Robust Workflows for the Expansion and Differentiation of Human Pluripotent Stem Cells as Aggregates in Suspension Culture

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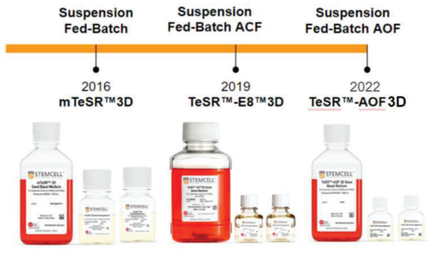
INTRODUCTION

STEMCELL's portfolio of TeSR™ 3D-based media products have been developed for robust and scalable suspension culture of human pluripotent stem cells (hPSCs) as aggregates. However, the field has been challenged by the lack of methods that can reproducibly scale hPSCs cultures without prior adaptation of cells to the higher shear levels imposed in stirred suspension. A critical balance exists between the agitation rate to maintain aggregates in suspension and the generation of shear. Agitation methods were tested for their ability to maintain aggregate suspensions and cell growth rates. Experiments were conducted with 6 different cell lines (3 PSC and 3 iPSC) in which cells were serially expanded in suspension cultures up to 500 mL. Aggregates were passaged non-enzymatically by dissociation using Gentle Cell Dissociation Reagent and filter-based trituration. The only system that gave reproducible growth across cell lines had a low-shear Vertical-Wheel® impeller design. With this workflow, hPSCs underwent a greater than 1.5- to 1.9-fold expansion per day (cell line dependent) with > 85% viability, > 90% expression of OCT4 and TRA-1-60, the capacity to differentiate to three germ layers, and a normal karyotype. To verify the utility of this workflow, 3 hPSC lines were further differentiated into polyploid megakaryocytes (MKs) in 3D suspension cultures. Differentiation used established 2D protocols with a 12-day endothelial-to-hematopoietic transition phase, and a 5-day progenitor-to-mature MK stage. At the end of the protocol, 45 - 75% of cells expressed CD41a, 25 - 65% of the cells co-expressed CD41a and CD42b, and 10 - 60 CD41a+CD42b+ cells were generated per seeded hPSC (n = 9). The DNA ploidy profile of the CD41a+CD42b+ cells generated showed 26% and 9% of cells had 4N and 8N+ DNA ploidy, respectively. The combination of TeSR 3D workflows and low-shear bioreactors provides a robust system suitable for the expansion of a wide range of hPSC lines.

Flexible Family of Fed-Batch Optimized Media

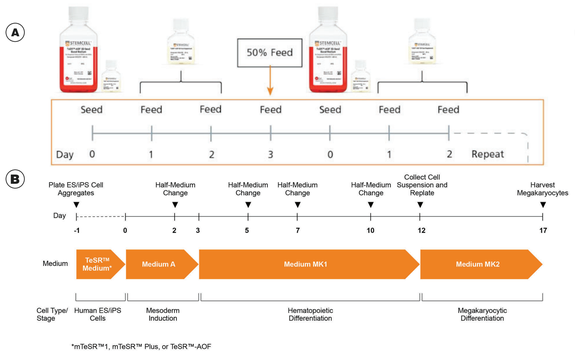


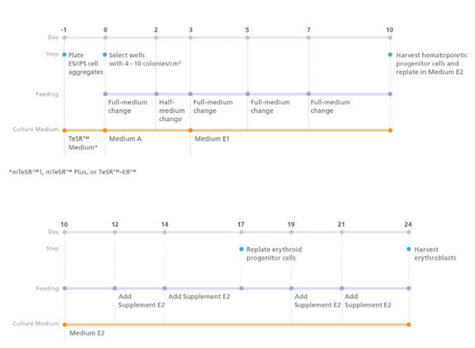
3D Suspension Media Design Objectives

- ✔ No adaptation step required from 2D to 3D suspension culture
- ✔ Cells exhibit a sustained daily expansion
- ✔ Fed-batch protocols to minimize culture handling and disruption
- ✔ Non-enzymatic filter based passaging
- ✔ Culture viabilities at the end of each passage > 85%
- ✔ Protocols maintain high glucose and low lactate concentrations
- ✔ After 5 passages in suspension: equivalent expression of markers of undifferentiated hPSCs; functional pluripotency as measured by trilineage differentiation; stable karyotype
- ✔ Lower cost per cell produced compared to traditional 2D culture media

METHODS

(A) High-quality adherent hPSC cultures are dissociated non-enzymatically to clumps using Gentle Cell Dissociation Reagent (GCDR; Catalog #100-0485). Cell clumps are resuspended at 0.5 - 1 x 10^5 viable cells/mL in seed medium + 10 µM Y-27632 (Catalog #72302). Fed-batch feed supplement is added on days 1 and 2, starting 24 hours after inoculating cell clumps. A half-medium change is performed on day 3 of a 4-day passage. After 3 or 4 days, aggregates are recovered using a 37 µm strainer (Catalog #27215/27250), then incubated in GCDR at 37°C for 6 minutes. GCDR is removed and aggregates are resuspended in seed medium + 10 µM Y-27632. Immediately after resuspension, aggregates are forced through a 37 µm strainer to generate cell clumps. Clumps are re-seeded into a fresh culture vessel at 0.5 - 1 x 10^5 viable cells/mL. Cells in 3D suspension culture maintain high expression of markers of the undifferentiated state OCT4 and TRA-1-60. **(B)** After three passages in suspension culture, the aggregates are passaged and seeded as 2 mL suspension cultures. Megakaryocyte differentiation is initiated using the off-the-shelf STEMdiff™ Megakaryocyte Kit and protocol (Catalog #100-0900), with harvests on days 12 and 17. **(C)** After three passages in suspension culture, the aggregates are passaged and seeded as 2 mL suspension cultures. Erythroblast differentiation is initiated using the off-the-shelf STEMdiff™ Erythroid Kit and protocol (Catalog #100-0074), with harvest on days 10, 17 and 24.





RESULTS

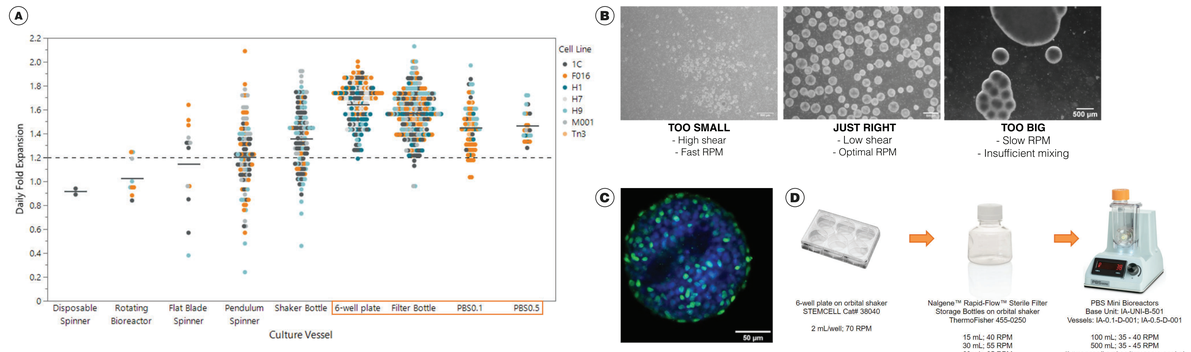


FIGURE 1. Optimizing Suspension Culture Operation

(A) Daily fold expansion of 7 cell lines in culture vessels tested from 2019 - present. Robust vessels outlined in orange having consistent daily fold expansion above 1.2. **(B)** 'Good' and 'Bad' aggregate morphology in different culture environments. Optimal aggregates are uniform in size and below 500 µm in diameter at day 4 of culture. They have a dimpled morphology in bright-field images resulting from multiple small voids distributed throughout the aggregate. **(C)** The immunofluorescent image shows an optically cleared confocal cross-section through a typical PSC aggregate showing these void spaces (blue:DAPI; green:OCT4). **(D)** Cells can be readily scaled up from 2D cultures to 2 mL suspension cultures in 6-well plates, from there up to 60 mL in an orbital shaker bottle, and then into 100 and 500 mL cultures in PBS-MINI Bioreactors (Catalog #100-1006 and #100-1007, respectively).

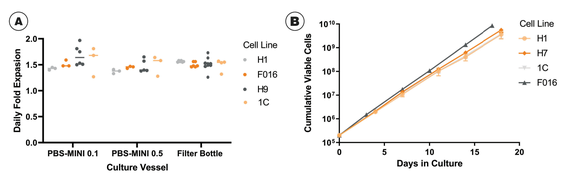


FIGURE 2. Reproducible growth in the PBS-MINI Bioreactors

(A) Daily fold expansion for hES and hiPS aggregates in mTeSR™3D and TeSR™-AOF 3D in the PBS-MINI alongside filter bottle control cultures. **(B)** Consistent expansion of hES and hiPS cell lines as aggregates in TeSR™-AOF 3D across multiple culture vessels. Greater than 10^9 cells produced after 5 passages in suspension culture with no adaptation passage.

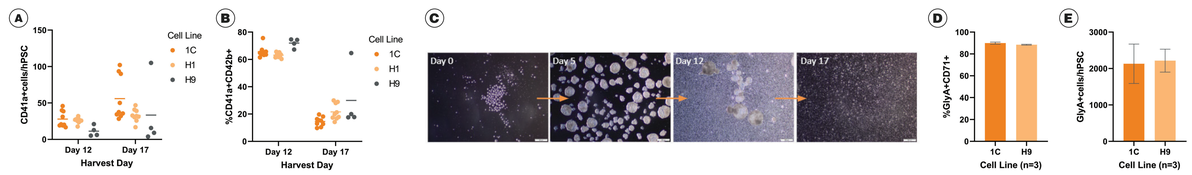


FIGURE 3. Hematopoietic Differentiation in Suspension Culture

(A) The number of CD41a+ cells generated per input hPSC and **(B)** the frequency of CD41a+CD42b+ MKs after culturing hPSCs for 12 and 17 days in suspension culture using the STEMdiff™ Megakaryocyte Kit. **(C)** Morphology evolution of the MK aggregates over the first 12 days and of the reseeded single cells over the last 5 days of the MK differentiation in suspension culture. **(D)** Frequency of GlyA+CD71+ erythroblasts and **(E)** the number of GlyA+ erythroid cells generated per hPSC after culturing for 24 days in suspension culture using the STEMdiff™ Erythroid Kit.

CONCLUSION

- Using STEMCELL's TeSR™ 3D family of media, hPSC expansion workflows have been optimized for consistent growth and aggregate morphology in suspension culture, starting from 2 mL in a 6-well plate to 500 mL in a PBS-MINI Bioreactor. With robust workflows in place, focus can be shifted to differentiation in suspension culture for cell therapy, as demonstrated with the megakaryocyte and erythroid protocols.