Bioproduction

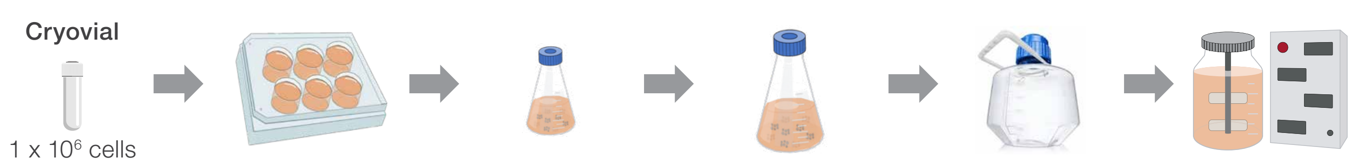
Scalable expansion of pluripotent stem cells using a seed train with a Nalgene 5 L shake flask

Introduction

Efficient and scalable expansion of pluripotent stem cells (PSCs) is a present challenge for PSC-derived allogeneic therapies that require large numbers of cells. Although liter-scale bioreactors can support the rapid generation of billions of PSCs grown as spheroids in suspension culture, they require large numbers of starting cells to seed the vessel. As PSCs are commonly cryopreserved at 1–4 x 10^6 cells per mL, thawing a sufficient number of vials to directly inoculate a multi-liter culture is impractical. To overcome this hurdle, “seed trains” are an effective means to ramp up PSC production gradually (Figure 1) and achieve the number of PSCs needed to seed a bioreactor.

A variety of shake flask sizes from 20 mL to 100 mL fill volumes can be used to support PSC scale-up on an orbital shaker platform. When culturing PSCs for seeding a liter-scale bioreactor, however, the number of shake flasks necessary to achieve these cell yields becomes difficult to maintain on a single orbital shaker platform. To address this challenge, we utilize a single **Thermo Scientific™ Nalgene™ 5-Liter Shake Flask with Faceted Bottom** to culture PSCs as spheroids in either 500 mL or 1 L culture volumes. The cell yields from a single 5 L shake flask after a single passage are sufficient to seed a 3 L bioreactor.

Described below is a protocol that guides suspension-based culture of PSC spheroids using Gibco™ Cell Therapy Systems™ (CTS™) StemScale™ PSC Suspension Medium in the 5 L shake flask. CTS StemScale medium enables robust expansion of PSCs in suspension for translational and clinical applications. Additionally, specific product-use statements, full documentation traceability, and convenient access to our Drug Master File (DMF) are available for CTS StemScale medium to support its use in clinical manufacturing of PSC-derived therapies.



	6-well plate	125 mL shake flask	500 mL shake flask	5 L shake flask	3 L bioreactor
Culture volume	2 mL (per well)	20 mL	100 mL	500–1,000 mL	2,000–3,000 mL
Required number of cells for seeding	8 x 10^5 cells (for 2 wells)	4 x 10^6 cells	2 x 10^7 cells	1–2 x 10^8 cells	4–6 x 10^8 cells
Estimated cell yield at passaging	6 x 10^6 cells (for 2 wells)	3 x 10^7 cells	1.5 x 10^8 cells	1–1.5 x 10^9 cells	3–5 x 10^9 cells

Figure 1. Scaling up to a liter-scale bioreactor using a shake flask seed train. When starting from a 6-well plate or 125 mL shake flask, it is possible to generate enough cells to seed a 3 L bioreactor within a few short passages. The cell yields from a smaller well plate or shake flask can be utilized to seed a larger shake flask for further scale-up.

We also offer suggestions to support downstream differentiation of PSC spheroids cultured in the 5 L shake flask. Billions of neural stem cells (NSCs) can be grown in a 5 L shake flask (Figure 2), compared to the millions of NSCs that can be grown in a 125 mL shake flask. As a result, the choices of flask size provide flexibility to ensure efficient and economical production of high-quality PSCs and terminally differentiated cells thereafter, within the requirements of the intended application. Based on the cell yields, the use of a 5 L shake flask for the culture and differentiation of PSC spheroids can be considered as an intermediate scale compared to smaller vessels or larger bioreactors.

Procedure overview

In this protocol, PSCs are maintained in suspension culture as spheroids grown on an orbital shaker platform. PSCs are initially seeded as single cells into an appropriately sized well plate or shake flask containing CTS StemScale medium, supplemented with a rho-associated protein kinase (ROCK) inhibitor and Invitrogen™ DNase I. Once cells have nucleated into spheroids, typically after 24 hours, PSC suspension cultures are then fed daily using a 50% medium exchange strategy. The nucleated spheroids will continue to grow while in culture. Once the spheroids reach an average diameter of 400 µm, typically within 5–6 days, they are dissociated back into single cells using diluted Gibco™ CTS™ TrypLE™ Select Enzyme. During a single passage in CTS StemScale medium, the number of PSCs is expected to expand 5- to 10-fold from the original number of seeded cells.

After dissociation, PSCs can be resuspended in CTS StemScale medium to resume PSC expansion in a larger shake flask. This process can be repeated until the required number of PSCs (~100 million–200 million PSCs) needed to seed into the 5 L shake flask is obtained. Once these PSCs have expanded as spheroids, they can then be harvested for seeding into a liter-scale bioreactor, cryopreserved, or used in downstream differentiation (Figure 2).

Section 1: PSC spheroid seed train expansion in CTS StemScale medium

Important notes

All cell culture protocols should be performed using proper aseptic techniques. Always use personal protective equipment.

This protocol was optimized using the Thermo Scientific™ CO_2 Resistant Shaker with a 19 mm orbit diameter. Other shaker platforms with different orbit diameters may require additional optimization of parameters (e.g., agitation speed, seeding density, media volume).

Additional guidance for suspension culture in CTS StemScale medium can be found in the CTS StemScale PSC Suspension Medium user guide (MAN1000080). Refer to the “Guidelines for orbital shaker hPSC cultures” and “Troubleshooting” sections in the user guide for tips and suggestions for optimizing spheroid growth.

For a visual reference, refer to the Gibco CTS StemScale PSC Suspension Medium how-to video series.

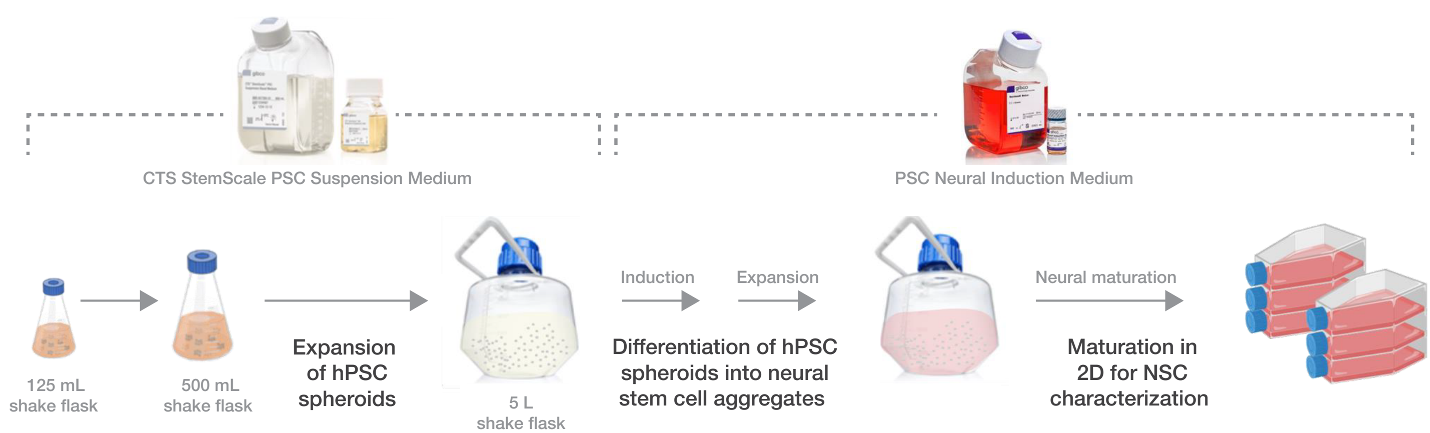


Figure 2. Schematic overview of neural stem cell differentiation in suspension. Spheroids scaled up to a 1 L culture undergo neural differentiation and expansion in a 5 L shake flask. PSC spheroids are induced into a neural stem cell state using Gibco™ PSC Neural Induction Medium and expanded in neural expansion medium. Finally, the aggregates can be dissociated and replated in monolayer culture to mature neurons in neural maturation medium.

Seeding PSCs into the suspension culture vessel

1. PSCs can be directly seeded into CTS StemScale medium by harvesting from adherent culture, dissociating from existing spheroids in suspension culture, or thawing from cryopreserved cell banks.
2. Fill the well plate or shake flask with the appropriate volume of CTS StemScale medium (Table 1).

Note: The 250 mL shake flask is not included as part of the seed train in Figure 1, although it may be substituted for the 125 mL shake flask or inserted as part of the seed train. The cell yield from the 125 mL shake flask is typically sufficient to seed the 500 mL shake flask.

3. Using the volumes in Table 1, supplement the CTS StemScale medium with final concentrations of 10 µM Y-27632 and 0.1 U/mL DNase I.

Table 1. Preparation of CTS StemScale medium plus Y-27632 and DNase I.

Vessel	CTS StemScale medium culture volume (mL)	10 mM Y-27632 volume (µL)	1 U/µL DNase I volume (µL)
6-well plate	2 (per well)	2 (per well)	0.2 (per well)
125 mL shake flask	20	20	2
250 mL shake flask	40	40	4
500 mL shake flask	100	100	10
5 L shake flask	500	500	50
	1,000	1,000	100

4. Store the vessel in a 37°C, 5% CO_2 incubator until PSCs are ready to be seeded.
5. After performing one of the methods in Step 1, count the single-cell suspension and calculate the volume required to seed cells into the suspension culture vessel to a density of 2.0×10^5 viable cells/mL.
6. Add the calculated volume of cells to the suspension culture vessel, then place the flask on an orbital shaker in a 37°C, 5% CO_2 incubator.
7. Set the orbital shaker to rotate continuously according to the recommended revolutions per minute (RPM) listed in Table 2.

Table 2. Recommended RPM for culturing spheroids in a well plate or shake flask.

Vessel	CTS StemScale medium culture volume (mL)	Recommended RPM
6-well plate	2 (per well)	70
125 mL shake flask	20	
250 mL shake flask	40	
500 mL shake flask	100	
5 L shake flask	500	55
	1,000	60

Note: The RPM values listed in Table 2 are general recommendations for initiating spheroid cultures at specific volumes in the well plates or shake flasks. Cell line–dependent optimization may require additional adjustments to RPM, depending on spheroid nucleation efficiency. Guidance and suggestions for agitation optimization are provided in the appendix. Additional information can be found in the "Guidelines for orbital shaker hPSC cultures" and "Troubleshooting" sections of the **CTS StemScale PSC Suspension Medium user guide (MAN1000080)**.

Exchanging medium in the suspension culture vessel

1. Remove the suspension culture vessel from the orbital shaker platform, and position it at a 45° angle to allow all spheroids to settle via gravity sedimentation according to the following recommendations:
   - For 6-well plates and 125 mL, 250 mL, and 500 mL shake flasks, wait 5 minutes for spheroids to settle.
   - For 5 L shake flasks, tilt the flask so the angled portion of the vessel is flat against the work surface and wait approximately 10 minutes for spheroids to settle. See Figure 3 for an example of a shake flask resting on its angled bottom.
2. Once spheroids have settled to the bottom of the shake flask,

Figure 3. Example of a 5 L shake flask undergoing a 50% medium exchange.

carefully perform a 50% medium exchange:

- Medium should be removed by manually pipetting from the back corners of the shake flask and withdrawing from the upper part of the liquid suspension.
- Avoid pipetting medium from the bottom of the vessel, as this is where spheroids will settle.
- After removing half of the spent medium from the vessel, add an equal volume of fresh medium to ensure the volume in the flask remains constant between feedings.

3. Return the vessel to the orbital shaker in the 37°C, 5% CO_2 incubator. Refer to Table 2 for recommended RPM settings based on the volume inside the shake flask.

Note: For additional guidance on feeding and the passage duration of spheroids cultured in CTS StemScale medium, refer to the “Feed the hPSC suspension cultures” section in the **CTS StemScale PSC Suspension Medium user guide (MAN1000080)**. For a visual reference, refer to the **Gibco CTS StemScale PSC Suspension Medium how-to video series**.

Passaging spheroids in the suspension culture vessel

After 5–6 days of culture in the CTS StemScale medium, the average diameter of the PSC spheroids will be around 400 µm (refer to Figure 4 for morphology, growth, and maintenance of pluripotency of high-quality spheroids grown in both small and large shake flasks). When the spheroids reach ~400 µm (assessed via daily sampling to measure spheroid size), proceed to passage the PSC spheroid suspension culture as described below:

1. Using a serological pipette, transfer the spheroid suspension from the culture vessel to an appropriately sized conical tube capable of holding the entire culture volume.
   - To reduce the total volume transferred, especially in the 5 L shake flask, consider allowing the spheroids to settle for up to 10 minutes (as described in “Exchanging medium in the suspension culture vessel”) before aspirating 60–80% of the spent medium from the flask. Once the total volume has been reduced, carefully transfer the remaining medium and all the spheroids to a conical tube, using a serological pipette.
2. Wait 5–10 minutes for spheroids to settle to the bottom of the conical tube. Once the spheroids have settled, carefully aspirate the supernatant, being careful to not disturb the spheroid pellet.

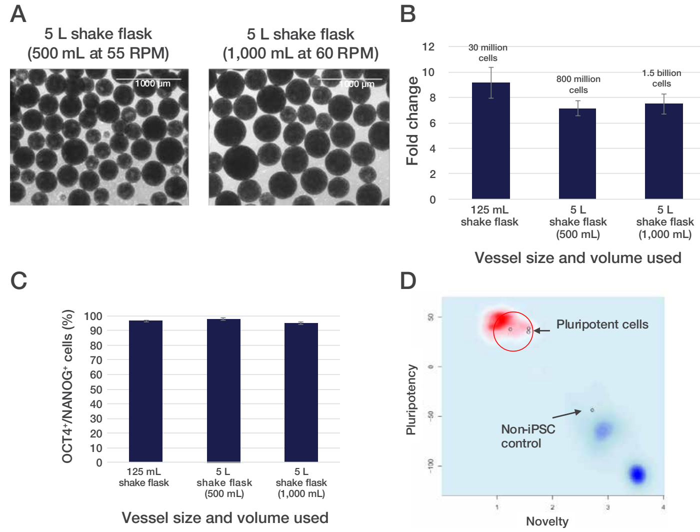


Figure 4. Characterization of PSCs expanded as spheroids in shake flasks. (A) Spheroids expanded in the 5 L shake flasks exhibit a rounded morphology in both the 500 mL (at 55 RPM) and 1 L (at 60 RPM) cultures in CTS StemScale medium. **(B)** Spheroid growth over 5 days. Fold expansion of the cells from the 5 L shake flasks was similar to the fold expansion from the 125 mL shake flask. **(C)** Spheroid pluripotency, day 5. PSCs harvested from all flasks maintained a high percentage of cells expressing pluripotency markers, as assessed via flow cytometric analysis of OCT4/NANOG double-positive cells. **(D)** Pluripotency was further confirmed by running all spheroid samples from the 125 mL shake flask and both 5 L shake flask fill volumes through the Applied Biosystems™ PluriTest™ Assay, a global assessment of pluripotency status against a validated and well-characterized reference set.

3. After aspirating the supernatant, proceed to dissociate the spheroids. Refer to Table 3 for suggestions on the volume of diluted CTS TrypLE Select Enzyme to add to the spheroids. Once the diluted enzyme has been added, incubate the tubes in a 37°C water bath as indicated below.

Table 3. Dissociation parameters for shake flask suspension cultures.

Vessel	CTS StemScale medium culture volume (mL)	Diluted CTS TrypLE Select Enzyme volume (mL)*	Dissociation time (min)**
6-well plate	2	1	5
125 mL shake flask	20	5	
250 mL shake flask	40	10	10
500 mL shake flask	100	20	10–15
5 L shake flask	500	50	20–25
	1,000	75	25–30

* Use 0.25X CTS TrypLE Select Enzyme (diluted in Gibco™ DPBS, no calcium, no magnesium) for the 6-well plate and the 125 mL and 250 mL shake flasks. Use 0.5X CTS TrypLE Select Enzyme for the 500 mL and 5 L shake flasks.

** Dissociation times may vary according to the continued presence of visible spheroids during the dissociation process. The dissociation time can be extended as necessary.

4. Once the spheroids have finished dissociating into single cells, add 3 mL of CTS StemScale medium for every 1 mL of diluted enzyme used during the dissociation process.
5. Centrifuge the cells at 200 x *g* for 4 min. After centrifugation, aspirate the supernatant and prepare the cell pellet for counting.
6. Once the cells have been counted, they can be seeded into new suspension culture vessels, cryopreserved, or used in subsequent downstream applications.
   - Refer to Figure 1 for recommendations on a shake flask seed train to achieve cell yields capable of seeding a liter-scale bioreactor.
   - For an example of downstream differentiation, the harvested PSCs from a 500 mL shake flask were used to seed a 5 L shake flask in preparation for neural differentiation. Refer to the next section ("Neural stem cell differentiation using a 5 L shake flask") for guidance and results.

Note: For additional guidance on passaging spheroids grown in CTS StemScale medium, refer to the "Passage the hPSC suspension cultures" section in the **CTS StemScale PSC Suspension Medium user guide (MAN1000080)**. For a visual reference, refer to the **Gibco CTS StemScale PSC Suspension Medium how-to video series**.

Section 2: Neural stem cell differentiation using a 5 L shake flask

For specific guidance on neural differentiation of the PSC spheroids, refer to the application note "**Neural differentiation of PSC spheroids grown in StemScale medium**". This application note will guide the user through neural induction, neural expansion, and neural maturation (Figure 5). The application note also has recommendations on medium reconstitution, medium exchange, and duration of neural differentiation.

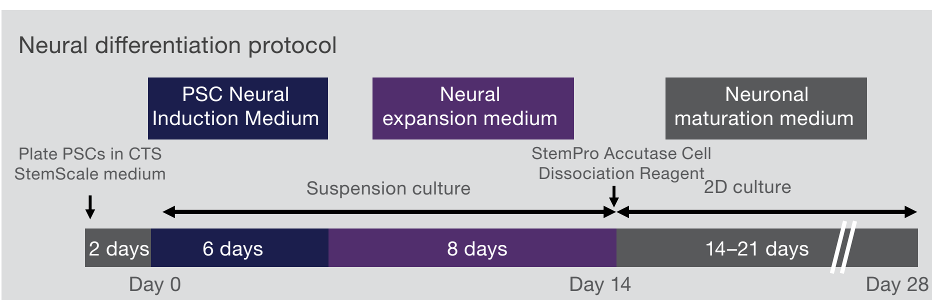


Figure 5. Neural differentiation process overview. Spheroids are grown for 1–2 days in the 5 L shake flask before starting the neural differentiation protocol. All 80% medium exchanges performed throughout this process are calculated using the CTS StemScale medium culture volumes for the appropriate vessel, as listed in Table 1.

Neural induction

At day 6 of induction, cells should be >90% SOX1/nestin co-positive and are ready for transition into neural expansion medium. Figure 6 provides a visual example of aggregates grown in the 5 L shake flask after 6 days of neural induction. Figure 7 highlights the NSC protein marker expression.

Neural expansion

On day 14 (day 8 of expansion), aggregates can be dissociated and plated in 2D for neuronal maturation, cryopreserved in neural expansion medium with 10% DMSO, or continued to be expanded. Figure 6 provides a visual example of aggregates grown in the 5 L shake flask after 8 days of neural expansion. Figure 7 highlights the NSC protein marker expression. Figure 8 demonstrates the total NSC yields after aggregate dissociation into single cells.

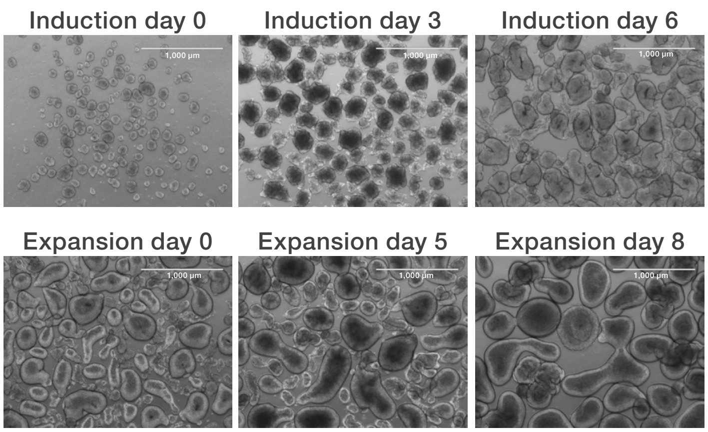


Figure 6. Cell aggregate morphology during neural induction and neural expansion. Changes in aggregate morphology will occur during the 6 days of neural induction and the subsequent 8 days of neural expansion. During neural induction, aggregates will transition from a rounded morphology on day 0 to an irregular morphology by day 6. During neural expansion, aggregates will begin to adopt neuroepithelial-like characteristics.

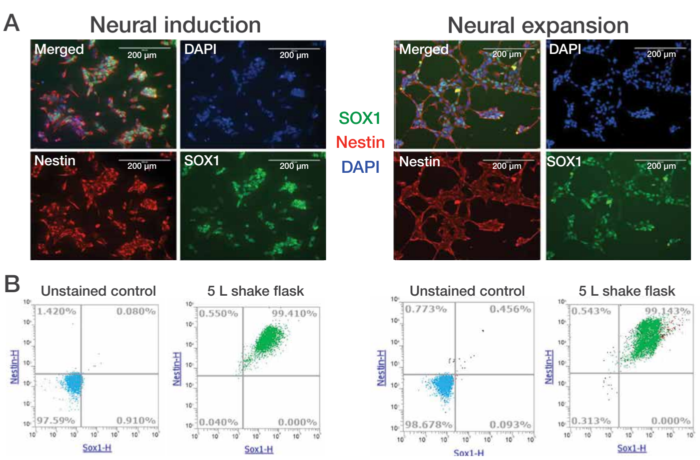


Figure 7. NSC marker expression in adherent cells after neural induction and neural expansion in suspension. After neural induction and neural expansion in suspension culture, NSC aggregates were dissociated, plated in 2D, and stained for NSC markers to assess the differentiation efficiency of these cultures. Cells cultured in the 5 L shake flask yield high-purity (>90%) SOX1/nestin co-positive cells, as demonstrated by **(A)** immunocytochemistry and **(B)** flow cytometric analysis.

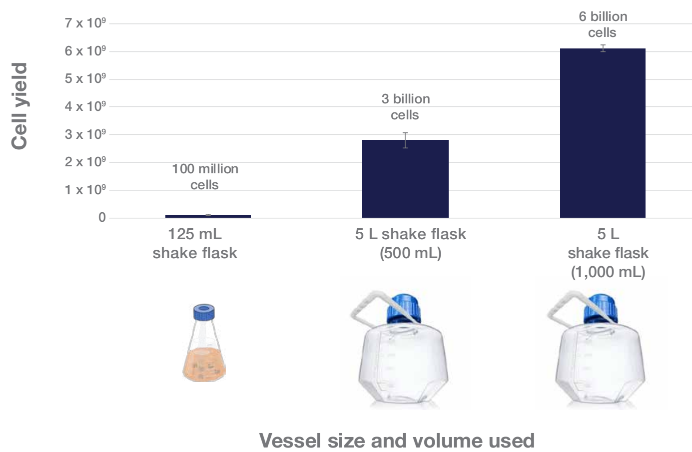


Figure 8. Total NSC yields from harvesting aggregates after neural expansion. After completion of neural expansion, the NSC aggregates were dissociated to calculate the total cell yield. Cell yields in the 5 L shake flask reached up to 6 billion NSCs in a 1,000 mL culture volume at 60 RPM. Compared to the 100 million NSCs generated in a 125 mL shake flask, growth in the 5 L shake flask enables scaling with the need for additional optimization at large volumes.

Neuronal maturation in 2D monolayer culture

On day 34 (day 20 of maturation), the culture population will be enriched with mature neurons. Refer to Figure 9 for expected results after 20 days of maturation.

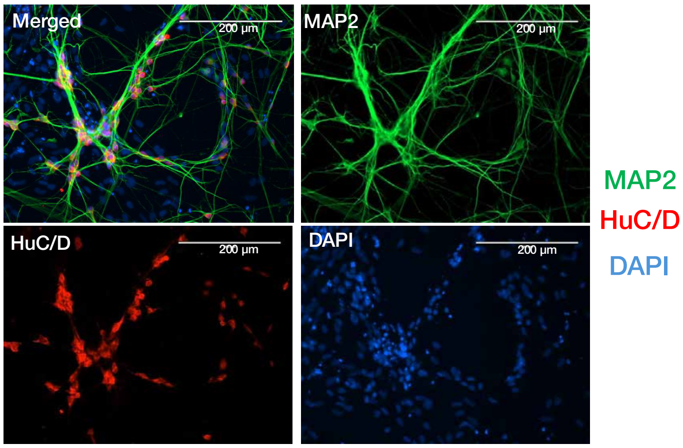


Figure 9. Maturation markers after 20 days of neural maturation. The population is enriched with mature neurons as assessed by HuC/D and MAP2 expression.

Appendix

Optimization of RPM in the 5 L shake flask

- The orbital shaker speeds specified in this guide are based on an orbital shaker platform with a 19 mm orbit diameter. Optimization of RPM speeds on the orbital shaker platform may be required to obtain appropriately sized spheroids. Refer to the PSC spheroid morphology images in Figure 10 for examples of RPM speeds set too low, too high, or at an ideal RPM in the 5 L shake flask.
- Additional guidance for orbital shaker platform RPMs can be found in the **CTS StemScale PSC Suspension Culture Medium user guide (MAN1000080)**. Refer to "Guidelines for orbital shaker hPSC cultures" and "Troubleshooting" for tips and suggestions for optimizing spheroid growth.

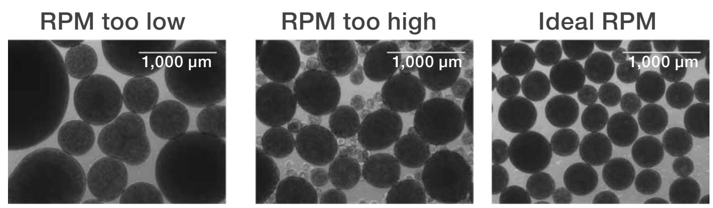


Figure 10. Orbital shaker platform RPM troubleshooting. If the RPM is set too low or too high for the 5 L shake flask, spheroids will form improperly over the duration of culture. For RPM values set too low, spheroids tend to aggregate into large clumps that are significantly greater than 400 µm in diameter. If the RPM is only slightly too low, then normal-sized spheroids will be visible among the large spheroids. For RPM values set too high, spheroids will form a range of small and large clusters. The small spheroids result from the high RPM, while the large spheroids begin to appear as aggregates collecting near the center of the flask and fuse into larger clumps.

Ordering information

Product	Quantity	Cat. No.
Nalgene 5-Liter Shake Flasks with Angled or Faceted Bottom	4/case	4115-5000/4115-5001
Nalgene Single-Use PETG Erlenmeyer Flasks with Plain Bottom: Sterile	24/case	4115-0125
Nunc Cell Culture Treated 6-Well Plate	75/case	140675
StemScale PSC Suspension Medium	1 L	A4965001
CTS StemScale PSC Suspension Medium	1 L	A5869601
CTS TrypLE Select Enzyme	100 mL	A1285901
PSC Neural Induction Medium	500 mL	A1647801
StemPro Accutase Cell Dissociation Reagent	100 mL	A1110501
DNase I, RNase-free	1,000 units	EN0521
CO_2 Resistant Shakers	1	88881103
DPBS, no calcium, no magnesium	500 mL	14190144
EVOS cell imaging systems	–	Various

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