TECHNICAL MANUAL

# Expansion of Human Pluripotent Stem Cells as Aggregates in Suspension Culture Using TeSR™-AOF 3D

STEMCELL™ TECHNOLOGIES

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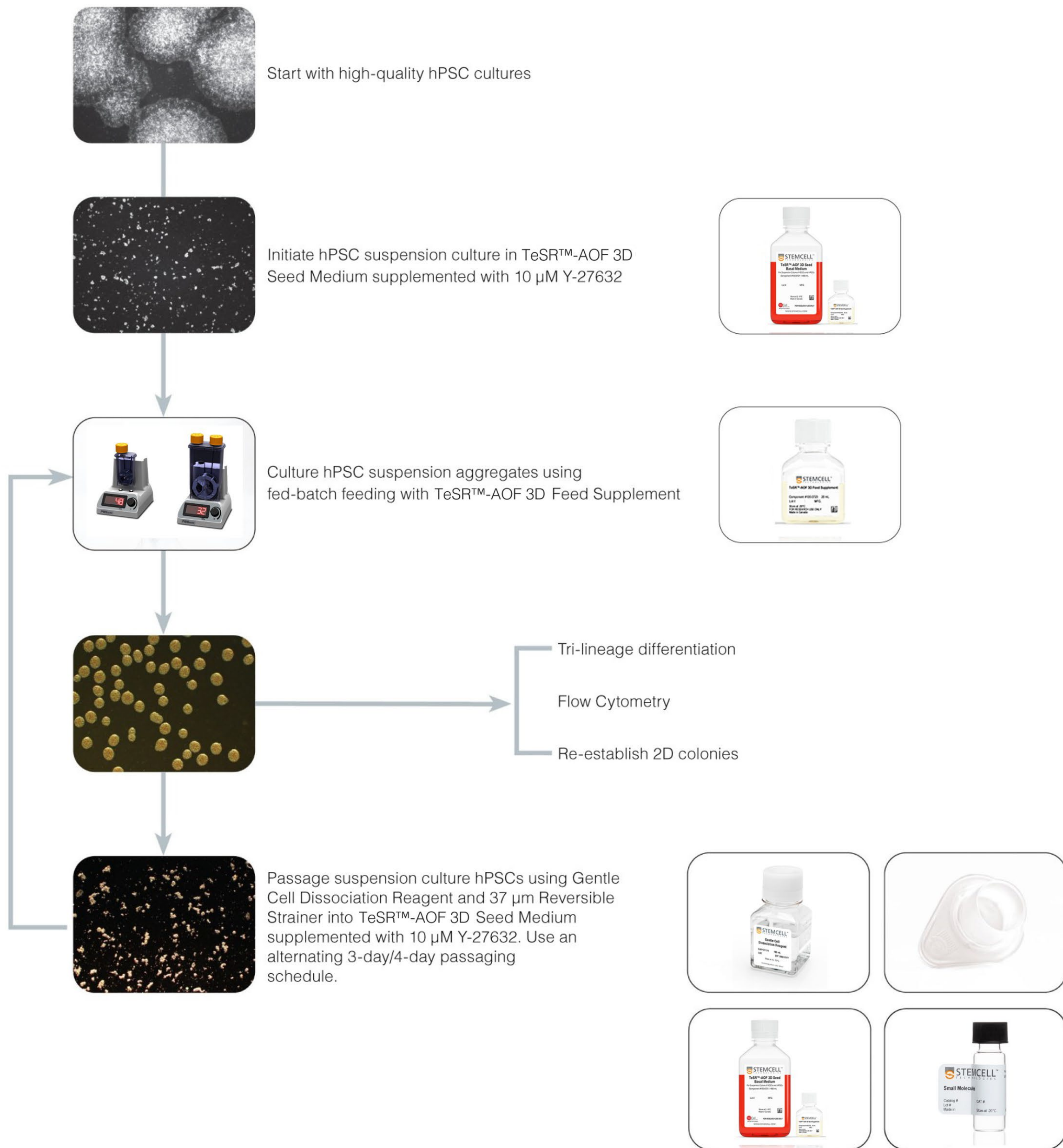
## 1.0 TeSR™-AOF 3D Workflow

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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 (embryonic stem [ES] cells and induced pluripotent stem [iPS] cells) 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, TeSR™-E8™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. TeSR™-AOF 3D is a low-protein, animal origin-free (AOF) formulation optimized for the robust expansion of undifferentiated hPSCs in a 3D suspension culture system. This medium is recommended when robust, AOF culture conditions are required for downstream applications.

TeSR™-AOF 3D uses a fed-batch strategy in which all the necessary nutrients and growth factors are added daily, with only a single medium change per week of culture. The TeSR™-AOF 3D kit consists of a Seed Medium (Seed Basal Medium + Seed Supplement) to initiate cultures and perform one medium change per week, and a Feed Supplement for daily 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. TeSR™-AOF 3D Seed Medium requires the 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. Matrigel®). By contrast, in the TeSR™-AOF 3D 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 daily medium replacements
- Manual selection and removal of differentiated cells is not required
- Can be easily automated using liquid handling robots and facilitates high-throughput experiments

Suspension hPSCs cultured in TeSR™-AOF 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, mTeSR™ Plus, TeSR™-E8™, or TeSR™-AOF cultures
- Have robust expansion across several hPSC lines
- Express equivalent levels of OCT4 and TRA-1-60 (assessed by flow cytometry) in comparison with cells grown in 2D mTeSR™1, mTeSR™ Plus, TeSR™-E8™, or TeSR™-AOF cultures
- Maintain more consistent glucose levels and lower lactate levels compared to 2D cultures
- Maintain differentiation capacity to the 3 germ layers, similar to 2D mTeSR™1, mTeSR™ Plus, TeSR™-E8™, or TeSR™-AOF cultures
- Have normal hPSC colony morphology when re-plated as 2D cultures

# 3.0 Materials, Reagents, and Equipment

## 3.1 TeSR™-AOF 3D (Catalog #100-0720)

For component storage and stability information, refer to the Product Information Sheet (PIS) for TeSR™-AOF 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 |
|---|---|---|
| 100-0721 | TeSR™-AOF 3D Seed Basal Medium | 480 mL |
| 100-0722 | TeSR™-AOF 3D Seed Supplement | 20 mL |
| 100-0723 | TeSR™-AOF 3D Feed Supplement | 20 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) |
| | Non-tissue culture-treated 6-well plates** | e.g. 27147 or 38040 |
| | Corning® Matrigel® hESC-Qualified Matrix | Corning 354277 |
| | Vitronectin XF™ | 07180 |
| | CellAdhere™ Dilution Buffer | 07183 |
| | CellAdhere™ Laminin-521 | 200-0117 |
| | mTeSR™1 | 85850 |
| | TeSR™-E8™ | 05990 |
| | mTeSR™ Plus | 100-0276 |
| | TeSR™-AOF | 100-0401 |
| | D-PBS (Without Ca++ and Mg++) | 37350 |
| | Gentle Cell Dissociation Reagent (GCDR) | 100-0485 |
| Materials Required for 3D Suspension Culture | Y-27632 (Dihydrochloride) | 72304 |
| | Gentle Cell Dissociation Reagent (GCDR) | 100-0485 |
| | 37 µm Reversible Strainer | 27250 (Large)<br>27215 (Small) |
| | Conical tubes | 38009 (15 mL)<br>OR<br>38010 (50 mL) |
| | Serological pipettes | 38003 (5 mL)<br>OR<br>38005 (25 mL)<br>OR<br>38006 (50 mL) |

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

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## 3.3 Vessels for hPSC Suspension Culture

*Note: The following vessels have been evaluated by STEMCELL Technologies. Additional culture vessels may be suitable for TeSR™-AOF 3D suspension culture.*

| CATEGORY | PRODUCT | CATALOG # |
|---|---|---|
| Suspension Culture Vessels | 6-Well Flat-Bottom Plate, Non-Treated | 38040 |
| | Nalgene™ Rapid-Flow™ Sterile Filter Storage Bottles | Thermo Fisher 455-0250 |
| | PBS-MINI MagDrive Bioreactor Base Unit | 100-1005 |
| | PBS-MINI 0.1 MAG Single-Use Vessel<br>OR<br>PBS-MINI 0.5 MAG Single-Use Vessel | 100-1006<br>OR<br>100-1007 |

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

## 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-MINI MagDrive Bioreactor, 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 TeSR™-AOF 3D Seed Medium

Use sterile technique to prepare TeSR™-AOF 3D Seed Medium (Seed Basal Medium + Seed Supplement + Y-27632). The following example is for preparing 500 mL of TeSR™-AOF 3D Seed Medium. If preparing other volumes, adjust accordingly.

1. Thaw TeSR™-AOF 3D Seed 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: Precipitate may be visible; warm to room temperature until dissolved. Performance will not be affected.*

   *Note: If not used immediately, store TeSR™-AOF 3D Seed Supplement in one of the following containers:*

   - *TeSR™-AOF 3D Seed Supplement bottle*
   - *50 mL polypropylene tubes (e.g. Catalog #38010)*
   - *2 mL or 0.5 mL polypropylene micro tubes (e.g. Sarstedt Catalog #72.694.006 or 72.730.005)*

   *Do not use other storage containers. Store TeSR™-AOF 3D Seed Supplement at -20°C for up to 3 months. Do not exceed the shelf life of the supplement. After thawing the aliquots, use immediately. Do not re-freeze.*

2. Add 20 mL of TeSR™-AOF 3D Seed Supplement to 480 mL of TeSR™-AOF 3D Seed Basal Medium. Mix thoroughly. If desired, TeSR™-AOF 3D Seed Medium can be filtered using a 0.2 µm low-protein binding filter.

   *Note: If not used immediately, store TeSR™-AOF 3D Seed Medium (without Y-27632) in one of the following containers:*

   - *TeSR™-AOF 3D Seed Basal Medium bottle*
   - *50 mL polypropylene tubes (e.g. Catalog #38010)*

   *Do not use other storage containers. Store TeSR™-AOF 3D Seed Medium (without Y-27632) 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 aliquots, proceed to step 3 and use immediately, or store at 2 - 8°C for up to 2 weeks. Do not re-freeze.*

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

## 4.2 TeSR™-AOF 3D Feed Supplement

Thaw TeSR™-AOF 3D Feed 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: Precipitate may be visible, and/or supplement may appear cloudy. Warm the supplement to room temperature until all precipitate has dissolved and the solution no longer appears cloudy. Performance will not be affected.*

*Note: If not used immediately, store TeSR™-AOF 3D Feed Supplement in one of the following containers:*

- *TeSR™-AOF 3D Feed Supplement bottle*
- *50 mL polypropylene tubes (e.g. Catalog #38010)*
- *2 mL or 0.5 mL polypropylene micro tubes (e.g. Sarstedt Catalog #72.694.006 or 72.730.005)*

*Do not use other storage containers. Store TeSR™-AOF 3D Feed Supplement 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 supplement. After thawing the aliquots, use immediately or store at 2 - 8°C for up to 2 weeks. Do not re-freeze.*

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## 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 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 a larger vessel such as the PBS-MINI MagDrive Bioreactor Single-Use Vessels. However, it is highly recommended to initiate 3D suspension cultures in non-tissue culture-treated 6-well plates before moving to a larger vessel. Refer to Table 1 for culture vessels evaluated by STEMCELL Technologies along with the recommended volumes and orbital shaker/impeller speeds.

**Table 1. Suspension Culture Vessels Evaluated by STEMCELL Technologies**

| CULTURE VESSEL | CATALOG # | RECOMMENDED INITIAL CULTURE VOLUME | RECOMMENDED 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<br>30 mL<br>60 mL | 40 RPM<br>55 RPM<br>60 RPM (2.5 cm orbital diameter) |
| PBS-MINI 0.1 MAG Single-Use Vessel | 100-1006 | 100 mL | 35 - 40 RPM |
| PBS-MINI 0.5 MAG Single-Use Vessel | 100-1007 | 500 mL | 35 - 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 is observed, increase the RPM.*

*Note: Agitation rates for the Nalgene™ Rapid-Flow™ Sterile Filter Storage Bottles are compatible with volumes ± 4 mL from the recommended volume. For example, 40 RPM is most suitable for 15 mL, but will also work for volumes ranging from 11 - 19 mL.*

*Note: The PBS-MINI 0.1 has a working range of 80 - 100 mL, and the 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 the PBS-MINI 0.1 and 0.5, respectively.*

*Note: As the 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 TeSR™-AOF 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 with Gentle Cell Dissociation Reagent 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 Manuals for mTeSR™1, mTeSR™ Plus, TeSR™-E8™, or TeSR™-AOF, available at www.stemcell.com, or contact us to request a copy.

High-quality hPSC cultures should express high levels (> 95%) of markers of the undifferentiated state, including OCT4, TRA-1-60, and SSEA-3, as assessed by flow cytometry. Cultures should also display normal colony morphology and a low percentage of differentiated cells, have 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 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

In the TeSR™-AOF 3D culture system, we recommend a non-enzymatic clump passaging protocol using Gentle Cell Dissociation Reagent (GCDR), which consistently yields undifferentiated aggregates in TeSR™-AOF 3D. Refer to section 5.2.1 for initiating a 3D suspension culture from 2D, and section 7.2 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 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, 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 TeSR™-AOF 3D Seed Medium and warm to room temperature (15 - 25°C).

   *Note: Do not warm TeSR™-AOF 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.

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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 TeSR™-AOF 3D Seed Medium. Gently detach the colonies by scraping with a serological glass pipette or a cell scraper.

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

   *Optional: Rinse the well with an additional 1 mL of TeSR™-AOF 3D Seed Medium 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 1: Counting Clumps). For recommended seeding density, see 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 TeSR™-AOF 3D Seed Medium 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. Refer to Table 2 for the seeding density recommended for each culture vessel, which should be optimal for most ES and iPS cell lines.

**Table 2. Recommended Seeding Densities**

| CULTURE VESSEL | CATALOG # | RECOMMENDED SEEDING DENSITY (VIABLE CELLS/mL) |
|---|---|---|
| 6-Well Flat-Bottom Plate, Non-Treated | 38040 | $1 \times 10^5$ |
| Nalgene™ Rapid-Flow™ Sterile Filter Storage Bottles | Thermo Fisher 455-0250 | $1 \times 10^5$ |
| PBS-MINI 0.1 MAG Single-Use Vessel | 100-1006 | $4 - 8 \times 10^4$ |
| PBS-MINI 0.5 MAG Single-Use Vessel | 100-1007 | $4 - 8 \times 10^4$ |

Some cell lines may undergo an adaptation phase during which they expand slowly in the first 1 - 2 passages in suspension culture. If this occurs, 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 TeSR™-AOF 3D

## 6.1 TeSR™-AOF 3D Feeding and Passaging Schedule

After initiating hPSC suspension culture in TeSR™-AOF 3D, passage the suspension culture on an alternating 3-day/4-day schedule as outlined in Table 3. 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 and 6.1.2 for details on addition of Feed Supplement and the half-medium change with TeSR™-AOF 3D Seed Medium, respectively. For the passaging protocol, refer to section 7.2.

**Table 3. Recommended Feeding and Passaging Schedules**

| DAY | STARTING WITH A 3-DAY PASSAGE | STARTING WITH A 4-DAY PASSAGE |
|---|---|---|
| 0 | **Initiate Culture** using TeSR™-AOF 3D Seed Medium + 10 µM Y-27632 | **Initiate Culture** using TeSR™-AOF 3D Seed Medium + 10 µM Y-27632 |
| 1 | Add TeSR™-AOF 3D Feed Supplement | Add TeSR™-AOF 3D Feed Supplement |
| 2 | Add TeSR™-AOF 3D Feed Supplement | Add TeSR™-AOF 3D Feed Supplement |
| 3 | **Passage and re-seed** into TeSR™-AOF 3D Seed Medium + 10 µM Y-27632 | **Half-medium change** with TeSR™-AOF 3D Seed Medium (without Y-27632) |
| 4 | Add TeSR™-AOF 3D Feed Supplement | **Passage and re-seed** into TeSR™-AOF 3D Seed Medium + 10 µM Y-27632 |
| 5 | Add TeSR™-AOF 3D Feed Supplement | Add TeSR™-AOF 3D Feed Supplement |
| 6 | **Half-medium change** with TeSR™-AOF 3D Seed Medium (without Y-27632) | Add TeSR™-AOF 3D Feed Supplement |
| 7 | **Passage** | **Passage** |

### 6.1.1 Addition of TeSR™-AOF 3D Feed Supplement

Add TeSR™-AOF 3D Feed Supplement to the hPSC suspension culture as described below, according to a recommended schedule in Table 3.

1. Thaw TeSR™-AOF 3D Feed Supplement (section 4.2).
2. Mix TeSR™-AOF 3D Feed Supplement by pipetting up and down.
3. Add TeSR™-AOF 3D Feed Supplement at 30 µL/mL of initial culture volume. Refer to Table 4 for the recommended volume of Feed Supplement to add to various culture vessels. Add the appropriate volume of Feed Supplement to the center of the culture. Do not remove any medium. Change pipette tips between cultures to prevent cross-contamination.

*Note: When feeding the PBS-MINI MagDrive Bioreactors, it is optional to remove a volume of spent medium equivalent to the volume of Feed Supplement to be added prior to feeding. This keeps the working volume constant and ensures that the maximum working volume is not exceeded. Alternatively, it is optional to seed the bioreactor at a lower volume such that the final volume is 100 mL or 500 mL.*

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**Table 4. Daily Volume of TeSR™-AOF 3D Feed Supplement for Various Culture Vessels**

| CULTURE VESSEL | INITIAL CULTURE VOLUME (mL) | DAILY VOLUME OF TeSR™-AOF 3D FEED SUPPLEMENT (mL)* |
|---|---|---|
| 6-Well Flat-Bottom Plate, Non-Treated | 2 | 0.06 |
| Nalgene™ Rapid-Flow™ Sterile Filter Storage Bottles | 15 - 60 | 0.45 - 1.8 |
| PBS-MINI 0.1 MAG Single-Use Vessel | 100 | 3 |
| PBS-MINI 0.5 MAG Single-Use Vessel | 500 | 15 |

* 30 μL of Feed Supplement per mL of initial culture volume.

### 6.1.2 Half-Medium Change with TeSR™-AOF 3D Seed Medium

On 4-day passages, perform a half-medium change on the day before passaging (i.e. third day of the 4-day passage). Use TeSR™-AOF 3D Seed Medium (without Y-27632). Do not add TeSR™-AOF 3D Feed Supplement on this day.

1. Prepare and aliquot a sufficient volume of TeSR™-AOF 3D Seed Medium (without Y-27632) and warm to room temperature (15 - 25°C).

   *Note: Do not warm TeSR™-AOF 3D Seed Medium in a 37°C water bath.*

2. Remove the culture vessel from the agitation platform and transfer to a sterile environment. Allow aggregates to settle in the culture vessel, then remove an appropriate volume of culture medium. For recommended medium change volumes, see Table 5.

**Table 5. Half-Medium Change Volumes for Various Culture Vessels**

| CULTURE VESSEL | INITIAL CULTURE VOLUME (mL) | VOLUME OF CULTURE MEDIUM TO REMOVE/ADD (mL) |
|---|---|---|
| 6-Well Flat-Bottom Plate, Non-Treated | 2 | 1 |
| Nalgene™ Rapid-Flow™ Sterile Filter Storage Bottles | 15 - 60 | 7.5 - 30 |
| PBS-MINI 0.1 MAG Single-Use Vessel | 100 | 50 |
| PBS-MINI 0.5 MAG Single-Use Vessel | 500 | 250 |

3. Add a volume of fresh TeSR™-AOF 3D Seed Medium (without Y-27632) equal to the volume of medium removed in step 2.

*Note: If the aggregates are settling slowly, the volume of culture medium removed can be passed over a 37 μm Reversible Strainer to capture the unsettled aggregates. The aggregates can then be washed back into the culture vessel when the fresh TeSR™-AOF 3D Seed Medium is added.*

## 6.2 hPSC Aggregate Morphology

When culturing hPSCs as aggregates in TeSR™-AOF 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, 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 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.

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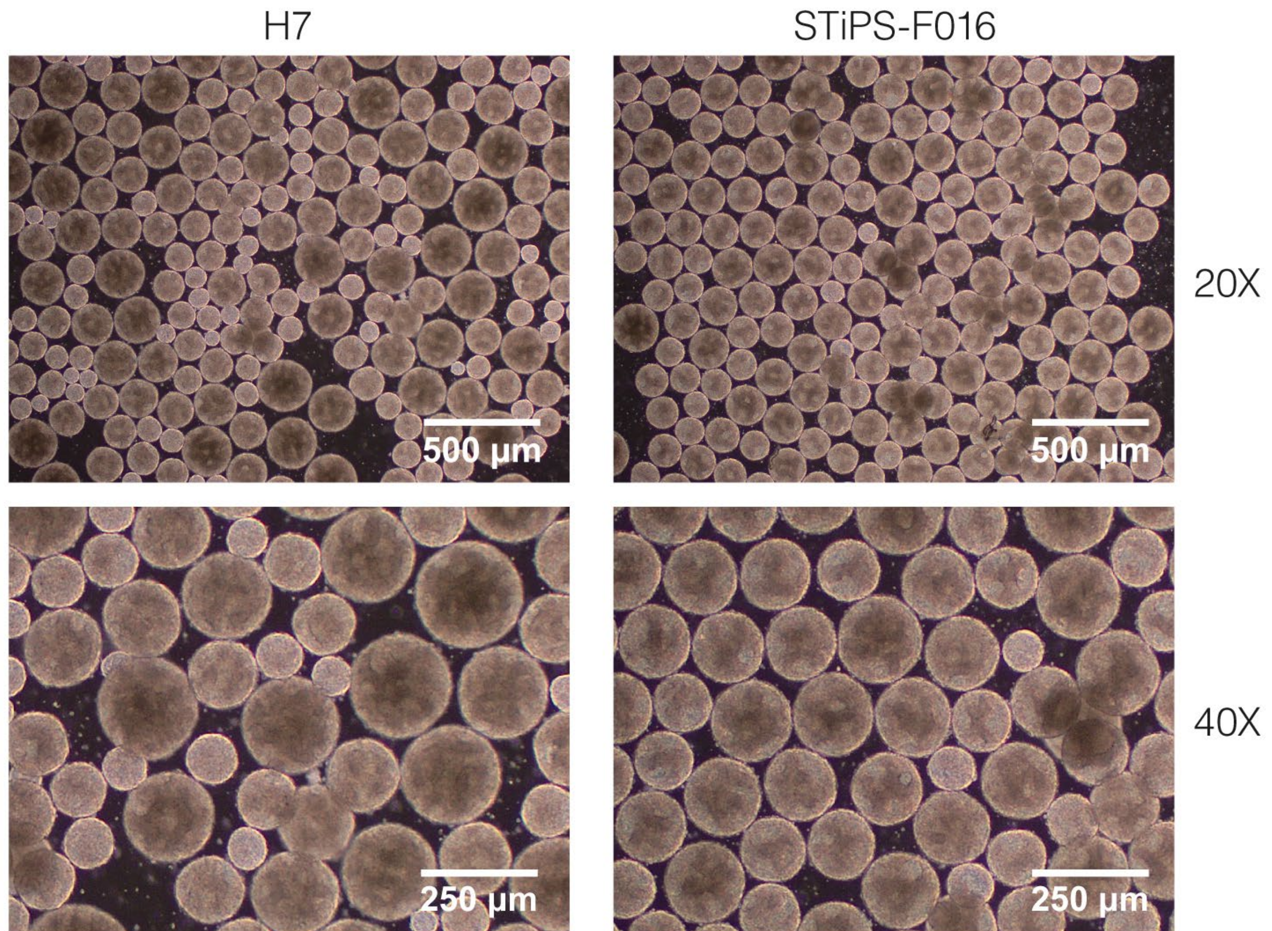


**Figure 1. Representative hPSC Aggregate Morphology of H7 ES Cell Line and STiPS-F016 Cell Line Prior to Passage in TeSR™-AOF 3D**

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

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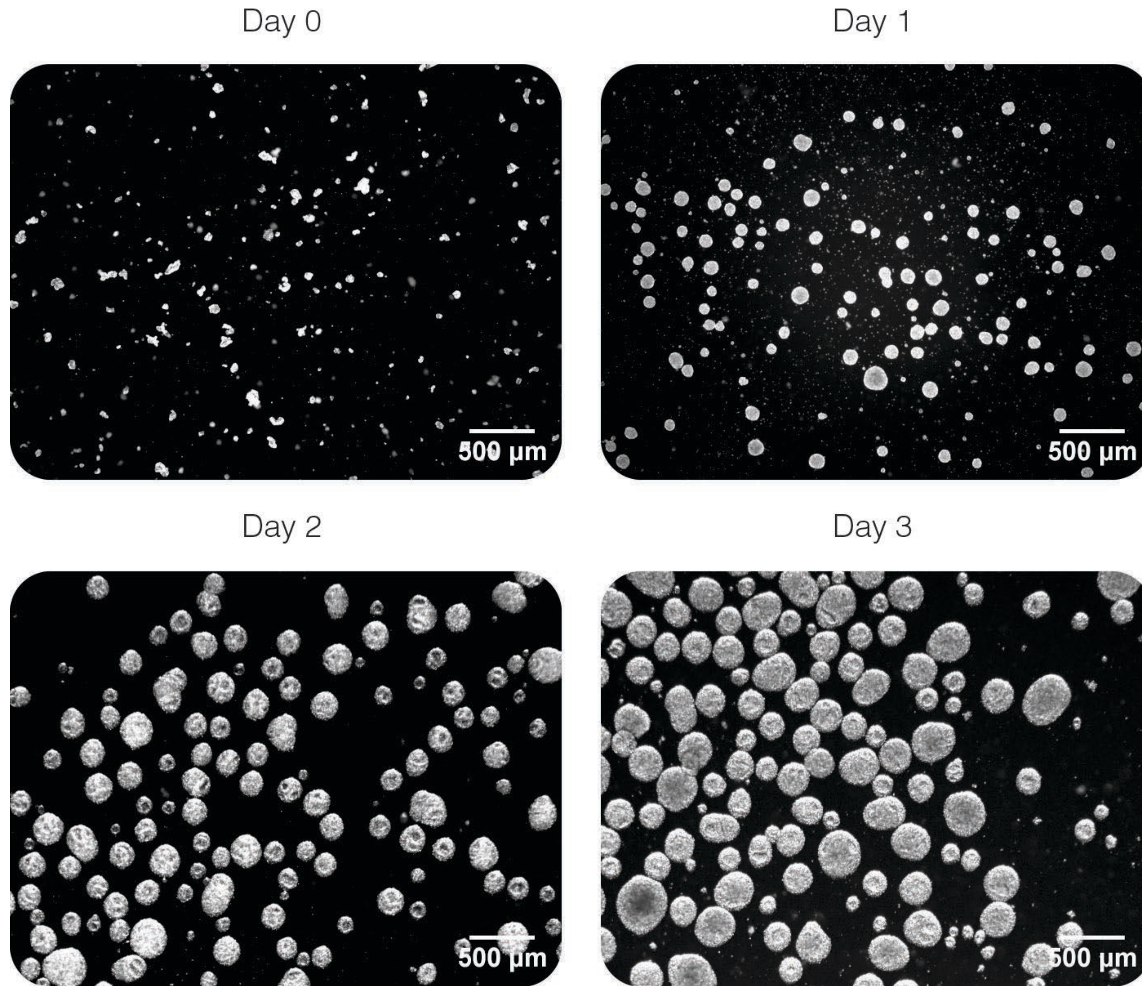


**Figure 2. H9 Aggregates on Days 0, 1, 2, and 3 in Suspension Culture**

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

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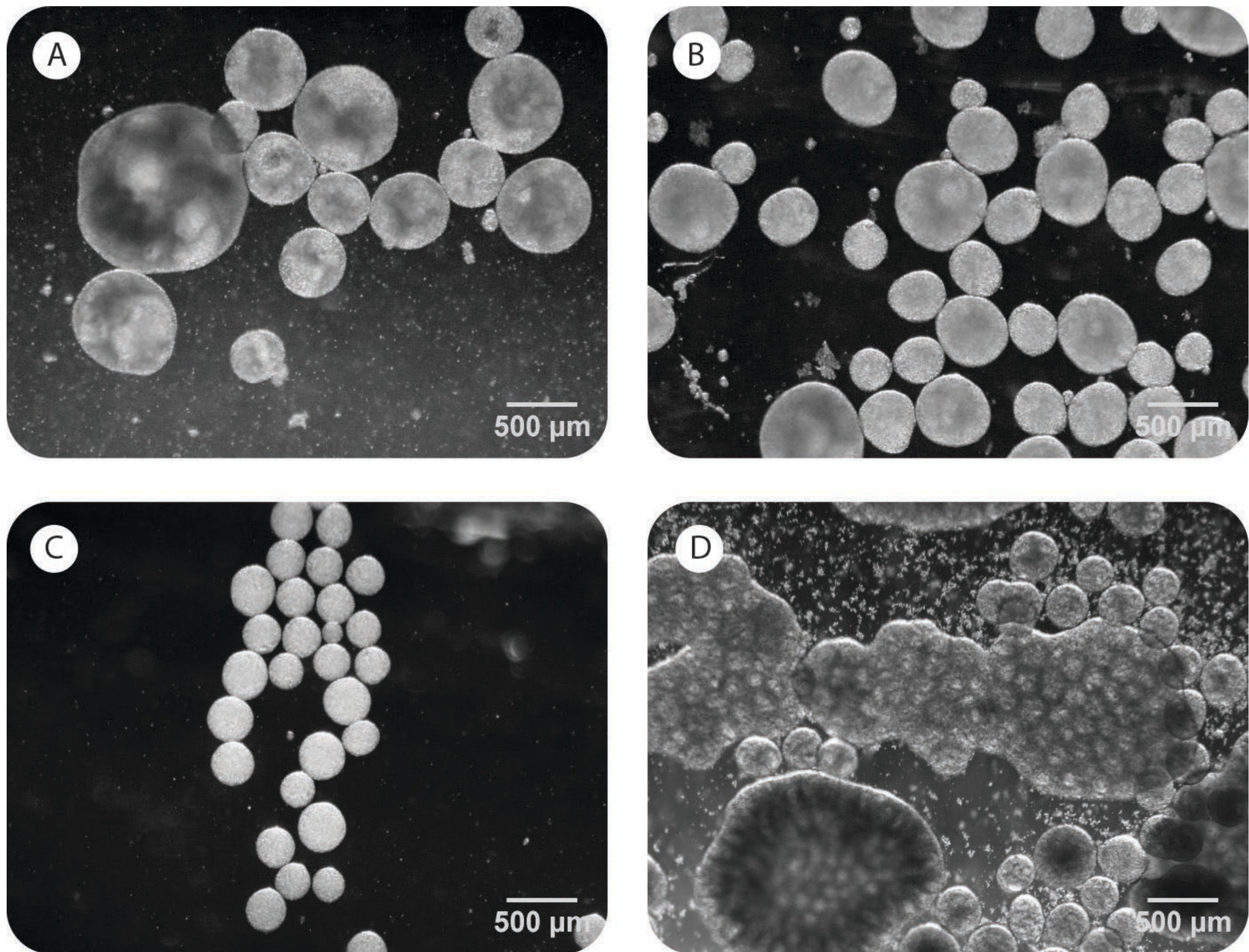


**Figure 3. Examples of hPSC Aggregates with Poor Morphology**

**(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 TeSR™-AOF 3D

## 7.1 Passaging Schedule

To prevent aggregates from getting excessively large (> 400 μm), we recommend an alternating 3-day/4-day passaging schedule (see section 6.1). 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 Passaging Aggregates as Small Clumps

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; 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 TeSR™-AOF 3D Seed Medium. The protocol is for the dissociation of aggregates from an initial culture volume of 15 mL and a day 3 or 4 volume of ~15.9 mL. For other culture volumes, refer to Table 6 for volumes of GCDR and TeSR™-AOF 3D Seed Medium.

**Table 6. Recommended Volumes of GCDR and TeSR™-AOF 3D Seed Medium When Dissociating Aggregates**

| INITIAL CULTURE VOLUME (mL) | DAY 4 CULTURE VOLUME (mL) | VOLUME OF GCDR (mL) | VOLUME OF TeSR™-AOF 3D SEED MEDIUM FOR RESUSPENSION (mL) |
|---|---|---|---|
| 2 | 2.12 | 1 | 1 |
| 15 | 15.9 | 5 | 5 |
| 60 | 63.6 | 15 | 20 |
| 100 | 106 | 20 | 25 |
| 500 | 530 | 30 | 40 - 50 |

1. Aliquot and warm 5 mL of GCDR to 37°C.
2. Bring TeSR™-AOF 3D Seed Medium to room temperature (15 - 25°C).
3. Prepare enough TeSR™-AOF 3D Seed Medium to resuspend and seed all conditions. See Table 6 for recommended resuspension volume.
4. Image the 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 or media 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. 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 (e.g. acridine orange (AO)/DAPI).*

   *Note: When passaging the 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 of the aggregates into the same conical tube in Step 6.*

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   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 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 another new 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. Using a serological pipette, slowly aspirate the GCDR, leaving ~0.5 mL to avoid removing any aggregates.
11. Add 5 mL of TeSR™-AOF 3D Seed Medium 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 TeSR™-AOF 3D Seed Medium 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.*

    *Note: Alternatively, a 5 mL serological with the tip broken off can be used for this step to increase the force-through surface area, preventing clogging.*
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, force 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 7).

    *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.*

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**Table 7. Stepwise Volumes of Resuspended Aggregates for Forcing Through a Strainer**

| INITIAL CULTURE VOLUME (mL) | SIZE OF 37 µm REVERSIBLE STRAINER | STEPWISE VOLUMES OF RESUSPENDED AGGREGATES |
|---|---|---|
| 2 | Small (15 mL tube) | 1 x 1 mL (5 mL serological pipette) |
| 15 | Large (50 mL tube) | 1 x 5 mL (25 mL serological pipette) |
| 60 | Large (50 mL tube) | 4 x 5 mL (25 mL serological pipette) |
| 100 | Large (50 mL tube) | 5 x 5 mL (25 mL serological pipette) |
| 500 | Large (50 mL tube) | 4 - 5 x 10 mL (25 mL serological pipette) |

14. *Optional:* Rinse the tube from step 12 with 5 mL of additional TeSR™-AOF 3D Seed Medium. 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 TeSR™-AOF 3D Seed Medium.*
15. Gently flick the tube to resuspend clumps. Remove a sample for counting viable cells or clumps (see Appendix 1: Counting Clumps).
16. Add the concentrated clump suspension to TeSR™-AOF 3D Seed Medium at room temperature in a new culture vessel. Top 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.*

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# 8.0 Troubleshooting

| PROBLEM | SOLUTION |
|---|---|
| Aggregates do not form after 24 hours | • Decrease RPM or increase culture volume<br>• Increase seeding density<br>• Increase initial clump size<br>• Ensure Y-27632 is present in TeSR™-AOF 3D Seed Medium |
| Aggregates are still small on day 4 | • Decrease RPM or increase culture volume<br>• Increase seeding density<br>• Increase initial clump size<br>• Increase length of early passages |
| Aggregates are merging into large conglomerates | • Increase RPM or decrease culture volume<br>• Ensure 2D maintenance culture is of high quality<br>• Decrease seeding density<br>• Use less shear force during passaging |
| Many single cells have not formed aggregates | • Ensure 2D maintenance culture is of high quality<br>• Monitor during additional passages (may improve)<br>• Decrease seeding density<br>• On day 1, use 37 µm Reversible Strainer to remove single cells from culture |
| Cells are not adapting to suspension culture | • Transition cells from 2D maintenance into a small-scale suspension culture in a 6-well plate<br>• Adjust RPM or culture volume to optimize mixing<br>• Some cell lines may require 1 - 2 passages to adapt to the suspension culture conditions |
| Aggregates are too large by end of passage | • Increase RPM or decrease culture volume<br>• Decrease seeding density<br>• Decrease clump size on day 0 |
| Aggregates will not dissociate to small clumps at passage | • Increase incubation time during dissociation<br>• Increase shear force during dissociation |
| Too many single cells post-dissociation | • Ensure 2D maintenance culture is of high quality<br>• Decrease incubation time during dissociation<br>• Decrease shear force during dissociation<br>• Ensure the half-medium change is performed on day 3 of a 4-day passage |
| Medium is very yellow by end of passage | • Decrease seeding density<br>• Ensure the half-medium change is performed on day 3 of a 4-day passage |
| Aggregates appear dense and dark | • Increase RPM or decrease culture volume<br>• Decrease seeding density<br>• Ensure maintenance culture is of high quality |
| Aggregates are irregular in shape | • Optimize culture conditions (e.g. RPM, initial culture volume, and seeding density)<br>• Ensure 2D maintenance culture is of high quality<br>• Start with more consistent clump size on day 0 |

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| PROBLEM | SOLUTION |
| --- | --- |
| Aggregates are non-uniform in size | • Optimize culture conditions (e.g. RPM, initial culture volume, and seeding density)<br>• Start with more consistent clump size on day 0 |
| Cells in aggregates lose expression of undifferentiated hPSC markers | • Ensure 2D maintenance culture is of high quality<br>• Optimize culture conditions (e.g. RPM and culture volume)<br>• Decrease seeding density<br>• 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<br>• Passage as clumps rather than as single cells<br>• Optimize passaging to maximize percentage of cells remaining in clumps |
| Cells from aggregates differentiate at lower efficiency than 2D mTeSR™1, mTeSR™ Plus, TeSR™-E8™, or TeSR™-AOF-derived cells | • See 'Cells in aggregates lose expression of undifferentiated hPSC markers'<br>• 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 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. 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 ($\mathbf{N_A}$) in the 5 µL sample.
5. Calculate the concentration of clumps (**C**) and the total number of clumps ($\mathbf{N_T}$) using the total volume of the clump suspension in µL ($\mathbf{V_T}$):

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

$$N_T = C \times V_T$$

6. Determine the target number of clumps to seed ($\mathbf{N_p}$) by multiplying the optimal seeding density (e.g. $1 \times 10^3$ clumps/mL) by the culture volume.
7. Calculate the volume (in µL) of clump suspension to seed ($\mathbf{V_p}$) 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 ($\mathbf{V_P}$) to the culture vessel and add TeSR™-E8™3D Seed Medium 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.

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## 11.0 Appendix 2: Differentiating Suspension Cultures into Three Germ Layers

hPSCs grown in suspension culture with TeSR™-AOF 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 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.

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.

## 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 7.2).
2. Count dissociated clumps (Appendix 1).
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 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 2D colony morphology should be restored.

## 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 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 karyotypes 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.

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# 14.0 Appendix 5: Dissociating Aggregates to Single Cells for Flow Cytometry

The following are instructions for preparing a single-cell suspension from aggregate cultures grown in TeSR™-AOF 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 TeSR™-AOF 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 with 15 mM HEPES or TeSR™-AOF 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).

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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: 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 15.3 and 15.4 for detailed protocols).

## 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.

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4. While the samples are centrifuging, prepare a sufficient quantity of the primary antibody mix or the directly conjugated antibody mix (100 µL/sample) using the appropriate antibody at the predetermined optimal working dilution.
5. Carefully remove the supernatant without disrupting the cell pellet and resuspend cells in the primary antibody mix. Gently mix and incubate on ice for 15 - 60 minutes.

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

## 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 analysis by flow cytometry.*
7. While the samples are incubating, prepare a sufficient quantity of the primary antibody mix (100 µL/sample) at the predetermined optimal working dilution, using SPB as the diluent.
8. Centrifuge cells at 300 x *g* for 5 minutes.
9. Carefully remove the supernatant without disrupting the cell pellet and resuspend cells in the primary antibody mix (100 µL/sample). Gently mix and incubate on ice for 15 - 60 minutes. If using a directly conjugated antibody, protect samples from exposure to direct light.
10. Add 1 mL of SPB to each tube, gently mix and centrifuge at 300 x *g* for 5 minutes.
    - If using an unconjugated primary antibody: While the samples are centrifuging, prepare a sufficient quantity of the secondary antibody mix (100 µL/sample) using the appropriate secondary antibody at the predetermined optimal working dilution. Proceed to step 11.
    - If using a directly conjugated antibody, proceed to step 13.

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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 TeSR™-AOF 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 to 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 7.2, and seed suspension cultures at 1 - 3 x $10^5$ viable cells/mL in TeSR™-AOF 3D. After 24 - 48 hours, complete a full-medium change to the differentiation medium.

TeSR™-AOF 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 TeSR™-AOF 3D

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