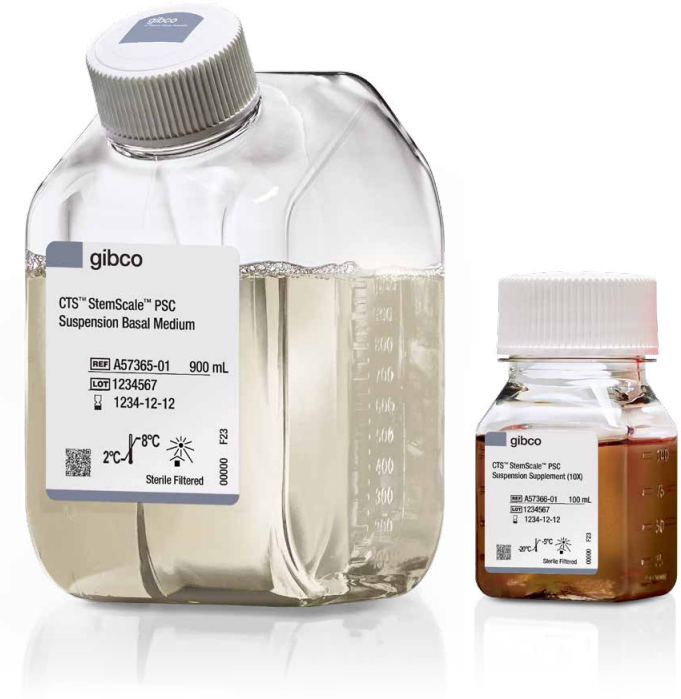
Stem cell research

Large-scale expansion of PSCs to support cell therapy manufacturing

Highlights

- CTS StemScale PSC Suspension Medium can achieve 5- to 10-fold expansion per passage across multiple PSC lines.
- CTS StemScale medium supports efficient and consistent growth of PSCs in various culture vessel sizes, providing a scalable solution for generating large numbers of hPSCs.
- CTS StemScale medium achieves similar cell yields as StemScale PSC Suspension Medium (RUO) when grown for an additional day, supporting the transition from bench to clinic.



Introduction

Scalable and efficient expansion of pluripotent stem cells (PSCs) continues to present a challenge for PSC-derived allogeneic therapies that require large numbers of cells. Three-dimensional (3D) suspension culture offers a promising solution for large-scale production of high-quality PSCs required for cell therapy manufacturing applications. However, a suspension culture medium that supports the transition from bench to clinic has not been commercially available. Therefore, we developed Gibco™ CTS™ StemScale™ PSC Suspension Medium, which promotes the self-aggregation of single cells into 3D spheroids that can be effectively scaled to multi-liter culture volumes with consistent performance across PSC lines. This PSC culture medium delivers similar benefits and performance as our research use only (RUO) Gibco™ StemScale™ PSC Suspension Medium but in a xeno-free formulation.

CTS StemScale medium overview

CTS StemScale PSC Suspension Medium is a xeno-free medium that enables robust expansion of PSCs in suspension for translational and clinical research. The medium is scalable and easy to use (Figure 1), utilizing the self-assembly method of spheroids to promote PSC growth without the use of microcarriers. PSCs expanded in CTS StemScale medium maintain pluripotency and genomic stability across multiple passages and can be differentiated to the three germ layers.

The benefits of CTS StemScale PSC Suspension Medium include:

- Enhanced expansion capability versus other PSC suspension media, reducing manufacturing time and cost
- Scalable expansion of PSCs with a simplified workflow that does not require a cell strainer
- Consistent growth of multiple PSC lines
- Supported regulatory filings with GMP manufacturing, traceability of raw materials, and regulatory documentation

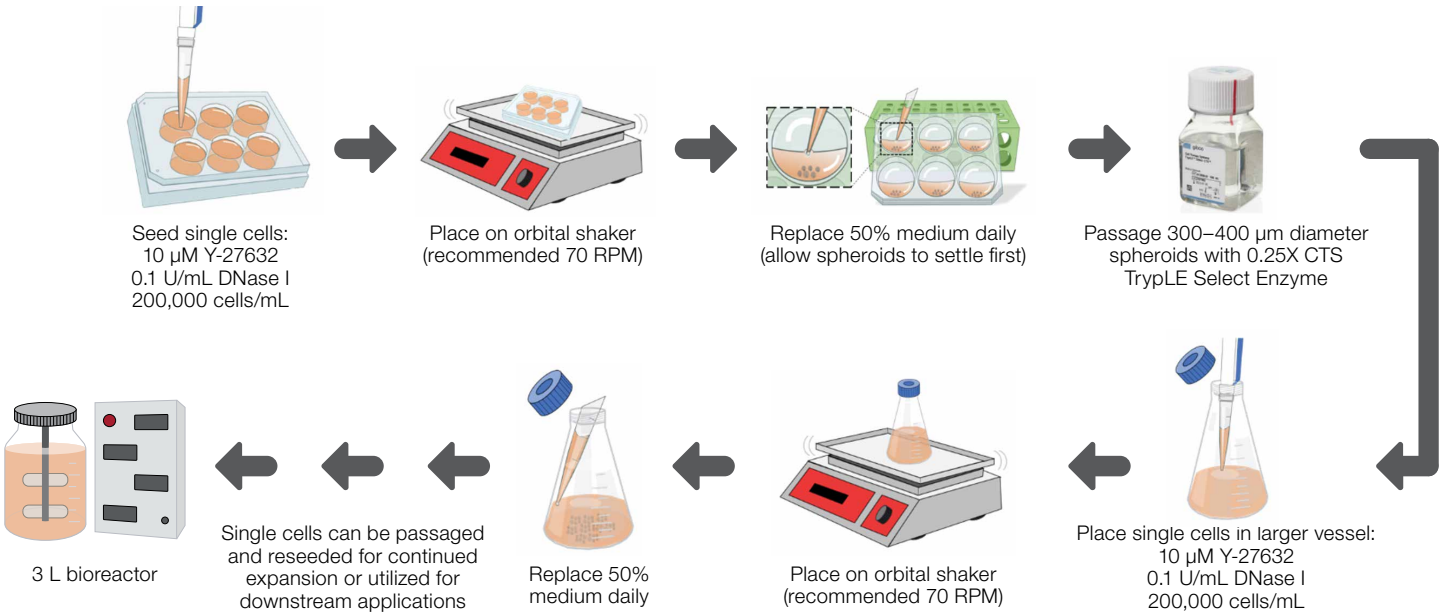


Figure 1. Simplified CTS StemScale PSC Suspension Medium workflow for initiation of small-scale cultures that can be scaled up. Cultures in CTS StemScale medium are initiated by seeding single cells in the presence of Y-27632 (to promote spheroid nucleation) and DNase I (to prevent undesirable cell aggregation from cell lysis during passaging). Cultures in CTS StemScale medium should be fed daily using 50% medium replacement. Once spheroids reach approximately 300–400 µm in diameter, they can be passaged using diluted (0.25X) Gibco™ CTS™ TrypLE™ Select Enzyme. The resulting single-cell suspension can then be scaled up into larger vessels or utilized in downstream applications.

RUO and CTS StemScale media comparison

Achieving consistent cell yields is critical for cell therapy manufacturing and for developing PSC-based therapies. CTS StemScale medium facilitates the transition to the clinic as it supports robust expansion of PSCs and comparable cell yields as the RUO StemScale medium. The formulation of CTS StemScale medium is similar to RUO StemScale medium but with specific modifications in line with regulatory guidance. These formulation changes necessitate a few minor protocol differences between the RUO and CTS versions of StemScale PSC Suspension Medium to provide similar performance; these protocol differences affect seeding density, feeding strategy, and days of growth (Table 1). For example, a higher seeding density is recommended for cultures in CTS StemScale medium as compared to cultures in RUO StemScale medium.

Furthermore, cultures in CTS StemScale medium will require an additional day of growth for spheroids to reach the recommended passaging metric of an average 300–400 µm spheroid diameter.

To align the CTS StemScale protocol with cell therapy manufacturing applications and to obtain similar cell yields as with the RUO StemScale medium, the CTS StemScale protocol incorporates two additional slight modifications to the RUO StemScale protocol (Table 1). The CTS StemScale protocol utilizes CTS TrypLE Select Enzyme during passaging rather than the Gibco™ StemPro™ Accutase™ Cell Dissociation Reagent. DNase I is also added while seeding new cultures in CTS StemScale medium.

Table 1. Protocol differences between RUO StemScale and CTS StemScale media. Modifications to the RUO StemScale protocol allow for similar cell yields to be obtained with the CTS StemScale medium.

	StemScale PSC Suspension Medium	CTS StemScale PSC Suspension Medium
Dissociation reagent	StemPro Accutase Cell Dissociation Reagent*	CTS TrypLE Select Enzyme (diluted**)
Inclusion of DNase I	DNase I not required	DNase I should be added
Days of growth	4–5 days†	5–6 days†
Feeding strategy	Every other day	Daily
Seeding density	150,000 cells/mL	200,000 cells/mL

* Contains an animal-origin component.

** CTS DPBS(–/–) can be used to dilute CTS TrypLE Select Enzyme to a lower concentration.

† Estimated time to achieve an average spheroid diameter between 300–400 µm.

Figure 2 shows a comparison between spheroids grown in RUO StemScale medium and spheroids grown in CTS StemScale medium from the same initial cell bank. Spheroids grown in RUO StemScale medium and CTS StemScale medium typically reach the upper limit (400 µm) within 5 days and 6 days, respectively (Figures 2A and 2C).

The additional day of growth for cultures in CTS StemScale medium enables the spheroids grown in this medium to achieve the same fold expansion as the spheroids grown in RUO StemScale medium (Figure 2B). Daily feeding of cultures with CTS StemScale medium will help ensure that spheroids remain highly viable through this additional day of growth. Cultures in CTS StemScale medium can also be seeded at a higher density to promote spheroid nucleation and greater cell yields.

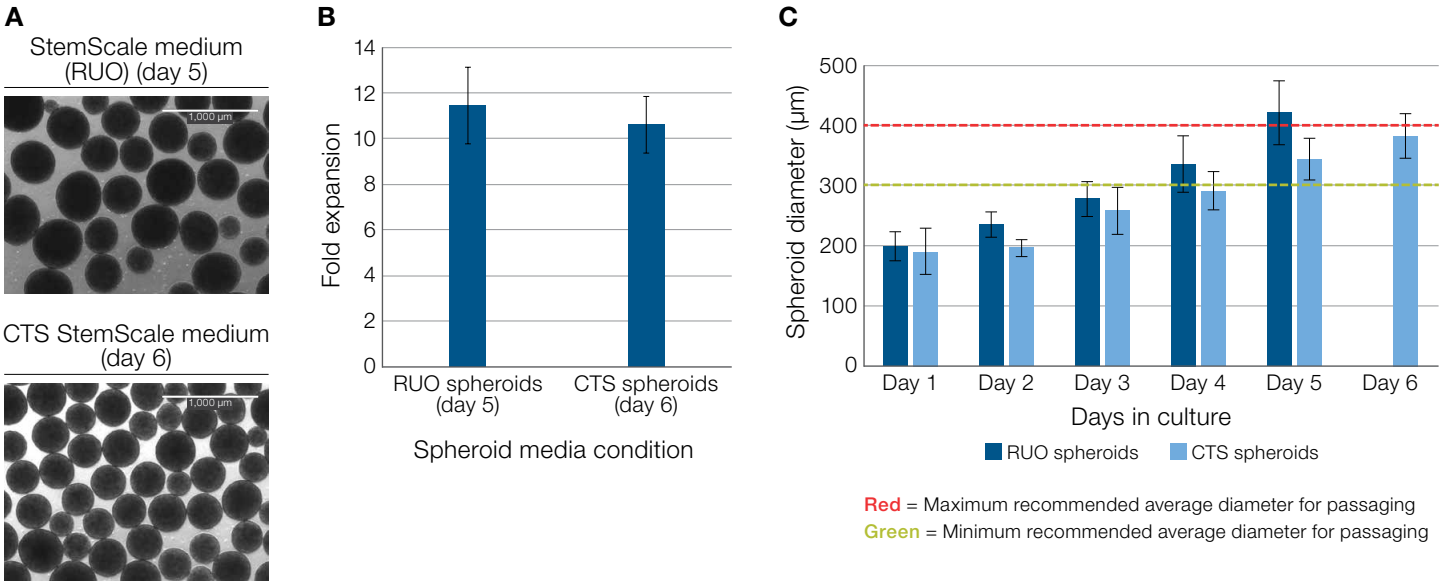


Figure 2. CTS StemScale medium provides similar performance to RUO StemScale medium. As indicated in Table 1, spheroids grown in CTS StemScale medium will require an additional day of growth as compared to spheroids grown in RUO StemScale medium to achieve similar cell yields. **(A)** Spheroid morphology on passage day. Spheroids grown in RUO StemScale medium will typically reach an average of 400 µm in diameter in 5 days, while spheroids grown in CTS StemScale medium will require an additional day to reach a similar diameter. **(B)** Cumulative cell expansion on passage day. By harvesting spheroids grown in RUO StemScale medium on day 5 and spheroids grown in CTS StemScale medium on day 6, it is possible to achieve similar total cell yields (reported as fold expansion). **(C)** Spheroid diameter comparison. The spheroid diameter of the spheroids grown in RUO StemScale medium and the spheroids grown in CTS StemScale medium are similar on the respective days of harvest, with both close to the upper recommendation of 400 µm in diameter.

For a cell therapy workflow, utilization of products designed for clinical applications helps minimize risk. As noted above, a change in the dissociation reagent was introduced to the CTS StemScale protocol as compared to the RUO StemScale protocol. Specifically, CTS TrypLE Select Enzyme, an animal origin–free reagent, is recommended for dissociating spheroids grown in CTS StemScale medium, rather than StemPro Accutase Cell Dissociation Reagent, which contains animal-origin components. Due to the nature of spheroid dissociation in CTS TrypLE Select Enzyme, however, it is important to add Thermo Scientific™ DNase I to the cultures in CTS StemScale medium when seeding dissociated cells on day 0. As shown in Figure 3A, cells dissociated using CTS TrypLE Select Enzyme can sometimes lyse and release genomic DNA (gDNA) into the cell culture environment, resulting in the undesirable aggregation of large clumps of spheroids. Inclusion of DNase I will help ensure that any potential gDNA is degraded. The addition of 0.1 U/mL DNase I is recommended for orbital shaker cultures (i.e., well-plate and shake-flask cultures), while large-scale vessels (i.e., 100 mL vessels up to liter-scale vessels) may require increased DNase I concentrations up to 1 U/mL. Figure 3B also shows the growth efficiency of spheroids dissociated in varying concentrations of CTS TrypLE Select Enzyme. Dilution of this enzyme to a concentration range of 0.1X–0.25X allows for spheroid nucleation and expansion most similar to that of control spheroids dissociated in StemPro Accutase reagent (Figures 3B and 3C). Notably, this dilution range works well for cultures grown on orbital shakers. When dissociating large quantities of spheroids in larger vessels, 0.5X–0.75X CTS TrypLE Select Enzyme will be necessary to account for the increased cell mass.

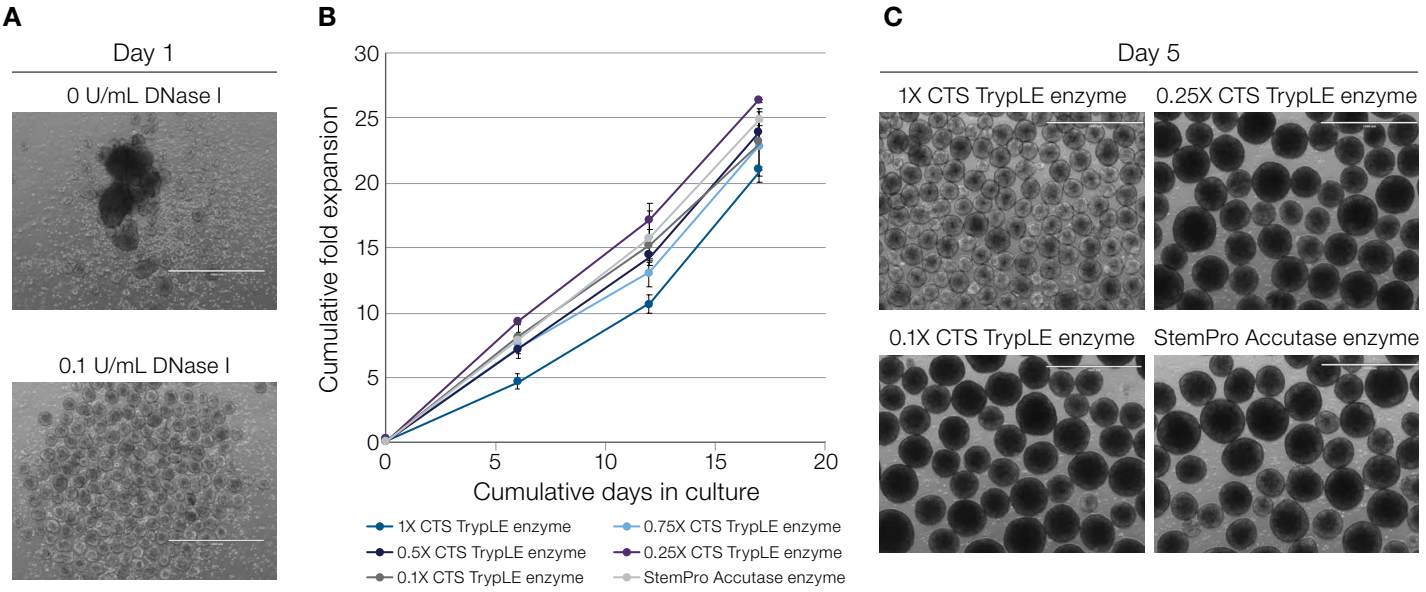


Figure 3. Spheroid dissociation with diluted CTS TrypLE Select Enzyme is similar to dissociation with StemPro Accutase reagent for orbital shaker cultures. (A) Day 1 spheroid nucleation after CTS TrypLE Select Enzyme dissociation. Cells that lyse during dissociation with CTS TrypLE Select Enzyme may release gDNA into the culture environment, leading to undesirable cell aggregation. Inclusion of 0.1 U/mL DNase I will help remove the gDNA from the culture environment and promote efficient spheroid nucleation. **(B)** Spheroid expansion over time after dissociation in various dilutions of CTS TrypLE Select Enzyme. Diluting the CTS TrypLE Select Enzyme to a lower concentration can promote improved spheroid growth and expansion. Spheroids dissociated with the lowest concentrations of CTS TrypLE Select Enzyme yielded higher cumulative fold expansion, ultimately generating results similar to spheroids dissociated using StemPro Accutase reagent. **(C)** Day 5 spheroid morphology after CTS TrypLE Select Enzyme dissociation. For orbital shaker cultures, CTS TrypLE Select Enzyme dilutions from 0.1X to 0.25X enable spheroids to nucleate and expand most similarly to cultures dissociated using StemPro Accutase reagent. Note that when moving beyond orbital shaker cultures, high quantities of spheroids will likely require increased CTS TrypLE concentrations (such as 0.5X–0.75X) for efficient dissociation of the increased cell mass.

Expansion capability of spheroids grown in CTS StemScale medium

For PSC lines that have differing nucleation efficiencies, reliable spheroid formation is necessary to produce cell yields required for clinical applications. In Figure 4, we illustrate the expansion of four different types of cell lines (iPSCs and embryonic stem cells (ESCs)) grown in CTS StemScale medium. Both iPSCs and ESCs readily nucleate into spheroids (Figure 4A). The cell lines that were tested also exhibited a range of 5x–10x expansion potential per passage (Figure 4B).

These results indicate that CTS StemScale medium supports the expansion capability of different PSC lines, with a typical cell line showing an average expansion potential per passage of 1 x 10^6–2 x 10^6 cells/mL.

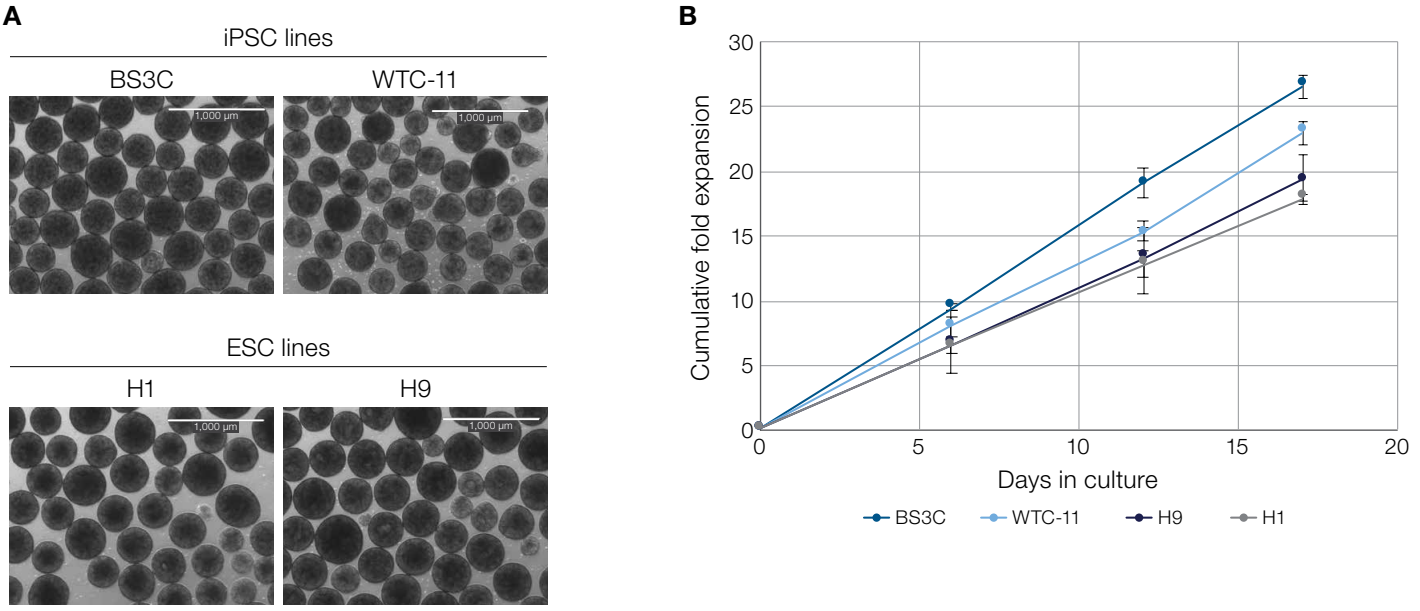


Figure 4. CTS StemScale medium supports the expansion of multiple cell lines. (A) Representative day 6 spheroid morphology**.** Although spheroid nucleation efficiency may vary between different cell lines, CTS StemScale medium enables all types of cell lines to nucleate and expand as spheroids. The images shown here are representative of spheroids after 6 days of growth in CTS StemScale medium. **(B)** Comparison of hPSC growth in CTS StemScale medium. Cell lines tested exhibited a range of 5x–10x expansion potential per passage. These results indicate that CTS StemScale medium supports the expansion capability of different PSC lines (BS3C, WTC-11, H9, and H1), with a typical cell line showing an average expansion potential per passage of 1 x 10^6–2 x 10^6 cells/mL. Since expansion is cell line dependent, it is important to become accustomed with how a particular line behaves when grown in CTS StemScale medium.

Although the expansion potential may vary, cell lines expanded in CTS StemScale medium exhibit consistent growth between passages. Therefore, estimating the average yield of a particular cell line is possible after becoming familiar with how the cell line performs in CTS StemScale medium. A user may therefore extrapolate the necessary culture volume required to obtain a desired number of cells. Figure 5 demonstrates the expansion of a PSC line grown for 30 consecutive passages in CTS StemScale medium. The evaluation was performed with spheroids fed daily or every other day. As seen in Figure 5A, both feed schedules were able to support growth of spheroids with consistent, rounded morphology. Toward the end of the study, however, the overall size of the spheroids fed daily tended to be larger than the overall size of the spheroids fed every other day. This difference in size contributed to the gradual difference in cumulative fold expansion. Notably, daily feeding of cultures in CTS StemScale medium yielded the highest cumulative fold expansion after 30 passages (Figure 5B). Although frequently skipping days for medium exchange contributes to a lower cumulative fold expansion, skipping one day periodically (such as one day on the weekend) will not significantly affect performance. Regardless of feeding strategy, all spheroids grown in CTS StemScale medium maintained pluripotency after 30 consecutive passages, as determined by flow cytometric analysis and the Thermo Scientific™ PluriTest™ Assay (Figures 5C and 5D). The cells harvested from the spheroids were also genomically stable and did not develop any new chromosomal aberrations over the duration of the 30 passages. Together, these results indicate that CTS StemScale medium can support stable and long-term growth of PSC lines.

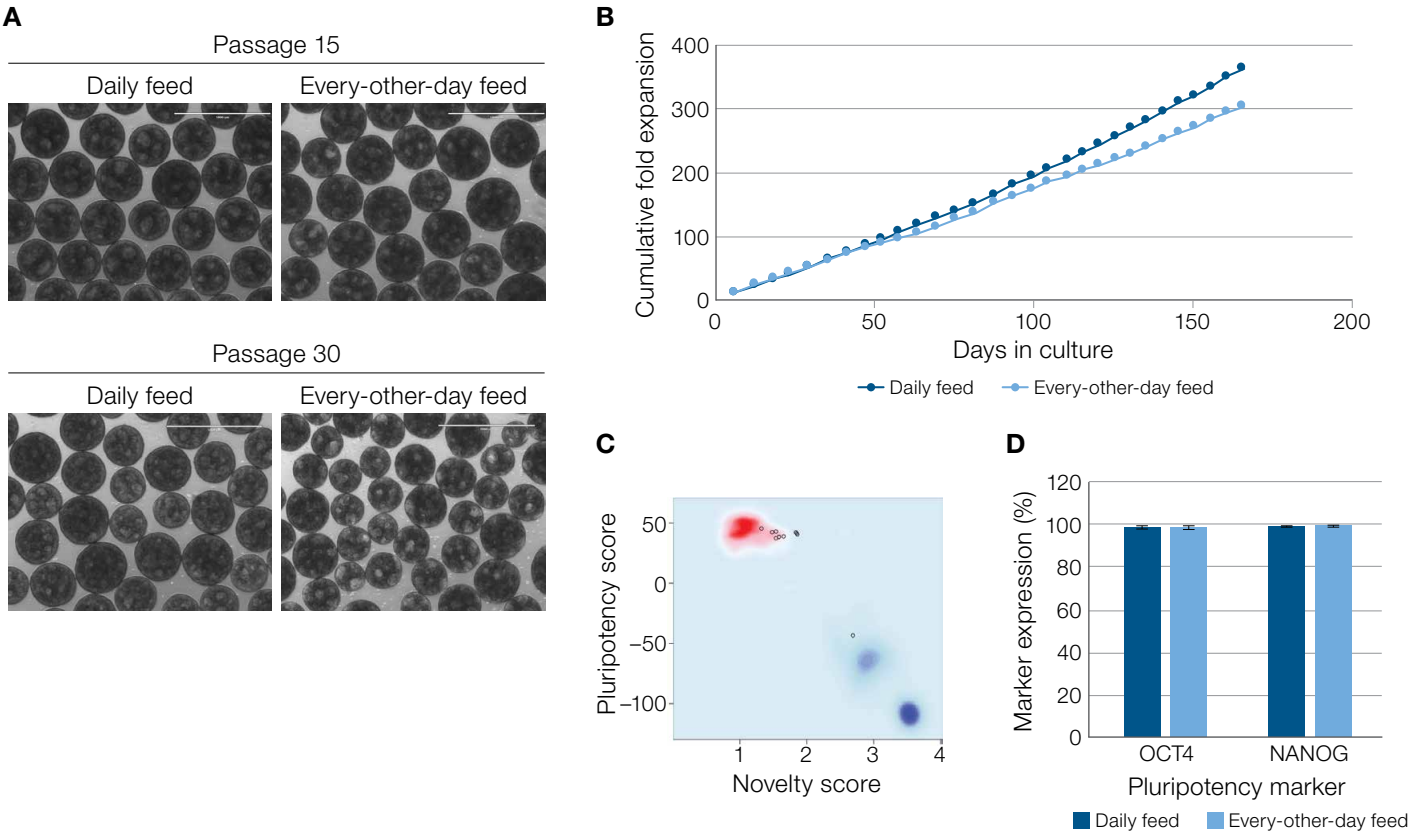


Figure 5. CTS StemScale medium maintains pluripotency and genomic stability during long-term expansion of spheroids. (A) Representative images of day 6 spheroid morphology. Cells grown as spheroids in CTS StemScale medium exhibit consistent growth over time, as evidenced by the 30-passage study. **(B)** Spheroid expansion over 30 consecutive passages. When expanded over 30 consecutive passages, the cultures fed daily had the most consistent growth, while the cultures fed every other day began to show reduced expansion after 7 passages. For this reason, it is recommended that cultures in CTS StemScale medium be fed daily to maximize expansion potential and cell viability. **(C)** Pluripotency assessment with the PluriTest Assay. Cells grown for 30 consecutive passages in CTS StemScale medium exhibited high expression of pluripotent markers as analyzed through the PluriTest Assay for specific gene expression. **(D)** Flow cytometric analysis of pluripotency markers. Cells maintained pluripotency as assessed by flow cytometric analysis of OCT4 and NANOG markers. Additionally, cell lines were confirmed to remain genomically stable, as no new chromosomal aberrations were detected at the end of the 30-passage study (data not shown).

Competitive analysis of CTS StemScale medium

As maximizing cell yields is advantageous for generating large numbers of cells in a cost-effective manner, the performance of CTS StemScale medium was evaluated against other commercially available PSC suspension media. Figure 6A compares the morphology of spheroids grown in CTS StemScale medium with spheroids grown in one such PSC suspension medium, i.e., medium "M". Both media followed their respective protocols in order to grow the spheroids over a 60-day duration. The spheroids grown in CTS StemScale medium required 10 passages in order to be grown for 60 consecutive days. In contrast, the spheroids grown in medium "M" required 18 passages to be grown for the same 60 consecutive days. Notably, spheroids grown in CTS StemScale medium were larger and tended to exhibit a more consistent spheroid morphology as compared to the spheroids grown in medium "M".

As shown in Figure 6B, CTS StemScale medium delivered the highest cumulative fold expansion in a 3-passage study as compared to the suspension media from other suppliers (medium "M", medium "A", and medium "B"). A long-term study was subsequently conducted with medium "M", as it exhibited higher fold expansion than either medium "A" or medium "B". In this second study, both suspension cultures were started from the same initial bank of cells and expanded 60 days, following the specific protocol for each medium. Analysis of the data revealed that CTS StemScale medium delivered 25% higher total cumulative cell yield than medium "M". Additionally, CTS StemScale medium required fewer passages to achieve this result, suggesting that CTS StemScale medium can offer more flexibility in maintaining suspension cultures over multiple passages (Figure 6C). These data demonstrate that CTS StemScale medium provides maximal expansion capability and higher expansion potential than other PSC suspension media options.

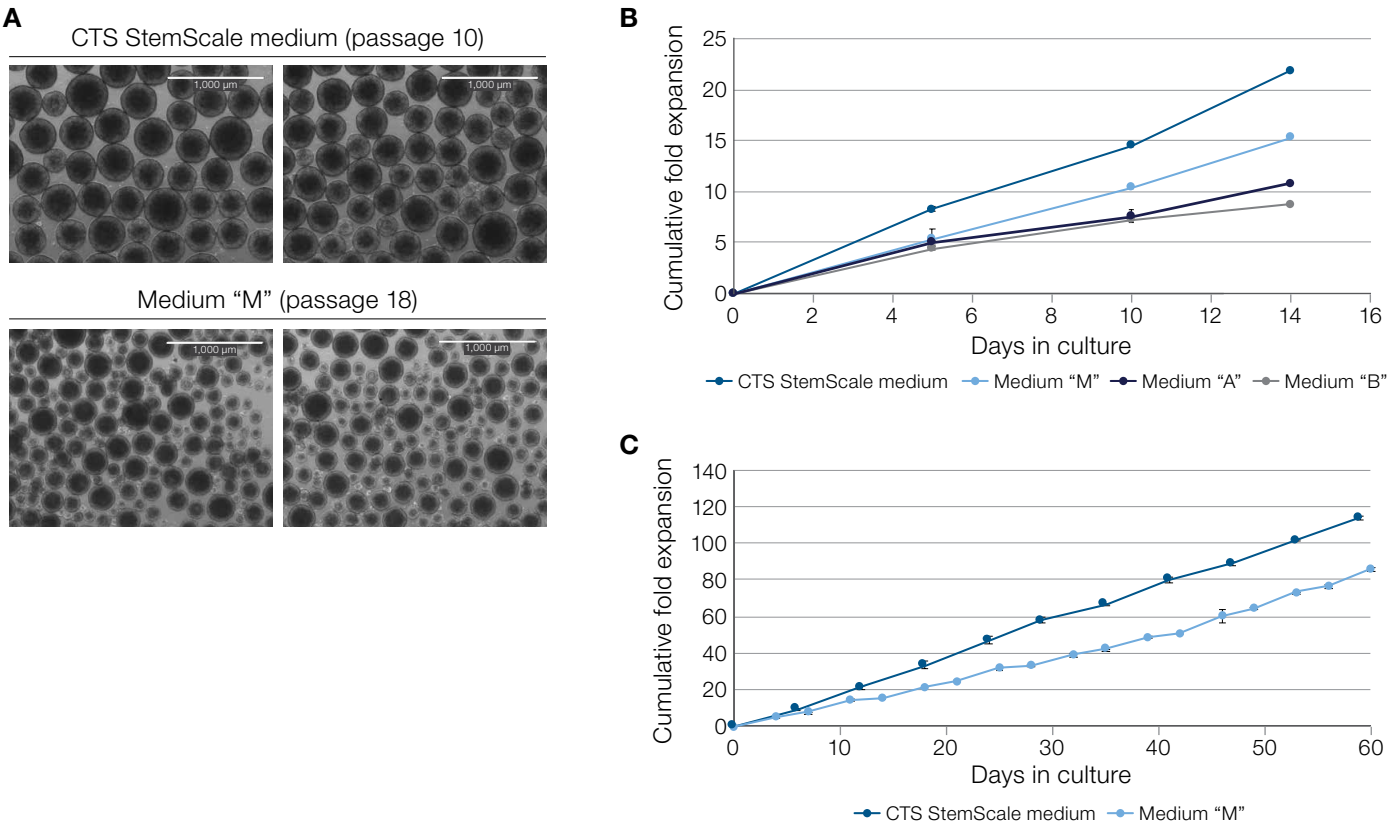


Figure 6. CTS StemScale medium delivers higher expansion potential than other PSC suspension media. (A) Representative spheroid morphology. CTS StemScale medium was compared against a commercially available suspension culture medium "M". Spheroids grown in CTS StemScale medium were larger and exhibited a more consistent spheroid morphology compared to the spheroids grown in medium "M". **(B)** Spheroid expansion in various PSC suspension culture media. Multiple PSC suspension media were selected for an initial 3-passage study against CTS StemScale medium. **(C)** Long-term competitive analysis: CTS StemScale medium vs. medium "M". After the initial 3-passage study, medium "M" was selected for a long-term study against CTS StemScale medium, with each medium using its respective protocol and passaging recommendation. In both studies **(B)** and **(C)**, CTS StemScale medium outperformed medium "M" and other media (medium "A" and medium "B") by exhibiting at least 25% greater expansion potential. CTS StemScale medium also required fewer passages compared to medium "M", reducing hands-on time and allowing for a more flexible culture schedule.

Scaling up with CTS StemScale medium

Another essential need for allogeneic therapies is scalability of the process for expanding PSCs. An example method for scaling up to large-scale vessels using CTS StemScale medium is shown in Table 2. One of the benefits of CTS StemScale medium is the ability to promote consistent spheroid growth in small-scale (i.e., <100 mL) and large-scale (i.e., >1 L) culture vessels. Since CTS StemScale medium does not require the use of cell strainers when dissociating spheroids, it is easy to adapt dissociation methods to large-scale vessels. Inclusion of cell strainers can be limiting when working with high volumes of liquids and large quantities of spheroids. Figure 7A shows a comparison of spheroid growth in CTS StemScale medium between vessels of different sizes. All vessels were seeded at the same concentration of 200,000 cells/mL and grown for the same number of days. The results indicate efficient growth can be achieved by maintaining the same culture settings in small-scale and large-scale vessels. In addition, pluripotency was assessed by flow cytometric analysis of OCT4 and NANOG expression. Cells expanded in CTS StemScale medium remained pluripotent in all culture vessels tested (Figure 7B).

Table 2. Scaling up with CTS StemScale PSC Suspension Medium. The numbers in this table represent a typical approach to scaling up to liter-scale vessels when using CTS StemScale medium. Average cell yield assumes a cell line that achieves ~8x expansion per passage.

Total days in culture	Culture vessel format	Culture vessel volume	Number of cells to seed vessel	Average cell yield (end of passage)	Total media consumed (mL)
5	6-well plate (full plate)	2 mL (per well)	2.4×10^6	19×10^6	36
10	125 mL shake flask	20 mL	4×10^6	32×10^6	60
15	100 mL spinner flask	100 mL	20×10^6	160×10^6	300
20	500 mL spinner flask	500 mL	100×10^6	800×10^6	1,500
25	3 L bioreactor	3 L	600×10^6	4.8×10^9	6,000

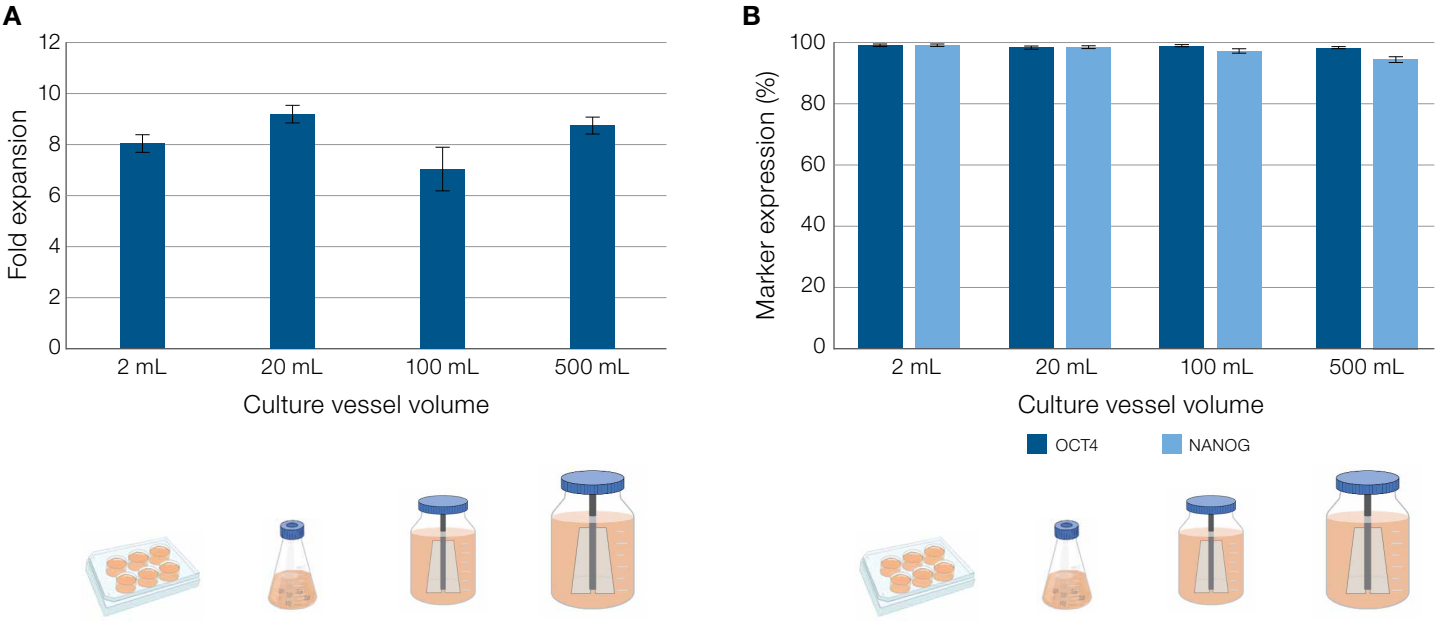


Figure 7. CTS StemScale medium supports efficient growth across multiple culture vessel sizes. (A) Cumulative growth after 6 days. The recommended seeding density of 200,000 cells/mL was used for cultures in CTS StemScale medium in all sizes of suspension culture vessels. Regardless of scale, cultures in CTS StemScale medium achieve a consistent high-fold expansion. **(B)** Spheroid pluripotency on passage day. Spheroids grown in CTS StemScale medium remain pluripotent in all different sizes of suspension culture vessels, as assessed through flow cytometric analysis of OCT4 and NANOG markers.

When scaling up for a cell therapy manufacturing workflow, it may be necessary to grow suspension cultures within a closed-system environment. Importantly, CTS StemScale medium is able to support spheroid growth within different types of closed-system liter-scale bioreactors. As shown in Figure 8A, CTS StemScale medium promotes the formation and expansion of PSC spheroids cultured in bioreactors with either a horizontal-blade or vertical-wheel impeller. Regardless of the impeller type, spheroids grown in CTS StemScale medium achieved similar yields across both bioreactor formats (Figure 8B) and also exhibited high expression of pluripotent markers as assessed via flow cytometric analysis (Figure 8C). Table 3 offers some suggestions for culture strategies inside these closed-system bioreactors.

After spheroids grown in CTS StemScale medium have been expanded in a bioreactor, it may be necessary to also harvest these spheroids within a closed-system environment. The Gibco™ CTS™ Rotea™ Counterflow Centrifugation System may be utilized to maintain this closed-system environment. Refer to this **application note** for details on how to harvest and dissociate spheroids by using the CTS Rotea system. With the CTS Rotea system, up to 5 x 10^9 cells (the typical yield from a 3L bioreactor culture in CTS StemScale medium) can be harvested at once. Although use of the CTS Rotea system was demonstrated with RUO StemScale medium, the application note provides a roadmap to adapt the protocol for a culture in CTS StemScale medium by switching to the CTS StemScale medium and using diluted CTS TrypLE Select Enzyme (with inclusion of DNase I) instead of StemPro Accutase reagent.

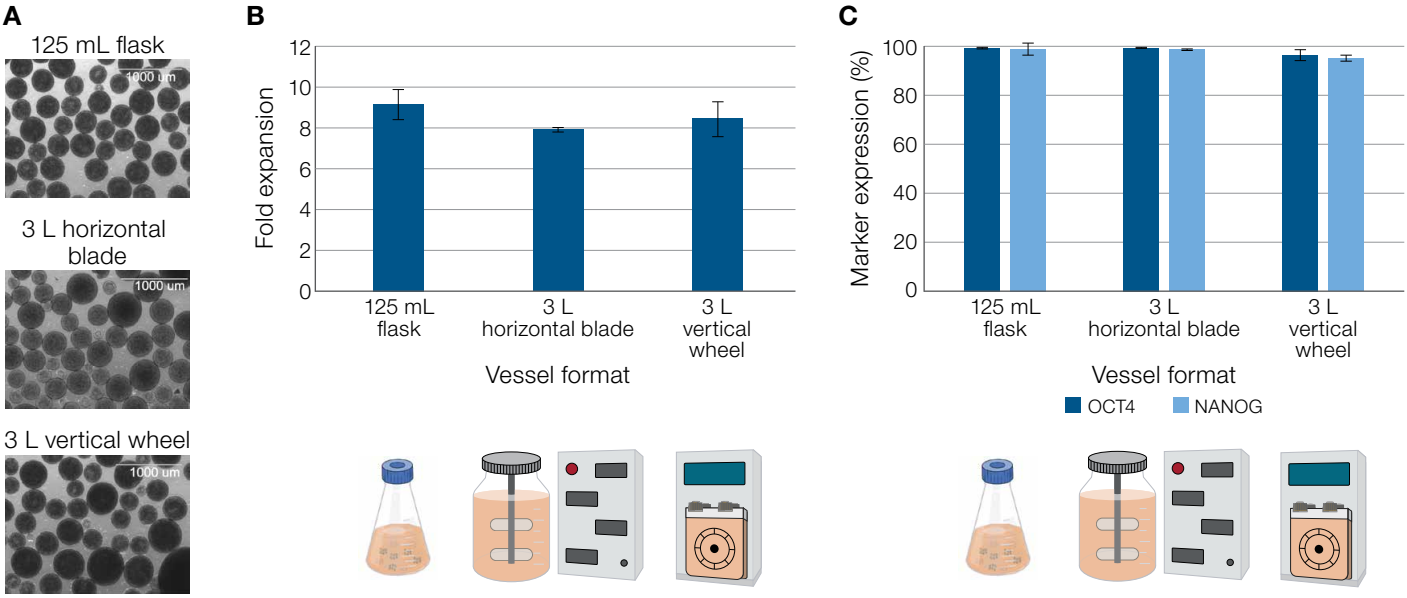
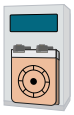
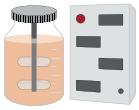


Figure 8. CTS StemScale medium promotes spheroid growth in liter-scale bioreactors. (A) Representative day 5 spheroid morphology. Spheroids grown in CTS StemScale medium continue to show high expansion potential even when grown in closed-system 3 L bioreactors. **(B)** Cumulative spheroid expansion. CTS StemScale medium supports spheroid growth in bioreactors with either a horizontal-blade or vertical-wheel impeller. The yields from these bioreactors are similar to cultures in CTS StemScale medium grown in small-scale vessels. **(C)** Bioreactor spheroid pluripotency assessment. The spheroids grown in bioreactors remain pluripotent, as assessed through flow cytometric analysis of OCT4 and NANOG markers.

Table 3. Bioreactor recommendations for growing spheroids in CTS StemScale medium. The RPM is the most critical parameter to establish and can be adjusted further from these recommendations depending on cell line performance. Spheroids can grow larger if RPM is reduced, but RPM should not be reduced to the point that large spheroids begin to fall out of suspension. Alternatively, increasing RPM will lead to nucleation of smaller spheroids.

Vessel format	Working volume (L)	Initial RPM considerations	Spheroid sedimentation time	TrypLE enzyme dilution	TrypLE enzyme dissociation volume	TrypLE enzyme dissociation time
 Vertical-wheel impeller	2–3	20–25	Up to 10 min	50–75%	50–60 mL	30–40 min
 Horizontal-blade impeller (dual-pitch blade)		Day 0: 65 Day 1–5: 130 (ramp up from 65)				

Conclusions

- CTS StemScale medium enables consistent growth of PSCs in suspension culture with a cell line–dependent 5x–10x cell expansion per passage.
- PSCs can be efficiently expanded across vessel formats at small scale (well plates and flasks) and large scale (bioreactors) using the same culture parameters.
- Cells grown for multiple passages in CTS StemScale medium maintain pluripotency and genomic stability.
- CTS StemScale medium delivers maximum expansion capability, with higher fold expansion than other PSC suspension media.

Learn more at **thermofisher.com/ctsstemscale**