

# StemScale™ PSC Suspension Medium

Catalog Number A4965001

Pub. No. MAN0019457 Rev. B.0



**WARNING!** Read the Safety Data Sheets (SDSs) and follow the handling instructions. Wear appropriate protective eyewear, clothing, and gloves. Safety Data Sheets (SDSs) are available from [thermofisher.com/support](https://www.thermofisher.com/support).

## Product description

Gibco™ StemScale™ PSC Suspension Medium (Cat. No. [A4965001](#)) is a scalable, easy-to-use medium that supports robust expansion of human pluripotent stem cells (hPSCs) in suspension. The medium uses a spheroid self-assembly method to promote PSC growth without the use of microcarriers. Cells are fed by 50% medium replacement and passaged using a simple enzymatic dissociation method. Pluripotent stem cells cultured in StemScale™ PSC Suspension Medium achieve greater than 4-fold expansion across multiple PSC lines and culture vessel formats.

Key features of the medium:

- Enhanced expansion capability—promotes greater than 4-fold expansion of hPSCs.
- Maintains pluripotency across cell lines—supports high expression of pluripotency markers for cells grown as spheroids.
- Flexible workflow—allows the option to skip feeding days and selectively determine when to passage suspension cultures based on the recommended 4- to 5-day passage schedule.
- Efficient media exchange method—eliminates the need for cell strainers.
- Scalable system—growth is supported across multiple culture vessel formats of different sizes.

Pluripotent stem cells cultured in the StemScale™ PSC Suspension Medium formulation were karyotyped using the Applied Biosystems™ KaryoStat™ Assay Kit and tested for pluripotency using OCT4 expression. Data show that the formulation maintains normal karyotype and pluripotency over multiple passages. Additionally, the formulation maintains trilineage differentiation potential of PSCs, as measured by the TaqMan™ hPSC Scorecard™ Panel.

## Procedure overview

In this procedure, adherent human PSCs are dissociated from the culture vessel, then seeded into StemScale™ PSC Suspension Medium plus ROCK inhibitor. The suspension hPSC cultures are fed by 50% medium exchange on day 1 and 3 after seeding, then passaged on day 4, using StemPro™ Accutase™ Cell Dissociation Reagent. If needed, the protocol can also be adapted to a 5-day passage schedule (see “Guidelines for suspension hPSC cultures” on page 3). After dissociation, the cells are resuspended in StemScale™ PSC Suspension Medium to resume hPSC expansion. At the third passage, hPSC spheroids can be harvested for use in downstream studies, or reseeded into suspension cultures for additional passages.

For more information on culturing PSCs in suspension using StemScale™ PSC Suspension Medium, go to [www.thermofisher.com/stemscale-elearn](https://www.thermofisher.com/stemscale-elearn).

## Contents and storage

Table 1 StemScale™ PSC Suspension Medium (Cat. No. [A4965001](#))

| Product                     | Cat. No. <sup>[1]</sup>  | Amount | Storage                                             | Shelf life <sup>[2]</sup> |
|-----------------------------|--------------------------|--------|-----------------------------------------------------|---------------------------|
| StemScale™ Basal Medium     | <a href="#">A4863001</a> | 900 mL | 2°C to 8°C<br>Protect from light.                   | 12 months                 |
| StemScale™ Supplement (10X) | <a href="#">A4876001</a> | 100 mL | –20°C to –5°C <sup>[3]</sup><br>Protect from light. |                           |

<sup>[1]</sup> StemScale™ PSC Suspension Medium is sold as a complete kit; individual components are not sold separately.

<sup>[2]</sup> Shelf-life duration is determined from Date of Manufacture.

<sup>[3]</sup> Store in a non-frost-free freezer.

## Culture conditions

**Media:** StemScale™ PSC Suspension Medium

**Cells:** Human pluripotent or embryonic stem cell (ESC) line

**Culture type:** Suspension

**Non-tissue culture-treated vessels:**

- 6-well plate
- 125-, 250-, or 500-mL shake flask

**Temperature range:** 36°C to 38°C

**Incubator atmosphere:**

- Humidified atmosphere of 5% CO<sub>2</sub>. Ensure that proper gas exchange is achieved in culture vessels.
- Place culture vessels on an orbital shaker platform with continuous rotation.

## Required materials not supplied

Unless otherwise indicated, all materials are available through [thermofisher.com](https://www.thermofisher.com). "MLS" indicates that the material is available from [fisherscientific.com](https://www.fisherscientific.com) or another major laboratory supplier.

Catalog numbers that appear as links open the web pages for those products.

**Table 2** Materials required for suspension hPSC cultures

| Item                                                                                                                                    | Source                                                                                                         |
|-----------------------------------------------------------------------------------------------------------------------------------------|----------------------------------------------------------------------------------------------------------------|
| <b>Equipment</b>                                                                                                                        |                                                                                                                |
| Countess™ II Automated Cell Counter                                                                                                     | AMQAX1000                                                                                                      |
| 37°C, 5% CO <sub>2</sub> incubator                                                                                                      | <a href="#">MLS</a>                                                                                            |
| Thermo Scientific™ CO <sub>2</sub> Resistant Shakers, or equivalent CO <sub>2</sub> -resistant orbital shaker with 19 mm orbit diameter | <a href="#">88881101</a>                                                                                       |
| EVOS™ XL Core Imaging System                                                                                                            | <a href="#">AMEX1000</a>                                                                                       |
| Centrifuge                                                                                                                              | <a href="#">MLS</a>                                                                                            |
| <b>Tubes, plates, and other consumables</b>                                                                                             |                                                                                                                |
| Serological pipettes                                                                                                                    | <a href="#">MLS</a>                                                                                            |
| Countess™ Cell Counting Chamber Slides                                                                                                  | <a href="#">C10228</a>                                                                                         |
| 15-mL conical tubes                                                                                                                     | <a href="#">MLS</a>                                                                                            |
| 50-mL conical tubes                                                                                                                     | <a href="#">MLS</a>                                                                                            |
| <b>Non-tissue culture-treated vessels, one of the following, or equivalent:</b>                                                         |                                                                                                                |
| Nunc™ Non-Treated Multidishes                                                                                                           | <a href="#">150239</a> (6-well)                                                                                |
| Nalgene™ Single-Use PETG Erlenmeyer Flasks with Plain Bottom: Sterile                                                                   | <a href="#">4115-0125</a> (125-mL)<br><a href="#">4115-0250</a> (250-mL)<br><a href="#">4115-0500</a> (500-mL) |
| <b>Reagents</b>                                                                                                                         |                                                                                                                |
| Gibco™ Human Episomal iPSC Line, or other human iPSCs or ESCs                                                                           | <a href="#">A18945</a>                                                                                         |
| Antibiotic-Antimycotic (100X)                                                                                                           | <a href="#">15240062</a>                                                                                       |
| StemPro™ Accutase™ Cell Dissociation Reagent (StemPro™ Accutase™ reagent)                                                               | <a href="#">A1110501</a>                                                                                       |
| Trypan Blue Solution, 0.4%                                                                                                              | <a href="#">15250061</a>                                                                                       |
| DPBS, no calcium, no magnesium                                                                                                          | <a href="#">14190250</a>                                                                                       |
| Y-27632 (ROCK inhibitor)                                                                                                                | Fisher Scientific™ <a href="#">129830-38-2</a>                                                                 |

## Suspension hPSC culture workflow

### Day 0: Seed suspension hPSC cultures

Seed suspension hPSC cultures from already-established adherent hPSC cultures (in a 6-well plate), or directly from cryopreserved hPSCs.

**Note:**

- To establish an adherent hPSC line from cryopreservation, see Appendix B, “Initiation and maintenance of adherent hPSC cultures”.
- To adapt suspension hPSC cultures to a weekend-free feeding schedule, see “Guidelines for adapting suspension hPSC cultures to a weekend-free feeding schedule” on page 4.

### Day 1 and Day 3: Feed the suspension hPSC cultures

Feed suspension hPSC cultures using a 50% medium exchange method to reduce metabolic waste in the medium while maintaining a constant culture volume to promote consistent spheroid growth.

**Note:** For information on weekend-free feeding, see “Guidelines for adapting suspension hPSC cultures to a weekend-free feeding schedule” on page 4.

### Day 4: Passage the suspension hPSC cultures

Passage the suspension hPSC cultures using StemPro™ Accutase™ reagent.

**Note:** If needed, the protocol can be adapted to a 5-day schedule (see “Guidelines for suspension hPSC cultures” on page 3).

### Repeat Day 1–Day 4 for the desired number of passages

Repeat passages can be used to scale-up suspension cultures into larger vessel formats. For optimal results, we recommend performing two consecutive passages to adapt cells into suspension culture, then harvesting the spheroids at the third passage.

### Harvest the hPSCs for downstream studies

Harvested hPSC spheroids can be used in a wide range of downstream studies, or reseeded into suspension cultures for additional passages.

## Guidelines for suspension hPSC cultures

- **IMPORTANT!** Perform all procedures inside a laminar flow hood, using aseptic techniques to prevent contamination.
- The volumes provided in this guide are sufficient to seed and passage a single multi-well plate or shake flask. To seed and passage multiple culture vessels, scale the volumes proportionally based on the number of vessels.
- This guide recommends scaling up and passaging suspension hPSC cultures over 3 consecutive passages. Suspension hPSC cultures can also be passaged for a shorter or longer duration, depending on your experimental requirements.
- This guide recommends feeding suspension hPSC cultures every other day using a 50% medium exchange method. Alternatively, suspension cultures can be fed daily or fed using >50% medium replacement to ensure spheroids maintain high-quality cells.
- **Note:** To adapt suspension hPSC cultures to a weekend-free feeding schedule, see “Guidelines for adapting suspension hPSC cultures to a weekend-free feeding schedule” on page 4.
- Seed suspension hPSC cultures from adherent hPSC colonies when the colonies are 50–80% confluent.
- Use non-tissue culture-treated plastics for suspension hPSC cultures.
- Seed hPSCs in StemScale™ PSC Suspension Medium, containing 10 μM ROCK inhibitor (Y-27632).

**IMPORTANT!** Do not use RevitaCell™ Supplement instead of ROCK inhibitor (Y-27632) (Cat. No. [129830-38-2](#)). Y-27632 targets ROCK for inhibition through a mechanism that is more favorable for hPSC spheroid formation with StemScale™ PSC Suspension Medium.

- Monitor spheroid morphology 24 hours after seeding to ensure proper formation of defined spheroidal structures.
- Record images of representative spheroids immediately before each passage, using the EVOS™ XL Core Imaging System or equivalent imaging system.

- Passage suspension hPSC cultures approximately every 4 days. The protocol can also be adapted to a 5-day passage schedule if needed (for example, to culture over the weekend or to culture cell lines that grow slowly). Ensure that suspension hPSC cultures are passaged before the average spheroid diameter grows larger than approximately 400  $\mu\text{m}$ .
- Do not use a cell strainer to passage suspension hPSC cultures. Use of a cell strainer can result in cell loss and contamination.
- For example images of hPSC spheroid morphology, see “Representative images of hPSC spheroid morphology” on page 10.

## Guidelines for adapting suspension hPSC cultures to a weekend-free feeding schedule

- For suspension hPSC cultures on 70-RPM orbital shaker platforms, we recommend increasing the culture volume for weekend-free cultures—On Friday, remove 50% of the spent medium, then add fresh StemScale™ PSC Suspension Medium at a volume 1.5X the volume removed.  
For example: If 1 mL of medium is removed, add 1.5 mL of fresh medium.
- (Alternative method) Perform a >75% medium exchange on Friday to avoid modifying the culture volume.

## Guidelines for shaking suspension hPSC cultures

- Ensure suspension hPSC cultures are continuously agitated to prevent aggregation and formation of clumps.

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**IMPORTANT!** Do not remove the culture vessel from the orbital shaker for more than 15 minutes.

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- Use a CO<sub>2</sub>-resistant orbital shaker (such as Cat. No. [88881101](#)) for optimal expansion of suspension hPSC cultures.

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**IMPORTANT!** Do not use a non-CO<sub>2</sub>-resistant shaker. Orbital shakers that are not designed for use in a CO<sub>2</sub> incubator can stop working overnight, leading to spheroid aggregation and cell death.

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- The orbital shaker speed specified in this guide is based on a shaker with a 19 mm orbit diameter. If an alternative orbit diameter is used, follow the manufacturer's guidelines to ensure proper speed conversion.
- Set the orbital shaker to continuously rotate at 70 RPM when using the culture vessel volumes specified in the following table.

**Table 3 Recommended orbital shaker speed for suspension hPSC cultures**

| Culture vessel | Culture volume | Recommended RPM <sup>[1]</sup> |
|----------------|----------------|--------------------------------|
| 6-well plate   | 2 mL per well  | 70 RPM                         |
| 125-mL flask   | 20 mL          |                                |
| 250-mL flask   | 40 mL          |                                |
| 500-mL flask   | 80 mL          |                                |

<sup>[1]</sup> Based on an orbital shaker with 19 mm orbital diameter.

## Before you begin

### Thaw reagents

- Thaw StemScale™ Supplement (10X) overnight at 2°C to 8°C.
- Thaw 10 mM ROCK inhibitor (Y-27632) at room temperature.  
Store Y-27632 in 50  $\mu\text{L}$  aliquots at –20°C. Avoid freeze-thaw cycles.

### Prepare StemScale™ PSC Suspension Medium

1. Gently invert the StemScale™ Supplement (10X) 5–7 times to mix.
2. Add the following reagents to a 900-mL bottle of StemScale™ Basal Medium.
  - StemScale™ Supplement (10X)—100 mL
  - (Optional) Antibiotic-Antimycotic (100X)—10 mL
3. Gently invert the bottle 5–7 times to mix.

Store the StemScale™ PSC Suspension Medium protected from light at 2°C to 8°C for up to 2 weeks, or aliquot the medium into smaller volumes and store at –20°C to –5°C for up to 6 months.

## Day 0: Seed suspension hPSC cultures

The following procedure provides guidance to seed suspension hPSC cultures from already-established adherent hPSC cultures (in a 6-well plate), or directly from cryopreserved hPSCs.

If you need to initiate an adherent hPSC culture before seeding hPSCs in suspension, see Appendix B, “Initiation and maintenance of adherent hPSC cultures”.

### Prepare StemScale™ PSC Suspension Medium + 10 µM Y-27632

Prepare the required volume of StemScale™ PSC Suspension Medium + 10 µM Y-27632 based on your suspension-culture vessel format.

1. Combine the following reagents in an appropriately-sized bottle or tube, according to the following table.

**Note:** For multiple culture vessels, scale the volumes proportionally based on the number of vessels.

**Table 4 Preparation of StemScale™ PSC Suspension Medium + Y-27632**

| Reagent                          | Volume <sup>[1]</sup> |              |              |              |
|----------------------------------|-----------------------|--------------|--------------|--------------|
|                                  | 6-well plate          | 125-mL flask | 250-mL flask | 500-mL flask |
| StemScale™ PSC Suspension Medium | 20 mL                 | 25 mL        | 50 mL        | 100 mL       |
| 10 mM Y-27632                    | 20 µL                 | 25 µL        | 50 µL        | 100 µL       |

<sup>[1]</sup> Volume required to resuspend dissociated hPSCs, then seed in the desired suspension-culture vessel.

2. Invert the bottle or tube 3–5 times to mix, then keep at room temperature until use.

### Prepare the suspension-culture vessel

1. Transfer the appropriate volume of StemScale™ PSC Suspension Medium + Y-27632 to each well or vessel, according to the following table.

**Table 5 Suspension-culture vessel preparation**

| Reagent                                    | Volume <sup>[1]</sup> |              |              |              |
|--------------------------------------------|-----------------------|--------------|--------------|--------------|
|                                            | 6-well plate          | 125-mL flask | 250-mL flask | 500-mL flask |
| StemScale™ PSC Suspension Medium + Y-27632 | 2 mL per well         | 20 mL        | 40 mL        | 80 mL        |

<sup>[1]</sup> Volumes are based on a 70-RPM orbital shaker agitation speed.

2. Store the prepared vessel in a 37°C, 5% CO<sub>2</sub> incubator until use.

Reserve the remaining StemScale™ PSC Suspension Medium + Y-27632 to resuspend cell pellets (next section).

## Prepare hPSCs for seeding into suspension cultures

1. After preparing the suspension culture vessel, pre-warm the remaining StemScale™ PSC Suspension Medium + Y-27632 in a 37°C water bath.
2. Prepare hPSCs for seeding into suspension cultures.

| For...                                                              | Do this...                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                        |
|---------------------------------------------------------------------|-------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------|
| Established adherent hPSC cultures in a 6-well plate <sup>[1]</sup> | <p>Ensure the adherent hPSC cultures are 50–80% confluent before starting this procedure.</p> <ol style="list-style-type: none"> <li>1. Pre-warm the following reagents in a 37°C water bath. <ul style="list-style-type: none"> <li>• StemPro™ Accutase™ reagent</li> <li>• StemScale™ PSC Suspension Medium</li> </ul> </li> <li>2. Aspirate the medium from each well, then add 3 mL of DPBS, no calcium, no magnesium (–/–). Gently move the plate back-and-forth to rinse the cells.</li> <li>3. Aspirate the DPBS (–/–), then add 1 mL of pre-warmed StemPro™ Accutase™ reagent to each well.</li> <li>4. Incubate the plate in a 37°C, 5% CO<sub>2</sub> incubator for 5 minutes.</li> <li>5. Using a P1000 pipette, resuspend the cells by pipetting up and down 2–3 times.</li> <li>6. Combine the contents of each well in a single, 50-mL conical tube, then immediately add 3 mL of StemScale™ PSC Suspension Medium per 1 mL of StemPro™ Accutase™ cell suspension.</li> <li>7. Gently invert the tube several times to mix, then centrifuge at 200 × g for 4 minutes.</li> <li>8. Aspirate the supernatant, then resuspend the cells in 1 mL of StemScale™ PSC Suspension Medium + Y-27632 per well passaged.</li> </ol>                            |
| Cryopreserved hPSCs                                                 | <p>Thaw a sufficient number of vials to seed the suspension-culture vessels at a density of <math>1.5 \times 10^5</math> viable cells/mL.</p> <ol style="list-style-type: none"> <li>1. Remove cryopreserved vials of hPSCs from liquid nitrogen storage, then rapidly thaw in a 37°C water bath until only a small ice crystal remains.</li> <li>2. Spray the outside of the vials with 70% ethanol.</li> <li>3. Working in a laminar flow hood, gently resuspend the cells by pipetting up and down, using a P1000 pipette.</li> <li>4. Transfer the contents from the vials to a 50-mL conical tube.</li> <li>5. Rinse each vial with 1 mL of StemScale™ PSC Suspension Medium + Y-27632.</li> <li>6. Slowly transfer the rinse to the 50-mL tube, drop-wise, while rocking the tube back-and-forth to reduce osmotic shock to the cells.</li> <li>7. Repeat the rinse procedure to achieve a final ratio of 3 mL of StemScale™ PSC Suspension Medium + Y-27632 to 1 mL of cryopreservation medium.</li> <li>8. Cap the tube, then gently invert 3–5 times to mix.</li> <li>9. Centrifuge the cells at 200 × g for 4 minutes.</li> <li>10. Gently aspirate the supernatant, then resuspend the cells in StemScale™ PSC Suspension Medium + Y-27632.</li> </ol> |

<sup>[1]</sup> Larger adherent vessel formats, such as round cell-culture dishes, can also be used to initiate suspension cultures.

3. Proceed with the cell suspension to count, then transfer the hPSCs to the suspension-culture vessel (next section).

## Count the hPSCs, then transfer to the prepared suspension-culture vessel

1. Measure cell viability and perform duplicate cell counts using the Trypan Blue exclusion method on the Countess™ II Automated Cell Counter.
2. Calculate the volume of cell suspension required to seed cells at a density of  $1.5 \times 10^5$  viable cells/mL.
3. Mix the cell suspension by pipetting up and down 5 times, then transfer the calculated volume to each prepared suspension-culture vessel (well of a plate or other vessel format).

After each addition, pipet up and down 1–2 times in the vessel to flush any remaining cells from the pipette tip.

4. Gently move the vessel back-and-forth several times to ensure the cells are evenly distributed.
5. Place the vessel on an orbital shaker (set to continuously rotate at 70 RPM) in a 37°C, 5% CO<sub>2</sub> incubator, then incubate the culture overnight.

6. Maintain the suspension hPSC cultures, according to the following schedule.
  - Day 1 and Day 3: Feed the suspension hPSC cultures (page 7)
  - Day 4: Passage the suspension hPSC cultures (page 7)
  - Repeat Day 1–Day 4 for the desired number of passages.  
For optimal results, we recommend performing two consecutive passages, then harvesting the spheroids at the third passage.

## Day 1 and Day 3: Feed the suspension hPSC cultures

1. Pre-warm an aliquot of StemScale™ PSC Suspension Medium in a 37°C water bath.
2. Remove the culture vessel from the orbital shaker platform.
3. Observe the suspension hPSC cultures under a microscope to ensure formation of well-defined spheroidal structures.  
To image spheroids in a shake flask, transfer 2 mL of the spheroid suspension to a well of a 6-well plate.  
For representative images of spheroid morphology, see “Representative images of hPSC spheroid morphology” on page 10.
4. Allow the spheroids to settle by gravity sedimentation, then perform a 50% medium exchange.

| For...                   | Do this...                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                      |
|--------------------------|-----------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------|
| Cultures in plates       | <ol style="list-style-type: none"> <li>1. Gently swirl the plate in a clockwise motion to bring the cells toward the center of each well.</li> <li>2. Balance the plate at a 45° angle, using a conical tube rack or similar object.</li> <li>3. With the plate tilted, wait 5 minutes, or until the spheroids settle between the side and bottom of the wells.</li> <li>4. Remove 50% of the spent medium from each well, then return the plate to a flat position.</li> <li>5. Replace the medium that was removed with an equal volume of pre-warmed StemScale™ PSC Suspension Medium.</li> <li>6. Gently move the plate back-and-forth several times to ensure the cells are evenly distributed.</li> </ol> |
| Cultures in shake flasks | <ol style="list-style-type: none"> <li>1. Gently swirl the flask in a clockwise motion to bring the cells toward the center of the vessel.</li> <li>2. Loosen the cap of the flask, then wait 5 minutes, or until the spheroids settle to the bottom of the vessel.</li> <li>3. Remove 50% of the spent medium from the flask, then replace with an equal volume of pre-warmed StemScale™ PSC Suspension Medium.</li> <li>4. Gently move the flask back-and-forth several times to ensure the cells are evenly distributed.</li> </ol>                                                                                                                                                                          |

5. Carefully return the culture vessel to the orbital shaker (set to continuously rotate at 70 RPM) in the 37°C, 5% CO<sub>2</sub> incubator.

## Day 4: Passage the suspension hPSC cultures

The following procedure is based on a 4-day passage schedule. If needed, the suspension hPSC cultures can be grown for up to 5 days before passaging.

### Before you begin

Prepare reagents and materials

- Pre-warm StemPro™ Accutase™ reagent and StemScale™ PSC Suspension Medium in a 37°C water bath.
- Prepare the required volume of StemScale™ PSC Suspension Medium + 10 µM Y-27632—See “Prepare StemScale™ PSC Suspension Medium + 10 µM Y-27632” on page 5.
- Prepare the suspension-culture vessel—See “Prepare the suspension-culture vessel” on page 5.

Record images of representative spheroids, then estimate the average spheroid diameter

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**IMPORTANT!** Return the culture vessel to the orbital shaker within 15 minutes of starting this procedure.

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1. (Optional) If you are culturing hPSCs in a shake flask, transfer 2 mL of the spheroid suspension to a well of a 6-well plate.
2. Gently swirl the plate in a clockwise motion to bring the cells toward the center of each well.

3. Using an EVOS™ XL Core Imaging System, record images of representative spheroids from each well, then estimate the average spheroid diameter.  
For representative images of spheroid morphology, see “Representative images of hPSC spheroid morphology” on page 10.
4. Based on the average spheroid diameter measured in step 3, proceed in one of the following ways.
  - If the average spheroid diameter is <400 µm—Grow the spheroids for one additional day (optional) or proceed to “Passage the suspension hPSC cultures” on page 8.
  - If the average spheroid diameter is ≥400 µm—Proceed to “Passage the suspension hPSC cultures” on page 8.

### Passage the suspension hPSC cultures

For optimal results, we recommend performing two consecutive passages, then harvesting the spheroids at the third passage (see “Harvest the hPSCs for downstream studies” on page 9).

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**IMPORTANT!** Do not triturate the spheroid suspension using a P1000 pipette until after the spheroids are dissociated into single cells. Triturating mature spheroids can reduce cell viability and contribute to cell death.

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1. Transfer the spheroid suspension from each culture vessel to a 15- or 50-mL conical tube.
  - (Optional) For multi-well plates, combine individual well cultures into a single suspension.
  - For large culture vessels, split the spheroid suspension between multiple 50-mL conical tubes.
2. Rinse each vessel with StemScale™ PSC Suspension Medium, then transfer the rinse to the tube containing the spheroid suspension. Use 50% of the culture volume as the rinse volume.
3. Centrifuge the tube at 200 × g for 4 minutes.
4. Aspirate the supernatant, then add the appropriate volume of StemPro™ Accutase™ reagent to each tube.

| Reagent                    | Volume        |              |              |              |
|----------------------------|---------------|--------------|--------------|--------------|
|                            | 6-well plate  | 125-mL flask | 250-mL flask | 500-mL flask |
| StemPro™ Accutase™ reagent | 1 mL per well | 5 mL         | 10 mL        | 20 mL        |

5. Incubate in a 37°C water bath for the specified time, according to your culture vessel format.
  - 6-well plate—10 minutes
  - 125-mL shake flask—10–15 minutes
  - 200-mL shake flask—15 minutes
  - 500-mL shake flask—15–20 minutes

At 2-minute intervals, gently mix or flick each tube to assess the level of dissociation. Spheroids should easily break apart as the incubation time approaches completion.
6. Gently triturate the StemPro™ Accutase™ suspension 5–7 times, or until the spheroids break apart into single cells or small clusters.
7. To each tube, immediately add 3 mL of StemScale™ PSC Suspension Medium per 1 mL of StemPro™ Accutase™ cell suspension.
8. Gently invert the tube several times to mix, then centrifuge at 200 × g for 4 minutes.
9. Aspirate the supernatant, then resuspend each cell pellet in StemScale™ PSC Suspension Medium + Y-27632.  
We recommend resuspending the cell pellet in the same volume of media that was used during the dissociation step (see step 4).
10. For each cell suspension, measure cell viability and perform duplicate cell counts using the Trypan Blue exclusion method on the Countess™ II Automated Cell Counter.
11. Calculate the volume of cell suspension required to seed the cells at a density of 1.5 × 10<sup>5</sup> viable cells/mL.
12. Mix each cell suspension by pipetting up and down 5 times, then transfer the calculated volume to each prepared suspension-culture vessel (well of a plate or other vessel format).  
After each addition, pipet up and down 1–2 times in the vessel to flush any remaining cells from the pipette tip.
13. Gently move the vessel back-and-forth several times to ensure the cells are evenly distributed.

14. Carefully place the vessel on an orbital shaker (set to continuously rotate at 70 RPM) in a 37°C, 5% CO<sub>2</sub> incubator.

15. Repeat Day 1–Day 4 for the desired number of passages.

## Harvest the hPSCs for downstream studies

Human PSC spheroids can be harvested on the day of the third passage or at a later passage, depending on your experimental requirements.

### Before you begin

Prepare reagents and materials

- Pre-warm StemPro™ Accutase™ reagent and StemScale™ PSC Suspension Medium in a 37°C water bath.
- Prepare the required volume of StemScale™ PSC Suspension Medium + 10 µM Y-27632 based on your suspension-culture vessel format.
  - a. Combine the following reagents in an appropriately-sized bottle or tube, according to the following table.

**Note:** For multiple culture vessels, scale the volumes proportionally based on the number of vessels.

**Table 6 Preparation of StemScale™ PSC Suspension Medium + Y-27632**

| Reagent                          | Volume <sup>[1]</sup> |              |              |              |
|----------------------------------|-----------------------|--------------|--------------|--------------|
|                                  | 6-well plate          | 125-mL flask | 250-mL flask | 500-mL flask |
| StemScale™ PSC Suspension Medium | 6 mL                  | 5 mL         | 10 mL        | 20 mL        |
| 10 mM Y-27632                    | 6 µL                  | 5 µL         | 10 µL        | 20 µL        |

<sup>[1]</sup> Volume required to resuspend dissociated hPSCs, then seed in the desired suspension-culture vessel.

- b. Invert the bottle or tube 3–5 times to mix, then keep at room temperature until use.

Record images of representative spheroids

**IMPORTANT!** Return the culture vessel to the orbital shaker within 15 minutes of starting this procedure.

1. (Optional) If you are culturing hPSCs in a shake flask, transfer 2 mL of the spheroid suspension to a well of a 6-well plate.
2. Gently swirl the plate in a clockwise motion, then record images of representative spheroids from each well using an EVOS™ XL Core Imaging System.

### Harvest the suspension hPSC cultures

**IMPORTANT!** Do not triturate the spheroid suspension using a P1000 pipette until after the spheroids are dissociated into single cells. Triturating mature spheroids can reduce cell viability and contribute to cell death.

1. Transfer the spheroid suspension from each culture vessel to a 15- or 50-mL conical tube.
  - (Optional) For multi-well plates, combine individual well cultures into a single suspension.
  - For large culture vessels, split the spheroid suspension between multiple 50-mL conical tubes.
2. Rinse each vessel with StemScale™ PSC Suspension Medium, then transfer the rinse to the tube containing the spheroid suspension. Use 50% of the culture volume as the rinse volume.
3. Centrifuge the tube at 200 × g for 4 minutes.
4. Aspirate the supernatant, then add the appropriate volume of StemPro™ Accutase™ reagent to each tube.

| Reagent                    | Volume        |              |              |              |
|----------------------------|---------------|--------------|--------------|--------------|
|                            | 6-well plate  | 125-mL flask | 250-mL flask | 500-mL flask |
| StemPro™ Accutase™ reagent | 1 mL per well | 5 mL         | 10 mL        | 20 mL        |

5. Incubate in a 37°C water bath for the specified time, according to your culture vessel format.

- 6-well plate—10 minutes
- 125-mL shake flask—10–15 minutes
- 200-mL shake flask—15 minutes
- 500-mL shake flask—15–20 minutes

At 2-minute intervals, gently mix or flick each tube to assess the level of dissociation. Spheroids should easily break apart as the incubation time approaches completion.

6. Gently triturate the StemPro™ Accutase™ suspension 5–7 times, or until the spheroids break apart into single cells or small clusters.

7. To each tube, immediately add 3 mL of StemScale™ PSC Suspension Medium per 1 mL of StemPro™ Accutase™ cell suspension.

8. Gently invert the tube several times to mix, then centrifuge at 200 × *g* for 4 minutes.

9. Aspirate the supernatant, then resuspend each cell pellet in StemScale™ PSC Suspension Medium + Y-27632.

We recommend resuspending the cell pellet in the same volume of media that was used during the dissociation step (see step 4).

10. For each cell suspension, measure cell viability and perform duplicate cell counts using the Trypan Blue exclusion method on the Countess™ II Automated Cell Counter.

11. Calculate and record the fold-change in cell count and the percent cell viability.  
Harvested hPSC spheroids are ready for use in a wide range of downstream studies.

12. (Optional) Reseed hPSCs into suspension cultures for additional passages.

## Representative images of hPSC spheroid morphology

Healthy spheroids should appear rounded, well-defined, and exhibit little to no “pitting”. The size and number of spheroids can differ depending on the bank recovery from cryopreservation. The important characteristic is the formation of defined spheroidal structures.

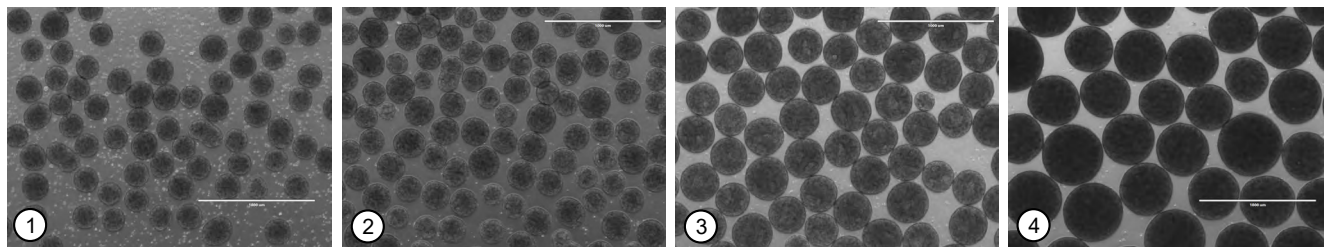


**Figure 1** Day 1–4: Expected morphology and growth of hPSC spheroids cultured in StemScale™ PSC Suspension Medium

① Day 1

② Day 2

③ Day 3

④ Day 4

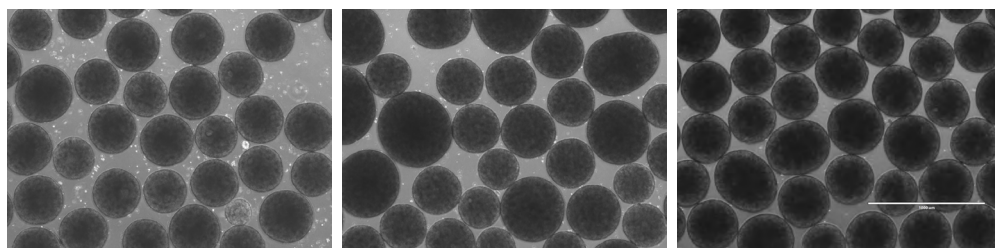


**Figure 2** Healthy hPSC spheroids on the day of passaging

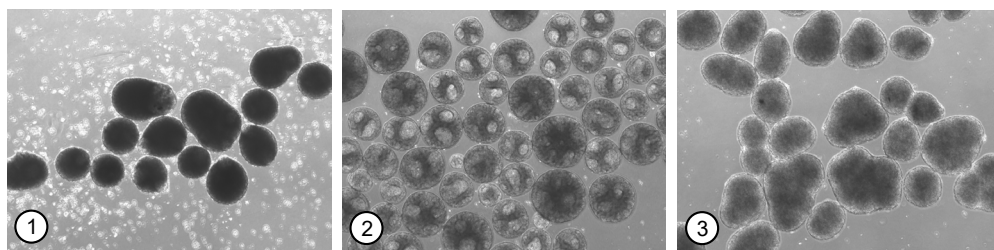
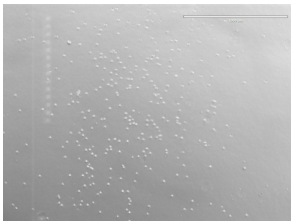
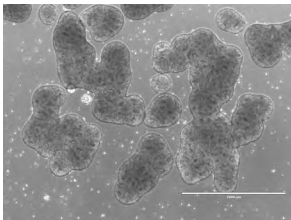
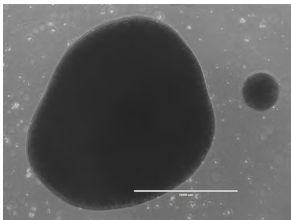
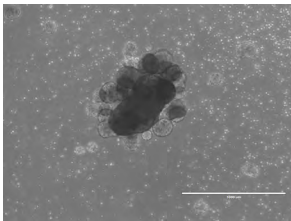


**Figure 3 Unhealthy hPSC spheroids on the day of passaging**

- ① Spheroids exhibiting minimal expansion in culture      ③ Spheroids with an uneven, misshapen structure
- ② Spheroids with a "pitted" appearance

## Appendix A Troubleshooting

| Observation                                                                                                                                                | Possible cause                                                                               | Recommended action                                                                                                                                                                 |
|------------------------------------------------------------------------------------------------------------------------------------------------------------|----------------------------------------------------------------------------------------------|------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------|
| Human PSCs are not forming spheroidal structures in suspension culture<br> | Human PSCs were seeded in StemScale™ PSC Suspension Medium without ROCK inhibitor (Y-27632). | Seed hPSCs in StemScale™ PSC Suspension Medium, containing 10 $\mu$ M ROCK inhibitor (Y-27632).                                                                                    |
|                                                                                                                                                            | The suspension cultures were agitated at a higher RPM than recommended.                      | Follow the procedure as described. Ensure the orbital shaker is set to continuously rotate at the correct speed (see "Guidelines for shaking suspension hPSC cultures" on page 4). |
| Spheroids are starting to form large clusters<br>                         | The suspension hPSC cultures were agitated improperly.                                       | Follow the procedure as described. Ensure the orbital shaker is set to continuously rotate at the correct speed (see "Guidelines for shaking suspension hPSC cultures" on page 4). |
|                                                                                                                                                            |                                                                                              | Ensure suspension hPSC cultures are continuously agitated to prevent aggregation and formation of clumps.                                                                          |
|                                                                                                                                                            |                                                                                              | Do not remove the culture vessel from the orbital shaker for more than 15 minutes.                                                                                                 |
|                                                                                                                                                            |                                                                                              | Use the recommended orbital shaker, or equivalent CO <sub>2</sub> -resistant orbital shaker with 19 mm orbit diameter.                                                             |
|                                                                                                                                                            |                                                                                              | Follow "Guidelines for shaking suspension hPSC cultures" on page 4.                                                                                                                |
| Spheroids are aggregating into large clumps<br>                          | More than the recommended volume of medium was used in the suspension culture.               | Follow the procedure as described. See Table 5 on page 5.                                                                                                                          |
|                                                                                                                                                            | The suspension hPSC cultures were agitated at a lower RPM than recommended.                  | Follow the procedure as described. Ensure the orbital shaker is set to continuously rotate at the correct speed (see "Guidelines for shaking suspension hPSC cultures" on page 4). |
|                                                                                                                                                            |                                                                                              | Ensure suspension hPSC cultures are continuously agitated to prevent aggregation and formation of clumps.                                                                          |
|                                                                                                                                                            |                                                                                              | Do not remove the culture vessel from the orbital shaker for more than 15 minutes.                                                                                                 |
|                                                                                                                                                            |                                                                                              | Use the recommended orbital shaker, or equivalent CO <sub>2</sub> -resistant orbital shaker with 19 mm orbit diameter.                                                             |
|                                                                                                                                                            |                                                                                              | Follow "Guidelines for shaking suspension hPSC cultures" on page 4.                                                                                                                |
| Spheroids are improperly forming after 24 hours in culture<br>           | An alternative cell dissociation reagent was used.                                           | Use StemPro™ Accutase™ Cell Dissociation Reagent to passage suspension hPSC cultures. See "Required materials not supplied" on page 2.                                             |

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## Appendix B Initiation and maintenance of adherent hPSC cultures

### Guidelines for adherent hPSC cultures

- Recommended substrate—Use Geltrex™ LDEV-Free, hESC-Qualified, Reduced Growth Factor Basement Membrane Matrix (Geltrex™ Matrix) (Cat. No. [A1413302](#)).
- To promote cell survival, thaw hPSCs in StemFlex™ Medium with RevitaCell™ Supplement (Cat. No. [A2644501](#)).
- Feed hPSCs with StemFlex™ Medium the day after seeding, then on alternating days until the cells reach the desired confluency.
- Split adherent hPSC cultures (for routine passaging or to seed suspension hPSC cultures) when adherent hPSC colonies are 50-80% confluent.

**Note:** Routine passaging is only required if adherent hPSC cultures are needed for subsequent assays. See “(Optional) Perform routine passaging of adherent hPSC cultures” on page 14.

### Before you begin

#### Thaw reagents

- Thaw the following reagents overnight at 2°C to 8°C.
  - Geltrex™ Matrix (1 aliquot)
  - StemFlex™ Supplement 10X

#### Coat a 6-well plate with Geltrex™ Matrix

**Note:** Geltrex™ Matrix gels in 5–10 minutes when kept above 15°C. Keep partial tubes and aliquots on ice to prevent premature gelling.

1. Gently invert an aliquot of Geltrex™ Matrix 3–5 times to mix, then place on ice.
2. Transfer 10 mL of pre-chilled DMEM/F-12, GlutaMAX™ Supplement to a 15-mL conical tube.
3. Add 100 µL of Geltrex™ Matrix to the DMEM/F-12, GlutaMAX™ Supplement.
4. Invert the tube 3 times, or until the solution is thoroughly mixed.
5. Transfer 1.5 mL of the Geltrex™ Matrix coating solution to each well of a 6-well tissue-culture plate.
6. Gently move the plate back-and-forth several times to ensure the plate is evenly coated.
7. Incubate the plate in a 37°C, 5% CO<sub>2</sub> incubator for a minimum of 1 hour.
8. At the time of use, aspirate and discard the supernatant above the Geltrex™ Matrix coating, then immediately seed cells in the appropriate pre-warmed medium.

#### Prepare StemFlex™ Medium

1. Gently invert the StemFlex™ Supplement 10X 5–7 times to mix.
2. Add the following reagents to a 450-mL bottle of StemFlex™ Basal Medium.
  - StemFlex™ Supplement 10X—50 mL
  - Antibiotic-Antimycotic (100X)—5 mL
3. Gently invert the bottle 5–7 times to mix.

Store the StemFlex™ Medium protected from light at 2°C to 8°C for up to 2 weeks.

#### Prepare StemFlex™ Medium + RevitaCell™ Supplement

- Prepare StemFlex™ Medium + RevitaCell™ Supplement at the time of use. Do not prepare in advance.
- Prepare 20 mL of StemFlex™ Medium + RevitaCell™ Supplement for each Geltrex™ Matrix-coated 6-well plate.

1. Add the following reagents to a 50-mL conical tube.
  - StemFlex™ Medium—20 mL

- RevitaCell™ Supplement—200 µL

2. Gently invert the tube 5–7 times to mix, then place in a 37°C water bath.

## Seed adherent hPSC cultures

The following sections provide guidance to establish an adherent hPSC line from cryopreservation.

### Prepare the Geltrex™ Matrix-coated 6-well plate for seeding

1. Aspirate and discard the supernatant above the Geltrex™ Matrix coating.
2. Add 1.5 mL of StemFlex™ Medium + RevitaCell™ Supplement to each well of the plate.

Keep the Geltrex™ Matrix-coated plate at room temperature until use.

### Thaw hPSCs, then seed adherent hPSC cultures

1. Pre-warm StemFlex™ Medium + RevitaCell™ Supplement in a 37°C water bath.
2. Remove cryopreserved vials of hPSCs from liquid nitrogen storage, then rapidly thaw in a 37°C water bath until only a small ice crystal remains.
3. Spray the outside of the vials with 70% ethanol.
4. Working in a laminar flow hood, gently resuspend the cells by pipetting up and down, using a P1000 pipette.
5. Transfer the contents from the vials to a 50-mL conical tube.
6. Rinse each vial with 1 mL of StemFlex™ Medium + RevitaCell™ Supplement.
7. Slowly transfer the rinse to the 50-mL tube, drop-wise, while rocking the tube back-and-forth to reduce osmotic shock to the cells.
8. Repeat step 6 and step 7 for a final ratio of 3 mL of StemFlex™ Medium + RevitaCell™ Supplement to 1 mL of cryopreservation medium.
9. Cap the tube, then gently invert 3–5 times to mix.
10. Centrifuge the cells at  $200 \times g$  for 4 minutes.
11. Gently aspirate the supernatant, then resuspend the cells in StemFlex™ Medium + RevitaCell™ Supplement.
12. Measure cell viability and perform duplicate cell counts using the Trypan Blue exclusion method on the Countess™ II Automated Cell Counter.
13. Calculate the volume of cell suspension required to seed cells at the appropriate densities.  
We recommend seeding the wells at the following densities:
  - $4 \times 10^4$  viable cells/cm<sup>2</sup>
  - $6 \times 10^4$  viable cells/cm<sup>2</sup>
  - $8 \times 10^4$  viable cells/cm<sup>2</sup>
14. Mix the cell suspension by pipetting up and down 5 times, then transfer the calculated volumes to duplicate wells of the prepared Geltrex™ Matrix-coated plate.
15. Gently move the plate back-and-forth several times to ensure the cells are evenly distributed.
16. Carefully place the plate into a 37°C, 5% CO<sub>2</sub> incubator, then incubate the adherent hPSC cultures overnight.

Record the vial information for the hPSC line.

## Feed the adherent hPSC cultures

Feed hPSCs with StemFlex™ Medium the day after seeding, then on alternating days until the cells reach the desired confluency.

1. For each 6-well plate, warm a 13-mL aliquot of StemFlex™ Medium in a 37°C water bath for 5 minutes.
2. Aspirate the spent medium from the plate, then add 2 mL of pre-warmed medium to each well.
3. Return the plate to the incubator.

Dissociate the adherent hPSC cultures to seed suspension hPSC cultures when the adherent hPSC colonies are 50-80% confluent. See “Day 0: Seed suspension hPSC cultures” on page 5.

### **(Optional) Perform routine passaging of adherent hPSC cultures**

The following procedure describes routine passaging of adherent hPSC cultures using Versene Solution. Versene Solution-passaging is only required if adherent hPSCs will be used for subsequent assays.

#### **Before you begin**

- Ensure adherent hPSC cultures are 50–80% confluent.
- Coat a 6-well plate with Geltrex™ Matrix—See “Coat a 6-well plate with Geltrex™ Matrix” on page 12.
- Pre-warm a 25-mL aliquot of StemFlex™ Medium in a 37°C water bath.
- Prepare the Geltrex™ Matrix-coated 6-well plate for seeding.
  - a. Aspirate and discard the supernatant above the Geltrex™ Matrix coating.
  - b. Add 1.5 mL of pre-warmed StemFlex™ Medium to each well of the plate.
 Keep the Geltrex™ Matrix-coated plate at room temperature until use.

#### **Passage adherent hPSC cultures using Versene Solution**

1. Select one well to passage.  
If needed, preserve the remaining wells to seed suspension hPSC cultures (see “Day 0: Seed suspension hPSC cultures” on page 5).
2. Aspirate the media from the selected well, then add 3 mL of DPBS, no calcium, no magnesium (–/–).
3. Gently tilt the plate back-and-forth to rinse the well, then aspirate the DPBS (–/–).
4. Add 1 mL of Versene Solution, then incubate the plate in a 37°C, 5% CO<sub>2</sub> incubator for 5 minutes, or until small holes form in the colonies.

---

**IMPORTANT!** Do not scrape hPSCs from the culture vessel or allow the colonies to float off the surface of the plate during passaging.

---

5. Aspirate the Versene Solution from the well, then resuspend the cells in 3 mL of StemFlex™ Medium.
6. Transfer the cell suspension to a 15-mL conical tube, then dilute with 5 mL of StemFlex™ Medium.
7. Using a 5-mL serological pipette, mix the cell suspension by pipetting up and down 2–3 times, then transfer the indicated volumes to duplicate wells of the coated plate.

**Note:** Volumes can be adjusted if a different split ratio is desired.

- 1 mL (1:8 split ratio)
  - 667 µL (1:12 split ratio)
  - 500 µL (1:16 split ratio)
8. Gently move the plate back-and-forth several times to ensure the cells are evenly distributed.
  9. Carefully place the plate into a 37°C, 5% CO<sub>2</sub> incubator, then incubate the adherent hPSC cultures overnight.
    - Feed the adherent hPSC cultures with StemFlex™ Medium the day after seeding, then on alternating days until the next passage.
    - Passage adherent hPSC cultures when the hPSC colonies reach 50–80% confluency, approximately every 3 days.

## Appendix C Documentation and support

### Related products

Unless otherwise indicated, all materials are available through [thermofisher.com](https://thermofisher.com).

| Product                                                                            | Catalog No.              |
|------------------------------------------------------------------------------------|--------------------------|
| StemFlex™ Medium Kit <sup>[1]</sup>                                                | <a href="#">A3349401</a> |
| Geltrex™ LDEV-Free, hESC-Qualified, Reduced Growth Factor Basement Membrane Matrix | <a href="#">A1413302</a> |
| RevitaCell™ Supplement (100X)                                                      | <a href="#">A2644501</a> |
| Versene Solution                                                                   | <a href="#">15040066</a> |

<sup>[1]</sup> Includes StemFlex™ Basal Medium and StemFlex™ Supplement 10X.

### Customer and technical support

Visit [thermofisher.com/support](https://thermofisher.com/support) for the latest service and support information.

- Worldwide contact telephone numbers
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  - Product FAQs
  - Software, patches, and updates
  - Training for many applications and instruments
- Order and web support
- Product documentation
  - User guides, manuals, and protocols
  - Certificates of Analysis
  - Safety Data Sheets (SDSs; also known as MSDSs)

**Note:** For SDSs for reagents and chemicals from other manufacturers, contact the manufacturer.

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**Revision history:** Pub. No. MAN0019457

| Revision | Date             | Description                                                                                                                                                                                                                                                               |
|----------|------------------|---------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------|
| B.0      | 11 February 2021 | <ul style="list-style-type: none"><li>• Updated the product name and catalog numbers.</li><li>• Added "Guidelines for adapting suspension hPSC cultures to a weekend-free feeding schedule" on page 4.</li><li>• Made minor wording updates to improve clarity.</li></ul> |
| A.0      | 28 July 2020     | New document for Early Access users.                                                                                                                                                                                                                                      |

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