Scalable Enzyme-Free Passaging of Human Pluripotent Stem Cells Cultured in mTeSR™1 or TeSR™-E8™ Without Scraping

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Introduction

Recent progress in human pluripotent stem cell (hPSC) research has been facilitated by the availability of defined culture media such as mTeSR™1 and TeSR™-E8™ that permit the maintenance of the pluripotent state in the absence of feeder cells. Both of these media were developed based on publications from James Thomson's laboratory [Ludwig *et al.*, 2006; Chen *et al.*, 2011], and can be used with enzyme-free passaging reagents such as Gentle Cell Dissociation Reagent (GCDR). However, a key limitation of this and other passaging reagents has been the need to mechanically scrape the culture surface to generate cell aggregates. Repeated generation and re-seeding of appropriately sized hPSC aggregates is critical, since repeated passaging as single cells can lead to the accumulation of chromosomal abnormalities [Mitalipova *et al.*, 2005]. We have developed ReLeSR™, an improved enzyme-free passaging reagent for the passaging of hPSC aggregates without the need for selection of differentiated cells, scraping, or complicated manipulation to obtain the desired aggregate size. ReLeSR™ enables the use of culture flasks and other closed vessels where the use of a cell scraper is not practical, thus facilitating culture scale-up and automation. Here we evaluate ReLeSR™ by assessing its performance using two human embryonic stem cell (hESC) lines (H1, H9) and two human induced pluripotent stem cell (hiPSC) lines (STiPS-M001, WLS-4D1) 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 hPSC with both ReLeSR™ and GCDR for multiple passages in either mTeSR™1 or TeSR™-E8™. Experiments were performed on two hESC and two hiPSC lines, and representative data is shown in the results section below.

Standard 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™ (STEMCELL/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™.

GCDR: The culture medium was aspirated and replaced with 1 mL of GCDR, 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™ was added to each well and immediately removed (i.e. colonies were exposed to a thin film of liquid). After 6 minutes incubation at 37ºC (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 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, expression of hPSC markers using flow cytometry, plating efficiency (# aggregates seeded ÷ # aggregates attached at >48h), percentage of undifferentiated colonies (# undifferentiated colonies ÷ by total # of colonies), and cell expansion (# aggregates harvested ÷ # aggregates seeded).

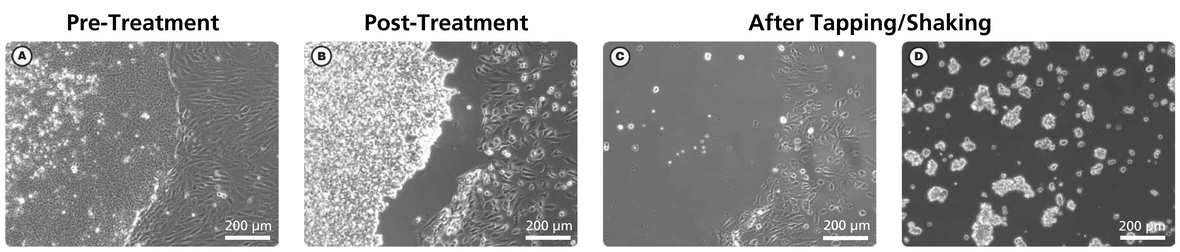

Figure 2. A) An hPSC culture ready for passaging. Note the presence of differentiated cells at the edge of the undifferentiated hPSC colony. **B)** Following incubation with ReLeSR™, the undifferentiated hPSC colony starts to lift off of the cultureware. The differentiated cells remain attached to the 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.

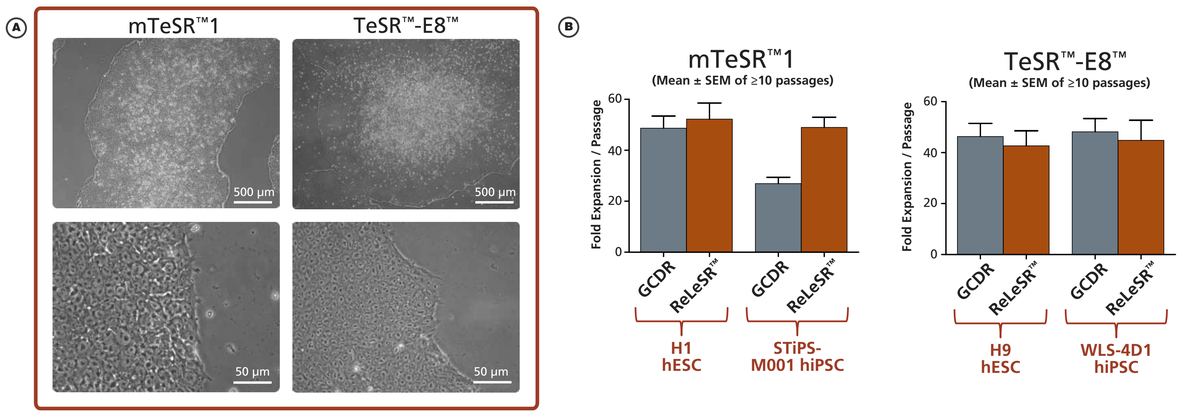

Figure 3. A) Undifferentiated human iPS (STiPS-M001) cells passaged with either GCDR or ReLeSR™ in mTeSR™1 or TeSR™-E8™ medium. Human ES and iPS cells cultured in mTeSR™1 or TeSR™-E8™ and passaged using ReLeSR™ retained the prominent nucleoli and high nuclear-to-cytoplasm ratio characteristic of these cell types. 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)** Graph shows 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.

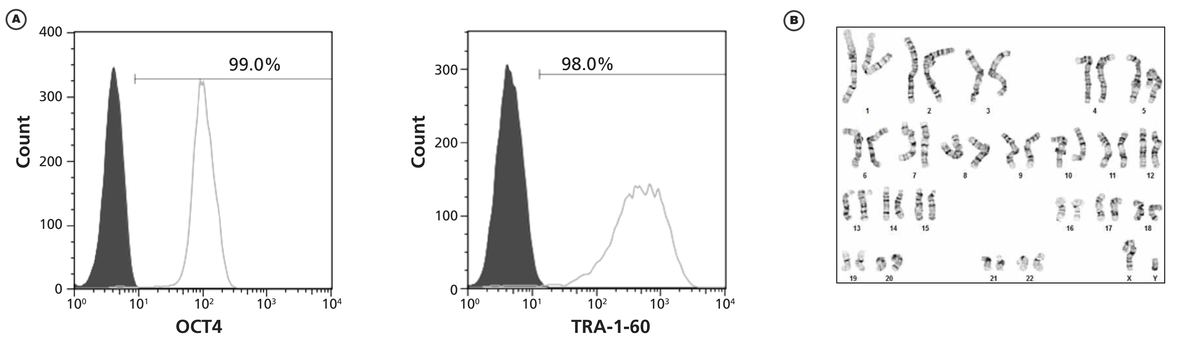

Figure 4. A) Representative histogram analysis of the markers of the undifferentiated state (Oct4 and Tra-1-60) for STiPS-M001 hiPSCs (white = sample, gray = secondary antibody only). Marker expression was assessed by flow cytometry after culturing for 10 passages in mTeSR™1 using ReLeSR™. **B)** Representative karyogram obtained for STiPS-M001 hiPSCs 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.

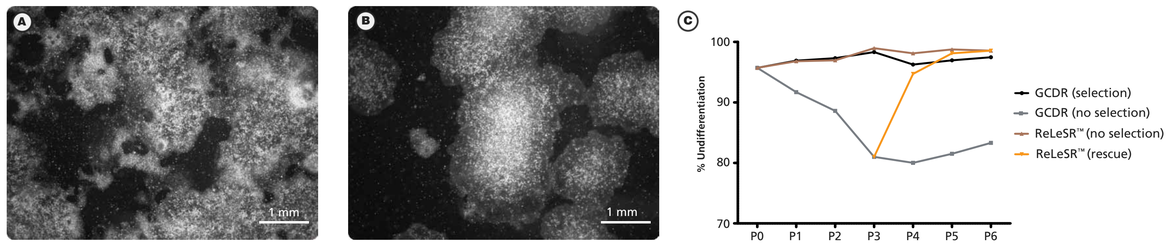

Figure 5. A) A poor quality hPSC culture containing ~50% undifferentiated cells. **B)** Following treatment of cultures shown in (A) with ReLeSR™ and subsequent replating, the differentiated cells were largely eliminated from the culture, with >90% undifferentiated cells present at the end of the next 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 passaged 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).

Summary

ReLeSR™

- An improved, enzyme-free reagent for passaging hPSCs
- Selectively detaches undifferentiated cells
- Easily generates optimally-sized aggregates without manual scraping
- Compatible with culture processes involving closed vessels
- Compatible with mTeSR™1, TeSR™-E8™, Vitronectin XF™ and Corning® Matrigel®

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