Single cell passaging methods

Introduction

Pluripotent stem cells (PSCs) are foundational tools for basic research and applied applications, including regenerative therapy, drug discovery, and toxicological assessment. Although the advent of feeder-free culture systems greatly improved the scalability of PSCs, the use of clump passaging techniques (e.g., using reagents such as dispase, collagenase, or EDTA) has often been incompatible with downstream experimental applications, such as high-throughput screening, gene editing, and directed differentiation.

Single cell culture is a standardized and accepted practice in stem cell culture research. By employing techniques to passage using single cell dissociation, scientists are able to have dependable cells for downstream applications and a basis of comparison for clone selection, imaging, cell sorting, and for work in suspension cultures where cell recovery and cell number are critical to success. Gibco® RevitaCell™ Supplement was developed for use with Essential 8™ Medium to enable maximum cell viability when performing single cell passaging. Unlike other commercially available formulations, RevitaCell™ Supplement includes a rho-associated protein kinase (ROCK) inhibitor that is more selective than traditional ROCK inhibitors (e.g., Y-27632 and thiazovivin) and is coupled with molecules containing antioxidant and free-radical scavenger properties. This cocktail minimizes the impact of stress from single cell passaging and greatly improves overall cell survival, which helps ensure improved consistency of cell supply and viability in downstream experiments.

Materials and methods

PSC culture and expansion

H9 human embryonic stem cells (hESCs) and human induced pluripotent stem cells (hiPSCs; generated with episomal vectors) were maintained under feeder-free conditions, culturing in Essential 8™ Medium on vitronectin (VTN-N)-coated plates. hiPSCs were passaged every 3 to 5 days upon reaching ~80% confluency.

Table 1. Materials used in these experiments.

Product name	Cat. No.
Alexa Fluor® 488 goat anti–mouse IgG (H+L)	A11029
Alexa Fluor® 594 goat anti–rabbit IgG (H+L)	A11012
DPBS, no calcium, no magnesium (10X)	14200-075
Essential 8™ Medium	A1517001
Gibco® RevitaCell™ Supplement (100X)	A2644501
Image-iT® Fixation/Permeabilization Kit	R37602
Mouse anti-Tra-1-81 (cl.26)	41-1100
PSC Cryomedium	A2644401
StemPro® Accutase® Cell Dissociation Reagent	A11105-01
Vitronectin, truncated recombinant human (VTN-N)	A14700
TrypLE™ Select Enzyme, no phenol red (1X)	12563-029

Single cell passaging of hiPSCs using TrypLE™ Select Enzyme or StemPro® Accutase® Cell Dissociation Reagent

For single cell passaging experiments (refer to workflow in Figure 1), cells were rinsed with DPBS without calcium or magnesium and subsequently treated with prewarmed TrypLE™ Select Enzyme (1 mL per well of a 6-well plate). Cells were incubated for 5 minutes in a 37°C, 5% CO_2 incubator. Following incubation, cells were triturated with a Pipetman® P1000 5 to 10 times to efficiently singularize the cells. Cell suspensions were then transferred to conical vials containing 3 mL of fresh Essential 8™ Medium to dilute the dissociation solution, and spun down at 200 x *g* for 4 minutes. Medium was aspirated, being careful to not disturb the cell pellet, and cells were recovered and cultured (at the cell seeding densities noted in Figure 1) in Essential 8™ Medium supplemented with 1X RevitaCell™ Supplement for 18 to 24 hours post-split, followed by daily feeding with Essential 8™ Medium alone for the remainder of the culture.

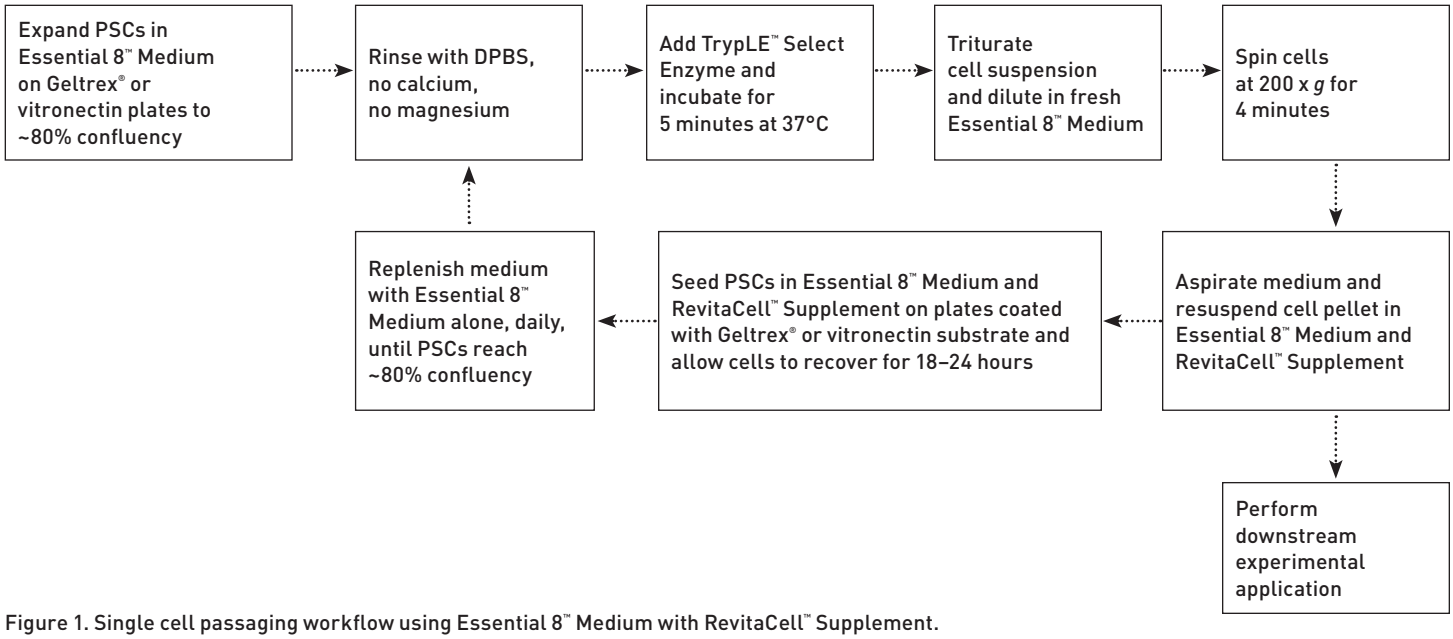

Figure 1. Single cell passaging workflow using Essential 8™ Medium with RevitaCell™ Supplement.

Kinetic growth and morphology assessment

To determine the kinetic growth of the cells and morphology changes following single cell passaging, images were acquired by phase-contrast microscopy using the IncuCyte™ ZOOM (Essen Biosciences) live-cell imaging platform at regular intervals. Cell masks were generated to determine the percentage change in confluency of the cells over time, providing a label-free method of determining the proliferation of cells after passaging.

Normal pluripotency assessment

hiPSCs and H9 ESCs were passaged using TrypLE™ Select Enzyme and cryopreserved using PSC Cryomedium. After thawing, cells were recovered using Essential 8™ Medium supplemented with 1X RevitaCell™ Supplement for 18 to 24 hours, followed by daily feeding with Essential 8™ Medium alone until cells reached ~80% confluency. Cells were then passaged using TrypLE™ Select Enzyme for a total of 10 passages, and recovered post-split using Essential 8™ Medium and 1X RevitaCell™ Supplement for 18 to 24 hours, followed by feeding with Essential 8™ Medium alone for the remainder of the culture. At the end of the 10th passage, cells were fixed, permeabilized (only required for nanog staining), and blocked using the Image-iT® Fixation/ Permeabilization Kit. Cells were subsequently stained with primary antibody solutions, including mouse anti–TRA-1-81 (1:100) and rabbit anti-Nanog (D73G4) (1:400; Cell Signaling Technology, Inc.), followed by treatment with fluorescent dye–conjugated secondary antibodies, including Alexa Fluor® 488 goat anti–mouse IgG (H+L) (1:1,000) and Alexa Fluor® 594 goat anti–rabbit IgG (H+L) (1:1,000). Cultures were subsequently imaged using the IncuCyte™ ZOOM platform.

Normal karyotype assessment

hiPSCs and H9 ESCs were passaged using TrypLE™ Select Enzyme and cryopreserved using PSC Cryomedium. After thawing, cells were recovered using Essential 8™ Medium with 1X RevitaCell™ Supplement for 18 to 24 hours, followed by feeding with Essential 8™ Medium alone until cells reached ~80% confluency. Cells were then passaged using TrypLE™ Select Enzyme for a total of 30 passages, and recovered post-split using Essential 8™ Medium with 1X RevitaCell™ Supplement for 18 to 24 hours, followed by feeding with Essential 8™ Medium alone for the remainder of the culture. In the 30th passage, cells were sent to Cell Line Genetics for G-band karyotype analysis.

Kinase profiling of the prosurvival small molecule included in the cocktail

To determine the key pathways targeted by the prosurvival small molecule contained in RevitaCell™ Supplement, compared to traditional ROCK inhibitors, small molecules were screened using SelectScreen® Kinase Profiling Services at the following concentrations: 10 µM Y-27632, 2 µM thiazovivin, and 1X final concentration of the prosurvival small molecule contained in RevitaCell™ Supplement. Percent of inhibition of 319 kinases was analyzed, with kinases having >80% inhibition shown in Figure 6.

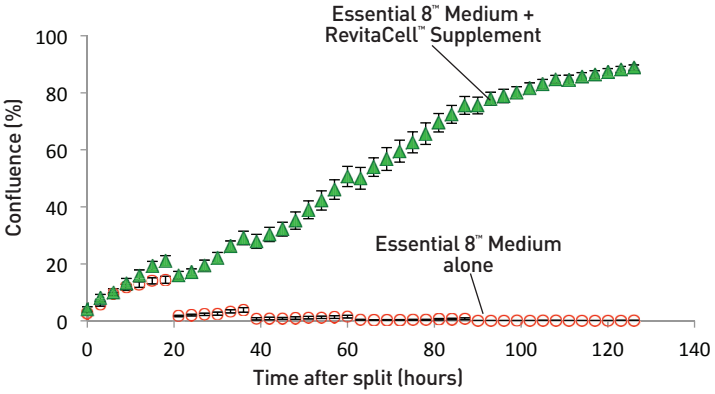

Figure 2. RevitaCell™ Supplement shows utility in single cell passaging using TrypLE™ Select Enzyme. iPSCs generated using episomal vectors were passaged using TrypLE™ Select Enzyme and seeded at a density of 25,000 viable cells/cm^2 onto culture plates coated with truncated recombinant human vitronectin, in Essential 8™ Medium with 1X RevitaCell™ Supplement. Following a 24-hour recovery, iPSCs were fed with Essential 8™ Medium alone for the remainder of their time in culture.

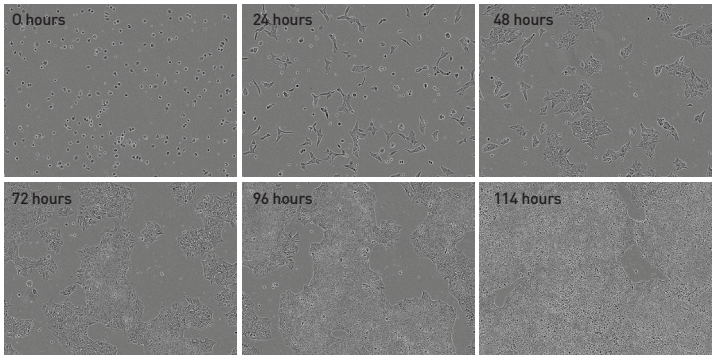

Figure 3. Normal morphology is retained following single cell passaging using Essential 8™ Medium plus RevitaCell™ Supplement. iPSCs generated using episomal vectors were passaged using TrypLE™ Select Enzyme and seeded at a density of 25,000 viable cells/cm^2 onto culture plates coated with truncated recombinant human vitronectin, in Essential 8™ Medium with 1X RevitaCell™ Supplement. Following a 24-hour recovery, iPSCs were fed with Essential 8™ Medium alone for the remainder of their time in culture.

Results

iPSCs generated with episomal vectors were passaged as single cells using TrypLE™ Select Enzyme. Following passaging, cells were allowed to recover for approximately 24 hours using Essential 8™ Medium, with or without the addition of 1X RevitaCell™ Supplement, followed by daily feeding with Essential 8™ Medium alone. Recovery of iPSCs was determined by monitoring their post-passage confluency using phase-contrast microscopy. Use of RevitaCell™ Supplement for 18 to 24 hours post-passaging provided vast improvement in cell survival and recovery of iPSCs (green triangles in Figure 2). Conversely, iPSCs grown in Essential 8™ Medium alone showed poor recovery (red open circles in Figure 2). Typical morphology of iPSCs cultured for approximately 24 hours in Essential 8™ Medium with 1X RevitaCell™ Supplement is shown in Figure 3. At 24 hours after passaging, cells have an elongated morphology. However, after recovery, iPSCs exhibited normal colony morphology. iPSCs cryopreserved using PSC Cryomedium and subsequently cultured according to the workflow shown in Figure 1 retained normal morphology and pluripotency (Figures 3 and 4). iPSCs retained expression of both nanog, an intracellular marker of pluripotency, and TRA-1-81, an extracellular marker of pluripotency. Furthermore, following single cell passaging according to the workflow in Figure 1 for 30 passages, PSCs retained normal karyotypes, indicating the robustness of this passaging method for use in long-term culture of PSCs (Figure 5).

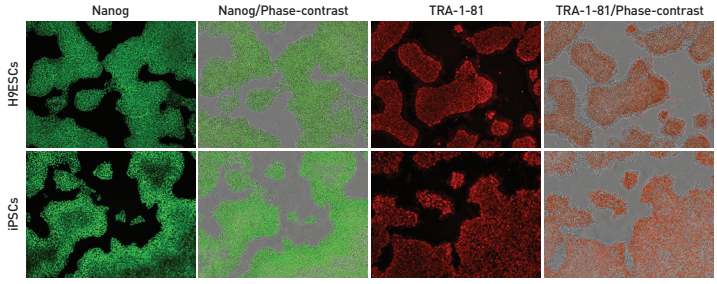

Figure 4. Normal pluripotency is retained following single cell passaging using Essential 8™ Medium plus RevitaCell™ Supplement. iPSCs generated using episomal vectors and H9 ESCs were passaged using TrypLE™ Select Enzyme and cryopreserved using PSC Cryomedium. After thawing, cells were recovered using Essential 8™ Medium with 1X RevitaCell™ Supplement for 18 to 24 hours, followed by feeding with Essential 8™ Medium alone until cells reached ~80% confluency. Cells were passaged using TrypLE™ Select Enzyme for a total of 10 passages, and recovered post-split using Essential 8™ Medium with 1X RevitaCell™ Supplement for 18 to 24 hours, followed by feeding with Essential 8™ Medium alone for the remainder of their time in culture. Morphology and pluripotency were assessed at the end of the 10th passage.

RevitaCell™ Supplement contains a prosurvival small molecule coupled with compounds having antioxidant and free-radical scavenger properties. SelectScreen® Kinase Profiling services were performed on the prosurvival small molecule contained in this cocktail to compare it with traditional ROCK inhibitors (10 µM Y-27632 and 2 µM thiazovivin; Figure 6). The prosurvival small molecule contained in RevitaCell™ Supplement is also a ROCK inhibitor. Additionally, the RevitaCell™ Supplement prosurvival small molecule exhibits >80% inhibition of only 7 kinases, whereas >80% inhibition is shown for 16 kinases by thiazovivin and 25 kinases by Y-27632, indicating that the prosurvival small molecule is a more selective ROCK inhibitor.

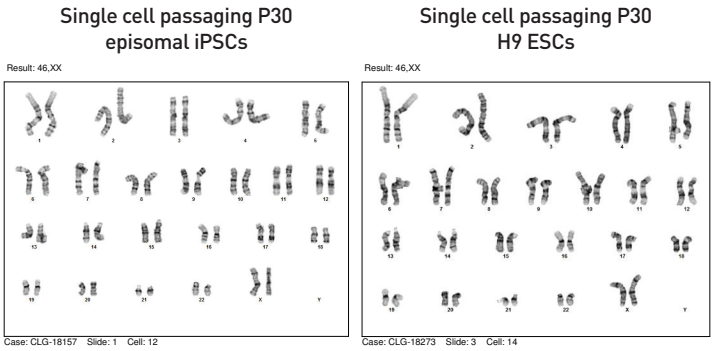

Figure 5. Normal karyotypes are retained following single cell passaging using Essential 8™ Medium plus RevitaCell™ Supplement at each passage for 30 passages after thawing. iPSCs generated using episomal vectors and H9 ESCs were passaged using TrypLE™ Select Enzyme and cryopreserved using PSC Cryomedium. After thawing, cells were recovered using Essential 8™ Medium with 1X RevitaCell™ Supplement for 18 to 24 hours, followed by feeding with Essential 8™ Medium alone until cells reached ~80% confluency. Cells were passaged using TrypLE™ Select Enzyme for a total of 30 passages, and recovered post-split using Essential 8™ Medium with 1X RevitaCell™ Supplement for 18 to 24 hours, followed by feeding with Essential 8™ Medium alone for the remainder of their time in culture. Karyotypes were assessed at the end of the 30th passage.

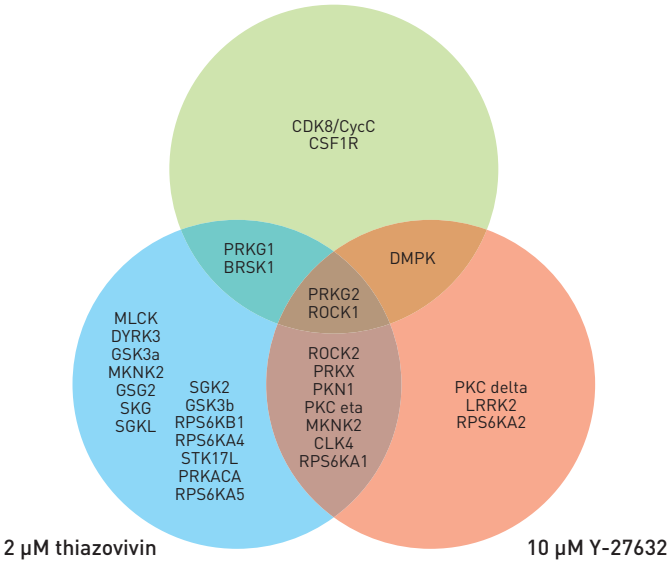

Figure 6. SelectScreen® Kinase Profiling of the prosurvival small molecule in RevitaCell™ Supplement shows it has more selectivity compared to traditional ROCK inhibitors. Small molecule compounds were tested against a panel of 319 kinases. Kinases exhibiting greater than 80% inhibition are shown.

Conclusion

RevitaCell™ Supplement was developed to improve the efficiency of post-thaw recovery of PSCs, and minimize apoptosis and necrosis over the first 24 hours after thawing. RevitaCell™ Supplement can also be used for routine single cell passaging of PSCs in Essential 8™ Medium, providing efficient recovery of cells while retaining normal morphology, pluripotency, and karyotype. Furthermore, the prosurvival small molecule contained within this cocktail is shown to be a more selective ROCK inhibitor, minimizing the off-target pathways affected. Thus, this system may provide a foundation for downstream experiments, including high-throughput screening and differentiation of PSCs to various cell types, as well as enabling gene editing applications.

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