ThermoFisher SCIENTIFIC

Protocol guide: CTS StemScale PSC Suspension Medium

Frequently asked questions and troubleshooting

Contents

Preparation and storage

1. **How should I store complete Gibco™ CTS™ StemScale™ PSC Suspension Medium?**
   Following reconstitution, the complete medium can be stored at 2°C–8°C for up to 2 weeks.

2. **Can I aliquot and freeze CTS StemScale PSC Suspension Supplement?**
   Yes. The CTS StemScale supplement may be aliquoted into smaller volumes upon receipt and stored at –5°C to –20°C for up to 6 months. Avoid repeated freeze-thaw cycles.

3. **Can the frozen aliquots of CTS StemScale PSC Suspension Supplement undergo multiple freeze-thaw cycles?**
   No. Each aliquot of CTS StemScale PSC Suspension Supplement should undergo only one freeze-thaw cycle. Once an aliquot is thawed, it should not be frozen a second time.

4. **Will freezing CTS StemScale PSC Suspension Supplement affect performance?**
   No. Aliquots of CTS StemScale PSC Suspension Supplement show no significant decrease in performance after one freeze-thaw cycle. Multiple freeze-thaw cycles, however, are not recommended.

Protocol recommendations

5. **How many components does the CTS StemScale PSC Suspension Medium kit consist of?**
   The CTS StemScale PSC Suspension Medium kit consists of two components: a basal medium and a supplement. Following reconstitution, the complete medium is used for all steps, from initiating cultures to feeding cultures.

6. **Is the formulation of CTS StemScale PSC Suspension Medium the same as that of RUO StemScale PSC Suspension Medium?**
   The formulation of CTS StemScale medium is similar to that of RUO StemScale medium but with specific modifications in line with regulatory guidance. These changes necessitate a few minor protocol differences between the RUO and CTS versions of StemScale medium to provide similar cell yields.

7. **What are the differences between the protocols for using RUO StemScale PSC Suspension Medium and CTS StemScale PSC Suspension Medium?**
   The major differences between the RUO and CTS StemScale medium protocols are listed in the following table.

	RUO 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
Estimated days of growth*	4–5 days	5–6 days
Feed strategy	Every other day	Daily
Seeding density	150,000 cells/mL	200,000 cells/mL

* To grow 300–400 µm diameter spheroids.

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8. **How can I prevent the spheroids in my suspension cultures from aggregating into large clumps when I remove the cultures from the orbital shaker?**
   When suspension cultures are removed from agitation, spheroids may begin to aggregate into large clumps if they remain settled for extended periods of time. To avoid undesirable aggregation, ensure that suspension cultures are not removed from the agitation source for than 15 minutes.

9. **Do you have any recommendations for handling suspension cultures when imaging?**
   Suspension cultures grown in well plates can easily be imaged under a microscope. For larger vessels such as shake flasks and spinner flasks, a small sample of spheroids should be removed and placed into a 6-well plate for imaging.

   Once in a 6-well plate, the suspension culture can be gently swirled in a circular motion to draw spheroids toward the center of the well. Images should then be acquired in a timely manner to avoid undesirable spheroid aggregation. After acquiring images, gently agitate the plate to redisperse spheroids throughout the well.

10. **How can I acquire images of suspension cultures that are not already growing in a well-plate format?**
    For suspension cultures that are growing in shake flasks or spinner flasks, remove a small volume of medium (~2–3 mL) containing spheroids from the flask. This aliquot of the spheroids can be placed into a 6-well plate for imaging.

11. **Will the time required for gravity sedimentation cause my spheroids to clump?**
    The amount of time the spheroids take to settle via gravity sedimentation should be minimized to avoid undesirable spheroid aggregation. The 5-minute gravity sedimentation recommended for orbital shaker cultures in the feeding protocol has no adverse effect on spheroids.

12. **Do I need to wait the full 5 minutes for gravity sedimentation to occur?**
    When spheroids are small and not easily visible by eye (i.e., on days 1–2 of culture), we recommend waiting the full 5 minutes to ensure all spheroids will settle to the bottom of the vessel.

    When spheroids are large and easily visible by eye (i.e., on days 3–5 of culture), the spheroids do not require the full 5 minutes of gravity sedimentation. The medium can be replaced once you can visually confirm all spheroids have settled at the bottom of the well.

    Notably, larger vessels (≥100 mL) may require some additional sedimentation time to avoid aspiration of small spheroids within the first 2 days of culture. Consider extending the sedimentation time in larger vessels by a few minutes if spheroids still remain suspended. Extending the total sedimentation time in larger vessels to 10 minutes should not adversely affect spheroid growth.

13. **How can I control the size of my spheroids?**
    The recommended method for controlling spheroid size is changing the rotation speed (RPM). When initiating cultures, a higher RPM will lead to nucleation and expansion of smaller spheroids over the culture duration. A lower RPM will result in nucleation and expansion of larger spheroids over the culture duration.

14. **Are there other variables that can affect the size of my spheroids?**
    For orbital shaker cultures, the suspension culture's volume is another metric that can influence spheroid size. When initiating cultures, a larger volume will lead to nucleation and expansion of larger spheroids over the culture duration. Therefore, it is critical to ensure that suspension cultures be maintained at a constant volume after a medium exchange. Fluctuations in volume can potentially cause undesirable changes in spheroid size. The volume in a 6-well plate, for example, should never exceed a total volume of 2.3 mL.

15. **Will spheroids be more uniform in size at higher RPM, given the same volume of medium?**
    Yes. While spheroids will be smaller at higher RPM, they will also be more uniform in size.

16. **If I do not have the Thermo Scientific™ CO_2 Resistant Shaker, how will this affect the RPM settings?**
    The RPM may need to be altered for other orbital shakers, depending on the orbital diameter. It is possible to estimate the recommended RPM for another shaker with the equation:

$$RPM_2 = \sqrt{(RPM_1^2 \times \frac{d_1}{d_2})}$$

    Where d_1 = orbital diameter of the Thermo Scientific CO_2 Resistant Shaker (19 mm); d_2 = orbital diameter of the alternative orbital shaker; RPM_1 = recommended RPM for the Thermo Scientific CO_2 Resistant Shaker; RPM_2 = recommended RPM for the alternative orbital shaker.

    Additional testing will be necessary to determine the optimal RPM based on this initial estimate.

17. **How do I obtain greater numbers of spheroids in the same culture vessel?**
    A greater number of spheroids can be obtained by simply increasing the initial seeding density. Increasing the seeding density too high (i.e., >300,000 cells/mL) is not recommended, as the medium will be depleted far more rapidly than at the recommended seeding density. Consequently, necrotic cores may develop more easily at higher seeding densities.

For orbital shaker cultures, an alternative is to adjust both RPM and culture volume simultaneously. By increasing both RPM and culture volume together, the number of cells used to seed a suspension culture can be increased while maintaining the recommended seeding density of 200,000 cells/mL and ensuring that spheroids do not fuse into a large mass of cells. Increasing both the RPM and culture volume results in a greater number of spheroids that can be harvested from the same type of orbital shaker culture vessel.

Example using a 125 mL shake flask:

- Standard recommended conditions: 70 RPM and a 20 mL culture volume
- To obtain greater numbers of spheroids: 90 RPM and a 40 mL culture volume

In general, shake flasks should have their culture volume doubled as the shaker speed increases from 70 RPM to 90 RPM.

18. How do I clean glass vessels for suspension cultures?
The steps of the recommended cleaning procedure are described below:

- Glass vessels should first be soaked in a 1% solution of 7X™ detergent for at least 2 hours, up to overnight.
- After removing the detergent solution, glass vessels should be scrubbed lightly with a brush and rinsed 3–4 times with tap water. Vessels should air-dry completely in preparation for the next step.
- Once the detergent cleaning has been completed, glass vessels must be siliconized to prevent cells from adhering to their surfaces. Addition of Thermo Scientific™ Water-Soluble Siliconizing Fluid for 5 minutes is sufficient to achieve siliconization.
- After removal of the siliconizing fluid, rinse vessels 3–4 times with tap water and air-dry.
- Place the glass vessels into sterilization bags and autoclave to ensure the glass vessels are sterile for future experiments.

19. I routinely use Gibco™ CTS™ RevitaCell™ Supplement for applications with adherent cultures. To initiate suspension cultures, can I also use CTS RevitaCell Supplement instead of Y-27632 when preparing 6-well plates or shake flasks with CTS StemScale PSC Suspension Medium?
Continue to use CTS RevitaCell Supplement for applications with your **adherent** cultures. For optimal spheroid nucleation in suspension cultures, however, we specifically recommend Y-27632.

20. How confluent should my adherent cultures be before initiating my suspension culture?
For best results, your adherent cultures should be 50–80% confluent. While all ranges of confluency are capable of forming spheroids, the overall growth and expansion of your suspension cultures may decrease if overly confluent adherent cultures are used for seeding.

21. How many total cells are needed to seed an entire 6-well plate?
Using our recommended seeding density of 200,000 cells/mL, a total of 2.4 x 10^6 viable cells are necessary to seed an entire 6-well plate.

22. What is the minimum final volume of medium necessary in each well when seeding a 6-well plate?
The recommended final volume of medium when seeding a 6-well plate is 2 mL per well at 70 RPM. We do not recommend using volumes less than 1.7 mL or more than 2.3 mL at 70 RPM. These volumes risk spheroids fusing into a large mass of cells.

23. What changes do I need to make to the protocol if I am using plates with wells smaller than those of a 6-well format?
When using smaller wells than those of a 6-well plate to grow suspension cultures, the culture volume and RPM will need to be adjusted. In general, the culture volume will decrease while the RPM will increase. The table below shows recommendations for other plate formats:

Vessel format	Culture volume	Rotation
6-well plate	2 mL per well	70–80 RPM
12-well plate	1 mL per well	90–100 RPM
24-well plate	350 µL per well	120–130 RPM
48-well plate	200 µL per well	150–160 RPM

24. Compared to the RUO StemScale PSC Suspension Medium protocol, the CTS StemScale PSC Suspension Medium protocol calls for using CTS TrypLE Select Enzyme instead of Gibco™ StemPro™ Accutase™ Cell Dissociation Reagent. What is the reason for the change?
For a cell therapy workflow, utilization of products designed for clinical applications helps minimize risk. CTS TrypLE Select Enzyme, an animal origin–free reagent, is recommended for dissociating spheroids grown in CTS StemScale medium. StemPro Accutase reagent is not recommended, as it contains animal origin components.

25. Why does the CTS StemScale PSC Suspension Medium protocol recommend diluting CTS TrypLE Select Enzyme with Gibco™ CTS™ DPBS (–/–)?
Cells can be very sensitive to dissociation with CTS TrypLE Select Enzyme, leading to some cells becoming stressed or lysed during dissociation. To minimize the sensitivity of these cells to the enzyme, we recommend diluting CTS TrypLE Select Enzyme to a lower concentration with CTS DPBS (–/–).

Dilution of CTS TrypLE Select Enzme will help maintain highly viable suspension cultures post-dissociation. For orbital shaker cultures, we recommend diluting CTS TrypLE Select Enzyme to a final concentration of 0.25X. This can be done by adding 3 mL of CTS DPBS (–/–) for every 1 mL of CTS TrypLE Select Enzyme.

For larger culture volumes (i.e., ≥100 mL), we recommend a range of 0.5X–0.75X CTS TrypLE Select Enzyme. This higher concentration accounts for the increased cell mass during dissociation.

26. If I am using diluted CTS TrypLE Select Enzyme, do I still need to include DNase I with my suspension cultures?
Yes. It is important to always add DNase I when working with CTS TrypLE Select Enzyme. Even when the CTS TrypLE Select Enzyme is diluted, lysed cells may release genomic DNA and cause spheroid aggregation issues. We recommend adding DNase I to suspension cultures at a concentration of 0.1 U/mL. Higher concentrations (such as 1 U/mL) may also be used in large-scale vessels.

27. During passaging, is it necessary to perform a wash with CTS DPBS (–/–) prior to addition of the diluted CTS TrypLE Select Enzyme?
No. Washing with CTS DPBS (–/–) before adding the diluted CTS TrypLE Select Enzyme is not necessary.

28. How do I determine final cell concentration using a hemocytometer?
The equation to use for a hemocytometer is:
Final cell concentration (in cells/mL) = (average cell count from one corner square) x (dilution factor) x (10,000)

29. When imaging spheroids, will I be able to see indications of necrosis or estimate the size of necrotic cores?
While it can be difficult to determine the presence of a necrotic core, a good indication is the coloration of the spheroid. Spheroids become darker in color as they grow and begin to form a necrotic core. We recommend passaging spheroids when the average spheroid diameter is between 300 and 400 µm. Beyond this size range, the likelihood of a necrotic core forming will increase.

Large spheroids that begin to appear dark are not unusual and should be passaged, as they are likely near the point of starting to develop a necrotic core. Healthy spheroids tend to have a dark appearance as the diameter gets closer to 400 µm.

Small spheroids that appear dark are likely experiencing stress and may require more frequent medium exchanges or a lower initial seeding density. When spheroids are small, they should have a brighter appearance compared to larger spheroids.

30. What volume of diluted CTS TrypLE Select Enzyme should I use, and how long should I incubate the spheroids in the enzyme at 37°C?
The volume of diluted CTS TrypLE Select Enzyme to use and the incubation time will vary depending on the culture size and vessel. In general, larger vessels with greater numbers of spheroids will require higher volumes of the enzyme to account for the increased cell mass. Guidance is provided in the following table. Incubation times can vary and may be extended for longer than listed in the table if the spheroids are not completely dissociated.

Vessel format	CTS TrypLE Select Enzyme concentration	CTS TrypLE Select Enzyme volume	CTS TrypLE Select Enzyme incubation time
6-well plate	0.25X	1 mL per well	5 min
24-well plate	0.25X	1 mL per well	5 min
125 mL shake flask	0.25X	5 mL	10 min
250 mL shake flask	0.25X	10 mL	10–15 min
500 mL shake flask	0.25X	20 mL	15–20 min
100 mL vertical wheel	0.5X	15 mL	15–20 min
500 mL vertical wheel	0.5X	50 mL	20–25 min
125 mL spinner flask	0.25X	10 mL	10–15 min

31. Can I centrifuge spheroids during routine feeding to assist with medium exchange?
No. The spheroids should not be centrifuged during routine feeding. Centrifugation may damage the large spheroids and reduce cell viability.

32. During passaging, is it necessary to place the conical tube with the spheroids in a water bath or incubate at 37°C after adding the diluted CTS TrypLE Select Enzyme?
Yes. Dissociation with CTS TrypLE Select Enzyme occurs faster at 37°C, which helps break apart spheroids more rapidly.

33. How do I assess the degree of spheroid dissociation during incubation with the CTS TrypLE Select Enzyme? How do I know if dissociation is complete?
Dissociation can be assessed by visual inspection. The longer the spheroids are exposed to the enzyme, the more easily they will break apart with gentle agitation of the conical tube; floating clumps should not be present. Dissociation is complete when there is a homogeneous, cloudy single-cell suspension.

Typically, spheroids will need to be exposed to the CTS TrypLE Select Enzyme for at least half of the recommended dissociation time before they begin to noticeably break apart. By gently agitating the tube every 2–3 minutes, it is possible to estimate whether the spheroids are at the point of breaking down into single cells. Toward the end of the recommended dissociation time, the spheroids can be more vigorously agitated to promote their full dissociation.

It is important to never triturate whole spheroids using a 1,000 µL pipette tip, as doing so will negatively impact their viability and may lead to cell death. Instead, wait for the spheroids to go through the gentle dissociation into single cells, and then proceed to use a 1,000 µL pipette tip to ensure the remaining clumps are fully broken apart.

34. How should I passage my cultures that are larger than 20 mL? Should I use multiple 50 mL conical tubes for dissociation and cell counting?

Multiple 50 mL conical tubes may be required for large-scale cultures. Large-volume conical tubes (i.e., 250 mL) can also be utilized if your centrifuge is able to accommodate them.

Once the supernatant has been removed from the pelleted spheroids, we recommend combining all the spheroids into a single conical tube prior to dissociating them with the diluted CTS TrypLE Select Enzyme. This will reduce hands-on time during dissociation. However, multiple conical tubes can be used for dissociation if you prefer. For cell counting, all of the dissociated cell suspensions should be combined into a single conical tube prior to centrifugation.

35. Does the CTS StemScale passaging protocol require a cell strainer to dissociate spheroids?

No, the CTS StemScale passaging protocol does not require a cell strainer, as spheroids are sufficiently dissociated into single cells through exposure to the CTS TrypLE Select Enzyme.

36. How many 6-well plates or culture vessels can I feed at the same time?

When first growing suspension cultures in well plates or other culture vessels, we recommend feeding these vessels one at a time. As you become more experienced and familiar with the performance of their cell lines in suspension, you may choose to increase the number of cultures that are fed simultaneously. Experienced users can become comfortable feeding multiple 6-well plates or 125 mL shake flasks simultaneously. As long as the medium is exchanged in all vessels in a timely fashion, the risk of settled spheroids aggregating into a large mass will be minimized.

37. When feeding 6-well plates, how much medium should I remove from each well?

We recommend removing 50% of the spent medium from each well and replacing it with an equal volume of fresh medium. Depending on the cell line, or if the seeding density was higher than recommended, you may choose to replace more than 50% of the medium.

38. When feeding a shake flask, can I swirl the flask before letting the spheroids settle to the bottom?

Yes. Swirling the flask before letting the spheroids settle can help collect the spheroids in the center at the bottom of the flask. This placement may allow for easier removal of medium. However, be sure that the flask is not disturbed further once you are ready to remove the spent medium.

39. Do you have any helpful tips for feeding my suspension cultures in shake flasks?

Yes. The suggestions below may be helpful when feeding your suspension cultures:

- Loosen the cap on the shake flask before preparing your serological pipette so that the cap can be easily removed with one hand.
- After removing the spent CTS StemScale medium, be sure to add the new medium down the side of the flask to avoid dispensing directly on top of the spheroids settled at the bottom of the flask. This technique helps prevent the formation of bubbles in the medium.

40. Do you have recommendations for culturing spheroids in liter-scale bioreactors?

Culturing spheroids in bioreactors is similar to culturing spheroids in small-scale vessels. The seeding density should be the same (200,000 cells/mL), along with the inclusion of Y-27632 and DNase I. Following these recommendations, the spheroids can be grown for approximately 5 days. For additional considerations, the following table has some recommendations for different types of bioreactors:

	Vertical wheel bioreactor	Horizontal blade impeller bioreactor
Working volume	2–3 L	
RPM	20–25 RPM	65 RPM initial for seeding (day 0) 130 RPM final for growth (ramp up on day 1)
Spheroid sedimentation time	Up to 10 min	
CTS TrypLE Select Enzyme concentration	0.5X–0.75X	
CTS TrypLE Select Enzyme dissociation volume	50–60 mL	
CTS TrypLE Select Enzyme dissociation time	30–40 min	

Performance

41. Does CTS StemScale PSC Suspension Medium maintain the pluripotency of hPSCs in suspension culture?

Yes. CTS StemScale medium maintains the pluripotency of hPSCs grown as spheroids in suspension culture, as assessed by both flow cytometric analysis and the PluriTest™ Assay.

42. Does CTS StemScale PSC Suspension Medium maintain normal karyotypes of hPSCs in suspension culture?

Yes. hPSCs grown as spheroids in CTS StemScale medium are genomically stable, as assessed by the Applied Biosystems™ KaryoStat™ Assay.

43. Is CTS StemScale PSC Suspension Medium able to maintain high viability of hPSCs in suspension culture?

Yes. Trypan blue staining of single cells obtained from dissociated spheroids indicates that viable cell counts of suspension cultures remain high (>90%).

44. Does CTS StemScale PSC Suspension Medium maintain the trilineage differentiation potential of hPSCs in suspension culture?
Yes. CTS StemScale medium maintains the trilineage differentiation potential of hPSCs, as assessed by the Applied Biosystems™ TaqMan™ hPSC Scorecard™ kit.

45. Is CTS StemScale PSC Suspension Medium compatible with different cell lines?
Yes. We have evaluated multiple iPSC and ESC cell lines, all of which were demonstrated to be compatible with CTS StemScale medium. Notably, growth in CTS StemScale medium is cell-line dependent, as some lines are observed to expand in suspension more effectively than other lines.

46. What vessels are suitable for my suspension cultures grown in CTS StemScale PSC Suspension Medium?
A variety of culture vessels are suitable for growing suspension cultures in CTS StemScale medium, including non–tissue culture treated well plates, plain-bottom shake flasks, spinner flasks, and liter-scale bioreactors.

47. Does CTS StemScale PSC Suspension Medium require the use of microcarriers to form spheroids?
No. CTS StemScale medium promotes the formation of spheroids through self-aggregation in the presence of a ROCK inhibitor.

48. On day 1 after seeding in CTS StemScale PSC Suspension Medium, spheroids are forming, but they are not perfectly round. Is this normal?
Yes. In suspension cultures grown using an orbital shaker, spheroids that are not perfectly round on day 1 may be observed. By day 2, these spheroids will take on a more uniform rounded shape.

49. What is the fold expansion per passage that I can expect using CTS StemScale PSC Suspension Medium?
On average, we have observed 5–10x expansion per passage, but this fold expansion can vary by cell line.

50. What is the recommended passaging schedule when using CTS StemScale PSC Suspension Medium?
The CTS StemScale PSC Suspension Medium protocol recommends passaging every 5–6 days, depending on spheroid size. We recommend passaging suspension cultures when the average spheroid diameter is 300–400 µm.

Passaging cultures earlier is also an option but may result in a lower cell yield. Early passaging is an option for avoiding passaging on a weekend.

51. Do I need to feed my cultures daily with CTS StemScale PSC Suspension Medium?
Yes. The CTS StemScale medium feeding schedule recommends daily feeding to maximize cell health. It is possible to skip one day on the weekend if desired, but otherwise cultures growing in CTS StemScale medium should always be fed daily.

52. Should I be concerned about waste products accumulating in my suspension cultures when using the CTS StemScale PSC Suspension Medium feeding method?
No. The CTS StemScale PSC Suspension Medium feeding method involves replacing 50% of the spent medium with fresh medium. This feeding method prevents spheroids from growing in a medium that is accumulating significant quantities of waste products.

53. Can I thaw frozen cells directly in CTS StemScale PSC Suspension Medium?
Yes. Cells that have been cryopreserved can be thawed directly in CTS StemScale medium. The same protocol for thawing cells for adherent cultures can be utilized for suspension cultures. Once cell counts have been obtained, the thawed cells can then be seeded into suspension cultures using the recommended seeding conditions for CTS StemScale medium.

Users should expect cells to require one passage in suspension before recovering from cryopreservation. After this recovery passage, suspension cultures will perform similarly to those seeded from adherent cultures.

54. Can I freeze my spheroids?
No. We recommend that suspension cultures be cryopreserved as single cells.

Troubleshooting

55. Spheroids are not forming. What should I do?
First, confirm that ROCK inhibitor (Y-27623) was added to the culture on day 0. Spheroids will not form without the presence of a ROCK inhibitor. If a ROCK inhibitor was added, then reduce the RPM applied to the culture vessel. Spheroids are unable to form if the RPM is too high.

56. The spheroids are forming large clumps. Is there anything I can do?
First, confirm that non–tissue culture treated vessels, which prevent spheroids from adhering to the bottom of the vessels, are being used.

Next, confirm that DNase I was added on the day of seeding the suspension culture vessels. Without DNase I, it is possible that genomic DNA from lysed cells is causing the clumping.

After confirming the above, if spheroids are still forming large clumps, then the RPM applied to the culture vessel may be too low. Increasing the RPM will prevent spheroids from aggregating into large clumps.

Alternatively, for orbital shaker cultures, the volume of medium in the culture vessel may be too large for the applied RPM, preventing spheroids from staying in suspension. Decreasing the volume of the medium will prevent spheroids from aggregating into large clumps.

57. **The spheroids were growing well but have suddenly formed large clumps. How can this be prevented?**
First, confirm that the suspension cultures have not been removed from agitation for extended periods of time. When imaging spheroids, cultures must not be removed from agitation for more than 15 minutes.

For orbital shaker cultures, confirm that the culture volume has not increased since day 0. Increasing the culture volume makes it more difficult for spheroids to be sufficiently agitated. If the culture volume has drifted, spheroids may no longer remain suspended at the current RPM.

Confirm that the orbital shaker was continuously powered. A power outage may have occurred and interrupted agitation temporarily. Spheroids require constant agitation to grow properly. Having the orbital shaker connected to a battery backup will ensure that the cultures will be continuously agitated even in the event of a power outage.

58. **Is it normal to see small dots present between the spheroids during imaging?**
Yes. These are single cells that have not been incorporated into spheroids. It is normal for suspension cultures to still have numerous single cells visible in the background. The presence of single cells after spheroid nucleation will not interfere with spheroid expansion over the culture duration.

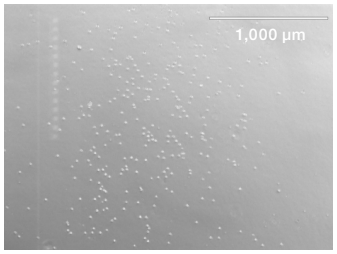
59. **My spheroids looked healthy before passaging, but failed to form spheroids in the next passage even though I included ROCK inhibitor. What happened?**
It is likely that the spheroids were damaged during the dissociation step of the passaging protocol. First, confirm that the large spheroids themselves were never triturated with a 1,000 µL pipette or serological pipette. The pipette should only be used once with large spheroids—to gently transfer the spheroids from a vessel to a conical tube. Further handling of large spheroids using a pipette will damage the spheroids and reduce cell viability.

Additionally, confirm that the spheroids were not overexposed to the dissociation enzyme. It is important to stop dissociation once the spheroids have broken down into single cells and the liquid suspension is visibly cloudy. Overexposure to the dissociation enzyme risks further damaging the cells and reducing their viability.

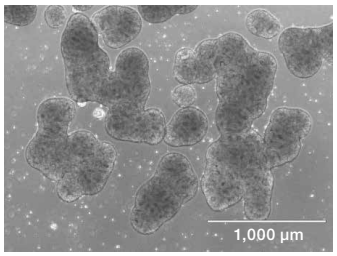
60. **Why is there less medium in my culture even if I am adding back the same volume that I remove when feeding?**
A lower volume of medium may be due to evaporation. Additional fresh medium may need to be added during the next feed day to account for the evaporated volume. To help minimize evaporation, keep the incubator water pan full.

61. **What do the suspension cultures look like:**

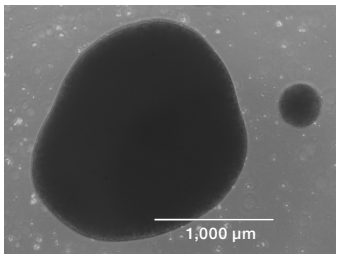
a. **If I do not add Y-27632 at day 0?**
Spheroids will not form without the presence of a ROCK inhibitor on day 0.



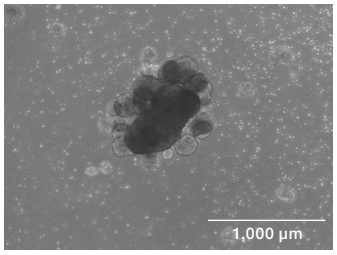
b. **If I have removed my cultures from agitation for an extended time period?**
Spheroids will have a tendency to aggregate into large clumps. The image below illustrates how individual spheroids are beginning to form large clusters.



c. **If the volume of medium in my culture is too high or the RPM is too low?**
If the cells in the culture vessel are not sufficiently agitated, they will all aggregate into a large clump.



d. **If I use CTS TrypLE Select Enzyme without including DNase I?**
Some cells may lyse and release genomic DNA into the suspension culture environment during dissociation with CTS TrypLE Select Enzyme. Without DNase I, the presence of small quantities of genomic DNA will cause some spheroids to aggregate into a single clump.



Comparison to other commercially available products

62. Managing multiple components in a medium kit can be challenging. Does the CTS StemScale PSC Suspension Medium kit have a similar number of components compared to other kits?

The CTS StemScale PSC Suspension Medium kit consists of two components: a basal medium and a supplement. Following reconstitution, the complete medium is used for all steps, from initiating cultures to feeding cultures.

Other suspension culture medium kits sometimes consist of multiple components, which may be used at different points in the suspension culture process.

63. Are there advantages to the CTS StemScale feeding method, compared to those for other suspension culture media?

The CTS StemScale medium feeding method involves replacing 50% of the spent medium with fresh medium. This feeding method prevents spheroids from being cultured in a medium that is accumulating significant quantities of waste products.

Feeding methods that use a fed-batch or overlay strategy do not remove the spent medium from suspension cultures. These methods reduce hands-on time when feeding cultures; however, the accumulation of waste products is likely to negatively impact the health of these cells.

The CTS StemScale medium feeding strategy also ensures a consistent volume throughout the growth period of the PSC spheroids. This is in contrast to batch-feeding methods where the volume increases incrementally. Culture volume is one of the determinants of shear force and spheroid size, and the 50% feeding strategy maintains the same volume throughout the growth period.

64. How does the CTS StemScale passaging protocol compare to protocols for other suspension culture media?

The CTS StemScale medium protocol recommends passaging every 5–6 days, depending on spheroid size. We recommend passaging suspension cultures when the average spheroid diameter is 300–400 µm, which occurs after approximately 5–6 days of growth. Passaging earlier is also an option, although the final cell yield will be lower than what is typically obtained.

Passaging protocols for other suspension culture media may be less flexible, depending on whether they utilize a fed-batch or overlay strategy. Generally, these cultures require a strict passaging schedule with little room for flexibility on weekends.

The CTS StemScale medium passaging protocol also does not require the use of cell strainers, while some other PSC suspension culture medium protocols may specifically require these tools.

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