Scalable Passaging of Human Pluripotent Stem Cells Cultured in Defined Media Using ReLeSR™

Erik B. Hadley[1], Jessica Norberg[1], Melanie Kardel[1], Vivian Lee, Terry E. Thomas[1], Allen C. Eaves[1,2], and Sharon A. Louis[1]
[1] STEMCELL Technologies Inc., Vancouver, Canada [2] Terry Fox Laboratory, BC Cancer Agency, Vancouver, B.C., Canada

Introduction

Current state-of-the-art culture methods for human pluripotent stem cells (hPSCs) use specialized and defined maintenance media including mTeSR™1 and TeSR™-E8™ to maintain an undifferentiated state in the absence of feeder cells. Both of these media were developed based on seminal publications from James Thomson's laboratory [Ludwig *et al.*, 2006; Chen *et al.*, 2011], and may be used with enzyme-based or enzyme-free passaging reagents such as Gentle Cell Dissociation Reagent (GCDR). The need to manually remove areas of spontaneous differentiation and to mechanically scrape cells from the cultureware following enzyme or calcium-chelation treatment are significant limitations that prevent scale-up to larger flasks or cell factories. We have developed an improved enzyme-free passaging reagent, ReLeSR™, which simplifies hPSC passaging by generating cell aggregates without the need for manual removal of differentiated cells, scraping, or complex manipulation to obtain the optimal aggregate size for efficient re-plating. This simplified protocol will greatly facilitate culture scale-up and automation. Here we assessed the performance of ReLeSR™ using human embryonic stem (ES) cell and induced pluripotent stem (iPS) cell lines cultured in either mTeSR™1 or TeSR™-E8™ medium. Additionally, we show that ReLeSR™ can also be used to rescue the quality of highly differentiated cultures in a single passage.

Materials & Methods

Following the methods described below, we were able to maintain high quality hPSCs with both ReLeSR™ and GCDR for multiple passages in either mTeSR™1 (Catalog #05850) or TeSR™-E8™ (Catalog #05940). Experiments were performed on 2 human ES cell lines (H1, H9) and 3 iPS cell lines (STiPS-M001, WLS-4D1, and STiPS-F016), and representative data is shown in the results section below.

Standard GCDR Protocol

Identify Differentiated Regions → Remove Differentiated Regions → Add Passaging Reagent → Incubate 6 - 8 minutes → Add TeSR™ → Scrape Colonies → Resize Cell Aggregates → Re-plate Cell Aggregates

ReLeSR™

Add & Remove ReLeSR™ → Incubate 5 - 9 minutes → Add TeSR™ → Tap/Shake Plate → Replate Cell Aggregates

Figure 1. The ReLeSR™ passaging protocol avoids difficult and time-consuming steps involved in hPSC culture.

Culture Matrices: Cultureware was coated with either a 10 µg/mL solution of Vitronectin XF™ (Catalog #07180/Primorigen Biosciences) or Matrigel® (Corning®) according to the supplier recommendations.

Passaging Cells: Cells were passaged every 5 - 7 days for up to 10 passages using a standard GCDR protocol (control) or a protocol developed for ReLeSR™.

Standard GCDR Protocol: The culture medium was aspirated and replaced with 1 mL of GCDR (Catalog #07174), followed by incubation at room temperature for 6 - 8 minutes. GCDR-treated aggregates were scraped, collected into 1 mL of fresh medium, and dissociated to the desired size by repeated pipetting.

ReLeSR™: The cells were first washed with 1 mL calcium- and magnesium-free PBS and then 1 mL of ReLeSR™ (Catalog #05872) was added to each well and immediately removed (i.e. colonies were exposed to a thin film of liquid). After 6 minutes incubation at 37°C (mTeSR™1 cultures), or 8 minutes incubation at room temperature (TeSR™-E8™ cultures), 1 mL of mTeSR™1 or TeSR™-E8™ was added. To dislodge the cell aggregates, the plate was held with one hand while the other hand was used to firmly tap the side of the plate for approximately 30 - 60 seconds. Alternatively, colonies were detached by placing the plate on a plate vortexer (Multi-Microplate Genie®, 120V; Model SI-4000; Scientific Industries) at 1,200 rpm and shaking for 2 - 3 minutes at room temperature (15 - 25°C). The cell aggregate suspension from either tapping or shaking was then transferred to a 15 mL tube without further manipulation of aggregate size by repeated pipetting.

Cell Plating: The dissociated aggregates were then diluted according to the desired split ratio and replated on a Corning® Matrigel® or Vitronectin XF™ pre-coated plate containing the appropriate maintenance medium.

Culture Assessment: At each passage, cells were characterized by observing cell morphology, percent undifferentiated cells ([# undifferentiated colonies/total # colonies] x 100), plating efficiency (# aggregates attached at 48 h/# aggregates seeded), and fold expansion (# aggregates harvested/# aggregates seeded). Expression of hPSC markers was assessed by flow cytometry at the end of each experiment.

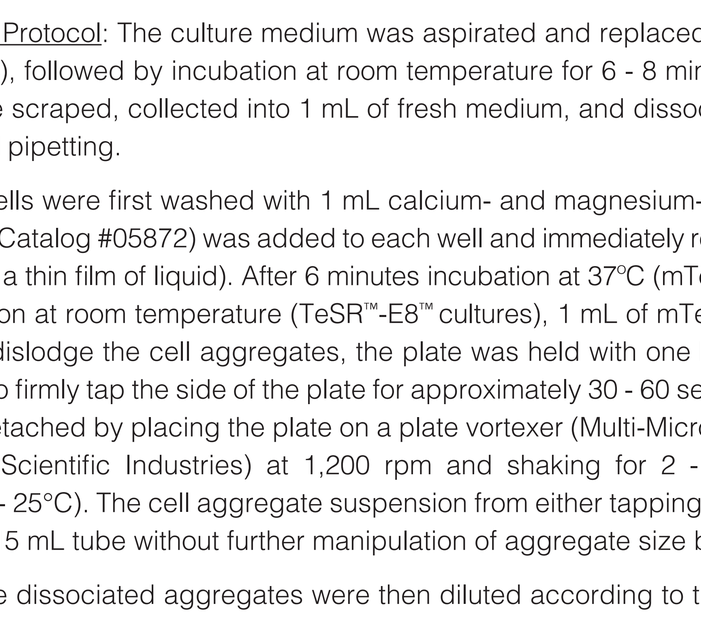

Figure 2. A) An hPSC culture ready for passaging. Note the presence of differentiated cells at the edge of the undifferentiated hPSC colony. **B)** Following incubation with ReLeSR™, the undifferentiated hPSC colony starts to lift off of the cultureware. The differentiated cells remain attached to the cultureware. **C)** Following tapping/shaking of the cultureware, the undifferentiated cells completely lift off of the cultureware. **D)** The undifferentiated hPSC colony is broken up into optimally-sized aggregates for replating.

Results

Characterization of hPSC passaged with ReLeSR™

Cells were passaged in either mTeSR™1 or TeSR™-E8™ medium for 10 passages and characterized using standard assays such as flow cytometry for markers of the undifferentiated state (OCT4 and TRA-1-60), expansion rate measurement, karyotype analysis and qualitative morphological assessment.

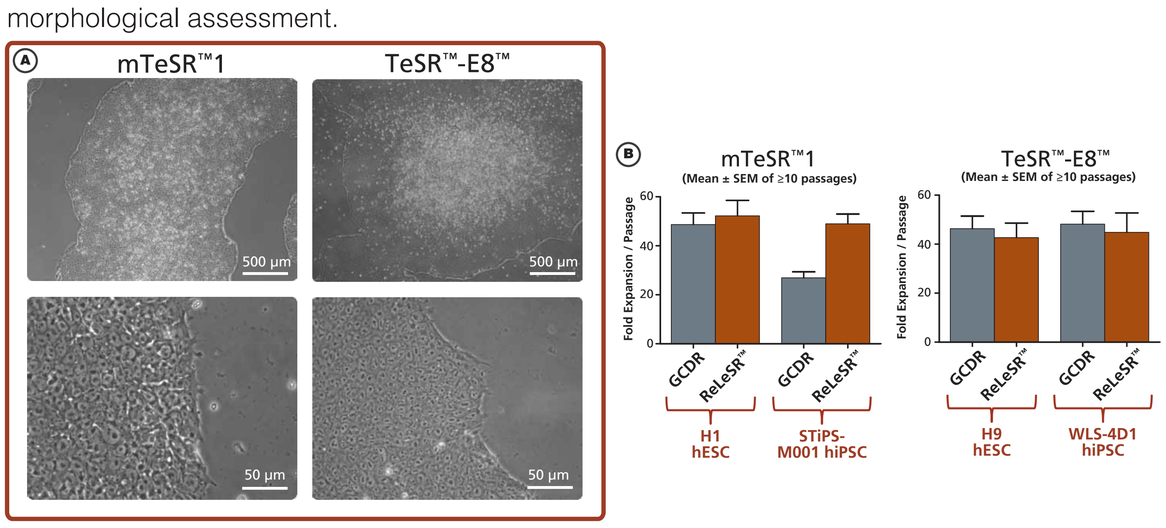

Figure 3. A) Undifferentiated human iPS (STiPS-M001) cells cultured in mTeSR™1 or TeSR™-E8™ media and dissociated with ReLeSR™ retain the prominent nucleoli and high nuclear-to-cytoplasm ratio characteristic of this cell type. Densely-packed cells and multilayering are prominent when cells are ready to passage. Images are representative of cultures at the optimal time of passaging. **B)** Graphs show the mean fold expansion per passage ± SEM obtained for human ES and iPS cells cultured in mTeSR™1 or TeSR™-E8™ with either GCDR (gray bars) or ReLeSR™ (brown bars) respectively over 10 passages. Note that the data is representative of cultures passaged every 5 - 7 days, however, the expansion is dependent on the duration of culture between the passages. Experiments were performed on 2 ES and 2 iPS cell lines, and representative data is shown.

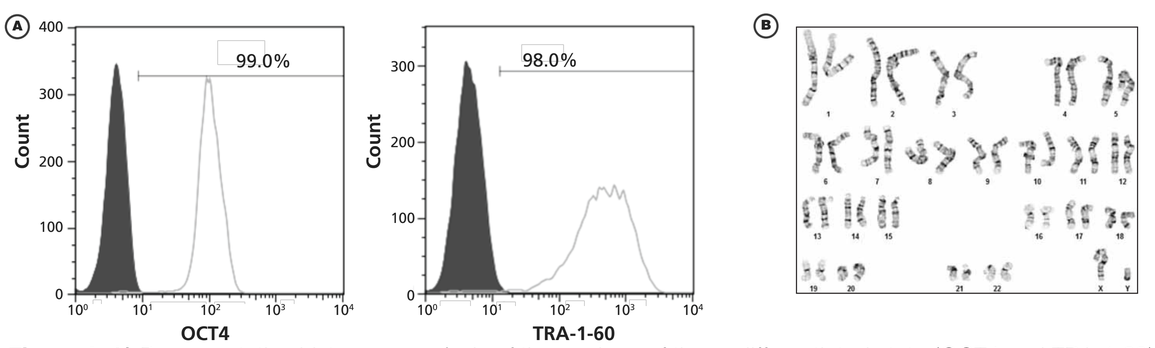

Figure 4. A) Representative histogram analysis of the markers of the undifferentiated state (OCT4 and TRA-1-60) for STiPS-M001 human iPS cells (white = sample, gray = isotype or secondary antibody only). Marker expression was assessed by flow cytometry after culturing for 10 passages in mTeSR™1 using ReLeSR™. **B)** Representative karyogram obtained for STiPS-M001 human iPS cells after culturing in mTeSR™1 for 10 passages using ReLeSR™ (20/20 cells analyzed were found to have a normal karyotype). Cells grown with each of the medium and passaging reagent combinations were found to have comparable performance and retained the accepted characteristics of high-quality hPSC cultures.

Rescue of Differentiated Cultures using ReLeSR™

Poor quality pluripotent stem cell cultures containing large proportions of differentiated cells can be rescued by passaging with ReLeSR™. During the dissociation step differentiated cells remain attached to the plate while the undifferentiated cells detach and are seeded for the next passage.

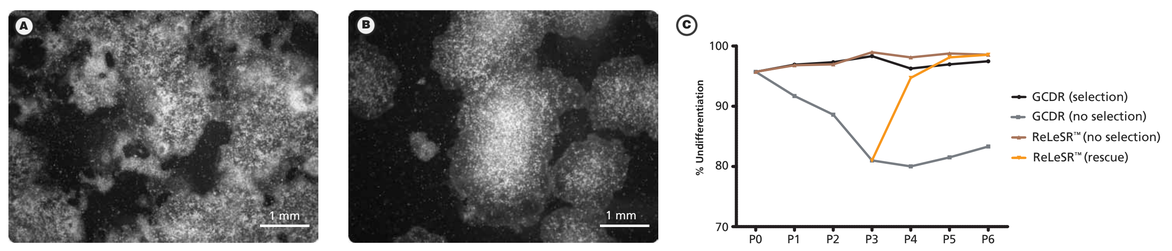

Figure 5. A) A poor quality hPSC culture containing ~50% undifferentiated cells. **B)** Following treatment of cultures shown in (A) with ReLeSR™ and subsequent replating, the differentiated cells were largely eliminated from the culture, with >90% undifferentiated cells present at the end of the next passage. **C)** Graph of percent undifferentiated colonies at each passage for cultures passaged with protocols as indicated. High proportions (>90%) of undifferentiated colonies were maintained using either GCDR with selection (black) or ReLeSR™ without selection (brown). Differentiation increased significantly when cells were dissociated using GCDR without selection (gray), however, the proportion of undifferentiated colonies was significantly reduced by treating this culture with ReLeSR™ starting at passage 3 (orange).

Differentiation of hPSCs Passaged With ReLeSR™

Human iPS cells (STiPS-F016) that were dissociated using ReLeSR™ could be differentiated into cells of all three germ layers after ≥15 passages. Cells were cultured in TeSR™-E8™ and dissociated using ReLeSR™ during every passage following their initial derivation.

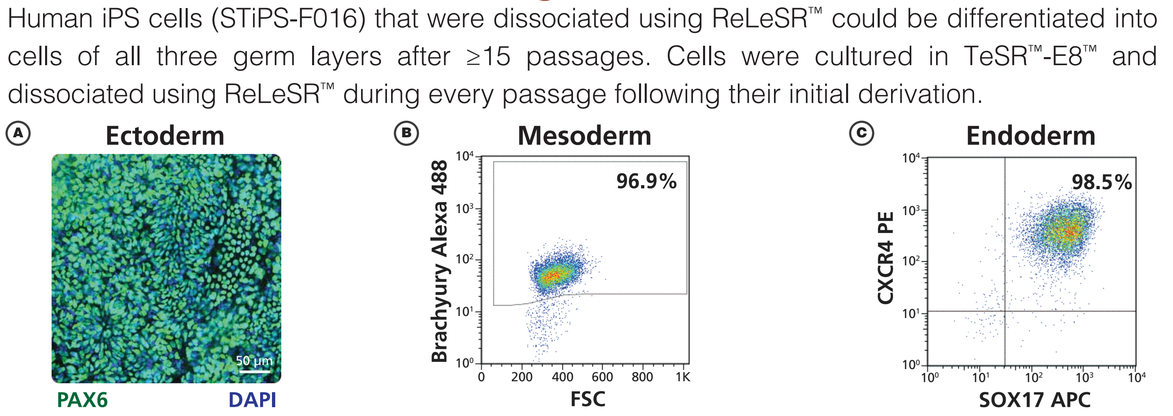

Figure 6. A) Immunocytochemistry image showing PAX6 (green) neural progenitor cells generated using STEMdiff™ Neural Induction Medium (Catalog #05831) for 9 days. Flow cytometry analysis showing differentiation into **B)** Brachyury+ early mesoderm cells using a protocol modified from Lian X, *et al.* 2012. **C)** SOX17+CXCR4+ definitive endoderm cells using the STEMdiff™ Definitive Endoderm Kit (Catalog #05110).

Summary

Our results indicate that ReLeSR™ is a suitable passaging reagent for the routine culture of hPSC in TeSR™ media: performance was comparable for all human ES and iPS lines tested, and the cells retained the hallmark characteristics of undifferentiated cells. ReLeSR™ simplifies passaging protocols for the maintenance of undifferentiated hPSCs in mTeSR™1 and TeSR™-E8™ by eliminating several steps requiring manual manipulation. Removal of these complex manipulation and selection steps gives operators the choice of selecting any cultureware from multiwell plates to flasks and closed vessels where these manipulations are not practical, thus enabling scale-up and automation.

PRIMORIGEN BIOSCIENCES® the stem cell company™

STEMCELL™ TECHNOLOGIES