TECHNICAL MANUAL

Expansion of Human Pluripotent Stem Cells as Aggregates in Suspension Culture Using mTeSR™3D

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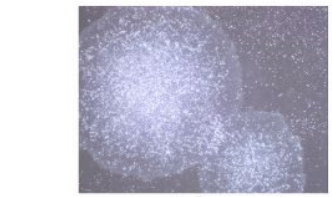
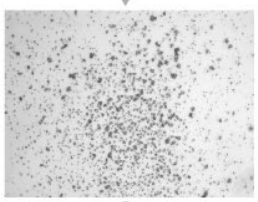
1.0 mTeSR™3D Workflow

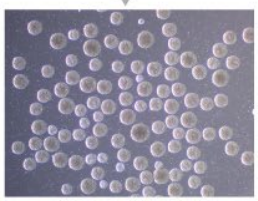


Start with high-quality hPSC cultures

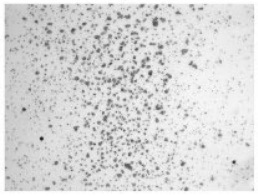


Initiate hPSC suspension culture in mTeSR™ 3D Seed Medium supplemented with 10 µM Y-27632

Culture hPSC suspension aggregates using fed-batch feeding with mTeSR™ 3D Feed Medium



- Tri-lineage differentiation
- Flow cytometry
- Re-establish 2D colonies



Passage suspension culture hPSCs using Gentle Cell Dissociation Reagent and 37 µm Reversible Strainer into mTeSR™ 3D Seed Medium supplemented with 10 µM Y-27632. Use an alternating 3-day/4-day passaging schedule.

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2.0 Introduction

A significant challenge in the field of human pluripotent stem cell (hPSC) research is the generation of large numbers of highly pure undifferentiated cells. Growing hPSCs in a two-dimensional (2D) adherent mTeSR™1, mTeSR™ Plus, TeSR™-E8™, or TeSR™-AOF system has long been the preferred method for maintenance of hPSCs; however, these systems are not conducive to large-scale production of hPSCs. To meet the growing demand for a robust, scalable system, mTeSR™3D and TeSR™-AOF 3D have been developed for the expansion of undifferentiated hPSCs in a three-dimensional (3D) suspension culture system as a standardized system to scale up hPSC production. mTeSR™3D is a defined, serum-free formulation optimized for the expansion of undifferentiated hPSCs in a 3D suspension culture system.

mTeSR™3D uses an optimized fed-batch strategy in which all the necessary nutrients and growth factors are added daily and spent medium is only removed prior to passaging. This novel workflow saves time and media, and cell yields are enhanced by minimizing opportunities for aggregate fusion and cell loss. The mTeSR™3D kit consists of a Seed Medium (Seed Basal Medium + Seed 5X Supplement) to initiate cultures and a Feed Medium (Feed Supplement A + Feed Supplement B) for daily fed-batch addition. These media contain recombinant human basic fibroblast growth factor (rh bFGF) and recombinant human transforming growth factor β (rh TGFβ). Addition of further growth factors is not required. mTeSR™3D Seed Medium requires addition of 10 µM Y-27632 for optimal aggregate formation when initiating and passaging cultures.

In a traditional 2D hPSC culture system, cells are grown as adherent colonies or as a monolayer on a 2D surface that is usually coated with inactivated mouse embryonic fibroblasts (iMEFs) or a feeder-free extracellular matrix (e.g. Corning® Matrigel®). By contrast, in the mTeSR™3D suspension culture system, cells grow as spherical aggregates in suspension without the addition of matrices or microcarriers.

Culturing cells in a 3D fed-batch suspension system has several advantages:

- Eliminates the need for daily medium changes between passages
- Reduces fluctuations in pH and in the concentration of nutrients and growth factors associated with a daily medium replacement
- No manual selection and removal of differentiated cells required
- Can be easily automated using liquid handling robots and facilitates high-throughput experiments

Suspension hPSCs cultured in mTeSR™3D:

- Transition efficiently from 2D cultures
- Display normal colony morphology when re-plated as 2D cultures
- Maintain differentiation capacity to the three germ layers, comparable to 2D hPSC cultures maintained in mTeSR™1
- Yield more cells per mL of medium used*
- Express equivalent levels of OCT4 and TRA-1-60 (assessed by flow cytometry)*

* Compared to 2D hPSC cultures maintained in mTeSR™1.

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3.0 Materials, Reagents, and Equipment

3.1 mTeSR™3D (Catalog #03950)

For component storage and stability information, refer to the Product Information Sheet (PIS) for mTeSR™3D, 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
03951	mTeSR™3D Seed Basal Medium	400 mL
03952	mTeSR™3D Seed 5X Supplement	100 mL
03953	mTeSR™3D Feed Supplement A	100 mL
03954	mTeSR™3D Feed Supplement B	12 mL

3.2 Materials Required for hPSC Suspension Culture

CATEGORY	PRODUCT	CATALOG #
Materials Recommended for 2D Maintenance Culture	Tissue culture-treated cultureware	e.g. 38016 (6-well plates)
	Corning® Matrigel® hESC-Qualified Matrix	Corning 354277
	mTeSR™1	85850
	mTeSR™ Plus	100-0276
	TeSR™-E8™	05990
	TeSR™-AOF	100-0401
	D-PBS (Without Ca++ and Mg++)	37350
	Gentle Cell Dissociation Reagent (GCDR)	100-0485
	Cell scrapers	e.g. 38065
Passaging Materials for 3D Suspension Culture	Y-27632 (ROCK inhibitor)	72304
	Gentle Cell Dissociation Reagent (GCDR)	100-0485
	37 µm Reversible Strainer	27250 (Large) 27215 (Small)
	Conical tubes	38009 (15 mL) OR 38010 (50 mL)
	Serological pipettes	38003 (5 mL) OR 38005 (25 mL) OR 38006 (50 mL)

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

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3.3 Vessels for hPSC Suspension Culture

The following suspension culture vessels are recommended by STEMCELL Technologies:

PRODUCT	CATALOG #
6-Well Flat-Bottom Plate, Non-Treated	38040
Nalgene™ Rapid-Flow™ Sterile Filter Storage Bottles	Thermo Fisher 455-0250
PBS-MINI 0.1 MAG and 0.5 MAG Bioreactor	Base Unit: 100-1005 Vessels: 100-1006, 100-1007

3.4 Equipment Required for hPSC Suspension Culture

- Vertical laminar flow hood certified for Level II handling of biological materials
- Incubator with humidity and gas control to maintain 37°C and 95% humidity in an atmosphere of 5% CO_2 in air
- Low-speed centrifuge with a swinging bucket rotor
- Pipette-Aid with appropriate serological pipettes
- Pipettors with appropriate tips
- Inverted microscope with a total magnification of 20X to 100X
- -20°C freezer
- Refrigerator (2 - 8°C)
- Orbital shaker (2.5 cm orbital diameter recommended), PBS Bioreactor Magnetic Drive Unit, or alternative suspension culture system

Optional

- Viable cell counter (e.g. ChemoMetec NucleoCounter® NC-250™) and viability stain (e.g. DAPI, Catalog #75004)

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4.0 Preparation of Reagents and Materials

4.1 mTeSR™3D Seed Medium

Use sterile technique to prepare mTeSR™3D Seed Medium (Seed Basal Medium + Seed 5X Supplement). The following example is for preparing 500 mL of mTeSR™3D Seed Medium. If preparing other volumes, adjust accordingly.

1. Thaw mTeSR™3D Seed 5X Supplement at room temperature (15 - 25°C) or overnight at 2 - 8°C. Do not thaw in a 37°C water bath. Mix thoroughly.

   Note: Supplement may appear slightly cloudy after thawing. If this is noted, place in a 37°C water bath for ~5 minutes, swirling occasionally until supplement becomes clear. Supplement must be free of cloudiness before adding to basal medium.

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

2. Add 100 mL of mTeSR™3D Seed 5X Supplement to 400 mL of mTeSR™3D Seed Basal Medium. Mix thoroughly.

   Note: If not used immediately, store mTeSR™3D Seed Medium at 2 - 8°C for up to 2 weeks. Alternatively, aliquot and store at -20°C for up to 6 months. Do not exceed the shelf life of the individual components. After thawing the aliquoted medium, use immediately or store at 2 - 8°C for up to 2 weeks. Do not re-freeze.

   Note: If desired, the medium can be filtered using a 0.2 µm low-protein binding filter.

3. Immediately before use, add 10 µM Y-27632 (Dihydrochloride). Mix thoroughly.

4.2 mTeSR™3D Feed Medium

Use sterile technique to prepare mTeSR™3D Feed Medium (Feed Supplement A + Feed Supplement B). The following example is for preparing 112 mL of mTeSR™3D Feed Medium. If preparing other volumes, adjust accordingly.

1. Thaw mTeSR™3D Feed Supplement A at room temperature (15 - 25°C) or overnight at 2 - 8°C. Do not thaw in a 37°C water bath. Mix thoroughly.

   Note: Once thawed, use 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 12 mL of mTeSR™3D Feed Supplement B to 100 mL of mTeSR™3D Feed Supplement A. Mix thoroughly.

   Note: Store mTeSR™3D Feed 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 Feed Medium, use immediately or store at 2 - 8°C for up to 2 weeks. Do not re-freeze.

   Note: If prepared aseptically, mTeSR™3D Feed Medium is ready for use. If desired, the medium can be filtered using a 0.2 µm low-protein binding filter.

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4.3 Suspension Culture Vessel Parameters

hPSCs are both shear-sensitive and agglomerate easily, thus requiring specific mixing dynamics. STEMCELL Technologies has evaluated many vessels/bioreactors for hPSC suspension culture and only recommends those which foster reproducible hPSC expansion across multiple passages and cell lines. For optimization of culture parameters in small-scale or high-throughput experiments, we recommend non-tissue culture-treated 6-well plates on an orbital shaker. For large-scale hPSC cultures, we recommend larger vessels such as Nalgene™ Storage Bottles or PBS-MINI MAG Single-Use Vessels. Refer to Table 1 for recommended volumes and orbital shaker/impeller speeds for each culture vessel.

Table 1. Recommended Suspension Culture Parameters for Culture Vessels Evaluated by STEMCELL Technologies

CULTURE VESSEL	CATALOG #	INITIAL CULTURE VOLUME	ORBITAL SHAKER/IMPELLER SPEED
6-Well Flat-Bottom Plate, Non-Treated	38040	2 mL	70 RPM (2.5 cm orbital diameter)
Nalgene™ Rapid-Flow™ Sterile Filter Storage Bottles	Thermo Fisher 455-0250	15 mL 30 mL 60 mL	40 RPM 55 RPM 65 RPM (2.5 cm orbital diameter)
PBS-MINI 0.1 MAG Single-Use Vessel	100-1006	100 mL	40 - 45 RPM
PBS-MINI 0.5 MAG Single-Use Vessel	100-1007	500 mL	40 - 45 RPM

Note: The culture volume and orbital shaker/impeller speed (RPM) may need to be optimized for individual cell lines. For example, if low expansion or small aggregate size is observed at the end of the passage, it is recommended to lower the RPM. However, if excessive merging and aggregation of aggregates are observed, increase the RPM.

Note: PBS-MINI 0.1 has a working range of 80 - 100 mL, and PBS-MINI 0.5 has a working range of 300 - 500 mL. However, operating conditions have been optimized for 100 mL and 500 mL cultures in PBS-MINI 0.1 and 0.5, respectively.

Note: As Nalgene™ Rapid-Flow™ Sterile Filter Storage Bottles do not have vented caps, open the caps slightly when placing the bottles in the incubator to allow gas exchange.

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5.0 Initiating hPSC Suspension Culture in mTeSR™3D

Adherent 2D cultures grown on Corning® Matrigel® with mTeSR™1, mTeSR™ Plus, TeSR™-E8™, or TeSR™-AOF can be transitioned directly into dynamic suspension culture with no separate adaptation step.

Note: Some cell lines may expand less rapidly during the first passage compared to later passages due to adaptation to the new culture environment. To minimize this adaptation step, it is highly recommended to initiate 3D suspension cultures in non-tissue culture-treated 6-well plates before moving to a larger vessel.

5.1 High-Quality hPSC Cultures

To successfully expand cells and maintain pluripotency in a suspension culture system, it is crucial to begin with high-quality hPSC cultures. Typically, hPSCs are maintained in 2D on Corning® Matrigel® with mTeSR™1, mTeSR™ Plus, TeSR™-E8™, or TeSR™-AOF, and are passaged using Gentle Cell Dissociation Reagent (GCDR) as clumps that are 50 - 100 µm in diameter. Cells cultured in other 2D matrix-medium combinations should also transition smoothly to 3D, but some optimization may be needed. For additional information on maintaining hPSCs as adherent colony cultures, refer to the Technical Manual: Maintenance of Human Pluripotent Stem Cells in mTeSR™1, available at www.stemcell.com, or contact us to request a copy.

High-quality hPSC cultures should express high levels of undifferentiated hPSC markers (> 95%) including OCT4, TRA-1-60, and SSEA-3 as assessed by flow cytometry. They should also display normal colony morphology, a low percentage of differentiated cells, normal growth rates, and retain expected karyotype as assessed by G-banding analysis or other method. Note that chromosomal and genetic aberrations may appear during long-term passaging in any in vitro system. It is important to periodically check hPSC maintenance cultures to ensure maintenance of expected karyotype; we recommend checking cultures every 5 - 10 passages. For routine screening to detect commonly observed karyotypic hPSC abnormalities, hPSC Genetic Analysis Kit (Catalog #07550) can be used.

5.2 Dissociating 2D Culture to Initiate 3D Suspension Culture

In the mTeSR™3D culture system, we recommend a non-enzymatic clump passaging protocol using GCDR. Passaging hPSCs as clumps supports long-term expansion of hPSCs while maintaining genetic stability. GCDR dissociation results in high cell viability and recovery and preserves the integrity of cell surface proteins that aid in hPSC aggregate formation. Refer to section 5.2.1 for initiating a 3D suspension culture from 2D, and section 7.1 for passaging aggregates as clumps.

5.2.1 Seeding Small Clumps into Suspension

The protocol for initiating suspension cultures with small clumps derived from 2D adherent colony cultures will be familiar to those who have passaged 2D colony cultures grown in mTeSR™1, mTeSR™ Plus, TeSR™-E8™, or TeSR™-AOF with GCDR. GCDR is an enzyme-free reagent for passaging hPSCs as clumps with manual scraping to generate small aggregates from 2D culture.

The following are instructions for passaging cells from one well of a 6-well plate cultured in mTeSR™1, mTeSR™ Plus, TeSR™-E8™, or TeSR™-AOF on Corning® Matrigel®. If using other cultureware, adjust volumes accordingly. Six wells of a confluent 6-well plate of hPSCs should generate 6 - 12 x 10^6 viable cells.

1. Prepare and aliquot a sufficient volume of mTeSR™3D Seed Medium + 10 µM Y-27632 and warm to room temperature (15 - 25°C).

   Note: Do not warm mTeSR™3D Seed Medium in a 37°C water bath. Add Y-27632 immediately before use (section 4.1).

2. Use a microscope to visually identify regions of differentiation. Mark these using a felt tip or lens marker on the bottom of the plate.
3. 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.

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Note: Selection may not be required if differentiation is < 5%. Selection should not exceed 20% of the well. For further information, refer to the Technical Manual: Maintenance of Human Pluripotent Stem Cells in mTeSR™1, available at www.stemcell.com, or contact us to request a copy.

4. Aspirate the spent medium from the well and add 1 mL of room temperature GCDR.
5. Incubate at room temperature for 6 - 8 minutes.
6. Aspirate GCDR. Add 1 mL of mTeSR™3D Seed Medium + 10 µM Y-27632 (prepared in step 1). Gently detach the colonies by scraping with a serological glass pipette or a cell scraper.

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

   Optional: Rinse the well with an additional 1 mL of mTeSR™3D Seed Medium + 10 µM Y-27632 to collect remaining clumps.
8. Carefully pipette the clump suspension up and down to break up the clumps as needed. A uniform suspension of aggregates approximately 50 - 100 µm in size is optimal; do not create a single-cell suspension.
9. Aliquot the cell suspension to count clumps manually or use an automated cell counting instrument such as a ChemoMetec NucleoCounter® NC-250™ (see Appendix 1: Counting Clumps). For recommended seeding densities, see section 5.3.
10. Add concentrated clump suspension to the culture vessel to obtain the desired number of total viable cells or clumps and then top up medium with mTeSR™3D Seed Medium + 10 µM Y-27632 to desired total volume.

5.3 Choosing an Optimal Seeding Density

Seeding a suspension culture at an appropriate density is important for aggregate formation efficiency, growth rates, metabolite concentrations, and maintenance of pluripotency. Different cell lines have intrinsically different growth rates and thus may have different optimal seeding densities.

It is recommended to seed at density ranging from **1 - 3 x 10^5 viable cells/mL**, which correlates to approximately 1 - 3 x 10^3 clumps/mL. Final harvested cell density at the end of a suspension culture passage should not exceed 1 - 2 x 10^6 viable cells/mL.

Some cell lines may experience an adaptation phase during which they expand slowly in the first 1 - 2 passages in suspension culture. In these instances, it may be desirable to seed at higher densities in early passages and decrease the seeding density once the cells have adapted to suspension culture.

It is recommended to optimize seeding densities for individual cell lines in a small-scale suspension system such as a non-tissue culture-treated 6-well plate, particularly for cell lines maintained in different 2D matrix-medium combinations.

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6.0 Culturing hPSC Suspension Aggregates Using mTeSR™3D

6.1 mTeSR™3D Feeding and Passaging Schedule

After initiating hPSC suspension culture in mTeSR™3D, passage the suspension culture on an alternating 3-day/4-day schedule as outlined in Table 2. The first passage after culture initiation can be either a 3-day or a 4-day passage, with the subsequent passages alternating passage length. See sections 6.1.1 for details on addition of the Feed Medium. For the passaging protocol, refer to section 7.1.

Table 2. Recommended Feeding and Passaging Schedules

DAY	STARTING WITH A 3-DAY PASSAGE	STARTING WITH A 4-DAY PASSAGE
0	**Initiate Culture** using mTeSR™3D Seed Medium + 10 µM Y-27632	**Initiate Culture** using mTeSR™3D Seed Medium + 10 µM Y-27632
1	Add mTeSR™3D Feed Medium	Add mTeSR™3D Feed Medium
2	Add mTeSR™3D Feed Medium	Add mTeSR™3D Feed Medium
3	**Passage and re-seed** into mTeSR™3D Seed Medium + 10 µM Y-27632	Add mTeSR™3D Feed Medium
4	Add mTeSR™3D Feed Medium	**Passage and re-seed** into mTeSR™3D Seed Medium + 10 µM Y-27632
5	Add mTeSR™3D Feed Medium	Add mTeSR™3D Feed Medium
6	Add mTeSR™3D Feed Medium	Add mTeSR™3D Feed Medium
7	**Passage**	**Passage**

6.1.1 Addition of mTeSR™3D Feed Medium

Add mTeSR™3D Feed Medium to the hPSC suspension culture as described below, according to a recommended schedule in Table 2.

1. Prepare mTeSR™3D Feed Medium (section 4.2).
2. Add mTeSR™3D Feed Medium at 112 µL/mL of initial culture volume to the center of the culture. Refer to Table 3 for the recommended volume of Feed Medium to add to various culture vessels. Change pipette tips between cultures to prevent cross-contamination.

Table 3. Daily Volume of mTeSR™3D Feed Medium for Various Culture Vessels

CULTURE VESSEL	INITIAL CULTURE VOLUME (mL)	DAILY VOLUME OF mTeSR™3D FEED MEDIUM (mL)
6-Well Flat-Bottom Plate, Non-Treated	2	0.224
Nalgene™ Rapid-Flow™ Sterile Filter Storage Bottles	15 - 60	1.68 - 6.72
PBS-MINI 0.1 MAG Single-Use Vessel	100	11.2
PBS-MINI 0.5 MAG Single-Use Vessel	500	56

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Note: When feeding PBS-MINI MAG Single-Use Vessels, it is required to remove a volume of spent medium equivalent to the volume of Feed Medium to be added prior to feeding. This keeps the working volume constant and ensures that the maximum working volume is not exceeded. Alternatively, the bioreactor can be seeded at a lower volume such that the final volume is 100 mL or 500 mL after feeding.

Note: If the culture medium appears yellow on day 3 of a 4-day passage, it is optional to perform a half-medium change with mTeSR™3D Seed Medium (without Y-27632) instead of a fed-batch feed to remove accumulated lactate.

6.2 hPSC Aggregate Morphology

When culturing hPSCs as aggregates in mTeSR™3D, monitoring the morphology of aggregates by microscopy and imaging is an important qualitative check. Divergence from typical aggregate morphology can indicate that the aggregates may be nutrient-limited, differentiated, or necrotic. This section includes images of typical undifferentiated hPSC aggregates over the course of a passage (Figure 1 & Figure 2), as well as images of hPSCs with poor aggregate morphology (Figure 3).

Typically, undifferentiated hPSC aggregates should not exceed 400 µm in diameter. If they grow much larger than this, they may experience nutrient deficiency in the core of the aggregate and subsequent differentiation and loss of expression of hPSC markers. See Troubleshooting (section 8.0) if aggregates are growing beyond this size in a standard passage.

Cell aggregates should be mostly spherical, with some loose packing of cells around the periphery. Healthy, high-quality aggregates exhibit shallow craters or pockmarks. This morphology is associated with high expression of hPSC markers and good expansion. If spheres appear to have large bulbs, fully translucent areas, or are smooth and spherical, this may indicate differentiation and loss of hPSC marker expression.

When the core of the aggregate appears slightly darkened, this has no detrimental effect on aggregate pluripotency. However, if the entire aggregate becomes very dark, this may indicate unhealthy aggregates that have been over-seeded and may be limited by low nutrient concentrations. Monitoring the morphology of aggregates over multiple passages, though qualitative, serves as an additional means to ensure high-quality aggregate cultures of pure undifferentiated hPSCs.

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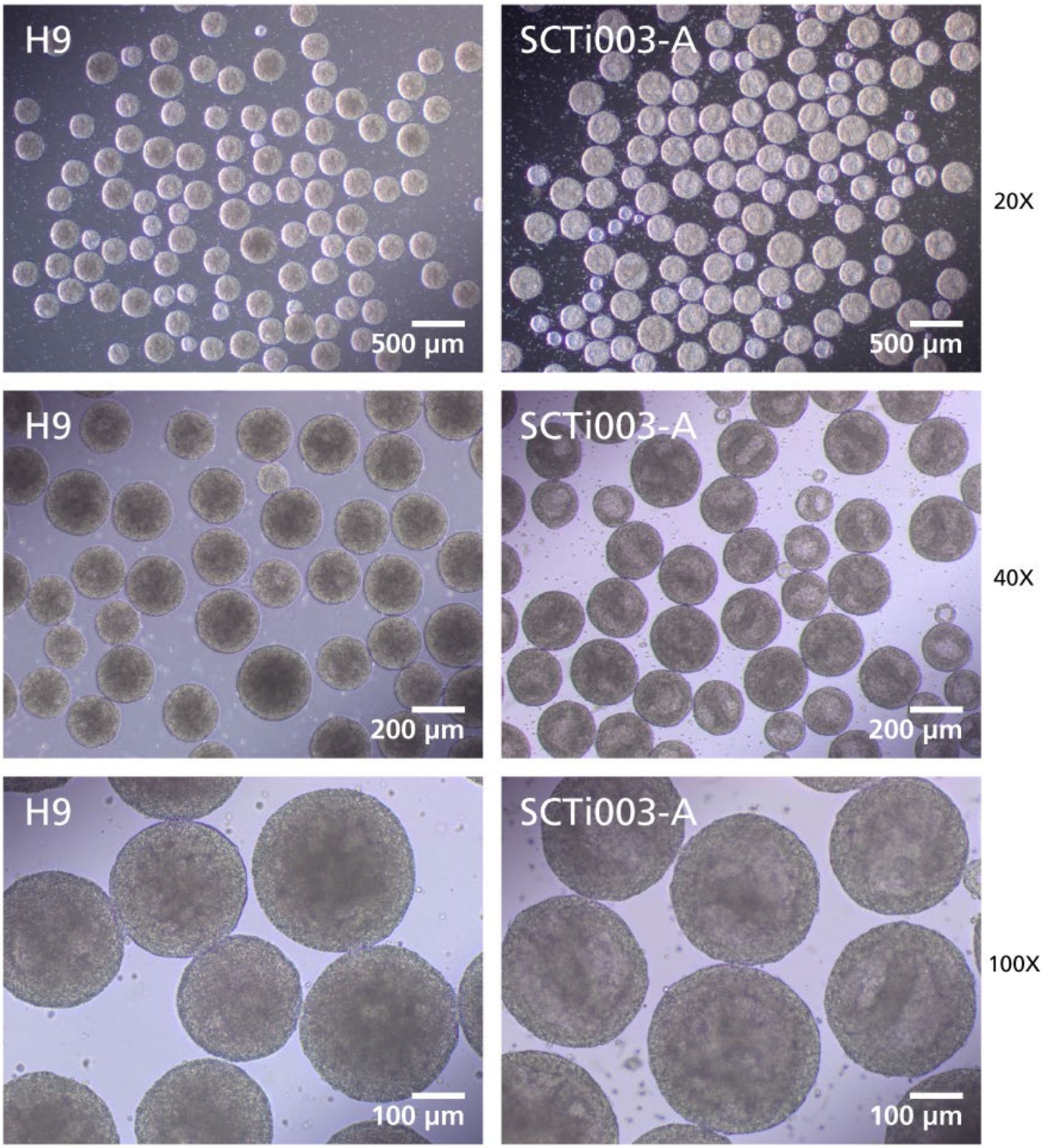

Figure 1. Representative hPSC Aggregate Morphology of H9 hESC Line and SCTi003-A hiPSC Line Prior to Passage in mTeSR™3D

Pluripotent aggregates are roughly spherical, with visible cratering across the surfaces, and diameters of 200 - 400 µm. Magnifications: 20X, 40X, and 100X.

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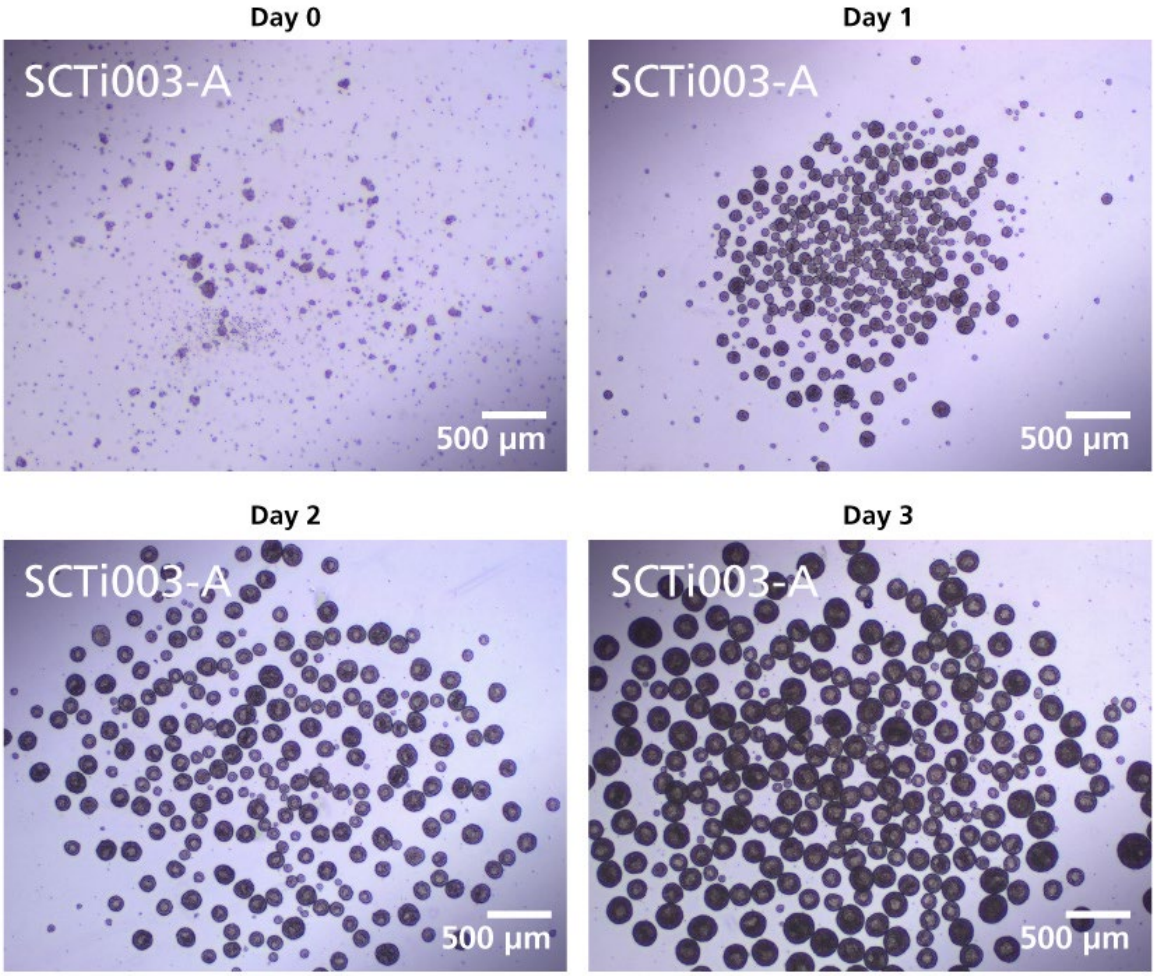

Figure 2. SCTi003-A Aggregates on Days 0, 1, 2, and 3 of Culture in mTeSR™3D

Aggregates form within 24 hours and grow in size over the course of a 3-day or 4-day passage. Magnification: 20X.

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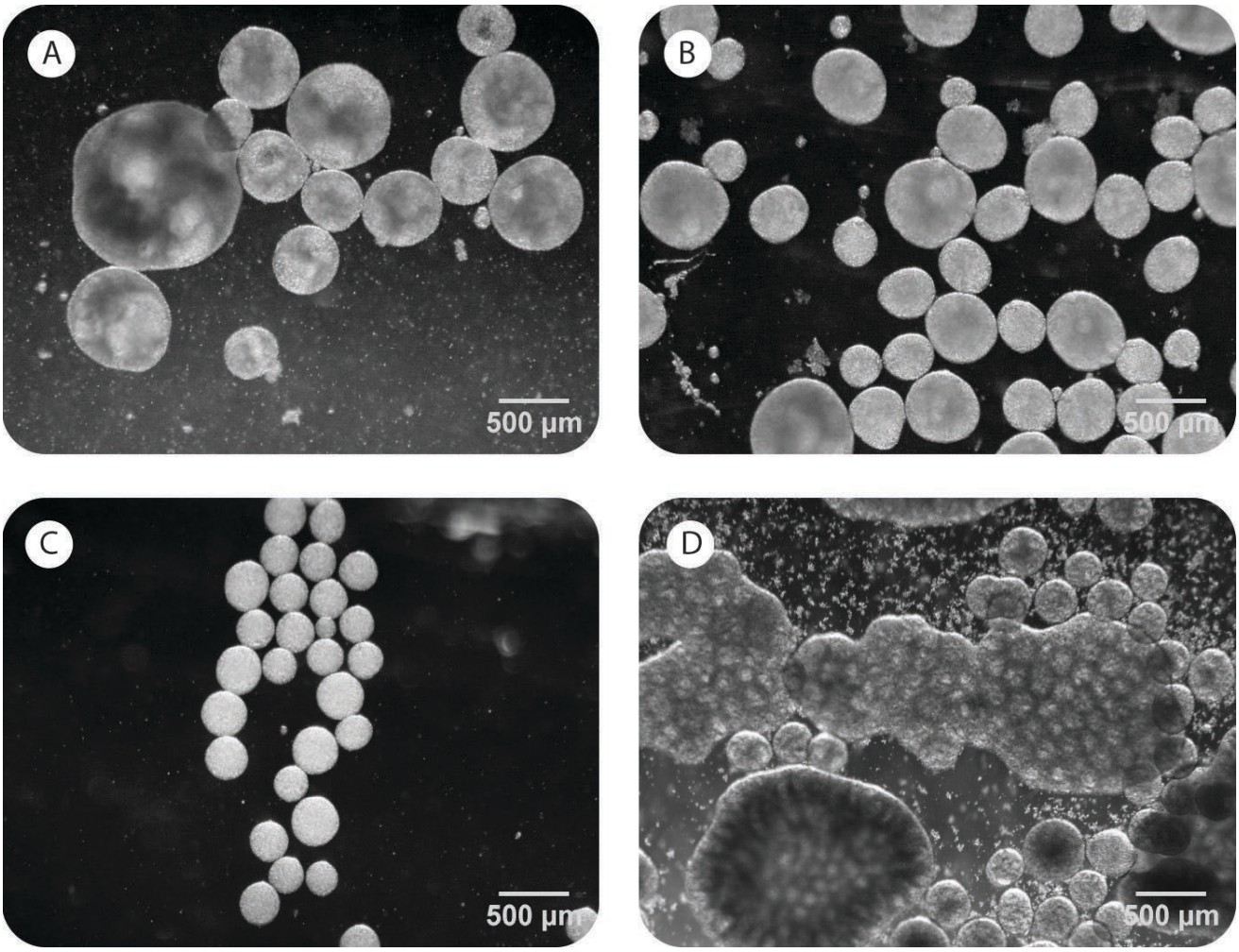

Figure 3. Examples of hPSC Aggregates with Poor Morphology

(A), **(B)**, & **(C)**: Smooth or excessively large aggregates as well as translucent patches may indicate differentiated or non-pluripotent cells resulting from non-optimal seeding densities or passaging length, or poor-quality starting cultures. **(D)** Excessive merging and aggregation of aggregates can occur if mixing is too slow or if there are stagnant zones in the culture vessel where aggregates can collect. Magnification: 20X.

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7.0 Passaging Suspension Culture hPSCs Grown in mTeSR™3D

7.1 Passaging Aggregates as Small Clumps

Aggregates in suspension grow in diameter over the course of a passage and must be dissociated and reseeded every 3 - 4 days. Dissociating aggregates to small clumps rather than single cells is recommended to minimize the probability of selecting for subpopulations of cells with abnormal karyotypes; these can gain proliferative advantages in stressful single-cell environments.

In the following passaging protocol, aggregates are dissociated to small clumps using Gentle Cell Dissociation Reagent (GCDR) and a 37 µm Reversible Strainer, then resuspended in mTeSR™ 3D Seed Medium + 10 µM Y-27632. GCDR dissociation results in high cell viability and recovery and preserves the integrity of cell surface proteins that aid in hPSC aggregate formation. The Reversible Strainer facilitates the generation of homogenous clump suspensions independent of culture density or volume. This protocol is for dissociating aggregates from an initial culture volume of 15 mL. For other culture volumes, refer to Table 4 for volumes of GCDR and mTeSR™3D Seed Medium + 10 µM Y-27632.

Table 4. Recommended Volumes of GCDR and Resuspension Medium When Dissociating Aggregates

INITIAL CULTURE VOLUME (mL)	VOLUME OF GCDR (mL)	VOLUME OF mTeSR™3D SEED MEDIUM + 10 µM Y-27632 FOR RESUSPENSION (mL)
2	1	1
15	5	5
60	15	20
100	20	25
500	30	40 - 50

1. Aliquot and warm 5 mL of GCDR to 37°C.
2. Bring mTeSR™3D Seed Medium to room temperature (15 - 25°C).
3. Prepare enough mTeSR™3D Seed Medium + 10 µM Y-27632 to resuspend and seed all conditions. See Table 4 for recommended resuspension volume.
4. Image cultures prior to passaging to assess aggregate size.
5. Filter out non-aggregated single cells by passing the entire volume of the culture through a large 37 µm Reversible Strainer with the arrow pointing up into a 50 mL conical tube or storage bottle for culture volumes > 45 mL.

   Note: For suspension cultures in a 6-well plate, the small 37 µm Reversible Strainer can be used with a 15 mL conical tube.

   Note: When passaging aggregates in a PBS-MINI 0.5 MAG Single-Use Vessel, modifications may be required for this step due to the large number of aggregates. Two options for modifications are as follows:

   a. *Use multiple 37 µm Reversible Strainers to collect the aggregates, then wash all aggregates into the same conical tube in step 6.*
   b. *Settle the aggregates at the bottom of the culture vessel, remove the spent medium, and add 10 mL D-PBS to rinse. Transfer the aggregates to the 50 mL conical tube from step 6, settle the aggregates and remove the D-PBS, then resuspend in GCDR.*
6. Flip the strainer onto a new 50 mL conical tube such that the arrow is pointing down and rinse with 5 mL of warm GCDR, gently tapping the strainer to dislodge all aggregates into the new tube.

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7. Flip the strainer onto another new 50 mL conical tube such that the arrow is pointing up and set aside. This strainer can be used to dissociate aggregates into small clumps (step 12).
8. Incubate the conical tube containing aggregates and GCDR in a 37°C water bath for 6 minutes (undisturbed).

   Note: In this step, the aggregates are partially dissociated by the GCDR in preparation for generation of small clumps (step 12).

   Note: Optimal incubation time may vary depending on the cell line.
9. Gently remove the conical tube from the water bath without disturbing the cell pellet.

   Note: For larger volumes of GCDR (> 5 mL), centrifuge the tube for 3 minutes at 100 x g to collect any aggregates that have not settled.
10. Using a serological pipette, slowly aspirate the GCDR, leaving ~0.5 mL to avoid removing any aggregates.
11. Add 5 mL of mTeSR™3D Seed Medium + 10 µM Y-27632 to the tube. Flick or gently swirl the tube to resuspend the aggregates.

    Note: If the culture has expanded > 5-fold over the course of the passage and aggregate density is high, consider doubling the volume of mTeSR™3D Seed Medium + 10 µM Y-27632 used to resuspend the aggregates prior to pushing through the 37 µm strainer.
12. Using a 25 mL serological pipette, remove the resuspended partially dissociated aggregates from the tube and place the serological in contact with the strainer on the conical tube left from Step 7. With the serological in a vertical orientation, with no gaps between it and the strainer, slowly force the partially dissociated aggregates through the strainer with the slowest setting on the Pipette-Aid (0.5 mL/second flow rate). This will generate clumps of appropriate size to initiate the subsequent passage.

    Note: Ensure there are no bubbles at the end of the pipette tip prior to pushing the aggregates through the strainer.

    Note: If the strainer appears clogged, slide the pipette laterally on the strainer while maintaining direct contact with it. Alternatively, increasing the flow rate slightly can help to prevent clogging, but use the lowest flow rate possible to minimize damage to the cells.

    Note: Force a maximum of 10 mL of aggregate suspension through the strainer at a time. For larger culture volumes, a stepwise procedure is recommended.
13. *Optional*: Rinse the strainer with 2 - 5 mL of additional mTeSR™3D Seed Medium + 10 µM Y-27632 to collect any clumps stuck on the bottom surface of the strainer.
14. Gently resuspend the clump suspension. Remove a sample for counting viable cells or clumps (see Appendix 1: Counting Clumps).
15. Add concentrated clump suspension to the culture vessel to obtain the desired number of total viable cells or clumps and then top up medium with mTeSR™3D Seed Medium + 10 µM Y-27632 to desired total volume.

    Note: Clumps may aggregate if left for extended periods of time (> 15 minutes) as a pellet. If very large clumps have formed (> 400 - 500 µm), gently triturate the entire clump suspension 1 - 2 times using a 25 mL serological pipette at a flow rate of ~1 mL per second immediately before seeding.

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8.0 Troubleshooting

PROBLEM	SOLUTION
Aggregates do not form after 24 hours	• Ensure maintenance culture is of high quality • Increase seeding density • Decrease RPM or increase culture volume • Ensure Y-27632 is present when seeding
Aggregates are still small on day 4	• Ensure maintenance culture is of high quality • Increase seeding density • Decrease RPM or increase culture volume
Aggregates are merging into large conglomerates	• Ensure maintenance culture is of high quality • Decrease seeding density • Increase RPM or decrease culture volume
Many single cells have not formed aggregates	• Ensure maintenance culture is of high quality • Monitor over more passages (may improve) • Ensure Y-27632 is present when seeding • On day 1 (i.e. 24 hours after seeding), use 37 µm Reversible Strainer to remove single cells from culture
Aggregates are getting too big by end of passage	• Decrease seeding density • Increase RPM or decrease culture volume
Aggregates will not dissociate at passage	• Increase incubation time during dissociation • Increase shear force during dissociation
Too many single cells post-dissociation	• Ensure maintenance culture is of high quality • Decrease incubation time during dissociation • Decrease shear force during dissociation
Medium is very yellow by end of passage	• Decrease seeding density • Perform a half-medium change on day 3 of a 4-day passage
Cells are sticking to the vessel wall	• Ensure maintenance culture is of high quality • Coat vessel with Anti-Adherence Rinsing Solution
Aggregates look dense and dark	• Ensure maintenance culture is of high quality • Increase RPM or decrease culture volume • Decrease seeding density
Aggregates are irregular in shape	• Ensure maintenance culture is of high quality • Start with more consistent clump size on day 0 • Optimize culture conditions (e.g. RPM, initial culture volume, and seeding density)
Aggregates are non-uniform in size	• Start with more consistent clump size on day 0 • Optimize culture conditions (e.g. RPM, initial culture volume, and seeding density)

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PROBLEM	SOLUTION
Cells in aggregates lose expression of undifferentiated hPSC markers	• Ensure maintenance culture is of high quality • Optimize culture conditions (e.g. RPM, initial culture volume, and seeding density) • Decrease seeding density • Passage when the aggregates are no larger than 350 - 400 µm in size
Cells in aggregates develop karyotype abnormalities	• Ensure maintenance culture is of high quality • Passage as clumps rather than as single cells • Optimize passaging to maximize percentage of cells remaining in clumps
Cells from aggregates differentiate at lower efficiency than 2D mTeSR™1-derived cells	• See 'Cells in aggregates lose expression of undifferentiated hPSC markers' • Try lower seeding densities in monolayer differentiation protocols, as reseeding from 3D may be more efficient than from standard 2D cultures

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10.0 Appendix 1: Counting Clumps

Accurate and precise quantification of clumps dissociated from 2D or 3D cultures is critical for measuring expansion and accurately seeding subsequent passages. Two methods for counting clumps are described below. The first method, Manual Clump Counting, can be performed by any lab with an inverted microscope. The second method, Viable Nuclei Counting, requires an image-based cell counting instrument such as the ChemoMetec NucleoCounter® NC-250™ in combination with a viability stain.

Manual Clump Counting

Count clumps ≥ 30 µm in diameter, as these are the most likely to form aggregates of ideal size.

1. Draw a "+" centered on the bottom of 2 wells of a 96-well flat-bottom plate (e.g. Catalog #38022) 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 dissociated and resuspended clump suspension to each well. Ensure that the clump suspension is well-mixed prior to removing the sample for counting.
4. Count the clumps in each well that are ≥ 30 µm in diameter. Average the results from the two wells to obtain the average number of clumps (N_A) in the 5 µL sample.
5. Calculate the concentration of clumps (C) and the total number of clumps (N_T) using the total volume of the clump suspension in µL (V_T):

$$C = (N_A / 5\ \mu L)$$

$$N_T = C \times V_T$$

6. Determine the target number of clumps to seed (N_p) multiplying the optimal seeding density (e.g. 1×10^3 clumps/mL) by the culture volume.
7. Calculate the volume (in µL) of clump suspension to seed (V_p) for each condition in your experiment:

$$V_P = N_P / C$$

8. Gently mix the clump suspension prior to seeding to ensure a uniform suspension.
9. Add calculated volume of clump suspension (V_P) to the culture vessel and add mTeSR™3D Seed Medium + Y-27632 to the desired volume.

Viable Nuclei Counting

Alternatively, clump cultures can be quantified as viable cells/mL using an image-based cell counting instrument such as the ChemoMetec NucleoCounter® NC-250™ in combination with a viability stain. Contact us at techsupport@stemcell.com for further information.

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11.0 Appendix 2: Differentiating Suspension Cultures into Three Germ Layers

hPSCs grown in suspension culture with mTeSR™3D can differentiate to the three germ layers: mesoderm, definitive endoderm, and ectoderm.

Cells derived from hPSC suspension culture tend to have a higher plating efficiency than those derived from 2D mTeSR™1 cultures when seeded as a monolayer for differentiation protocols. If cultures appear overgrown compared to 2D controls on day 1 of a differentiation protocol, consider lowering the seeding density to achieve desired confluency for differentiation.

Generate a single-cell suspension using Gentle Cell Dissociation Reagent (GCDR), then use STEMdiff™ Trilineage Differentiation Kit (Catalog #05230) to differentiate cells to the three germ layers.

12.0 Appendix 3: Re-Establishing 2D Colony Cultures

For suspension cultures that have been passaged as clumps:

1. Dissociate aggregates to small clumps (section 7.1).
2. Count dissociated clumps (see Appendix 1: Counting Clumps).
3. Plate 200 - 300 clumps/well onto a Corning® Matrigel®-coated 6-well plate in 2 mL of mTeSR™1, mTeSR™ Plus, TeSR™-E8™, or TeSR™-AOF. Maintain as a standard 2D hPSC culture.

   Note: For additional information on coating plates, maintaining 2D cultures, and passaging 2D to 2D, refer to the Technical Manuals for mTeSR™1 (Document #10000005505), mTeSR™ Plus (Document #10000007757), TeSR™-E8™ (Document #10000005516), or TeSR™-AOF (Document #10000008160), available at www.stemcell.com, or contact us to request a copy.
4. After one or two 2D clump passages, typical 2D colony morphology should be restored.

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13.0 Appendix 4: Assessing Karyotype

To assess whether chromosomal abnormalities have been acquired or have increased in prevalence in suspension cultures, it is recommended to assess the karyotype at the beginning and end of an experiment, as well as every 5 - 10 passages. Karyotype can be assessed by G-banding. Additional routine screening to detect the most common karyotypic abnormalities observed in hPSCs can be performed using the hPSC Genetic Analysis Kit (Catalog #07550).

The resolution of G-banding karyotype is limited to > 5 Mb. For higher resolution of variations in gene copy number, deletions, duplications, and other genotypic changes, consider using fluorescent in situ hybridization (FISH), SNP genotyping, RT-PCR, or microarrays.

G-banding karyotypes are also limited in the number of spreads analyzed. Due to the laborious nature of the protocol, only 20 - 30 cells are analyzed per culture. Low-frequency sub-populations of abnormal cells can go undetected in both maintenance and experimental cultures. Improper culture techniques may increase the frequency of genetically abnormal cells in culture.

14.0 Appendix 5: Dissociating Aggregates to Single Cells for Flow Cytometry or Downstream Applications

The following are instructions for preparing a single-cell suspension from aggregate cultures grown in mTeSR™3D with an initial culture volume of 15 mL. For other culture volumes, refer to Table 4 for volumes of GCDR and resuspension medium.

1. Aliquot and warm 5 mL of GCDR to 37°C.
2. Warm mTeSR™3D Seed Medium or medium for desired downstream application to room temperature (15 - 25°C).
3. Filter out non-aggregated single cells by transferring the entire volume of culture through a large 37 µm Reversible Strainer with the arrow pointing up into a 50 mL conical tube.
4. Flip the strainer onto a new 15 mL conical tube such that the arrow is pointing down and rinse with 5 mL of warm GCDR gently tapping the strainer to dislodge all aggregates into the new tube. Discard the strainer.
5. Incubate tube in a 37°C water bath for 15 - 20 minutes. Gently flick the tube to resuspend the aggregates every 3 - 5 minutes.

   Note: Optimal incubation time may vary depending on the cell line.
6. Centrifuge the tube for 4 minutes at 100 x g to pellet the aggregates.
7. Carefully aspirate the supernatant without disturbing the cell pellet.
8. Flick the tube to resuspend the pellet and then add 1 mL of mTeSR™3D Seed Medium or medium for desired downstream application.
9. Use a P1000 pipette to gently triturate the cells 5 - 15 times (optimal number of triturations may vary depending on cell line, GCDR incubation time, and density of culture).

   Note: If you are resuspending multiple pellets, perform steps 7 - 9 one tube at a time.
10. Top up to the volume of medium indicated in Table 4.

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15.0 Appendix 6: Flow Cytometry Methods

15.1 Reagents and Materials

15.1.1 Antibodies

Antibodies can be used to characterize hPSCs by flow cytometry. The tables below list a selection of antibodies available from STEMCELL Technologies that can be used to characterize undifferentiated hPSC cultures. For a complete list of antibodies, including other conjugates, sizes, and clones, visit www.stemcell.com/antibodies.

Surface Antigen Labeling

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

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

Intracellular Antigen Labeling

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

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

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15.1.2 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	100-0485
Dulbecco's Phosphate Buffered Saline with 2% Fetal Bovine Serum (2% FBS/PBS)	07905
Costar® Microcentrifuge Tubes, 1.7 mL	38038
Falcon® Round-Bottom Tubes, 5 mL	38007
Falcon® Conical Tubes, 15 mL	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. Sigma-Aldrich 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	2%
D-PBS (Without Ca++ and Mg++)	37350	to final volume

* Mix well and store at 2 - 8°C.

15.2 Preparation of a Single-Cell Suspension for Flow Cytometry

Prepare a single-cell suspension as indicated in Appendix 5. Perform a viable cell count using either Trypan Blue and a hemocytometer or AO/DAPI and a ChemoMetec NucleoCounter® NC-250™. The single-cell suspension may now be used for surface antigen and/or intracellular antigen labeling (see sections 15.3 and 15.4 for detailed protocols).

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15.3 Surface Antigen Labeling Protocol

Note: Optimal concentrations of antibodies must be predetermined by titration for each antibody.

Note: The staining protocol can also be complete in a 96-well round-bottom plate; adjust methodology accordingly.

1. Determine the number of samples required to perform flow cytometry, including labeling controls.
2. Aliquot approximately 2 x 10^5 cells per sample into a 5 mL tube or a 1.7 mL 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.

   Note: 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 tube if necessary.

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

15.4 Intracellular Antigen Labeling Protocol for OCT4

Note: Optimal concentrations of antibodies must be predetermined by titration for each antibody.

1. Determine the number of samples required to perform flow cytometry, including labeling controls.
2. Aliquot approximately 2 x 10^5 cells per sample into a 5 mL tube or a 1.7 mL tube.
3. Centrifuge cells at 300 x g for 5 minutes.
4. Carefully remove the supernatant without disrupting the cell pellet and resuspend cells in 250 µL of 2% Paraformaldehyde Solution/tube. Gently mix and incubate on ice for 15 - 30 minutes.
5. Add 1 mL of 2% FBS/PBS per tube. Gently mix and centrifuge at 300 x *g* for 5 minutes.
6. Carefully remove the supernatant without disrupting the cell pellet and resuspend cells in 500 µL of SPB/tube. Gently mix and incubate at room temperature (15 - 25°C) for 15 minutes.

   Note: Cells should remain in SPB until the final resuspension step, prior to flow cytometric analysis.
7. While the samples are incubating, prepare a sufficient quantity of the primary antibody mix (100 µL/sample) at the predetermined optimal working dilution, using SPB as the diluent.
8. Centrifuge cells at 300 x g for 5 minutes.

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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.

   Note: 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 tube if necessary.
14. Place samples on ice, while avoiding exposure to direct light, and analyze by flow cytometry as soon as possible.

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16.0 Appendix 7: Downstream Differentiation

hPSCs grown in suspension culture in mTeSR™3D can be differentiated into specific cell types using many of STEMCELL's STEMdiff™ kits. To ensure successful downstream differentiation hPSC aggregates should be assessed in terms of morphology, markers of undifferentiated cells, and karyotype before proceeding with the differentiation.

To complete the differentiation in 2D in accordance with the Product Information Sheet (PIS) for the specific differentiation kit, passage the high-quality hPSC suspension culture on day 3 or 4. Dissociate the aggregates to clumps or single cells and plate the cells as specified in the PIS. Note that cells derived from hPSC suspension cultures tend to have a higher plating efficiency than those derived from 2D mTeSR™1 or TeSR™-E8™ cultures when seeded as a monolayer for differentiation protocols. If cultures appear overgrown compared to 2D controls on day 1 of a differentiation protocol, consider lowering the seeding density to achieve desired confluency for differentiation.

Proof-of-concept experiments have also demonstrated compatibility of STEMCELL's STEMdiff™ kits with differentiation in suspension culture; however, optimization of culture parameters is required. It is recommended to evaluate and optimize the selected STEMdiff™ kit in a 6-well plate format before transitioning to large volumes. To complete differentiation in suspension culture, passage the high quality hPSC suspension culture on day 3 or 4 as detailed in section 7.1, and seed suspension cultures at 1 - 3 x 10^5 viable cells/mL in mTeSR™3D. After 24 - 48 hours, complete a full-medium change to the differentiation medium.

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mTeSR™3D is manufactured and sold under global exclusive license from Accellta for culture medium for hPSCs in suspension under feeder-free, non-adherent conditions.

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TECHNICAL MANUAL

Expansion of Human Pluripotent Stem Cells as Aggregates in Suspension Culture Using mTeSR™3D

STEMCELL™ TECHNOLOGIES

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