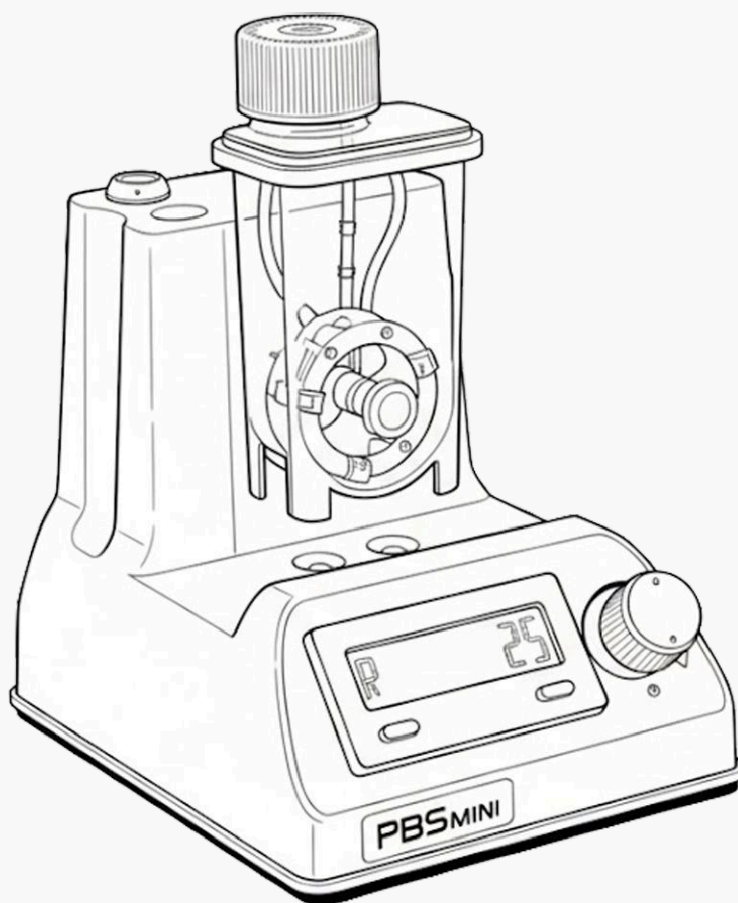


Protocol for Expansion of PSC Aggregates in the PBS-Mini Vertical-Wheel Bioreactor



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1. Overview

The scope of this protocol is to provide step-by-step guidance for forming and expanding pluripotent stem cell (PSC) aggregates, starting with 2D adherent cultures in T-flasks for a seed train followed by 3D suspension cultures in PBS-Mini Vertical-Wheel® bioreactors for aggregation and expansion. The recommended culture parameters were developed by PBS Biotech's CDO Lab through extensive optimization studies using multiple PSC lines and media formulations [1].

We recommend using the following methods and parameters as a starting point for optimization, as performance may vary between cell lines and specific processes. As indicated in Figure 1, these methods are specific to upstream processing and transitioning from 2D to 3D culture. Users can continue to develop manufacturing processes such as expansion, directed differentiation, and harvest.

Need help tailoring your process for optimal results? PBS Biotech's Application Engineers can assist with strategies to achieve your target aggregate size, cell density, or differentiation goals. Contact them at app.eng@pbsbiotech.com.

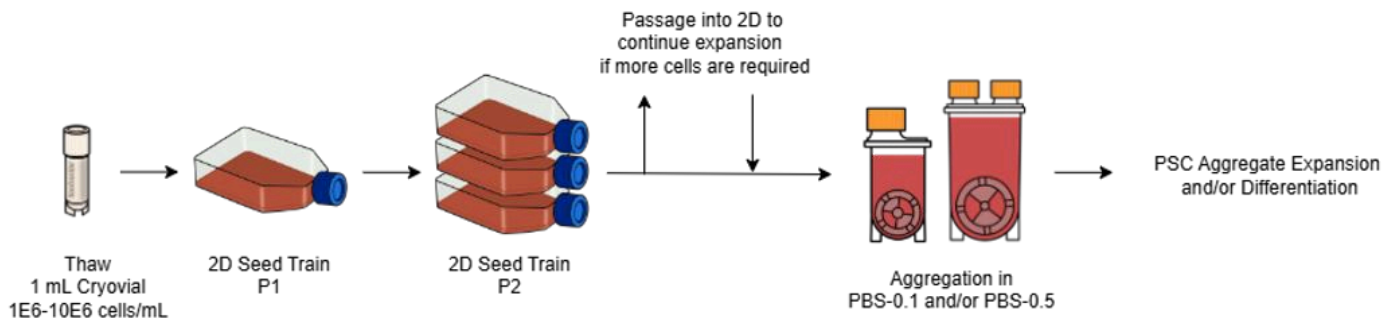


Figure 1. Sample 2D seed train and 3D expansion strategy for PSCs in the PBS-Mini

[1] Borys BS, Dang T, Worden H, Larijani L, Corpuz JM, Abraham BD, Gysel EJ, Malinovska J, Krawetz R, Revay T, Argiropoulos B, Rancourt DE, Kallos MS, Jung S. Robust bioprocess design and evaluation of commercial media for the serial expansion of human induced pluripotent stem cell aggregate cultures in vertical-wheel bioreactors. Stem Cell Res Ther. 2024 Jul 29;15(1):232. doi: 10.1186/s13287-024-03819-9. PMID: 39075528; PMCID: PMC11288049. [Link](#)

2. 2D Culture: Seed Train in T-Flask

The quality of your 2D seed train is extremely important for the downstream performance of your PSC culture in the PBS-Mini. We recommend a minimum of two passages in adherent culture before transitioning your cells to suspension culture, especially if you are starting with cryopreserved cells.

If more cells are required to seed into the bioreactors, additional passages can be included to reach the target cell number. Recommendations from the PBS Biotech CDO Lab for 2D PSC culture are noted in Table 1. We recommend using these parameters as a starting point for optimization, as performance may vary between cell lines and media formulations.

Passage	Key Process Parameter	Value
Thaw – Passage 1	Seeding density	15,000 viable cells/cm ²
	Culture duration	3 days
	Medium exchange regime	100% medium exchange daily
Passage 2+	Seeding density	5,000 viable cells/cm ²
	Culture duration	4 days
	Medium exchange regime	100% medium exchange daily
All 2D Culture	Medium volume to culture vessel surface area	0.32 mL/cm ²
	DPBS and enzyme volume to culture vessel surface area	0.08 mL/cm ²
	Number of DPBS washes prior to addition of enzyme	2
	Enzyme incubation time	5 to 20 minutes
	Centrifuge settings	500 RCF for 5 minutes

Table 1. 2D Culture Parameters

A. Inoculation in T-Flask

The following method and associated reagent volumes assume the starting material for culture is a 1 mL vial of cryopreserved PSCs at a density of 1×10^6 to 1×10^7 cells/mL. It also assumes standard incubator conditions of 85–95% humidity, 5% CO₂, and 37°C.

Cells / Reagents	Consumables
- Cryopreserved PSCs - PSC culture medium mTeSR1*, TeSR AOF, StemFlex, StemScale, E8, etc. - ROCK inhibitor Y-27632 or equivalent supplement - Coating substrate Matrigel, iMatrix-511, Laminin 521, etc.	- T-flasks - Serological pipettes - Aspirating pipettes 15 mL/50 mL conical tubes - Microcentrifuge tubes

*Reagent options used in optimized PSC process in **bold**

Preparation

1. Formulate PSC Thawing Medium: PSC culture medium + 10 μ M ROCKi.

It is important to follow the vendor recommendations for medium expiration dates after formulation. We also recommend aliquoting medium while still cold at the volumes required for different process days to avoid multiple heating and cooling cycles that can negatively impact the stability of medium components.

2. Prepare each T-flask with coating substrate, if required, according to the vendor's recommendations.
3. Batch each flask with appropriate volume of PSC Thawing Medium and set them inside the incubator.
4. Prewarm remaining PSC Thawing Medium in the incubator.

Thaw Cryovial

5. Thaw the cryovial containing frozen cells in a water bath at 37°C until only a small ice crystal remains.
6. Using a 5 mL serological pipette, gently transfer thawed cell suspension to a 15 mL conical tube containing 9 mL pre-warmed PSC Thawing Medium for a final volume of 10 mL.
7. Gently triturate the cell suspension 2 times while tilting the tube at a 45° angle to achieve a homogenous mixture.
8. Centrifuge resuspended cells in conical tube at 300 x g for 5 minutes.
9. After centrifugation, transfer the conical tube to BSC and aspirate supernatant, making sure not to disturb the cell pellet.
10. Resuspend cell pellet with 5 mL PSC Thawing Medium. Gently triturate 5 times with a 5 mL serological pipette or until the cell pellet is fully resuspended.
11. Add another 5 mL PSC Thawing Medium into the resuspended solution for a total volume of 10 mL.

Sample and Count

12. Triturate the cell suspension 3–5 times with a 5 mL serological pipette. On the final trituration, take one 0.5 mL sample in a 1.5 mL Eppendorf tube for counting.
 13. Repeat Step 1 and take a second 0.5 mL sample.
 14. Keep the conical tube containing the total cell suspension in the incubator while counting cells.
 15. Vortex Eppendorf tubes with samples for 3 seconds at 3000 rpm and take 2 counts from one aliquot. Repeat for a second sample and calculate the average of all counts (4 total counts).
- Getting the right initial seeding density is very important for the health of the culture so we recommend taking several counts. If there is more than 10% variance between the two samples, vortex both aliquots again and repeat this step.*

Seed Flask

16. Prepare cell suspension for seeding based on recommended densities in **Table 1**.

The table below includes sample volumes for seeding. Your values may be different based on your cells, media, and experimental conditions.

Vessel	Batching Volume	Target Seeding Density	Inoculum Density	Inoculum Volume	Final Culture Volume
T-25	6 mL	15,000 viable cells/cm ² (46,875 cells/mL)	187,500 viable cells/mL	2 mL	8 mL
T-75	18 mL			6 mL	24 mL

Sample calculations for 2D thaw and seeding

- 17. Using an appropriately-sized serological pipette (typically 5 mL or 10 mL), mix the inoculum suspension at least 5 times.
- 18. Seed inoculum into prepared culture flasks. Gently add cells by submerging the tip of serological pipette containing cells below each flask’s liquid level prior to pipetting in the inoculum suspension.
- 19. Gently rock and swirl each flask in a figure 8 motion to evenly disperse the cell suspension over the entire surface.
- 20. Observe cells under the microscope to confirm even dispersal and place flasks in the incubator.

B. Medium Exchange in T-Flask

General recommendations for passage length and medium exchange regime are listed in **Table 1** based on experiments performed by the CDO Lab using multiple PSC cell lines in adherent culture. However, the rate of expansion is cell-line dependent and should be characterized for any PSC line used. Daily 100% medium exchange is recommended, though some media formulations may vary, and vendor recommendations should be considered.

Materials

Cells / Reagents	Consumables
PSC culture medium mTeSR1*, TeSR AOF, StemFlex, StemScale, etc.	- Serological pipettes - Aspirating pipettes

Reagent options used in optimized PSC process in **bold*

Preparation

1. Formulate and prewarm PSC culture medium.
2. Prior to medium exchange, check the morphology and confluency of the PSCs under the microscope and take images at 4x and 10x magnification, noting confluency and any abnormalities.

Measuring the pH of the spent medium from the flask and comparing it to the pH of fresh medium can also show whether the cell density exceeds the medium pH buffering capacity. This data can also help PBS Application Engineers troubleshoot any issues with your seed train culture.

Medium Exchange

3. Transfer T-flasks to BSC.
4. Aspirate spent medium.
5. Transfer fresh, prewarmed medium into each flask.

Instead of dispensing medium directly onto cells, tilt the flask and dispense medium along the flask wall.

6. Transfer flasks to incubator.

C. Harvest from T-Flask

PSCs in 2D culture should be harvested once they reach 70-80% confluency to avoid spontaneous differentiation, help maintain pluripotency, and keep cells in a proliferative state while passaging. When passaged during the exponential growth period, PSCs have improved survival and can quickly recover to re-establish cell-to-cell interactions when seeded back into 2D culture or into 3D culture for aggregate formation.

Materials

Cells / Reagents	Consumables
• PSC culture medium mTeSR1*, TeSR AOF, StemFlex, StemScale, etc. • ROCK inhibitor Y-27632 or equivalent • Enzymatic dissociation reagent Accutase, TrypLE, etc. • DPBS without calcium and magnesium	• PBS-Mini 0.1 and/or 0.5 vessels • Serological pipettes • Aspirating pipettes • 15 mL/50 mL conical tubes • Microcentrifuge tubes

Reagent options used in optimized PSC process in **bold*

Preparation

1. Formulate PSC Inoculation Culture Medium: PSC culture medium + 10 μ M ROCKi.
2. Aliquot enzymatic dissociation reagent, formulate with 10 μ M ROCKi, then prewarm in the incubator or water bath.

Do not re-use enzymatic dissociation reagents that have been warmed to 37°C. PBS Biotech's CDO Lab has shown that the addition of ROCKi to the enzymatic dissociation reagent improves viability of PSCs after harvest.

3. Prior to harvesting, check the morphology and confluency of PSCs under the microscope and take images at 4x and 10x magnification, noting confluency and any abnormalities.

Measuring the pH of the spent medium from each flask and comparing it to the pH of fresh medium can also show whether the cell density is exceeding the medium pH buffering capacity. This data can also help PBS Biotech's Application Engineers troubleshoot any issues with your seed train culture.

Harvest

4. Transfer T-flasks to BSC and aspirate spent medium.
5. Add pre-warmed DPBS (0.08 mL/cm²) to each flask.
Instead of dispensing DPBS directly onto cells, tilt the flask and dispense DPBS along the flask wall.
6. Rinse the attached cell layer by gently rocking each flask back and forth several times.
7. Aspirate DPBS.
8. Repeat Steps 5 through 7 for two total rinses with DPBS.
9. Add pre-warmed enzymatic dissociation reagent (0.08 mL/cm²) to each flask.
10. Gently rock each flask to ensure that the attached cell layers are completely covered by enzymatic dissociation reagent.
11. Place flasks into the incubator at 37°C for 5 minutes, then check for cell detachment using a microscope. Greater than 95% of cells should be detached after incubation. If not, incubate for an additional 1 to 2 minutes.
Dissociation time may vary depending on the media formulation and/or coating substrate used during PSC expansion. If there are issues with detachment of cells from the flask surface, wash 1 to 2 times with DPBS—prior to adding the dissociation reagent or extend dissociation time. We do not recommend the use of cell scrapers for PSC detachment.
12. Collect the detached cells from each flask using a serological pipette and transfer into 50 mL conical tubes.
13. Add PSC Inoculation Culture Medium (0.08 mL/cm²) to each flask to rinse and collect remaining cells. Add medium and cells to 50 mL tubes already containing cells.
14. Centrifuge tubes at 500 x g for 5 minutes.
15. After centrifugation, transfer tubes to BSC and aspirate supernatant, making sure not to disturb the cell pellet.
16. Using a 5 mL serological pipette, gently resuspend the cell pellet in each tube with 10 mL prewarmed PSC Inoculation Culture Medium.

Sample and Count

1. Gently mix each tube by triturating 5 times. On the final trituration, take one 0.5 mL sample in a 1.5 mL Eppendorf tube for counting.
2. Repeat Step 1 and take a second 0.5 mL sample.
3. Keep the conical tubes containing the total cell suspension in the incubator while counting cells.
4. Vortex Eppendorf tubes with samples for 3 seconds at 3000 rpm and take 2 counts per aliquot. Repeat for second aliquot and calculate the average of all counts (4 total counts).

Getting the right initial seeding density is very important for the health of the culture so we recommend taking several counts. If there is more than 10% variance between the two samples, vortex both aliquots again and repeat this step.

Next Steps

1. For seeding back into 2D culture, refer to the previous section: [Inoculation in T-Flask → Seed Flask \(pg. 4\)](#)
2. For inoculation into PBS-Mini vessels, refer to the following section: [Inoculation in PBS-Mini \(pg. 11\)](#)

3. 3D Culture: Expansion in PBS-Mini

When designing a PSC aggregate process in the PBS-Mini, it is important to understand the effects of the various process parameters (i.e., agitation rate, seeding density, and medium exchange methods) and to identify your process goals (i.e., target yields, aggregate size, and pluripotency).

Further details on these topics can be found on our website at <https://www.pbsbiotech.com/webinars>.

Additionally, How-To videos for PBS-Mini processing are available on our website at <https://www.pbsbiotech.com/how-to-videos>.

While cell culture performance may vary depending on cell lines and media formulations, we recommend starting with the parameters listed in Table 2 which reliably meet the following criteria on Day 7:

- 1.5×10^6 to 2.0×10^6 cells/mL
- Average aggregate diameter $\leq 400 \mu\text{m}$ and 87% of all aggregates within $\pm 50 \mu\text{m}$

Need help tailoring your process for optimal results? PBS Biotech's Application Engineers can assist with strategies to achieve your target aggregate size, cell density, or differentiation goals. Contact them at app.eng@pbsbiotech.com.

Vessel	Key Process Parameter	Value
PBS-Mini 0.1	Agitation rate	60 RPM <i>Recommended scalable working range: 50 to 80 RPM</i>
	Seeding density	20,000 cells/mL
	Working volume	60 to 100 mL
	Medium exchange method	Discrete 50% medium exchange via in-vessel settling (D3, D4, D5, D6)
PBS-Mini 0.5	Agitation rate	40 RPM <i>Recommended scalable working range: 24 to 42 RPM</i>
	Seeding density	20,000 cells/mL
	Working volume	300 to 500 mL
	Medium exchange method	Continuous perfusion (0.5VVD to 1 VVD)*

Table 2. 3D Parameters for 7-Day PSC Expansion

* More information on continuous perfusion setup and parameter recommendations can be found on PBS Biotech's website:

- [PBS-Mini 0.5 Vessel with 60 \$\mu\text{m}\$ Perfusion Filter - User Manual](#)
- [Aggregate Perfusion Medium Exchange - Application Note](#)

A. Inoculation in PBS-Mini

The following inoculation method is related to the following harvest method sections:

- [Harvest from T-Flask \(pg. 7\)](#)
- [Harvest and Dissociation in Conical Tube \(pg. 16\)](#)
- [Harvest and Dissociation in PBS-Mini \(pg. 18\)](#)

It is recommended to prepare the reagents and materials listed in this section prior to harvesting to minimize the amount of time that cells are stationary prior to inoculation.

When transitioning from 2D to 3D, we also recommend creating a control culture by seeding a T25 or T75 flask with the same inoculum used to seed the PBS-Mini. This protocol assumes standard incubator conditions of 85 to 95% humidity, 5% CO₂, and 37°C.

Materials

Cells / Reagents	Consumables
• PSC culture medium mTeSR1*, TeSR AOF, StemFlex, StemScale, etc. • ROCK inhibitor Y-27632 or equivalent	• PBS-Mini 0.1 and/or 0.5 vessels • Serological pipettes • Aspirating pipettes • 15 mL/50 mL conical tubes • Microcentrifuge tubes

*Reagent options used in optimized PSC process in **bold**

Preparation

1. Decontaminate and install PBS-Mini Base(s) as necessary into the incubator.
When not in use, the PBS-Mini Base should not be stored in the incubator. Prolonged exposure to incubator conditions can shorten the instrument's lifespan.
2. Formulate PSC Inoculation Culture Medium: PSC culture medium + 10 µM ROCKi.
3. Prewarm PSC Inoculation Culture Medium and batch culture vessels (see Table 2), then place each vessel on a PBS-Mini Base set to the correct agitation inside the incubator.
We recommend batching medium for an extended period in a vessel prior to inoculation to allow medium to equilibrate temperature and dissolved gases. Equilibration overnight with agitation is best, but can be for a minimum of 2 hours for the PBS-Mini 0.1 vessel or 4 hours for the PBS-Mini 0.5 vessel. Other containers can be used but equilibration may take longer without venting or agitation.
4. Formulate enzymatic dissociation reagent with 10 µM ROCKi and prewarm in the incubator.
Do not re-use enzymatic dissociation reagents that have been warmed to 37°C. PBS Biotech's CDO Lab has shown that the addition of ROCKi to the enzymatic dissociation reagent improves viability of PSCs after harvest.

Inoculate PSCs in PBS-Mini

5. Prepare cell inoculum suspension based on values in **Table 2**.

The table below includes suggested values. Your values may be different based on your cells, medium, and experimental conditions. Prepare enough inoculum to inoculate all vessels from the same inoculum suspension to ensure consistency and accuracy between vessels.

Vessel	Batching Volume	Target Seeding Density	Inoculum Density	Inoculum Volume	Final Culture Volume
PBS-Mini 0.1	95 mL	20,000 viable cells/mL	400,000 viable cells/mL	5 mL	100 mL
PBS-Mini 0.5	475 mL			25 mL	500 mL

Sample calculation for 3D inoculation

6. Using an appropriately-sized serological pipette, mix the inoculum suspension at least 5 times, then add inoculum suspension into each prepared PBS-Mini vessel to reach the target seeding density.
7. Place each vessel onto a PBS-Mini Base inside the incubator.
8. Turn on agitation for each base and ensure the correct agitation speed is set. Check that each Vertical-Wheel impeller is spinning smoothly before closing the incubator.
9. After 2 to 4 hours of inoculation, take a small sample and count the cells to verify cell inoculation density and viability.

This is optional but strongly recommended. If cell viability is <80% the cause may be the quality of cell bank or the mixing and transferring of inoculum suspension.

B. Medium Exchange in PBS-Mini

Take extra precautions to maintain aseptic techniques during this process as it poses the greatest risk for contamination. For a PSC aggregate culture, 50% medium exchange on D3, D4, D5, and D6 of a 7-Day culture is recommended as a starting point. This schedule can be optimized based on cell growth and media analysis. Medium exchange on D1 or D2 is not recommended for cultures with low inoculation densities as smaller aggregates take longer to settle. If medium exchange is required before D3, spinning down the desired culture volume to be exchanged in a centrifuge tube, aspirating the spent medium, and resuspending the cell pellet in fresh medium is recommended.

Perform medium exchange for one PBS-Mini vessel at a time to minimize process time per vessel, aggregate settling time, and chances of cross-contamination. The settling time of aggregates should be minimized to reduce the changes of unwanted size heterogeneity. Optimizing your medium exchange protocol is one of the best ways to improve aggregate homogeneity. Do not use ROCKi-containing medium for medium exchange.

Materials

Cells / Reagents	Consumables
PSC culture medium mTeSR1*, TeSR AOF, StemFlex, StemScale, etc.	- Serological pipettes - Aspirating pipettes

Reagent options used in optimized PSC process in **bold*

Preparation

1. Formulate PSC culture medium.

For initial inoculation of bioreactors, cells are cultured in PSC complete medium + 10uM ROCKi. For every medium exchange thereafter, ROCKi is no longer required.

2. Prewarm an aliquot of PSC culture medium in the incubator overnight.

Medium Exchange

3. Turn off agitation on a PBS-Mini Base, remove the corresponding vessel, and place it on the incubator shelf.
4. Let the cells settle for 5 minutes inside the incubator.
5. 2 minutes into the settling period, tap the vessel gently to dislodge cells from the Vertical-Wheel impeller.
6. Carefully transfer the vessel to the BSC with minimal jostling to prevent resuspension of aggregates.
7. Slowly remove 50% of the spent medium from the vessel using a serological pipette from the top of the liquid level. Do not disturb the settled aggregates at the bottom.

To optimize the settling time, the spent medium can be centrifuged and counted to estimate cell loss during medium exchange.

8. Add fresh pre-warmed PSC culture medium at the same volume as the spent medium removed. Cap the vessel.
9. Put the vessel back onto the PBS-Mini Base inside the incubator and resume agitation.
10. Repeat Steps 3 through 9 for each vessel.

C. Sampling from PBS-Mini

Sampling can be done at the same time as medium exchange. The following method is used to take samples from a PBS-Mini vessel, image the samples to characterize aggregate morphology, dissociate aggregates into single cells, and then perform a cell count and viability assay using an automated cell counter. Counting single cells instead of aggregates allows for a more accurate method of determining the total number of cells in culture.

Materials

Cells / Reagents	Consumables
• PSC culture medium mTeSR1*, TeSR AOF, StemFlex, StemScale, etc. • Enzymatic dissociation reagent Accutase, TrypLE, etc.	• 5 mL/10 mL serological pipettes • Aspirating pipettes • 15 mL/50 mL conical tubes • 1.5 to 2 mL microcentrifuge tubes • 6-well or 12-well flat-bottom plate • P1000 micropipette tips

Reagent options used in optimized PSC process in **bold*

Preparation

1. Prepare aliquots of enzymatic dissociation reagent (1.5 mL per sample) and PSC culture medium (1 to 3 mL per sample) and place them in the incubator for 40 to 60 minutes prior to sampling.
TrypLE, Accumax, and other enzymatic dissociation reagents can be used, but the aggregate dissociation timing in this protocol is optimized for Accutase. Check manufacturer's recommendations if using a different reagent. Enzyme-free dissociation reagents are not recommended.
2. Decontaminate and install a PBS-Mini Base in the BSC for sampling.
We strongly recommend placing a base in the BSC to allow sampling under continuous agitation to ensure accurate sample collection. If a second base is not available to provide agitation during sampling, the aggregate or cell count may not accurately represent the suspended cell distribution.

Sample Aggregates

3. Transfer a PBS-Mini vessel from the incubator to the BSC and install it onto a base. Agitate at 110 to 120% the speed used for culture (e.g., culture agitation = 60 rpm, sampling agitation = 66 to 72 rpm) to improve mixing throughout the total vessel volume and ensure a representative sample is taken.
4. Allow culture to mix for 10 to 20 seconds prior to sampling to ensure homogeneity of the suspension.
5. Insert a 5 mL serological pipette vertically through the single port (PBS-Mini 0.1 vessel) or the left-side port (PBS-Mini 0.5 vessel) until the tip is just above the side of the Vertical-Wheel impeller (near the middle of the vessel).
6. Remove a sample and transfer to one of the following containers:
 - A. For morphology analysis, take a 1 mL sample and transfer to a flat-bottom 6- or 12-well plate.
 - B. For pH measurement, take a sample volume that is suitable for your pH analyzer and transfer to a tube.
 - C. For metabolite analysis, take a sample volume that is suitable for your metabolite analyzer and transfer it to a tube.
 - D. For dissociation, cell counting, and viability analysis, take a 3 mL sample and transfer it to a 15 mL conical tube.

Do not dispense multiple samples from a single serological pipette draw. Each sample must be collected independently, as aggregates may settle within the pipette. The same serological pipette may be used to take multiple samples if perfect aseptic technique is maintained. To minimize contamination risk, use a new serological pipette for each sample whenever possible.

Morphology Analysis

7. Transfer the well plate with 1 mL sample to the microscope. Gently swirl the plate in a circular motion until all aggregates have collected in the center, then image at 4x and 10x. Morphological images should be taken within 5 minutes of sampling to avoid aggregate merging or other deviations from their morphology in culture.

We recommend taking a minimum of 3 images across the cluster of aggregates to get a better representation of aggregate diameter and morphology across the entire sample. After swirling, larger aggregates tend to collect in the center of the well, while smaller aggregates tend to gather toward the outer edges. To quantify aggregate concentration (aggregates per milliliter), the sample may need to be diluted or reduced in volume to ensure all aggregates are visible within the microscope's field of view. PSC aggregates should not exceed 400 μm diameter to maintain even distribution of nutrients and oxygen to all cells within each aggregate.

pH Analysis

8. Measure the pH of the sample immediately using a recently calibrated pH meter.

Metabolite Analysis

9. Measure the concentration of key nutrients and metabolites using your preferred metabolite analyzer.

Metabolite samples can be stored up to 1 month at 20°C or up to 1 year at 80°C. Once thawed, samples should not be re-frozen.

Dissociation into Single Cells

10. Centrifuge the 15 mL conical tube containing 3 mL of sample at 500 x g for 5 minutes.
11. Using a P1000 micropipette, carefully remove as much of the supernatant as possible without disturbing the cell pellet.
12. Add 1 mL of enzymatic dissociation reagent to the tube. Gently resuspend the pellet to disperse the aggregates while avoiding harsh trituration.
13. Place the tube in a water bath at 37°C and incubate for at least 20 minutes.
14. Remove the tube from the water bath and triturate each sample with a 1 mL serological pipette until aggregates are fully dissociated. Avoid creating bubbles while triturating.
15. Quench the enzymatic dissociation reagent to stop its activity by adding an equal volume of PSC culture medium (1:1 dilution).

Cell Counting and Viability Analysis

16. Triturate the cell suspension 3 times with a P1000 micropipette. On the final trituration transfer 500 µL into an Eppendorf tube.
17. Repeat Step 16 and take a second 500 µL sample.
18. Vortex Eppendorf tubes with samples for 3 seconds at 3000 rpm and take 2 counts from one sample. Repeat for the second sample and calculate the average of all counts (4 total counts).
Cell viability during PSC aggregate culture should be ≥85%. If viability falls below 85%, handling during sampling, dissociation, and counting should be reviewed. If viability remains below 85% after optimizing handling techniques, culture parameters should be investigated.

D. Harvest and Dissociation in Conical Tube

This method describes transferring PSC aggregates from a PBS-Mini vessel into a conical tube before dissociation into a single cell suspension for continued expansion, differentiation, or final harvest. The reagent volumes used in this method are for the harvest of the entire culture volume of the PBS-Mini vessel and can be scaled down if a partial harvest is performed. The enzymatic dissociation reagent volume and dissociation time should be optimized based on your cells and process.

Materials

Cells / Reagents	Consumables
• PSC culture medium mTeSR1*, TeSR AOF, StemFlex, StemScale, etc. • Enzymatic dissociation reagent Accutase, TrypLE, etc. • ROCK inhibitor Y-27632 or equivalent • Cryopreservation medium (if applicable)	• Serological pipettes • Aspirating pipettes • 15 mL/50 mL conical tubes • Microcentrifuge tubes • 50 mL/500 mL flat-bottom bottle • Orbital shaker

*Reagent options used in optimized PSC process in **bold**

Preparation

1. Formulate PSC Passaging Medium: PSC culture medium + 10 µM ROCKi. Recommended volumes at each scale can be found in **Table 3**.
It is important to follow the vendor recommendations for medium expiration dates after formulation. We also recommend aliquoting medium while still cold at the volumes required for different process days to avoid multiple heating and cooling cycles that can negatively impact the stability of medium components.
2. Aliquot enzymatic dissociation reagent, formulate with 10 µM ROCKi, then prewarm in the incubator or water bath. Recommended volumes at each scale can be found in **Table 3**.
Tryple, Accumax, and other enzymatic dissociation reagents can be used, but the dissociation timing in this protocol is optimized for Accutase. Check manufacturer's recommendations if using a different reagent. Enzyme-free dissociation reagents are not recommended.

Vessel Size	Key Process Parameter	Value
PBS-Mini 0.1	Enzymatic dissociation reagent - Accutase	20 mL
	PSC Passaging Medium	Up to 150 mL
PBS-0.5	Enzymatic dissociation reagent - Accutase	100 mL
	PSC Passaging Medium	Up to 750 mL

Table 3. *Parameters for Aggregate Dissociation in Conical Tube*

Harvest and Dissociation

- Transfer a PBS-Mini vessel from the incubator to the BSC and place it on a base set to 110 to 120% of the culture agitation set point (e.g., culture agitation = 60 rpm, sampling agitation = 66 to 72 rpm).
- Insert a 25 mL serological pipette vertically through the single port (PBS-Mini 0.1 vessel) or the left-side port (PBS-Mini 0.5 vessel) until the tip is just above the side of the Vertical-Wheel impeller (near the middle of the vessel).
- Collect desired volume of aggregates and medium and transfer to an appropriately-sized conical tube. Once the Vertical-Wheel impeller is no longer fully submerged, turn off agitation and continue to collect remaining volume, gently pipetting up and down 1 to 2 times if needed to resuspend aggregates. To reach the last 5 to 10 mL of volume, switch to a 5 mL serological pipette to reach between the wheel and vessel wall.
- Centrifuge samples at 500 x g for 5 minutes.
- Using a 5 mL serological pipette, carefully remove as much of the supernatant as possible without disturbing the cell pellet.
- Add enzymatic dissociation reagent to conical tube following the recommended volumes in **Table 3**. Gently resuspend the pellet to disperse the aggregates while avoiding harsh trituration. Transfer suspended cells to the flat-bottom bottle.
- Place the bottle on the orbital shaker inside the incubator at 70 rpm for 20 minutes.
- Bring the bottle to the BSC and gently triturate with a 10 mL serological pipette until aggregates are no longer visible. Make sure not to create any bubbles while triturating. Take a small sample to visualize dissociation under the microscope. If the majority of cells are in a single cell suspension, proceed to Step 11. If not, place the bottle back in the incubator for 5 minutes.
- When dissociation is confirmed, transfer the cell suspension into appropriately-sized conical tube(s).
- Rinse the empty bottle with an equal volume of PSC Passaging Medium to enzymatic dissociation reagent and transfer the rinse volume into conical tube(s) containing cells to quench enzyme activity.
- Centrifuge the conical tube(s) at 500 x g for 5 minutes.

14. Aspirate the supernatant, being careful not to disturb the cell pellet.
15. Use an appropriately-sized serological pipette to resuspend the cell pellet with PSC Passaging Medium (or cryopreservation medium) at a cell density within the working range of the cell counter (e.g., 2×10^5 to 2×10^6 cells/mL).

Sample and Count

16. Triturate the cell suspension 3-5 times with a 5 mL serological pipette. On the final trituration, transfer one 1 mL sample to an Eppendorf tube.
17. Repeat Step 16 and take a second 1 mL sample.
18. Vortex Eppendorf tubes with samples for 3 seconds at 3000 rpm and take 2 counts from one sample. Repeat for a second sample and calculate the average of all counts (4 total counts).
Cell viability during PSC aggregate culture should be $\geq 85\%$. If viability falls below 85%, handling during sampling, dissociation, and counting should be reviewed. If viability remains below 85% after optimizing handling techniques, culture parameters should be investigated.

Next Steps

For cryopreservation, resuspend cells in cryopreservation medium at a resuspension volume that corresponds to the desired cell density (i.e., 1×10^6 to 1×10^7 cells/mL).

For continued expansion, refer to the previous section: [Inoculation in PBS-Mini \(pg. 12\)](#)

E. Harvest and Dissociation in PBS-Mini

This method describes the steps to dissociate aggregates within a PBS-Mini vessel to simplify passaging and harvesting and to eliminate the need for transfer to external containers for dissociation. The agitation speed, enzymatic dissociation reagent volume, and dissociation time should be optimized based on your cells and process.

Materials

Cells / Reagents	Consumables
- PSC culture medium mTeSR1*, TeSR A0F, StemFlex, StemScale, etc. - Enzymatic dissociation reagent Accutase, TrypLE, etc. - ROCK inhibitor Y-27632 or equivalent	- Serological pipettes - Aspirating pipettes 15 mL/50 mL conical tubes - Microcentrifuge tubes

*Reagent options used in optimized PSC process in **bold**

Preparation

1. Formulate PSC Passaging Medium: PSC culture medium + 10 μ M ROCKi. Recommended volumes at each scale can be found in **Table 4**.

It is important to follow the vendor recommendations for medium expiration dates after formulation. We also recommend aliquoting medium while still cold at the volumes required for different process days to avoid multiple heating and cooling cycles that can negatively impact the stability of medium components.

2. Aliquot enzymatic dissociation reagent, formulate with 10 μ M ROCKi, then prewarm in the incubator or water bath. Recommended volumes at each scale can be found in **Table 4**.

Tryple, Accumax, and other enzymatic dissociation reagents can be used, but the dissociation timing in this protocol is optimized for Accutase. Check manufacturer's recommendations if using a different reagent. Enzyme-free dissociation reagents are not recommended.

Vessel Size	Key Process Parameter	Value
PBS-Mini 0.1	Enzymatic dissociation reagent - Accutase	20 mL
	PSC Passaging Medium	Up to 150 mL
	Agitation speed for dissociation	80 RPM
PBS-0.5	Enzymatic dissociation reagent - Accutase	100 mL
	PSC Passaging Medium	Up to 750 mL
	Agitation speed for dissociation	40 RPM

Table 4. Parameters for In-Vessel Aggregate Dissociation

Harvest and Dissociation

3. Turn off agitation on a PBS-Mini Base, remove the corresponding vessel, and place it on the incubator shelf
4. Let the cells settle for 5 minutes inside the incubator.
5. 2 minutes into the settling period, tap the vessel gently to dislodge cells from the Vertical-Wheel impeller.
6. Carefully transfer the vessel to the BSC with minimal jostling to prevent resuspension of aggregates.
7. Remove as much spent medium from the vessel as possible using an appropriately-sized serological pipette without disturbing settled cells at the bottom. Use a 10 mL or 5 mL serological pipette to further remove additional culture medium without disturbing settled cells.

It may be difficult to remove all of the spent medium via serological pipette while not disturbing the settled aggregates. We have found that leaving a small amount of medium in the vessel does not interfere with digestion enzyme activity. If desired, a DPBS wash step can be added prior adding the enzymatic dissociation reagent.

Harvest and Dissociation

8. Add prewarmed enzymatic dissociation reagent to the vessel following the recommended volumes listed in **Table 4**.
9. Place the vessel back on its base in the incubator and start agitation at the recommended agitation speeds listed in **Table 4**.
After adding the enzymatic dissociation reagent at the recommended volumes, the Vertical-Wheel impeller will be exposed to air during agitation. This provides increased shear forces within the liquid to mechanically dissociate the aggregates in conjunction with the enzymatic dissociation.
10. After 8 minutes, transfer the vessel to the BSC and gently triturate with a serological pipette until aggregates are no longer visible. Take a small sample to visualize dissociation under the microscope. If the majority of cells are in a single cell suspension, proceed to Step 11. If not, place the vessel back on its base within the incubator for another 5 minutes.
11. When dissociation is confirmed, transfer the cell suspension into an appropriately-sized conical tube.
12. Rinse the empty vessel with an equal volume of PSC Passaging Medium to enzymatic dissociation reagent and transfer the rinse volume into the conical tube containing cells to quench enzyme activity.
13. Centrifuge the conical tube at 500 x g for 5 minutes.
14. Aspirate the supernatant, being careful not to disturb the cell pellet.
15. Use an appropriately-sized serological pipette to resuspend the cell pellet with PSC Passaging Medium (or cryopreservation medium) at a cell density within the working range of the cell counter (e.g., 2×10^5 to 2×10^6 cells/mL).

Sample and Count

16. Triturate the cell suspension 3 to 5 times with a 5 mL serological pipette. On the final trituration, transfer one 1 mL sample to an Eppendorf tube.
17. Repeat Step 15 and take a second 1 mL sample.
18. Vortex Eppendorf tubes with samples for 3 seconds at 3000 rpm and take 2 counts from one sample. Repeat for a second sample and calculate the average of all counts (4 total counts).
Cell viability during PSC aggregate culture should be $\geq 85\%$. If viability falls below 85%, handling during sampling, dissociation, and counting should be reviewed. If viability remains below 85% after optimizing handling techniques, culture parameters should be investigated.

Next Steps

For cryopreservation, resuspend cells in cryopreservation medium at a resuspension volume that corresponds to the desired cell density (i.e., 1×10^6 to 1×10^7 million cells/mL).

For continued expansion, refer to the previous section: [Inoculation in PBS-Mini \(pg. 12\)](#)

STILL HAVE QUESTIONS?

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