TECHNICAL MANUAL

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

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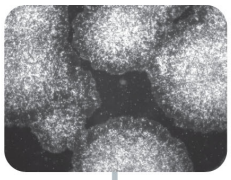
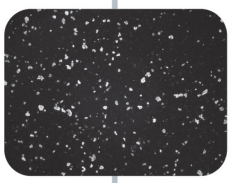
1.0 mTeSR™3D Workflow

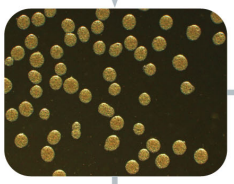


Start with high-quality hPSC cultures

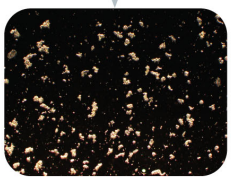


Initiate hPSC suspension culture in mTeSR™3D Seed Medium supplemented with 10 µM Y-27632

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.

2.0 Introduction

A significant challenge in the field of human pluripotent stem cell research is the generation of large numbers of highly pure undifferentiated cells. Although growing human pluripotent stem cells (hPSCs; embryonic stem [ES] cells and induced pluripotent stem [iPS] cells) in a two-dimensional (2D) adherent mTeSR™1 system has long been the preferred method for maintenance of hPSCs, a standardized system to scale up hPSC production has yet to be established. To meet this growing demand, we have developed mTeSR™3D, a defined, serum-free formulation optimized for the expansion of undifferentiated hPSCs in a three-dimensional (3D) suspension culture system.

mTeSR™3D uses a fed-batch strategy in which all the necessary nutrients and growth factors are added daily and spent medium is only removed prior to passaging. The mTeSR™3D kit consists of a Seed Medium (Basal Medium + 5X Supplement) to initiate cultures and a Feed Medium (Supplement A + Supplement B) for daily fed-batch addition. The 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.

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. Matrigel®). By contrast, in the mTeSR™3D suspension culture system, cells grow as spherical aggregates in suspension without the addition of matrices or microcarriers.

Compared to 2D, growing cells in a 3D fed-batch suspension system has a number of advantages:

- Eliminates the need to replace medium daily 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 have been shown to:

- Transition efficiently from 2D cultures
- Yield more cells per mL of medium used than cells grown in 2D mTeSR™1 cultures
- Express equivalent levels of OCT4, TRA-1-60, and SSEA-3 (assessed by flow cytometry) as cells grown in 2D mTeSR™1 cultures
- Have similar metabolic profiles compared to 2D mTeSR™1 cultures (glucose-to-lactate yield, cell-specific glucose uptake rate, cell-specific lactate production rate)
- Maintain differentiation capacity to the three germ layers, similar to 2D mTeSR™1 cultures
- Have normal hPSC colony morphology when re-plated as 2D cultures

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
	D-PBS (Without Ca++ and Mg++)	37350
	Gentle Cell Dissociation Reagent (GCDR)	07174
	Cell scrapers	e.g. 38065
Passaging Materials for 3D Suspension Culture	Y-27632 (ROCK inhibitor)	72304
	Gentle Cell Dissociation Reagent (GCDR)	07174
	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 human ES and iPS cell research available from STEMCELL Technologies Inc., visit www.stemcell.com.

3.3 Vessels and Coatings for hPSC Suspension Culture

Note: The following vessels and coatings have been evaluated by STEMCELL Technologies. Additional culture vessels may be suitable for mTeSR™3D suspension culture.

CATEGORY	PRODUCT	CATALOG #
Suspension Culture Vessels	6-Well Flat-Bottom Plate, Non-Treated	38040
	Corning® 125 mL Polycarbonate Erlenmeyer Flask with Vent Cap	Corning 431143
	300 mL Polycarbonate Round Bottles	Qorpak AKM-3002-0007
	PFEIFFER CELLSPIN Spinner Flask with 1 Pendulum, 100 mL	PFEIFFER 182 023
	PFEIFFER CELLSPIN Spinner Flask with 2 Pendula, 250 mL	PFEIFFER 182 026
	Applikon miniBio 500 bioreactor	V3LP070201
Vessel Coatings (see Appendix 1)	Anti-Adherence Rinsing Solution	07010
	DMEM/F-12 with 15 mM HEPES	36254
	Sodium hydroxide (NaOH)	Sigma 221465
	Sigmacote®	Sigma SL2

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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, spinner flask platform, or alternative suspension culture system
- Autoclave for re-usable glass spinner flasks, if applicable

Optional

- Viable cell counter (e.g. ChemoMetec NucleoCounter® NC-250™) and viability stain

4.0 Preparation of Reagents and Materials

4.1 mTeSR™3D Seed Medium

Use sterile technique to prepare mTeSR™3D Seed Medium (Basal Medium + 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. 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.

   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 (section 5.2.2).

4.2 mTeSR™3D Feed Medium

Use sterile technique to prepare mTeSR™3D Feed Medium (Supplement A + 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.

   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.

4.3 Suspension Culture Vessel Parameters

A variety of culture vessels can be used for growing hPSCs in suspension, depending on the desired culture scale and the available systems in each laboratory. For small-scale optimization or high-throughput experiments, we recommend non-tissue culture-treated 6-well plates (Catalog #38040). For large-scale hPSC cultures, we recommend a larger vessel such as the Applikon miniBio 500 bioreactor. Refer to Table 1 for culture vessels evaluated by STEMCELL Technologies along with the recommended volumes and orbital/impeller speeds.

For more information, refer to Appendix 1: Preparing Suspension Culture Vessels.

Table 1. Suspension Culture Vessels Evaluated by STEMCELL Technologies

CULTURE VESSEL	CATALOG #	RECOMMENDED INITIAL CULTURE VOLUME	RECOMMENDED ORBITAL SHAKER/PENDULUM/IMPELLER SPEED
6-Well Flat-Bottom Plate, Non-Treated	38040	2 mL	70 rpm (1.9 cm orbital diameter)
Corning® 125 mL Polycarbonate Erlenmeyer Flask with Vent Cap	Corning 431143	20 mL	70 rpm (1.9 cm orbital diameter)
Qorpak® 300 mL Polycarbonate Round Bottles	Qorpak AKM-3002-0007	24 mL	65 rpm (2.5 cm orbital diameter)
PFEIFFER CELLSPIN Spinner Flask with 1 Pendulum, 100 mL	PFEIFFER 182 023	50 mL	50 - 55 rpm
PFEIFFER CELLSPIN Spinner Flask with 2 Pendula, 250 mL	PFEIFFER 182 026	100 mL	40 - 50 rpm
Applikon miniBio 500 bioreactor	Applikon V3LP070201	200 mL	70 - 90 rpm

5.0 Initiating hPSC Suspension Culture in mTeSR™3D

Adherent 2D cultures grown on Corning® Matrigel® with mTeSR™1 can be transitioned directly into dynamic suspension culture with no separate adaptation step. Note that some cell lines might expand less rapidly during the first passage than in later passages.

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, and are passaged with Gentle Cell Dissociation Reagent as clumps that are 50 - 100 µm in diameter. Cells cultured in other 2D matrix-media 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 and a low percentage of differentiated cells, have normal growth rates, and retain 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 normal karyotype; we recommend checking cultures every 5 - 10 passages. For routine screening to detect the most common karyotypic abnormalities observed in hPSCs, the hPSC Genetic Analysis Kit (Catalog #07550) can be used.

5.2 Dissociating 2D Culture to Initiate 3D Suspension Culture

5.2.1 Choosing a Dissociation Method

hPSCs efficiently form aggregates in mTeSR™3D in suspension whether seeded as single cells or as small clumps. One method may be preferred depending on the cell line and intended downstream applications. In Table 2, clump passaging and single-cell passaging methods are compared.

A review of hPSC suspension culture literature reveals a diverse array of dissociation protocols, including using ACCUTASE™[1,2,3] or trypsin/EDTA[4,5] to generate single cells; combinations of collagenase IV, collagenase B, and TrypLE™ Select[3,6,7,8]; or mechanical dissociation to small clumps[5,6].

In the mTeSR™3D culture system, we recommend a non-enzymatic clump passaging protocol using Gentle Cell Dissociation Reagent (GCDR), which consistently yields undifferentiated aggregates in mTeSR™3D. Refer to section 5.2.2 for initiating a 3D suspension culture from 2D, and section 7.3 for passaging aggregates as clumps.

Table 2. Comparison of Clump Passaging and Single-Cell Passaging for hPSC Aggregate Cultures

CLUMP PASSAGING	SINGLE-CELL PASSAGING
Initiating suspension culture as small clumps dissociated from adherent colonies with GCDR reduces the number of single cells entering the system.	Even in the presence of ROCK inhibitor Y-27632, the single-cell environment can direct cells towards apoptosis or senescence.[9]
Lower probability of selecting for sub-populations of cells with karyotype abnormalities.	Passaging hPSCs enzymatically to single cells may select for the growth of sub-populations carrying karyotype abnormalities.[9,10]
hPSC aggregates may be slightly larger by the end of the passage as compared to those formed from single cells, and may require optimization of seeding density and/or passaging schedule.	Passaging hPSCs as single cells with enzymatic dissociation methods for up to 21 and 57 passages, respectively, with normal karyotype has been shown.[1,4]
ROCK inhibitor Y-27632 promotes optimal aggregate formation and morphology.	ROCK inhibitor Y-27632 supports the survival and proliferation of single cells as well as aggregate formation.

5.2.2 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 with Gentle Cell Dissociation Reagent (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 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, which is sufficient to seed a 20 - 50 mL mTeSR™3D culture at a seeding density of 1 - 5 x 10^5 viable cells/mL. Refer to Table 3 for information about recommended seeding densities.

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. Always use fresh mTeSR™3D Seed Medium + Y-27632.

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.

   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 D-PBS (Without Ca++ and Mg++) at room temperature.
5. Aspirate D-PBS from the well and add 1 mL of GCDR at room temperature.
6. Incubate at room temperature for 6 - 8 minutes.
7. Aspirate GCDR. Add 1 mL of mTeSR™3D Seed Medium + 10 µM Y-27632. Gently detach the colonies by scraping with a serological glass pipette or a cell scraper.

   Note: Take care to avoid scraping the colonies into clumps that are too small.

8. 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.

9. 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.
10. Aliquot the cell suspension to count clumps manually or use an automated cell counting instrument such as a ChemoMetec NucleoCounter® NC-250™ (see Appendix 2: Counting Clumps). For recommended seeding densities, refer to Table 3 in section 5.3.
11. 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. Typically, seeding densities range from 1 - 5 x 10^5 viable cells/mL, which correlates to approximately 1 - 5 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.

Table 3. Optimal Seeding Densities for ES and iPS Cell Lines Evaluated by STEMCELL Technologies

CELL LINE	OPTIMAL SEEDING DENSITY (VIABLE CELLS/mL)	OPTIMAL SEEDING DENSITY (CLUMPS/mL)
H1 ES	1 x 10^5	1 x 10^3
H7 ES	2 x 10^5	2 x 10^3
H9 ES	5 x 10^5	5 x 10^3
WLS-1C (iPS)	3 x 10^5	3 x 10^3
STiPS M001*	1 x 10^5	1 x 10^3
STiPS F016*	2 x 10^5	2 x 10^3

*in-house derived iPS cell lines

6.0 Culturing hPSC Suspension Aggregates Using mTeSR™3D

6.1 Fed-Batch mTeSR™3D Feed Medium Addition

1. Prepare mTeSR™3D Feed Medium (section 4.2).
2. For recommended daily feed volumes for various culture vessels, see Table 4.

   Note: The ratio of feed volume to initial culture volume has been optimized as 0.112 mL of mTeSR™3D Feed Medium per mL of mTeSR™3D Seed Medium to provide sufficient nutrients and growth factors daily.
3. Mix mTeSR™3D Feed Medium prior to use by pipetting up and down. Add the appropriate volume of mTeSR™3D Feed Medium to the center of the culture. Do not remove any medium. Change pipettes between cultures to prevent cross-contamination.

   Note: For best results, an alternating 3-day/4-day passaging schedule is recommended, with fed-batch addition of mTeSR™3D Feed Medium on non-passaging days.

Table 4. Daily Volume of mTeSR™3D Feed Medium for Various Culture Vessels

CULTURE VESSEL	VOLUME OF mTeSR™3D SEED MEDIUM (mL)	DAILY VOLUME OF mTeSR™3D FEED MEDIUM (mL)
6-Well Flat-Bottom Plate, Non-Treated	2	0.224
Corning® 125 mL polycarbonate Erlenmeyer flask	20	2.24
Qorpak® 300 mL Polycarbonate Round Bottles	24	2.7
PFEIFFER CELLSPIN Spinner Flask with 1 Pendulum, 100 mL	50	5.6
PFEIFFER CELLSPIN Spinner Flask with 2 Pendula, 250 mL	100	11.2
Applikon miniBio 500 bioreactor	200	22.4

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 (Figures 1 - 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, but not perfectly so, with some loose packing of cells around the periphery. If spheres appear to have large bulbs or fully translucent areas, these could be signs of differentiation. The appearance of shallow craters or pockmarks on the aggregate surface is associated with high expression of hPSC markers and good expansion. If aggregates appear smooth and spherical, this can indicate differentiation and loss of expression of hPSC markers.

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.

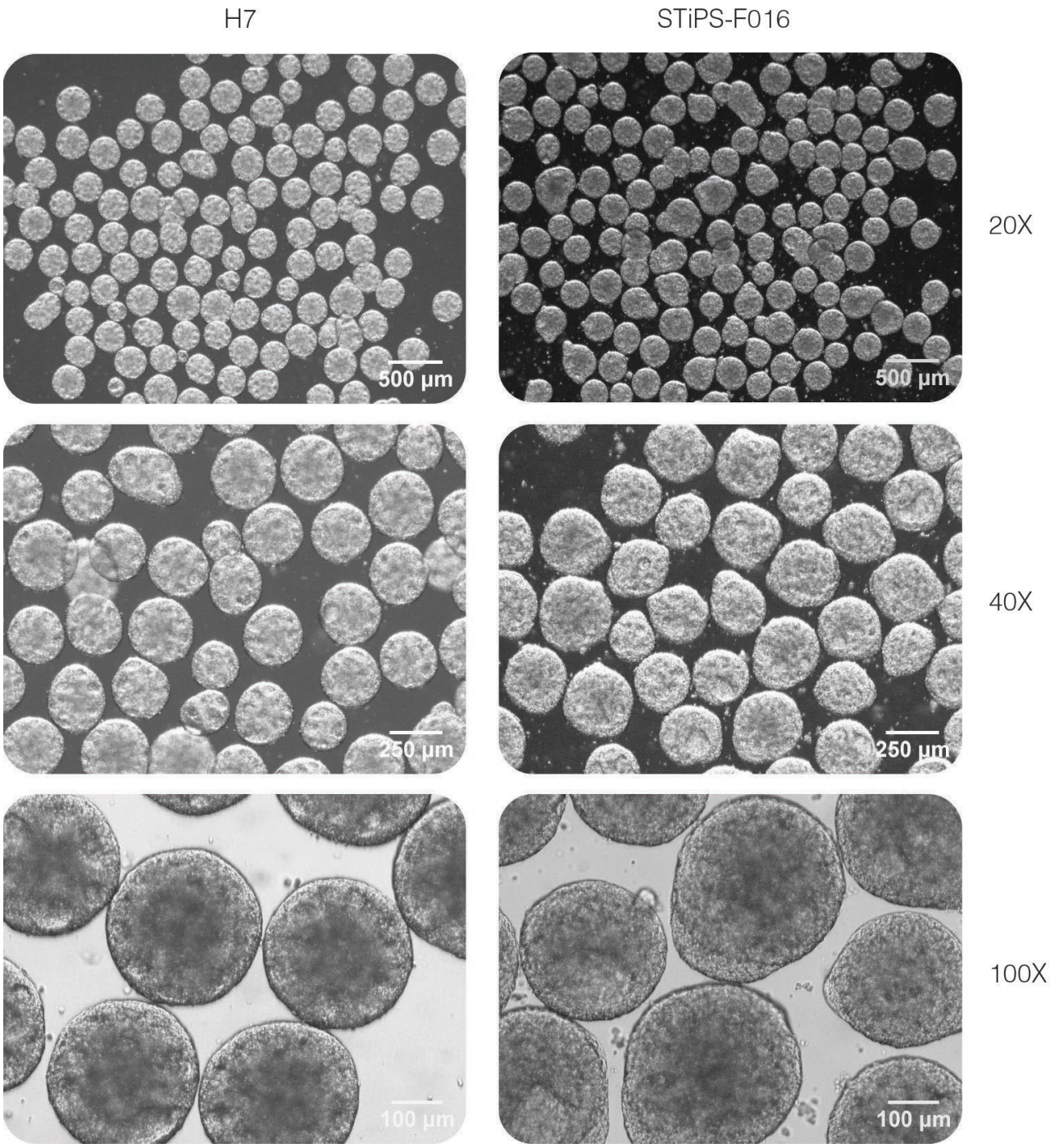

Figure 1. Representative hPSC Aggregate Morphology of H7 ES Cell Line and STiPS-F016 Cell Line Prior to Passage in mTeSR™3D

Pluripotent aggregates are roughly spherical, with visible cratering across the surfaces, and diameters between 200 and 300 µm. Magnifications: 20X, 40X, and 100X.

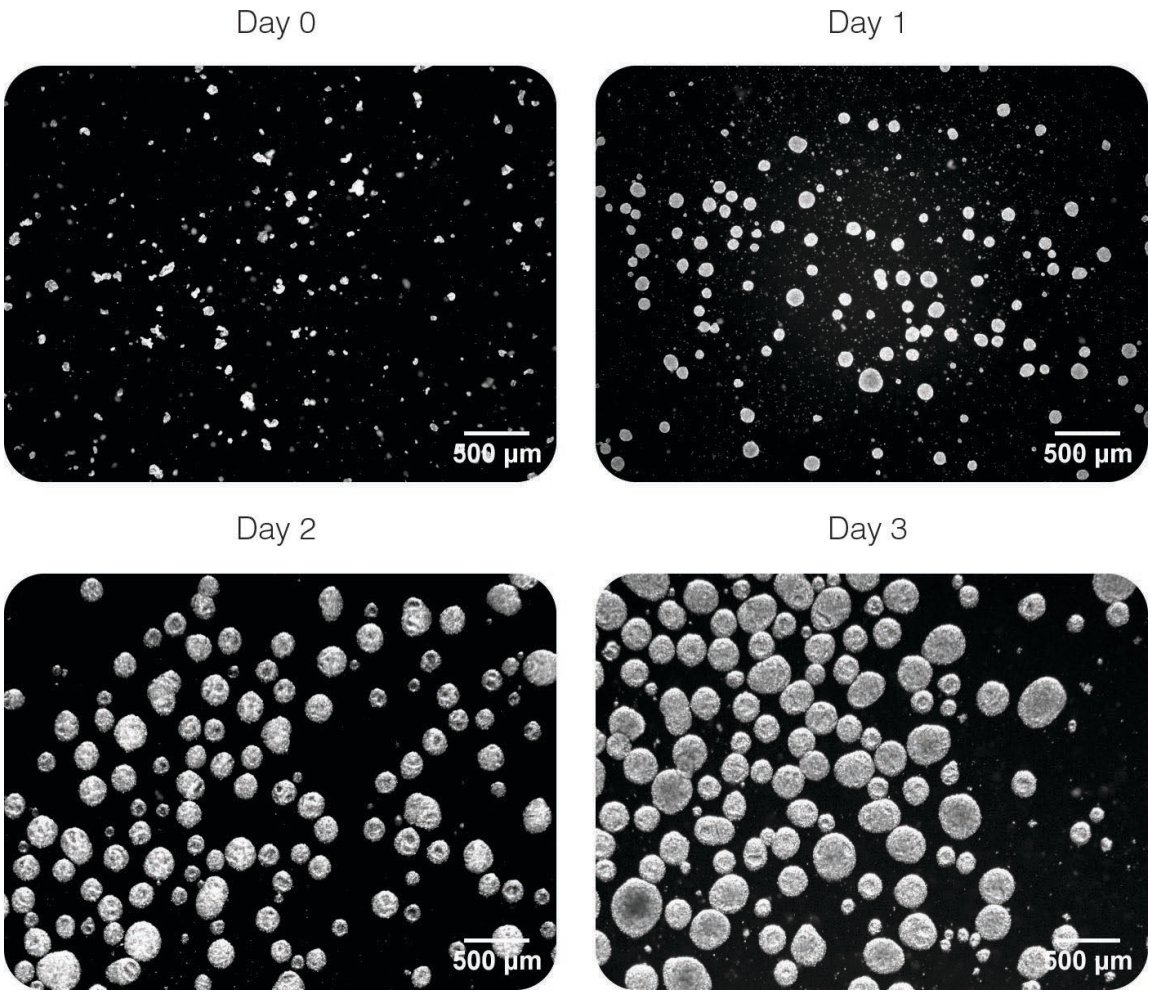

Figure 2. H9 Aggregates on Days 0, 1, 2, and 3 of Culture in mTeSR™3D

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

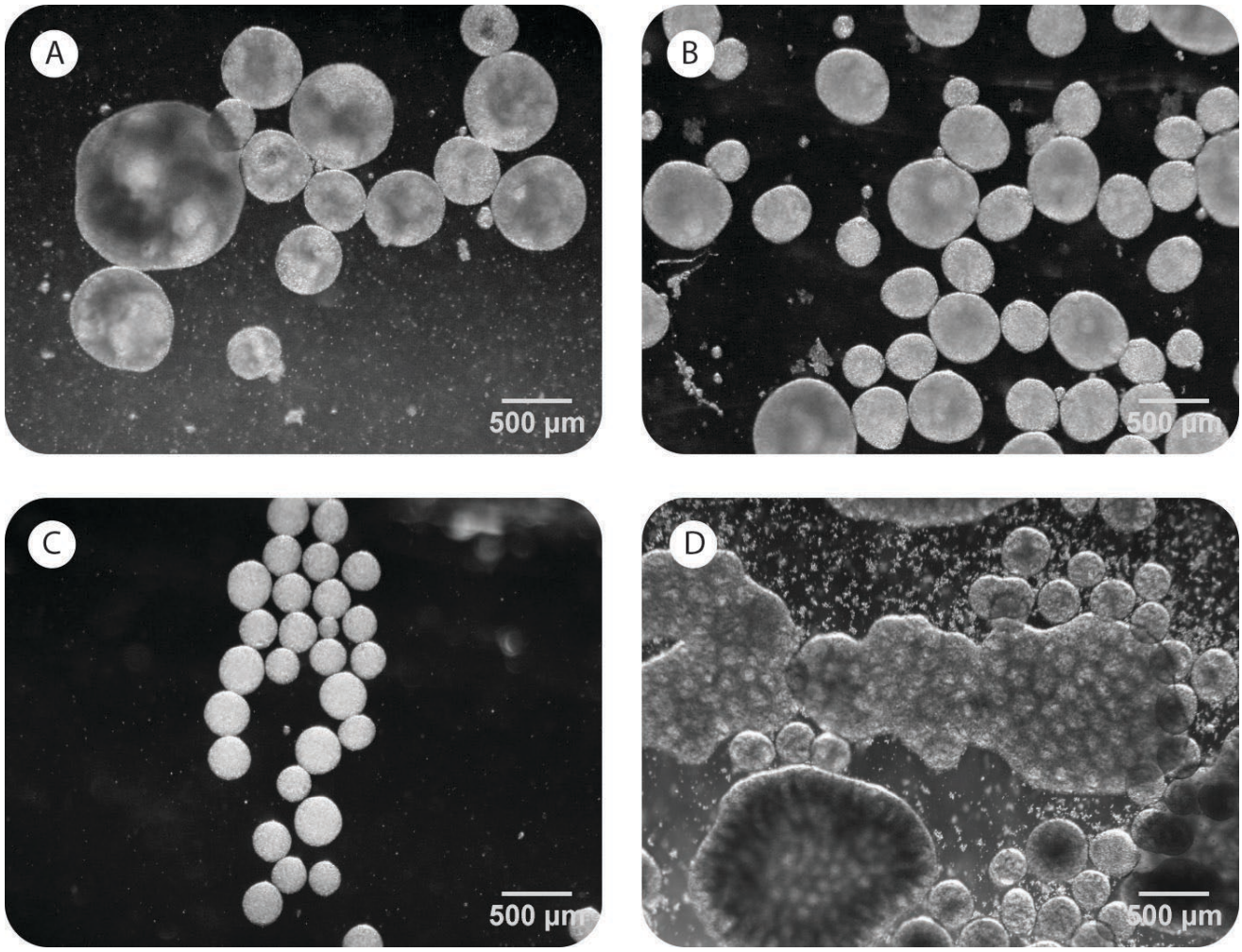

Figure 3. Examples of hPSC Aggregates with Poor Morphology

(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.

7.0 Passaging Suspension Culture hPSCs Grown in mTeSR™3D

7.1 Passaging Schedule

To prevent aggregates from getting excessively large (> 400 µm), we recommend an alternating 3-day/4-day passaging schedule. If timed properly, no passaging will be required on weekends. Ensure that the mean aggregate size does not exceed 400 µm; otherwise the core of the aggregates could become nutrient-deficient, resulting in differentiation or cell death.

7.2 Choosing a Passaging Method

Aggregates in suspension grow in diameter over the course of a passage and must be dissociated and re-seeded every 3 - 4 days. Dissociating aggregates to small clumps rather than single cells is recommended to minimize the probability of selecting for sub-populations of cells with abnormal karyotypes that can gain proliferative advantages in stressful single-cell environments. In section 7.3, a passaging method is described in which aggregates are dissociated to small clumps using Gentle Cell Dissociation Reagent and a 37 µm Reversible Strainer.

7.3 Passaging Aggregates as Small Clumps

Aggregates can be dissociated to small clumps (50 - 100 µm in diameter) using the following protocol, in which they are first treated with Gentle Cell Dissociation Reagent (GCDR), then resuspended in mTeSR™3D Seed Medium + 10 µM Y-27632, and finally pushed through a 37 µm Reversible Strainer to generate small clumps.

The described protocol is for the dissociation of aggregates from an initial culture volume of 20 mL and a day 4 volume of ~26.7 mL. Volumes of GCDR and mTeSR™3D Seed Medium + 10 µM Y-27632 can be scaled according to the culture volume (see Table 5).

Table 5. Recommended Volumes of GCDR and Resuspension Medium When Dissociating Aggregates

INITIAL CULTURE VOLUME (mL)	DAY 4 CULTURE VOLUME (mL)	VOLUME OF GCDR (mL)	VOLUME OF mTeSR™3D SEED MEDIUM + 10 µM Y-27632 FOR RESUSPENSION (mL)
2	2.7	1	1
20	26.7	5	5
50	66.8	15	15
100	133.6	30	30

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 5 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 into a 50 mL conical tube.

   Note: For suspension cultures in a 6-well plate, the small 37 µm Reversible Strainer can be used with a 15 mL conical tube. Collect aggregates on the smaller side of the strainer with the arrow pointing up.

   Note: If desired, cells in the filtrate can be quantified by centrifuging the collection tube, removing all but ~1 mL, and counting using a viability stain such as acridine orange (AO)/DAPI.

6. Flip the strainer onto a new 50 mL conical tube and rinse with 5 mL of warm GCDR, gently tapping the strainer to dislodge all aggregates into the new tube.
7. After the aggregates are rinsed off the strainer, flip the strainer onto a second 50 mL conical tube and set aside. This strainer can be used to dissociate aggregates into small clumps (step 13). Ensure that the strainer side that contacted the aggregates is facing up to prevent any non-dissociated aggregates from being re-seeded into the subsequent passage.
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 13).

   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 2 minutes at 100 x g to collect any aggregates that have not settled.

10. Slowly aspirate the GCDR using a serological pipette, 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.

    Note: Ensure there are no bubbles at the end of the pipette tip prior to pushing the aggregates through the strainer. Prevent bubbles by aspirating the aggregate suspension from the tube slowly. Remove bubbles by shifting the pipette to a diagonal angle and tapping the pipette lightly on a tube to force the bubbles upwards and away from the tip.

    Note: Work quickly to prevent the aggregates from settling to the bottom of the pipette before pushing through the strainer.

13. Using the strainer and new conical tube from step 7, place the pipette containing aggregates directly on top of the strainer in a vertical orientation so that the pipette is level, with no gaps between it and the strainer. With the slowest setting on the Pipette-Aid, pass the partially dissociated aggregates through the strainer (0.5 mL/second flow rate). This will generate clumps of appropriate size to initiate the subsequent passage. For larger culture volumes, a stepwise procedure is recommended (see Table 6).

    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.

Table 6. Scaled Protocols for Pushing GCDR-Treated Aggregates Through a 37 µm Reversible Strainer

INITIAL CULTURE VOLUME (mL)	SIZE OF 37 µm REVERSIBLE STRAINER	STEPWISE VOLUMES OF RESUSPENDED AGGREGATES FORCED THROUGH 37 µm REVERSIBLE STRAINER
2	Small (15 mL tube)	1 x 1 mL (5 mL serological pipette)
20	Large (50 mL tube)	1 x 5 mL (25 mL serological pipette)
50	Large (50 mL tube)	3 x 5 mL (25 mL serological pipette)
100	Large (50 mL tube)	3 x 10 mL (50 mL serological pipette)

14. *Optional*: Rinse the tube from step 12 with 5 mL of additional mTeSR™3D Seed Medium + 10 µM Y-27632. Pass through the strainer into the tube used in step 13 to collect any remaining aggregates from the conical tube after GCDR dissociation.

    Note: hPSC clumps may remain on the surface of the strainer after passing the partially dissociated aggregates through it. Minimize loss by adjusting the flow rate and volume of flow-through for individual cell lines. To increase yield, rinse the strainer with an additional 1 - 5 mL of mTeSR™3D Seed Medium + 10 µM Y-27632.

15. Gently resuspend the clump suspension. Remove a sample for counting viable cells or clumps (see Appendix 2: Counting Clumps).

16. Seed the clumps into a new culture vessel by adding the concentrated clump suspension to mTeSR™3D Seed Medium + 10 µM Y-27632 at room temperature (15 - 25°C) and topping up with additional medium to the desired final volume and cell density.

    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.

8.0 Troubleshooting

PROBLEM	SOLUTION
Aggregates do not form after 24 hours	• Increase seeding density • Decrease rpm • Increase initial clump size • Ensure Y-27632 is present in Seed Medium
Aggregates are still small on day 4	• Increase seeding density • Decrease rpm • Increase initial clump size • Increase length of early passages
Aggregates are merging into large conglomerates	• Ensure 2D maintenance culture is high quality • Decrease seeding density • Use less shear during passaging
Many single cells have not formed aggregates	• Ensure 2D maintenance culture is high quality • Monitor over more passages (may improve) • Decrease seeding density • On day 1 (t = 24 h), use 37 µm Reversible Strainer to remove single cells from culture
Aggregates are getting too big by end of passage	• Decrease seeding density • Decrease length of passage • Decrease clump size on day 0
Aggregates will not dissociate to small clumps at passage	• Decrease length of passage • Increase incubation time during dissociation • Increase shear force during dissociation
Aggregates will not dissociate to single cells at passage (if using single-cell passaging strategy)	• Decrease length of passage • Increase incubation time during dissociation • Increase shear force during dissociation • Use 37 µm Reversible Strainer to exclude undissociated aggregates from the culture
Too many single cells post-dissociation	• Ensure 2D maintenance culture is high quality • Decrease incubation time during dissociation • Decrease shear force during dissociation
Medium is very yellow by end of passage	• Decrease length of passage • Decrease seeding density
Cells are sticking to the vessel wall	• Ensure 2D maintenance culture is high quality • Decrease seeding density • Coat polycarbonate vessel with Anti-Adherence Rinsing Solution • Coat glass vessel with Sigmacote® • Decrease rpm
Aggregates look dense and dark	• Ensure maintenance culture is high quality • Decrease length of passage • Decrease seeding density

PROBLEM	SOLUTION
Aggregates are irregular in shape	• Ensure 2D maintenance culture is high quality • Start with more consistent clump size on day 0 • Optimize culture conditions such as rpm, initial culture volume, seeding density
Aggregates are non-uniform in size	• Start with more consistent clump size on day 0 • Optimize culture conditions such as rpm, initial culture volume, seeding density
Cells in aggregates lose expression of undifferentiated hPSC markers	• Ensure 2D maintenance culture is high quality • Decrease length of passage • 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 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

9.0 References

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10.0 Appendix 1: Preparing Suspension Culture Vessels

10.1.1 Coating Polycarbonate Vessels

If cells are sticking to the vessel walls of your polycarbonate culture vessel, it is recommended to coat the vessels with Anti-Adherence Rinsing Solution prior to inoculation. Anti-Adherence Rinsing Solution contains a hydrophobic surfactant that prevents cells from sticking to the vessel walls. Cell sticking can also be minimized by decreasing the shear in the system.

1. Obtain a new Corning® 125 mL Polycarbonate Erlenmeyer Flask or Qorpak® 300 mL Polycarbonate Round Bottle.
2. Add 20 mL Anti-Adherence Rinsing Solution and close the lid tightly.

   Note: If culturing > 20 mL, increase the volume of Anti-Adherence Rinsing Solution to equal the initial culture volume.
3. Swirl and rotate the bottle to rinse all surfaces, without allowing Anti-Adherence Rinsing Solution to go into the lid. Remove the Anti-Adherence Rinsing Solution.
4. Add an equal volume of room temperature (15 - 25°C) DMEM/F-12 with 15 mM HEPES. Close lid tightly, then swirl and rotate the bottle to rinse all surfaces without allowing DMEM/F-12 to go into the lid. Remove DMEM/F-12.
5. Close the lid tightly until ready to use.

10.1.2 Cleaning and Coating Glass Spinner Flasks

Clean reusable glass spinner flasks (e.g. PFEIFFER CELLSPIN models) with NaOH, then coat with Sigmacote® siliconizing reagent prior to adding cells, as described below. This will remove debris and prevent cells from sticking to the vessel walls.

Note: The full cleaning and coating procedure does not need to be repeated prior to each use. At a minimum, clean the glass with warm distilled water and a gentle brush between uses followed by autoclaving. Monitor the glass over the course of each experiment for cell and debris adherence. Repeat the full cleaning and coating procedure when the cell debris adherence is noticeable. Do not use detergents, or cleaning tools that have come in contact with detergents, on glass spinner flasks.

1. Ensure that the glass surface of the spinner flask is clean and free of debris.

   Note: Use warm distilled water and soft brush to remove debris if necessary.
2. Prepare a 0.1 M solution of NaOH in distilled water.
3. Add 0.1 M NaOH to the spinner flask and soak for 30 - 60 minutes at room temperature (15 - 25°C).

   Note: Volume of NaOH should be greater than or equal to the anticipated volume of suspension culture by the end of the culture passage.
4. Discard NaOH. Rinse thoroughly 3 times with an equal volume of distilled water.
5. Allow the glass to air dry until no moisture remains on surface.
6. Add 2 mL of Sigmacote® to the spinner flask. Using a serological pipette, rinse all surfaces that will contact cells, including the pendulum or impeller.

   Note: Work in a fume hood when using Sigmacote®. Excess Sigmacote® can be removed and reused. If disposing of excess Sigmacote®, do so in accordance with your institution's policies.
7. Leave the flask in a chemical fume hood overnight to allow the glass surface to dry completely.
8. Rinse with a large volume (to the level of the side-arms) of distilled water 3 times.
9. Autoclave the glass spinner flask in a standard dry autoclave cycle (121°C for 30 minutes).
10. Rinse the surface 3 times with DMEM/F-12 with 15 mM HEPES prior to use.

    Note: Rinse volume should be approximately 50% of the volume anticipated for cell culture.

11.0 Appendix 2: Counting Clumps

Accurate and precise quantification of clumps dissociated from 2D and 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 and is identical to the method used to count clumps for mTeSR™1 colony passaging. 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 ≥ 50 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 ≥ 50 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) by referring to Table 3 for the optimal seeding density and multiplying by 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 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.

12.0 Appendix 3: Differentiating Suspension Cultures into Three Germ Layers

hPSCs grown in suspension culture with mTeSR™3D have the ability to 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 an enzymatic reagent, then use STEMdiff™ Trilineage Differentiation Kit (Catalog #05230) to differentiate cells to the three germ layers.

13.0 Appendix 4: Re-Establishing 2D Colony Cultures

For suspension cultures that have been passaged as clumps:

1. Dissociate aggregates to small clumps following the protocol outlined in section 7.3.
2. Count dissociated clumps according to the protocol outlined in Appendix 2.
3. Plate 200 - 300 clumps/well onto a Corning® Matrigel®-coated 6-well plate in 2 mL of mTeSR™1 + 10 µM Y-27632. 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 Manual: Maintenance of Human Pluripotent Stem Cells in mTeSR™1, available at www.stemcell.com or contact us to request a copy.
4. After one or two 2D clump passages, typical mTeSR™1 colony morphology should be restored.

For suspension cultures that have been passaged as single cells:

1. Plate 4 x 10^5 cells/well onto a Corning® Matrigel®-coated 6-well plate in 2 mL of mTeSR™3D Seed Medium + 10 µM Y-27632.
2. When wells are ~80% confluent (3 - 4 days), clump passage cells using GCDR. Count dissociated clumps according to the protocol outlined in Appendix 2.
3. Plate 200 - 300 clumps/well onto a Corning® Matrigel®-coated 6-well plate in 2 mL of mTeSR™1 + 10 µM Y-27632. 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 Manual: Maintenance of Human Pluripotent Stem Cells in mTeSR™1, available at www.stemcell.com or contact us to request a copy.
4. After one or two 2D clump passages, typical mTeSR™1 colony morphology should be restored.

14.0 Appendix 5: 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.

15.0 Appendix 6: Dissociating Aggregates to Single Cells for Flow Cytometry

The following are instructions for preparing a single-cell suspension from aggregate cultures grown in mTeSR™3D in non-tissue culture-treated 6-well plates. If using other cultureware, adjust volumes accordingly. Cultures should be harvested at the time they would normally be ready for passaging.

1. Aliquot and warm an appropriate enzymatic cell dissociation reagent to 37°C.
2. Warm medium (DMEM/F-12 with 15 mM HEPES or mTeSR™3D Seed Medium) and D-PBS (Without Ca++ and Mg++) to room temperature (15 - 25°C) before use.
3. Filter out non-aggregated single cells by transferring the entire volume of culture through a small 37 µm Reversible Strainer into a 15 mL conical tube.

   Note: Collect aggregates on the smaller side of strainer with the arrow pointing up.
4. Flip the strainer onto a new 15 mL conical tube and rinse with 2 mL of D-PBS (Without Ca++ and Mg++), tapping strainer to release all aggregates.
5. Allow aggregates to settle for 3 - 5 minutes, then remove D-PBS.
6. Add 1 mL of warm enzymatic reagent. Incubate at 37°C for 7 minutes.

   Note: The incubation time may vary for different cell lines and enzymatic reagents.
7. Dissociate the aggregates by pipetting up and down to create a single-cell suspension.
8. Add 2 - 4 mL of medium (DMEM/F-12 or mTeSR™3D Seed Medium).
9. Centrifuge cells at 300 x *g* for 5 minutes.
10. Resuspend cells in an appropriate medium for desired downstream applications (e.g. 2% FBS/PBS for flow cytometry).

16.0 Appendix 7: Flow Cytometry Methods

16.1 Reagents and Materials

16.1.1 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.

16.1.2 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	07174
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
Optional: Nuclear stain (e.g. 1 mg/mL propidium iodide diluted 1 in 1000 in 2% FBS/PBS)	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	2%
D-PBS (Without Ca++ and Mg++)	37350	to final volume

*Mix well and store at 2 - 8°C.

16.2 Preparation of a Single-Cell Suspension for Flow Cytometry

Prepare a single-cell suspension as indicated in Appendix 6: Dissociating Aggregates to Single Cells for Flow Cytometry. 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 16.3 and 16.4 for detailed protocols).

16.3 Surface Antigen Labeling Protocol

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 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.

16.4 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 4 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.

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.

TECHNICAL MANUAL

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

STEMCELL™ TECHNOLOGIES

TOLL-FREE PHONE 1 800 667 0322
PHONE +1 604 877 0713
INFO@STEMCELL.COM
TECHSUPPORT@STEMCELL.COM
FOR GLOBAL CONTACT DETAILS VISIT WWW.STEMCELL.COM

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