An Animal Origin-Free Seeding Supplement to Enhance Human Pluripotent Stem Cell Survival in 2D and 3D Cell Culture Applications

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INTRODUCTION

Cell survival is a major bottleneck in workflows for manufacturing human pluripotent stem cells (hPSCs), particularly during stressful procedures, such as gene editing, cloning, and transitioning between culture environments. Poor cell survival reduces yields, increases batch-to-batch variability, and can elevate production costs impacting manufacturability. Derivation of clonal hPSC lines remains a major hurdle in several hPSC workflows, including clonal isolation of rare cell populations following CRISPR genome editing. To overcome these hurdles, we previously developed CloneR™ and CloneR™2, single-cell cloning supplements that have been widely adopted, yielding 20 and 40% hPSC cloning efficiencies, respectively. To further support the field with more defined product specifications and requirements, we have now developed CloneR™3, an animal origin-free supplement designed to support workflows utilizing TeSR™-AOF, hPSC maintenance medium. Additionally, we have demonstrated that CloneR™3 yields cloning efficiencies comparable to CloneR™2 in mTeSR™1 and mTeSR™ Plus.

METHODS

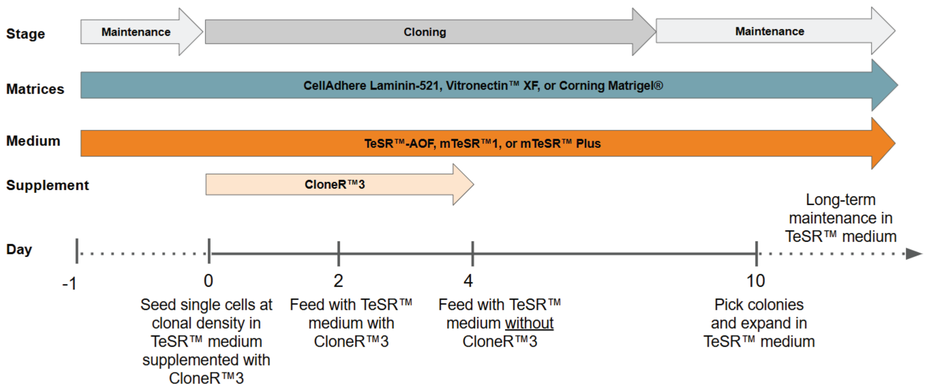


FIGURE 1. hPSC Cloning Workflow with CloneR™3, an Animal Origin-Free Cell Seeding Supplement

hPSCs can be seeded as single cells at clonal density (e.g. 50 cells/cm^2) or sorted at 1 cell/well in a 96-well plate in TeSR™ medium (TeSR™-AOF, mTeSR™1, or mTeSR™ Plus) supplemented with CloneR™3. On day 2, cells were fed with TeSR™ medium + CloneR™3. From day 4, cells were maintained and fed daily with TeSR™ medium without CloneR™3. Colonies were ready for selection from day 10. After picking, clonal cell lines can be expanded and maintained long-term in TeSR™ medium. For analysis, cells were fixed and stained with Hoechst nuclear stain, imaged on the ImageXpress® Micro 4 High Content Imaging System, and cell nuclei were quantified using ImageJ analysis. For other 2D culture applications, such as routine passaging, cells can be seeded in TeSR™ medium + CloneR™3 at the desired density, followed by a full-medium change with TeSR™ medium 24 hours after seeding.

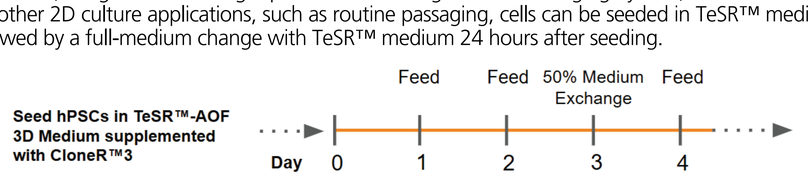


FIGURE 2. hPSC 3D Suspension Workflow with TeSR™-AOF 3D Supplemented with CloneR™3

- To adapt TeSR™-maintained hPSCs from 2D adherent culture to 3D suspension culture, colonies were dissociated with Gentle Cell Dissociation Reagent (GCDR) or ReLeSR™ into clumps and seeded at 5 x 10^4 cells/mL in TeSR™-AOF 3D supplemented with CloneR™3 or the Rho kinase inhibitor Y-27632 in non-tissue culture treated 6-well plates. Cells were incubated for four days on an orbital shaker (2.5 cm orbital diameter) set to 70 rpm.
- To expand hPSCs previously adapted to 3D suspension culture, aggregates were harvested and dissociated with GCDR at 37°C for 6 or 15 minutes to generate small clumps or single cells, respectively. For passaging on day 3 or 4, cells were seeded in TeSR™-AOF 3D + CloneR™3 or Y-27632 at 1 x 10^5 cells/mL or 5 x 10^4 cells/mL, respectively.
- Cryopreserved hPSCs may be seeded directly into 3D culture conditions at 1 x 10^5 cells/mL in TeSR™-AOF 3D + CloneR™3 or Y-27632 for harvest on day 4.

RESULTS

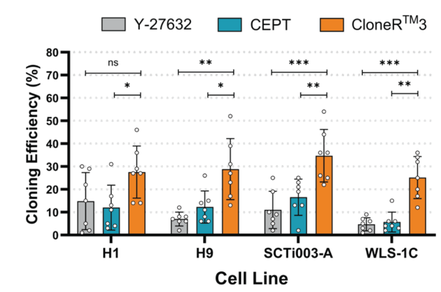
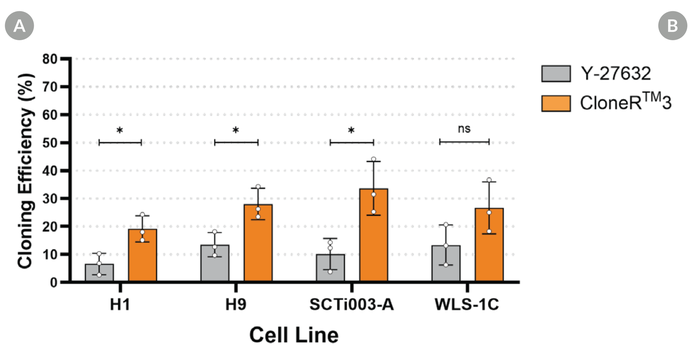


FIGURE 3. CloneR™3 Increases Survival of hPSCs Plated Using Single-Cell Deposition in Genome Editing Workflows

Four hPSC lines were seeded in 96-well plates at 1 cell/well using single-cell deposition via the BD FACSAria™. This technique is the gold standard of cloning in genome editing workflows and provides a stringent culture condition to showcase CloneR™3 efficacy. CloneR™3 conditions displayed an average cloning efficiency of 27.5 ± 10.9%, an increase by 5.7 and 4.4 folds over Y-27632 and CultureSure™ CEPT Cocktail (CEPT), respectively (mean ± SD, n ≥ 6 per cell line). Statistical analysis was performed using an unpaired Student's t-test (* = p value < 0.05, ** = p value < 0.01, *** = p value < 0.001, and ns, not significant.



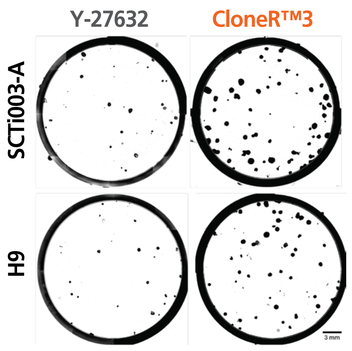


FIGURE 4. CloneR™3 Improves Cloning Efficiency Compared to Y-27632

Four hPSC lines were seeded at clonal density (50 cells/cm^2) on Vitronectin XF™ in TeSR™-AOF + Y-27632 or CloneR™3. **(A)** TeSR™-AOF + CloneR™3 increased the cloning efficiency of hPSCs compared to TeSR™-AOF + Y-27632. **(B)** Representative images of 200 cells (H9 and SCTi003-A) in 12-well plates grown in TeSR™-AOF on Vitronectin XF™ on day 10 after seeding showing homogeneous colony morphology in TeSR™-AOF + CloneR™3. Statistical analysis was performed using an unpaired Student's t-test (* = p value < 0.05).

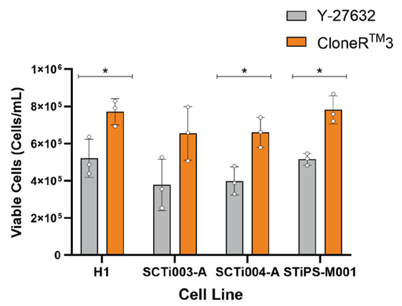


FIGURE 5. CloneR™3 Improves Cell Yields During Transition from 2D to 3D Suspension Culture

The transition from static 2D culture to 3D suspension culture is a bioprocessing bottleneck that can be stressful for hPSCs and may result in lower-than-expected expansion on the first passage. hPSC clumps seeded into TeSR™-AOF 3D + CloneR™3 exhibited cell yields 1.58-fold higher than Y-27632-supplemented cells after four days in culture. Across four cell lines tested, Y-27632 and CloneR™3 had an average cell yield of 4.5 x 10^5 cells/mL ± 11.7% and 7.2 x 10^5 cells/mL ±7.3%, respectively. Bars show the mean ± SD. Statistical analysis was performed using an unpaired Student's t-test (* = p value < 0.05).

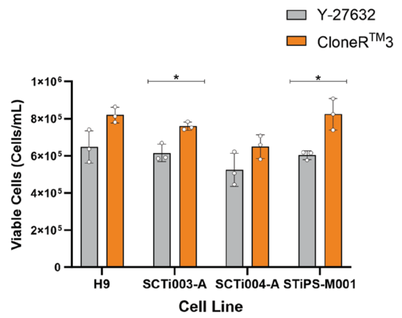


FIGURE 6. CloneR™3 Improves Cell Yields During Routine 3D Aggregate Suspension Culture in TeSR™-AOF 3D

To achieve sufficient cell yields, serial passaging of hPSCs, by employing a seed train scale-up or scale-out strategy may be necessary. Four hPSC lines adapted to 3D suspension culture for one passage were dissociated into small clumps, seeded in TeSR™-AOF 3D + CloneR™3, and passaged every three to four days for five passages. Cells seeded in TeSR™-AOF 3D + CloneR™3 demonstrated higher cell yields after four days in culture compared to cells cultured in TeSR™-AOF 3D + Y-27632. Across four cell lines tested, CloneR™3-supplemented cells had an average yield of 7.6 x 10^5 cells/mL ± 5.8%, compared to 6.0 x 10^5 cells/mL ± 6.3% for Y-27632-supplemented cells. Data points represent a total of three day-4 passages of five total passages in culture each averaged from two technical replicates. Bars show the mean ± SD. Statistical analysis was performed using an unpaired Student's t-test (* = p value < 0.05).

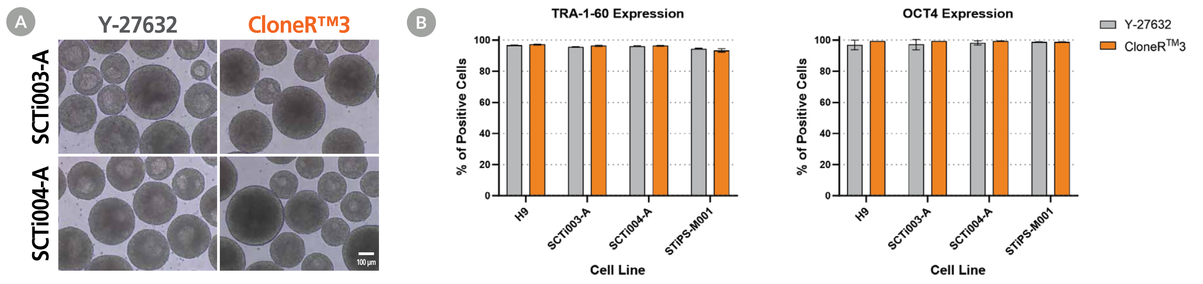


FIGURE 7. hPSCs Expanded in TeSR™-AOF 3D with CloneR™3 for Five Passages Exhibit High-Quality Aggregate Morphology and Express Markers of the Undifferentiated State

Four cell lines were cultured for five passages in TeSR™-AOF 3D supplemented with Y-27632 or CloneR™3. **(A)** Representative images of the SCTi003-A and SCTi004-A hPSC lines showing that 3D hPSC aggregates maintained typical spherical morphology with visible pock marking across the surface indicative of high-quality, healthy hPSCs. Scale bars = 100 µm. **(B)** Aggregates were dissociated to single cells, stained for undifferentiated markers, TRA-1-60 and OCT4, and analyzed by flow cytometry. Across four cell lines, > 90% of cells in both culture conditions expressed TRA-1-60 and OCT4. Bars represent the average of two technical replicates and error bars represent the standard deviation.

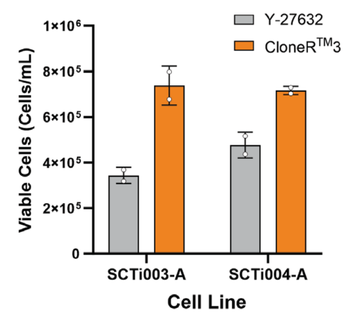


FIGURE 8. CloneR™3 Improves Post-Thaw Recovery of Cryopreserved Single Cells in 3D Suspension Culture

Cryopreserving and thawing hPSCs is a stressful event that typically results in significant cell loss and reduced expansion. hPSC aggregates that were cryopreserved as single cells then thawed and seeded into TeSR™-AOF 3D supplemented with CloneR™3 demonstrated markedly higher post-thaw recovery after four days in culture compared to cryopreserved single cells that were thawed and seeded into TeSR™-AOF 3D supplemented with Y-27632. Across four cell lines tested, Y-27632- and CloneR™3-supplemented cells had an average yield of 4.1 x 10^5 cells/mL ± 10.5% and 7.3 x 10^5 cells/mL ± 3.6%, respectively. Data points represent individual biological replicates each averaged from two technical replicates. Bars show the mean ± SD.

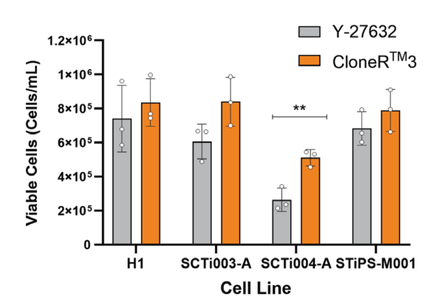


FIGURE 9. CloneR™3 Improves Cell Yields Following Single-Cell Passaging of 3D Aggregate Suspension Cultures

Passaging 3D hPSC aggregates as single cells can be more stressful than traditional clump passaging, especially when culturing more challenging cell lines or seeding at lower densities. 3D hPSC aggregates were dissociated to single cells using GCDR and re-seeded at 5 x 10^4 cells/mL. Single-cell hPSCs cultured in TeSR™-AOF 3D supplemented with CloneR™3 demonstrated significantly higher cell yields after four days in culture compared to Y-27632-supplemented cells. Across four cell lines tested, Y-27632- and CloneR™3-supplemented cells had an average yield of 5.7 x 10^5 cells/mL ± 19.3%, and 7.4 x 10^5 cells/mL ± 11.8%, respectively. Data points represent individual biological replicates (n = 3) each averaged from two technical replicates. Bars show the mean ± SD. Statistical analysis was performed using an unpaired Student's t-test (** = p value < 0.01).

SUMMARY

- CloneR™3 is an animal origin-free supplement developed to enhance hPSC survival during periods of cell stress.
- CloneR™3 outperforms Y-27632 and CEPT in improving the efficiency of hPSC single-cell deposition cloning.
- CloneR™3 significantly boosts cell yields during stressful 3D culture manipulations, including 2D-to-3D transition, single-cell passaging, and post-thaw recovery.
- hPSCs cultured in TeSR™-AOF 3D maintain high-quality aggregate morphology and express markers of the undifferentiated state, OCT4 and TRA-1-60.

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