCs from 2D aggregate cultures maintained in mTeSR™ Plus, mTeSR™1, TeSR™-AOF, TeSR™-E8™, or other feeder-free maintenance media to single-cell cultures expanded in eTeSR™. It is recommended to seed hPSCs at higher densities for the first 1 - 2 passages after transitioning to eTeSR™ to compensate for lower expansion rates.

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1.0 Introduction

The maintenance and expansion of human pluripotent stem cells (hPSCs) in feeder-free conditions requires the use of complex media formulations, in combination with careful handling techniques, to maintain high-quality cultures throughout long-term passaging. The use of feeder-free culture conditions eliminates the inherent biological variability of feeder cells, an undefined component of the culture system.

Recent efforts have focused on simplifying the technical requirements of hPSC culture protocols. Single-cell passaging of hPSCs is becoming more widely adopted for routine maintenance as it allows for simpler workflows, less technical training, and compatibility with several applications (e.g. automation, gene editing). However, the link between long-term single-cell passaging and increased genetic instability in hPSC cultures is well-documented,[5,6] and few labs have rigorous quality control measures in place to assess the quality of these hPSCs. The cellular stress exerted on hPSCs during single-cell passaging can result in up to 50% cell death within 24 hours of plating, allowing genetic variants with selective advantages to survive and rapidly take over a culture.

eTeSR™ is a stabilized, feeder-free cell culture medium optimized for the single-cell maintenance and expansion of hPSCs. eTeSR™ has been specifically developed to alleviate the selective pressure placed on hPSC cultures when performing routine single-cell passaging. hPSCs maintained in eTeSR™ display increased plating efficiencies and cell expansion, as well as significantly improved genetic stability throughout long-term single-cell passaging. To cope with the increased metabolic demand and cell stress associated with single-cell passaging, eTeSR™ has improved buffering capacity and optimized metabolites to produce high-quality hPSCs with enhanced genetic stability. With stabilized key components (e.g. FGF2), eTeSR™ is compatible with both daily and restricted feeding schedules while maintaining high cell quality and equivalent performance.

eTeSR™ is compatible with a variety of cell culture matrices for long-term routine culture, including Corning® Matrigel® hESC-Qualified Matrix and CellAdhere™ Laminin-521.

hPSCs maintained in eTeSR™ have been shown to:

- Have significantly improved long-term genetic stability compared to hPSCs maintained as single cells in mTeSR™ Plus, mTeSR™1, or other feeder-free media
- Display homogeneous and characteristic hPSC morphology
- Express markers associated with undifferentiated pluripotent stem cells (i.e. OCT4, SSEA-3, TRA-1-60, and NANOG)
- Have a high cloning efficiency when plated at a clonal density with CloneR™2 (Catalog #100-0691)
- Undergo directed differentiation to mature cell types from all three germ layers (i.e. mesoderm, endoderm, and ectoderm) using STEMdiff™ Trilineage Differentiation Kit (Catalog #05230)
- Require a minimal adaptation period when transferred from 2D-aggregate cultures maintained in mTeSR™1, mTeSR™ Plus, TeSR™-E8™, TeSR™-AOF, or other feeder-free maintenance media to single-cell culture in eTeSR™
- Require no adaptation period when transferred from single-cell cultures maintained in mTeSR™ Plus, mTeSR™1, or other feeder-free media to single-cell culture in eTeSR™

2.0 Materials, Reagents, and Equipment

2.1 eTeSR™ (Catalog #100-1215)

For component storage and stability information, refer to the Product Information Sheet (PIS) for eTeSR™, 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 NAME	COMPONENT #	SIZE
eTeSR™ Basal Medium	100-1216	450 mL
eTeSR™ 10X Supplement	100-1217	50 mL

2.2 Additional Materials for hPSC Culture

CATEGORY	PRODUCT	CATALOG #
Passaging Reagents	TrypLE™ Express Enzyme (1X), phenol red OR ACCUTASE™	Thermo Fisher 12605028 OR 07920
	Gentle Cell Dissociation Reagent (GCDR)	100-0485
Matrices and Related Materials	Corning® Matrigel® hESC-Qualified Matrix	Corning 354277
	CellAdhere™ Laminin-521	77003
	CellAdhere™ Dilution Buffer	07183
	Tissue culture-treated cultureware*	e.g. 38016 (6-well plates)
Cryopreservation Media	FreSR™-S	05859
Other Materials	DMEM/F-12 with 15 mM HEPES	36254
	Trypan Blue	07050
	Hausser Scientific™ Bright-Line Hemocytometer	100-1181
	Conical tubes, 15 mL and 50 mL	e.g. 38009 and 38010
	D-PBS (Without Ca++ and Mg++)	37350
	CloneR™2 OR Y-27632 (Dihydrochloride)	100-0691 OR 72302
	hPSC Genetic Analysis Kit	07550

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

For a complete list of products for hPSC research available from STEMCELL Technologies, visit www.stemcell.com.

2.3 Equipment Required for hPSC 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
- -20°C freezer
- Refrigerator (2 - 8°C)

For cryopreservation (optional):

- Corning® CoolCell® LX Cell Freezing Container (e.g. Catalog #200-0642)
- -150°C freezer or liquid nitrogen (LN_2) vapor tank
- -80°C freezer

3.0 Culturing hPSCs Using eTeSR™

The procedures described in this manual are for the 2D maintenance of hPSCs using single-cell passaging unless stated otherwise. These procedures are general and may require cell line-specific optimization.

3.1 Morphology of hPSCs Maintained in eTeSR™

hPSCs maintained in eTeSR™ form a tightly-packed, 2D monolayer and exhibit homogenous morphology with a high nuclear-to-cytoplasm ratio and prominent nucleoli (Figure 1A). eTeSR™ is compatible with Corning® Matrigel® and CellAdhere™ Laminin-521 matrices, with cells displaying comparable cellular morphology when cultured on either matrix (Figure 1B). Areas of loose-packed cells may be observed prior to the formation of a densely packed monolayer; this is expected and does not impact cell quality.

Spontaneous differentiation, characterized by irregular cell morphology either at the edge or within the monolayer, is rarely observed in eTeSR™-maintained hPSC cultures. Spontaneous differentiation may be observed during the initial transition from aggregate to single-cell passaging, but will be lost after subsequent passaging. High levels of spontaneous differentiation in eTeSR™-maintained cultures may indicate poor initial cell quality.

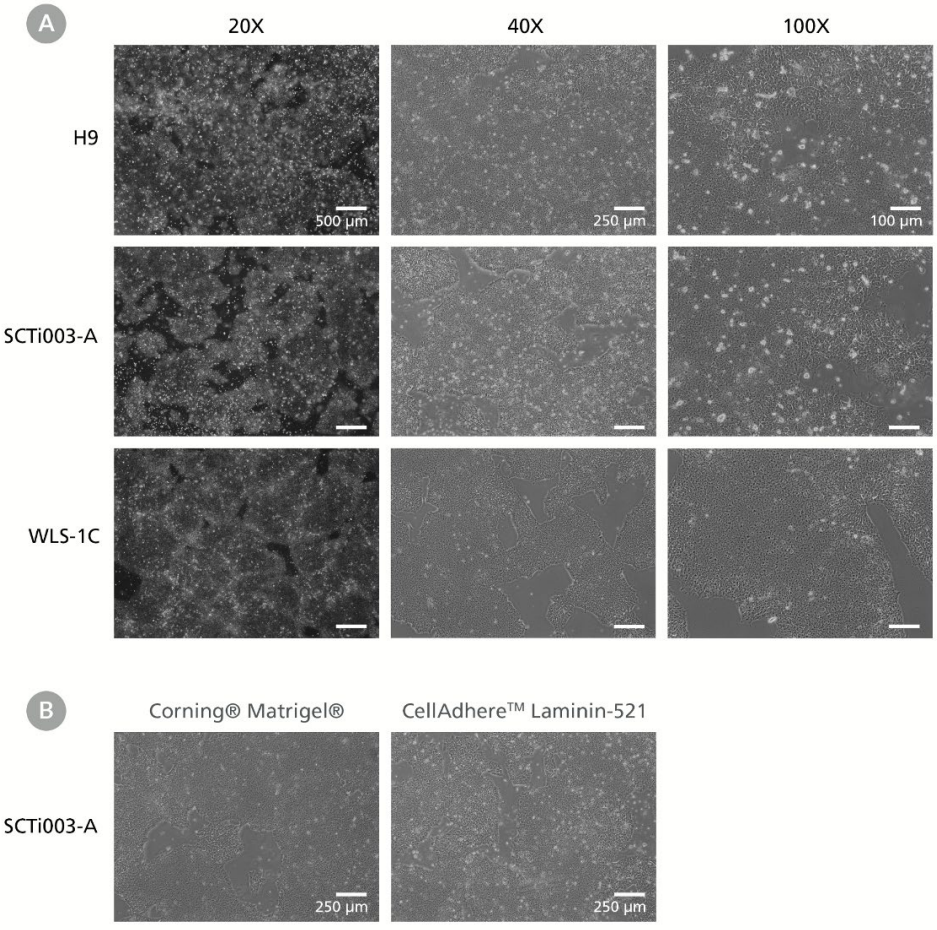

Figure 1. Cellular Morphology of hPSCs Maintained in eTeSR™

(A) Representative morphology of H9, SCTi003-A, and WLS-1C cell lines maintained in eTeSR™ on Corning® Matrigel®. Magnifications: 20X, 40X, and 100X. (B) SCTi003-A cultures display comparable cell morphology when maintained on Corning® Matrigel® or CellAdhere™ Laminin-521 matrices. All cell lines were maintained for 5 passages following a flexible feed schedule (section 6.0).

3.2 Assessing eTeSR™ Cultures to Determine Day of Passage

hPSCs maintained in eTeSR™ are ready to passage when the culture monolayer reaches a confluency of 70 - 95% (Figure 2). Passaging cells outside of this confluency range may impact plating efficiency and cell quality. Refer to Table 2 for recommended seeding density ranges to achieve optimal confluence on the desired day of passaging.

hPSC morphology will vary slightly as the culture becomes more confluent. 24 hours after seeding, the culture will consist of small, adherent clusters of 1 - 4 cells with elongated morphology. By day 2 - 3, cells begin to display classic hPSC morphology. Finally, by day 4 - 5, the cell clusters will merge to form a monolayer hPSCs with regular gaps that can allow the culture to expand if required. It is not recommended to allow the culture to form a tightly packed, > 95% confluent monolayer (Figure 2).

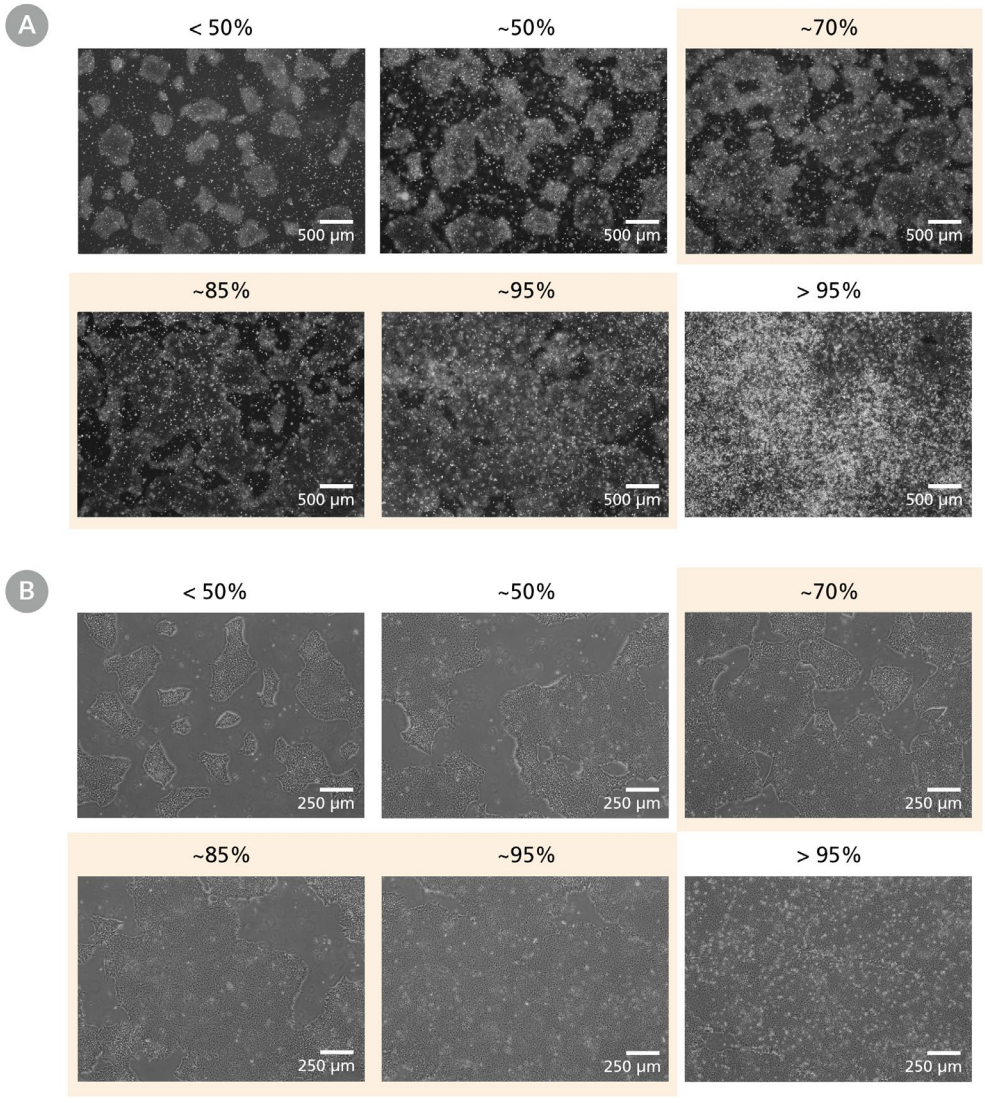

Figure 2. Optimal Confluency Range for Passaging eTeSR™ Maintained Cultures

Phase contrast images of hPSCs (H9) plated at a range of seeding densities then cultured for five days in eTeSR™ on Matrigel® coated plates. A and B show 20X and 40X magnification images respectively with approximate culture confluency indicated above images from low (< 50%) to high confluency (> 95%). Images highlighted in orange show cultures at an optimal density range for passaging (70 - 95%). Refer to Table 2 for additional guidance on optimal seeding densities.

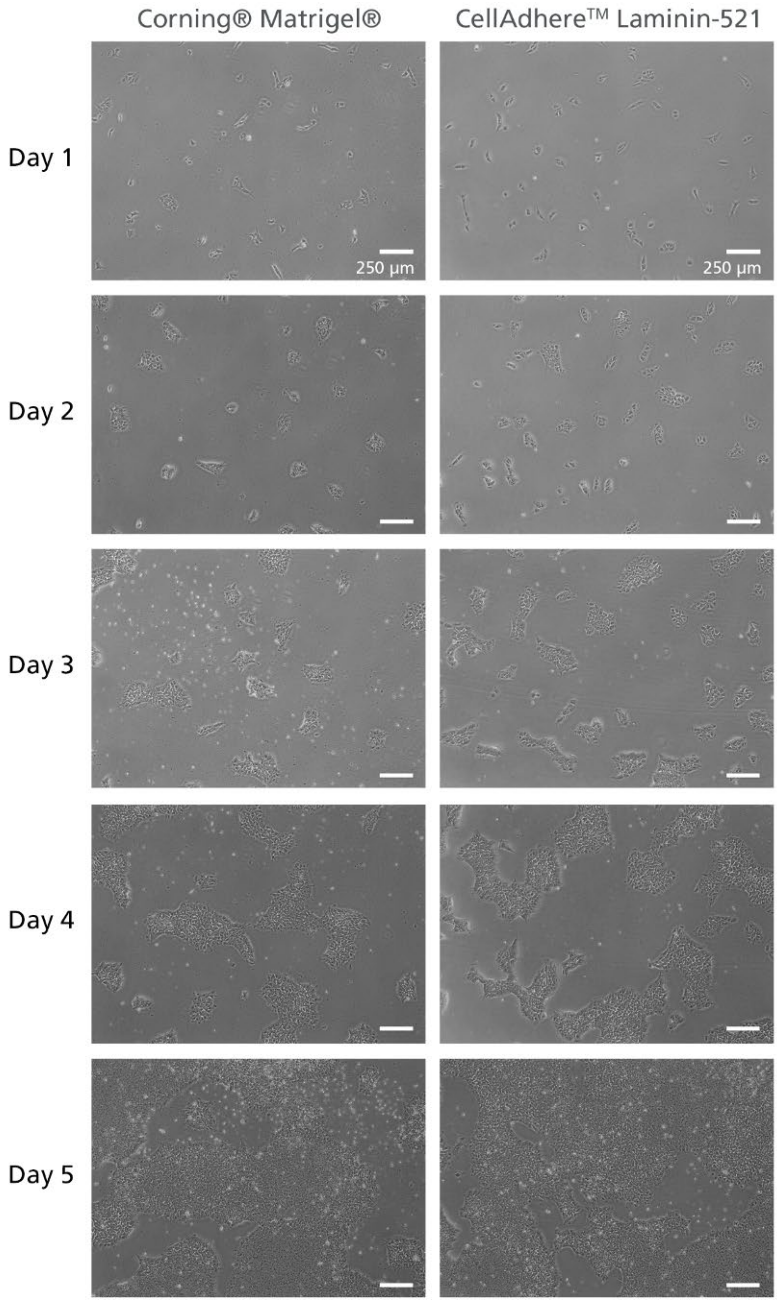

Figure 3. hPSCs Cultured in eTeSR™ on Corning® Matrigel® and CellAdhere™ Laminin-521 at Days 1 - 5 After Passaging

Phase contrast images show hPSCs (H9) maintained in eTeSR™ on cultureware coated with hESC-qualified Corning® Matrigel® (left) and CellAdhere™ Laminin-521 (right). Over time, hPSC cultures progress from individual cells to a confluent monolayer. hPSC lines exhibit comparable morphology on either matrix. Cultures were fed following a flexible feed schedule (section 6.0).

4.0 Preparation of Reagents and Materials

4.1 Complete eTeSR™ Medium

Use sterile technique to prepare complete eTeSR™ medium (Basal Medium + 10X Supplement).
The following example is for preparing 500 mL of complete medium. If preparing other volumes, adjust accordingly.

1. Thaw eTeSR™ 10X Supplement at room temperature (15 - 25°C) or overnight at 2 - 8°C. **Do not thaw in a 37°C water bath**. Mix thoroughly by swirling; do not shake.

   Note: eTeSR™ 10X Supplement may appear slightly cloudy after thawing. This will not affect performance.

   Note: Once thawed, use supplement immediately or aliquot and store at -20°C for up to 6 months. Do not exceed the shelf life of the supplement. After thawing the aliquoted supplement, use immediately. Do not re-freeze.

2. Add 50 mL of eTeSR™ 10X Supplement to 450 mL of eTeSR™ Basal Medium. Mix thoroughly by swirling; do not shake. Warm to room temperature before use (do not warm complete eTeSR™ medium in a 37°C water bath).

   Note: If not used immediately, store complete eTeSR™ 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 eTeSR™ 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 Preparation of Cell Culture Seeding Supplements

4.2.1 Y-27632 (Dihydrochloride)

Note: For complete instructions on preparing and using Y-27632 (Dihydrochloride) as a cell culture supplement, refer to the PIS (Document #10000002341), available at stemcell.com.

Prepare a stock solution by dissolving Y-27632 in D-PBS (Without Ca++ and Mg++) or water at a working concentration of 5 - 10 mM.

For example, to prepare 5 mM stock solution in D-PBS or water, resuspend 1 mg in 624 µL of D-PBS (pH 7.2) or water.

Prepare a stock solution fresh before use. If not used immediately, aliquot the stock solution and store at -20°C. Immediately before use, thaw the stock solution and add to complete eTeSR™ medium to a final concentration of 10 µM.

4.2.2 CloneR™2

Thaw CloneR™2 at room temperature (15 - 25°C). If CloneR™2 is not used immediately, store at 2 - 8°C. Do not exceed the shelf life of the supplement. Alternatively, aliquot and store at -20°C. Once aliquots are thawed, use immediately; do not re-freeze. For complete instructions, refer to the PIS for CloneR™2 (Document #10000011289), available at stemcell.com, or contact us to request a copy.

4.3 Matrices for Coating Cultureware

Successful single-cell culture of hPSCs in eTeSR™ requires the use of a suitable matrix to allow attachment of cells. hESC-qualified Corning® Matrigel® or CellAdhere™ Laminin-521 have been tested as suitable surface coating matrices with eTeSR™.

Use sterile technique when coating cultureware. Tissue culture-treated cultureware (e.g. Falcon® 6-Well Flat-Bottom Plate, Tissue Culture Treated, Catalog #38016) is required for use with Corning® Matrigel® or CellAdhere™ Laminin-521. For specific instructions for each matrix, refer to section 4.3.1 (Corning® Matrigel®), or 4.3.2 (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 COATING SOLUTION
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

4.3.1 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 24 mL of diluted matrix. Always keep Matrigel® on ice when thawing and handling to prevent it from gelling. If using other volumes, adjust accordingly.

1. Thaw one aliquot of Matrigel® on ice.
2. Dispense 24 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 thoroughly. 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.
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 for 30 minutes before use.

4.3.2 CellAdhere™ Laminin-521

1. Thaw CellAdhere™ Laminin-521 overnight at 2 - 8°C.

   Note: If not used immediately, store at 2 - 8°C for up to 3 months.
2. Dilute CellAdhere™ Laminin-521 in D-PBS (Without Ca++ and Mg++) to a final concentration of 10 - 20 µg/mL.

   Note: When adapting to a 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 the coated cultureware 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. Allow the cultureware to warm to room temperature for ≥ 1 hour before use.

5.0 Routine Passaging of hPSCs in eTeSR™

This protocol is for passaging hPSCs maintained in eTeSR™ on Corning® Matrigel® or CellAdhere™ Laminin-521 using enzymatic dissociation. TrypLE™ Express Enzyme or ACCUTASE™ may be used as a dissociation reagent.

hPSCs are ready to be passaged when the culture reaches approximately 70 - 95% confluency. Visually check the quality of the cultures before passaging. The culture should have minimal (< 2%) spontaneous differentiation and display characteristic hPSC morphology (i.e. high nuclear-to-cytoplasm ratio and prominent nucleoli).

5.1 Preparation

- At least 1 hour before passaging, coat tissue culture-treated plates with Corning® Matrigel® (section 4.3.1) or CellAdhere™ Laminin-521 (section 4.3.2).

  Note: If using pre-coated cultureware stored at 2 - 8°C, warm to room temperature (15 - 25ºC) for at least 1 hour before use.
- If using ACCUTASE™, thaw at room temperature, overnight at 2 - 8°C, or in a container of cool water before use. For storage and stability information, refer to the PIS for ACCUTASE™ (Document #10000000377).
- Prepare Y-27632 (Dihydrochloride) or CloneR™2 to be used as a plating supplement as described in sections 4.2.1 or 4.2.2, respectively.

5.2 Harvesting and Seeding hPSCs as Single Cells

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

1. Warm a complete eTeSR™ medium (section 4.1) coated culture plate(s) to room temperature (15 - 25°C). Add 2 mL of complete eTeSR™ medium to a 15 mL conical tube and set aside.

   Note: Do not warm complete eTeSR™ medium in a 37°C water bath.
2. Prepare Single-Cell Plating Medium by adding the desired plating supplement to an appropriate volume of complete eTeSR™ medium as follows:
   - CloneR™2: Add at a 1 in 10 dilution (e.g. add 10 mL of CloneR™2 to 90 mL of complete eTeSR™ medium).
   - Y-27632 (Dihydrochloride): Add to a final concentration of 10 µM.
3. Wash the well with 2 mL of D-PBS (Without Ca++ and Mg++). Discard the wash.
4. Add 0.5 mL of TrypLE™ Express Enzyme or ACCUTASE™.
5. Incubate at 37°C and 5% CO_2 for 4 - 8 minutes.

   Note: The incubation time may vary for different matrices. For cultures maintained on Corning® Matrigel®, 4 - 5 minutes is typically optimal for dissociation. A longer incubation time (i.e. 6 - 8 minutes) may be required for cultures maintained on CellAdhere™ Laminin 521 or for performing automated passaging protocols.
6. Add 1 mL of complete eTeSR™ medium (prepared in step 1) to the well.
7. Harvest the cells by carefully tilting the plate and gently pipetting up and down to detach the cells. Transfer the single-cell suspension to the 15 mL conical tube prepared in step 1.

   Note: Avoid excessive or harsh trituration, as this may adversely impact cell viability. If the cells do not readily detach, a longer incubation time may be required.
8. Centrifuge the cell suspension at 300 x *g* for 5 minutes.
9. Discard the supernatant and gently flick the tube 3 - 5 times to resuspend the cell pellet.

10. Add 1 - 2 mL of Single-Cell Plating Medium (prepared in step 2) to the cells. Mix gently.
11. Count cells using Trypan Blue and a Hausser Scientific™ Bright-Line Hemocytometer (or an automated cell counting method).

    Note: The cell suspension should contain little to no cell clumps, as this will impact the final cell count. Refer to Figure 4 for representative images of an ideal single-cell suspension of dissociated hPSCs. If the suspension appears too clumpy, further protocol optimization (e.g. plating densities, incubation time, etc.) may be required.

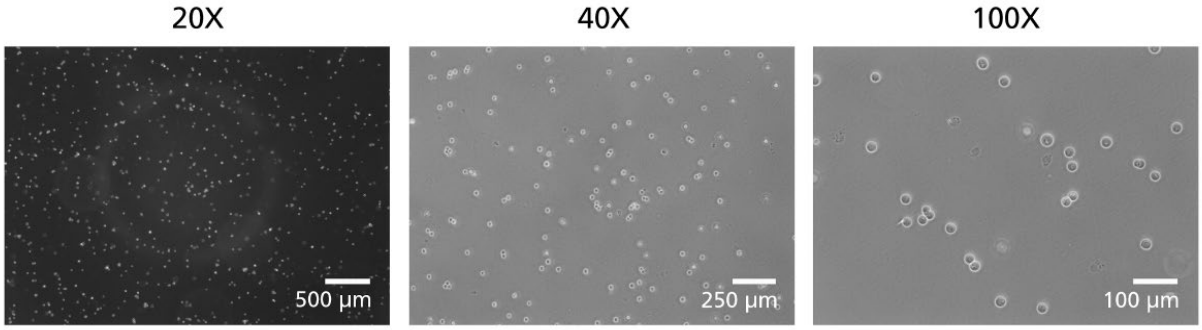

Figure 4. Single-Cell Suspension with Minimal Clumping

Representative images of a single-cell suspension of H9-ES cells after dissociation with TrypLE™ Express Enzyme. The suspension should consist primarily of single-cells with as few cell clumps as possible. Images were taken at 20X, 40X, and 100X magnifications.

12. Remove the coating matrix from the culture plate(s) prepared in step 1 and immediately add 2 mL/well of Single-Cell Plating Medium.
13. Seed the cells at the desired density. Refer to Table 2 for recommended seeding densities to achieve approximately 70 - 95% confluency for a day 4 or day 5 passaging schedule.

    Note: It is important to plate a range of cell densities to ensure that at least one well will reach 70 - 95% confluency on the desired day of passaging (i.e. 4 or 5 days after seeding).

    Note: Cell seeding densities are cell line-dependent and may need to be optimized for individual cell lines. Cultures grown in eTeSR™ may exhibit enhanced growth rates compared to those grown in other TeSR™ media. Therefore, users may need to reduce the seeding density as required when adapting cultures to eTeSR™.

Table 2 . Recommended Seeding Densities for hPSC Cultures to Reach 70 - 95% Confluence in 4 or 5 Days

DAYS BEFORE hPSCs REACH CONFLUENCE	EXAMPLE NUMBER OF CELLS SEEDED PER WELL OF A 6-WELL PLATE (~10 cm^2)		
	LOW DENSITY	MEDIUM DENSITY	HIGH DENSITY
4 Days	2 x 10^4	5.5 x 10^4	9.5 x 10^4
5 Days	1.8 x 10^4	4 x 10^4	6.5 x 10^4

14. Move the plate in several quick, short, back-and-forth and side-to-side motions to distribute the hPSCs across the surface of the wells. Incubate at 37°C and 5% CO_2. Do not disturb the plate for 24 hours.
15. Remove Single-Cell Plating Medium from the wells and add 2 mL of complete eTeSR™ medium per well. Incubate at 37°C and 5% CO_2 for 24 hours.
16. Perform medium changes as needed using complete eTeSR™ medium and visually assess cultures to monitor growth until the cells reach ~70 - 95% confluence. The medium can be changed daily or every other day. To skip two consecutive days of feeding, add twice the volume of medium (e.g. 4 mL/well of a 6-well plate).

6.0 Flexible Feeding and Passaging Schedules

eTeSR™ accommodates more flexible and restricted feeding schedules without affecting performance or cell quality. To determine a convenient schedule that suits your lab's routine, refer to Table 3 below. Any combination of feeding intervals can be used between passages when following these guidelines.

With the exception of the recommended medium change on Day 1 after passaging to remove the seeding supplements (CloneR™2 or Y-27632) from the culture, cells can be fed daily, every other day, or up to two consecutive days of feeding can be skipped when using eTeSR™. The feeding interval guidelines are shown below, and operators can design the feeding schedule accordingly to their experimental need.

Note: Highly proliferative or very confluent cultures may benefit from daily medium changes. For example, cultures may be fed 24 hours before passaging to accommodate the higher culture confluency.

Table 3. Flexible 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)

Single-cell hPSC cultures maintained in eTeSR™ should be passaged every 4 - 5 days once the cells reach 70 - 95% confluence. Passaging cultures on a Monday/Friday/Wednesday schedule allows for a weekend-free passaging protocol (see Table 4) that is compatible with the feeding schedules detailed in Table 3. We recommend performing a full-medium change 24 hours after passaging, as the prolonged presence of seeding supplements in the culture may affect cell quality. Additionally, cultures grown in eTeSR™ may exhibit improved growth rates compared to those grown in other TeSR™ media. To support cultures that are highly proliferative or confluent, more frequent medium changes are recommended.

Table 4. hPSC Maintenance Schedule with Minimal Weekend Work

WEEK 1	MONDAY	TUESDAY	WEDNESDAY	THURSDAY	FRIDAY	SATURDAY	SUNDAY
	Passage*	Feed	-	Feed	**Passage****	Feed	-
WEEK 2	MONDAY	TUESDAY	WEDNESDAY	THURSDAY	FRIDAY	SATURDAY	SUNDAY
	Feed	-	**Passage****	Feed	Double Feed	-	-

* Cells seeded to reach confluency in 4 days.
** Cells seeded to reach confluency in 5 days.

7.0 Additional Protocols

7.1 Transitioning Cells

7.1.1 hPSCs Routinely Passaged as Single Cells to eTeSR™

There is typically no adaptation step required when transitioning single-cell passaged hPSC cultures maintained in mTeSR™ Plus, mTeSR™1, or other feeder-free media to eTeSR™. An enhanced growth rate may be observed, resulting in higher confluence cultures sooner after passaging. Cultures should be monitored carefully after the transition to ensure that the optimal passaging day is not missed. Alternatively, hPSCs may be seeded at lower densities after transitioning to eTeSR™ to compensate for enhanced growth. It is important to plate a range of cell densities to ensure that at least one seeded well will reach optimal passaging confluence on the desired day. Additionally, a parallel culture may be maintained for 1 - 2 passages in the original feeder-free maintenance medium as a source of backup cells in the event that eTeSR™-transitioned cultures become over-confluent.

7.1.2 hPSCs Routinely Passaged as Aggregates to eTeSR™

An adaptation step may be required when transitioning hPSCs from 2D aggregate cultures maintained in mTeSR™ Plus, mTeSR™1, TeSR™-AOF, TeSR™-E8™, or other feeder-free maintenance media to single-cell cultures expanded in eTeSR™. It is recommended to seed hPSCs at higher densities for the first 1 - 2 passages after transitioning to eTeSR™ to compensate for lower expansion rates.

7.1.3 Monolayer to Aggregate Passaging

To transition monolayer cultures into aggregate cultures using enzyme-free dissociation reagents, refer to the Tech Tip: Sticking Together - Clump Passaging for ES and iPS Cell Maintenance, available at www.stemcell.com.

7.2 Cryopreserving and Thawing Cells

hPSCs maintained in eTeSR™ can be cryopreserved as single cells using FreSR™-S. Cells maintained in other feeder-free media (e.g. mTeSR™ Plus, mTeSR™1, TeSR™-E8™, or TeSR™-AOF) may be thawed into the same medium and conditions used prior to cryopreservation or thawed directly into eTeSR™.

7.2.1 FreSR™-S

FreSR™-S is a defined, serum-free, and animal component-free medium for cryopreserving human hPSCs as single cells. It is ready to use and contains cryoprotectant agents. For complete instructions, refer to the PIS for FreSR™-S (Document #10000000240), available at stemcell.com, or contact us to request a copy.

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 eTeSR™ in 6-well plates using FreSR™-S. If using other cultureware, adjust volumes accordingly. Cultures should be harvested and cryopreserved at the time they would normally be ready for passaging.

1. Prepare a single-cell suspension of hPSCs as described in section 5.0, steps 1 - 8.
2. Carefully remove the supernatant with a pipette, leaving a small amount of medium to ensure the cell pellet is not disturbed. Gently flick the tube 3 - 5 times to resuspend the cell pellet.
3. Add cold (2 - 8°C) FreSR™-S to obtain a cell suspension of 1 x 10^6 cells/mL and mix thoroughly.
4. Transfer 0.5 mL of the single-cell suspension to each cryovial.

5. 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

The following are instructions for thawing one vial of 1 x 10^6 frozen hPSCs into one well of a 6-well plate. If thawing additional vials or using other cultureware, adjust volumes accordingly. hPSCs should be thawed into coated cultureware (section 4.3). 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, warm eTeSR™ (15 - 25°C), and coated cultureware ready before starting the protocol to ensure that the thawing procedure is performed as quickly as possible.

   Note: Do not warm eTeSR™ in a 37°C water bath.
2. Prepare 8.5 mL of Single-Cell Plating Medium (section 5.2, step 2). Add 4 mL to a 15 mL conical tube and set aside.
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 swirling 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 pipette to slowly transfer the contents of the cryovial to the 15 mL conical tube prepared in step 2.
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. Gently flick the tube 3 - 5 times to resuspend the cell pellet.
10. Add 1 mL of Single-Cell Plating Medium to the tube. Mix gently.
11. Transfer the cell suspension into one well of a coated 6-well plate containing 2 mL of Single-Cell Plating Medium.
12. Gently disperse the cells throughout the well with several short, back-and-forth and side-to-side motions to distribute the hPSCs across the well surface. Incubate at 37°C and 5% CO_2. Do not disturb the plate for 24 hours.
13. Remove Single-Cell Plating Medium from the wells and add 2 mL of complete eTeSR™ medium per well. Incubate at 37°C and 5% CO_2 for 24 hours.
14. Perform medium changes as needed using complete eTeSR™ medium. Visually assess cultures to monitor growth and passage the cells once they reach ~70 - 95% confluence (section 5.0).

7.3 Gene Editing and Cloning

Seeding single cells after stressful events (e.g. transfection or single-cell cloning) may result in the selection of hPSCs harboring genetic changes conferring a survival advantage. When used in combination with CloneR™2, the cloning efficiency and survival of hPSCs can be further increased.

7.3.1 CloneR™2 and ArciTect™ CRISPR-Cas9 Genome Editing

For using eTeSR™ with CloneR™2 for gene editing workflows, refer to the PIS (Document #10000011289), available at www.stemcell.com, or contact us to request a copy. For genome editing with the ArciTect™ CRISPR-Cas9 System, refer to the Tech Tip: Genome Editing of Human Pluripotent Stem Cells Using the ArciTect™ CRISPR-Cas9 System, available at www.stemcell.com.

8.0 References

1. Thomson JA et al. (1998) Embryonic stem cell lines derived from human blastocysts. Science 282(5391): 1145–7.
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3. Draper JS et al. (2004) Recurrent gain of chromosomes 17q and 12 in cultured human embryonic stem cells. Nat Biotechnol 22(1): 53–4.
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5. International Stem Cell Initiative. (2011) Screening ethnically diverse human embryonic stem cells identifies a chromosome 20 minimal amplicon conferring growth advantage. Nat Biotechnol. 29(12): 1132–44.
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Appendix 1: Flow Cytometry Protocols

Reagents and Materials

Antibodies

Antibodies can be used to characterize hPSCs by flow cytometry. The tables below contain information about a selection of antibodies available from STEMCELL Technologies that can be used to characterize hPSCs. 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.

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 5.0. Perform a viable cell count using Trypan Blue and a Hausser Scientific™ Bright-Line Hemocytometer (or an automated cell counting method). The single-cell suspension may now be used for surface antigen and/or intracellular antigen labeling.

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 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) per 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.

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 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 6).

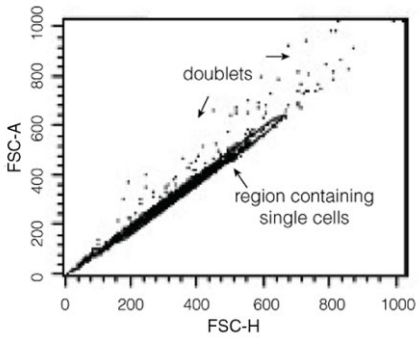

Figure 5. Example of Doublet Discrimination by Flow Cytometry

TECHNICAL MANUAL

Expansion of Human Pluripotent Stem Cells in eTeSR™

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