Enzyme-Free Protocols for Isolation and Maintenance of Human Induced Pluripotent Stem Cells (hiPSC) in a Completely Xeno-Free System and their Differentiation to Neural Cells

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Introduction

TeSR™-E7™ is a xeno-free (XF) medium that supports reprogramming of somatic cells to human induced pluripotent stem cells (hiPSC). Emerging hiPSC colonies can then be isolated and expanded in XF TeSR™-E8™ Medium with the Vitronectin XF™ matrix. A key barrier to scaling up this workflow is the need to mechanically generate cell aggregates during hiPSC isolation and passaging steps. We developed ReLeSR™, for the selective detachment of hiPSC aggregates without the need for scraping, removal of differentiated cells or complicated manipulation to obtain the desired aggregate size. ReLeSR™ enables the use of closed vessels where cell scraping is not practical, thus facilitating scale-up and automation. We evaluated ReLeSR™ by isolating and characterizing a new fibroblast derived hiPSC line in a completely XF workflow. Fibroblasts were cultured in MesenCult™-ACF Medium and reprogrammed to nascent hiPSC colonies in TeSR™-E7™ using an episomal vector system containing reprogramming factors.

Materials and Methods

Reprogramming: Fibroblasts were maintained and reprogrammed according to the method shown in **Figure 1**.

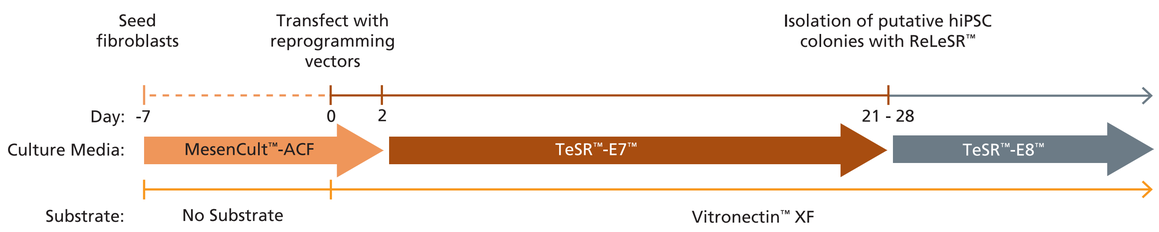
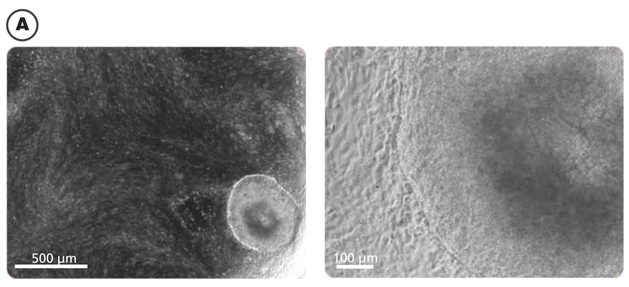


FIGURE 1: Schematic of reprogramming system using a xeno-free workflow
Newborn foreskin fibroblasts were expanded in MesenCult™-ACF with uncoated plates, and the media was changed once per passage. The fibroblasts were harvested and then transfected with episomal vectors containing OCT4, SOX2, KLF4, L-MYC and Lin28. Post transfection (day 0), cells were seeded on Vitronectin XF™ coated plates at cell densities of 250 - 500 cells per cm^2 in 24 well plates. Densities were chosen to maximize the number of wells with a single hiPSC colony (see **Figure 2B**). On day 2 post-transfection, media was changed to TeSR™-E7™ and changed daily until day 21 - 28, when hiPSC colonies were identified by microscopic examination of morphology. Colonies were isolated without manual selection using ReLeSR™ (see **Passaging Cells** for protocol), transferred to Vitronectin XF™ coated plates, and cultured in TeSR™-E8™ for further expansion of hiPSCs.



B

Seeding Density	Total Wells	# Wells >1 colony	# Wells = 1 colony
250 cells/cm^2	24	14	6
500 cells/cm^2	48	42	1

FIGURE 2: Newly reprogrammed hiPSC colony ready for selection using ReLeSR™
A) Day 23 hiPSC colony grown in xeno-free conditions. Using ReLeSR™ (see **Passaging Cells** for protocol) emerging hiPSC colonies were selectively detached and replated down in TeSR™-E8™ on Vitronectin XF™. **B)** The number of colonies per well at 250 and 500 cells per cm^2. Note that only wells containing one colony per well were harvested to generate clonal cell lines using the ReLeSR™ protocol.

Culture Matrices: Fibroblasts were cultured without a substrate. Following transfection, cells were seeded on 24-well plates coated with a 10 µg/ml solution of Vitronectin XF™.

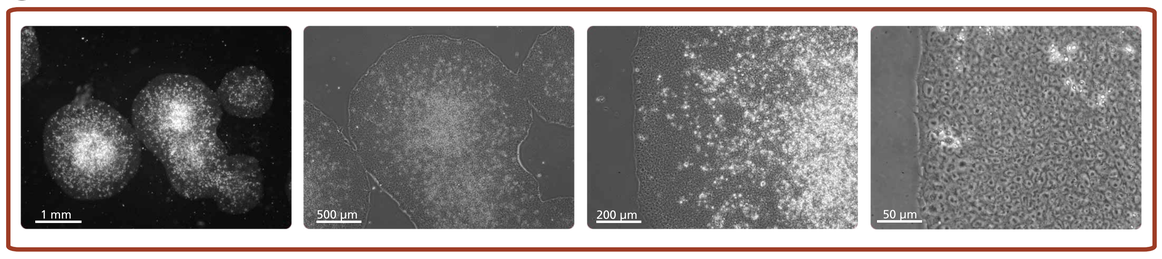
Passaging Cells: Cells were passaged every 5 - 7 days using the ReLeSR™ protocol. The cells were first washed with 1 mL calcium and magnesium-free PBS and then 1 mL of ReLeSR™ was added to each well and immediately removed. After 7 - 9 minutes incubation at room temperature, 1 mL of TeSR™-E8™ was added. Cell aggregates were dislodged by firmly tapping the plate for 30 - 60 seconds and transferring to a 15 mL tube for aggregate counts and replating.

Cell Plating: Nascent hiPSC colonies harvested with ReLeSR™ were seeded into the first passage at a 1:1 split ratio. In subsequent passages the dissociated aggregates were diluted according to the desired split ratio and replated onto Vitronectin XF™ coated plates in TeSR™-E8™.

Culture Assessment: At each passage, cultures were characterized by observing cell morphology, percentage of undifferentiation [(# undifferentiated colonies / total # colonies) x 100%], and fold expansion (# aggregates harvested / # aggregates seeded). After 5 passages cultures were characterized by assessing hiPSC markers using flow cytometry, karyotype analysis, and directed differentiation down the neural lineage.

Results

Characterization of hiPSCs reprogrammed using a xeno-free workflow: Performance was evaluated by cell morphology, fold expansion, expression of hiPSC markers, karyotype analysis and neural differentiation.



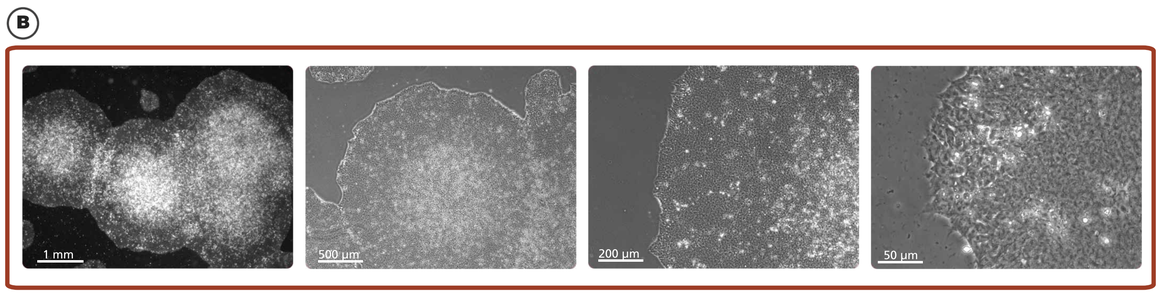
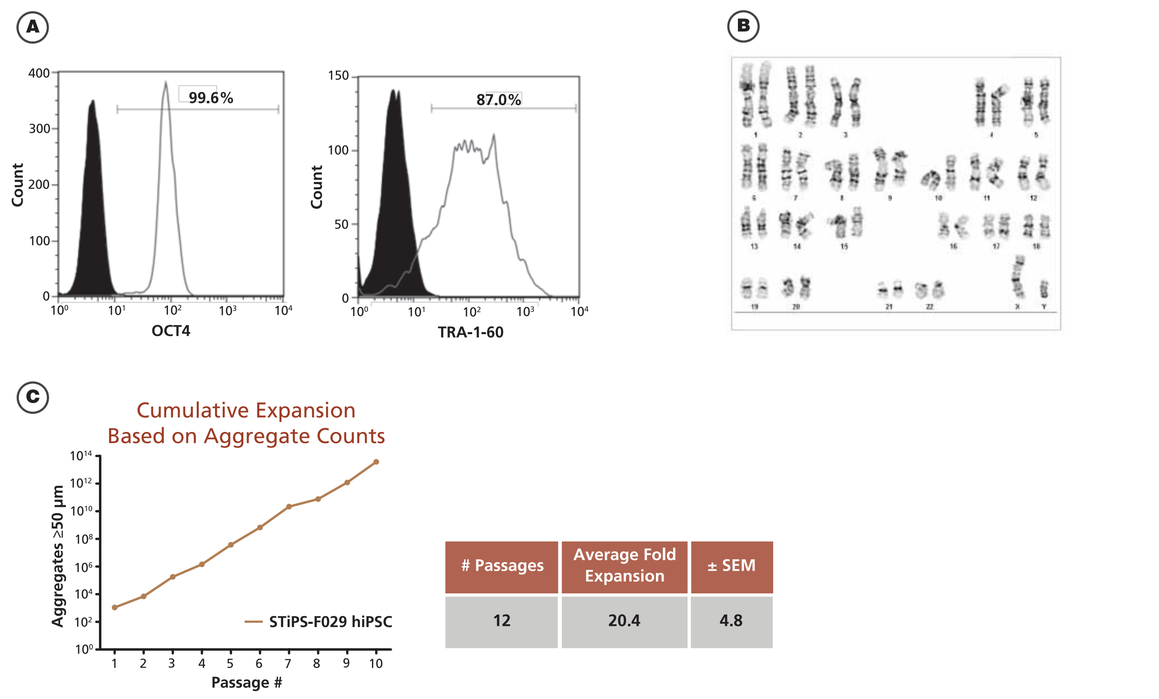


FIGURE 3: High quality morphology of hiPSC colonies derived using a xeno-free workflow
A) Undifferentiated hiPSCs cultured and expanded using a XF workflow compared microscopically to **B)** STiPS-F016, a well-characterized control cell line derived from normal human dermal fibroblast cells under non-XF conditions and maintained on Matrigel™ and TeSR™-E8™. Colonies derived using a XF workflow 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.



# Passages	Average Fold Expansion	± SEM
12	20.4	4.8

FIGURE 4: Cells grown using a XF workflow retain the accepted characteristics of high-quality hiPSC cultures
A) Representative histogram analysis of the markers of the undifferentiated state (OCT4 and TRA-1-60) for hiPSC colonies derived using a XF workflow (white = sample; black = isotype control). Marker expression was assessed by flow cytometry after culturing for 5 passages in TeSR™-E8™ and the ReLeSR™ protocol. **B)** Representative karyogram obtained from XF derived hiPSC cultures after 5 passages using ReLeSR™ (20/20 cells analyzed were found to have a normal karyotype). **C)** Average fold expansion of XF hiPSC line over 12 passages at 20.4 ± 4.8 (SEM).

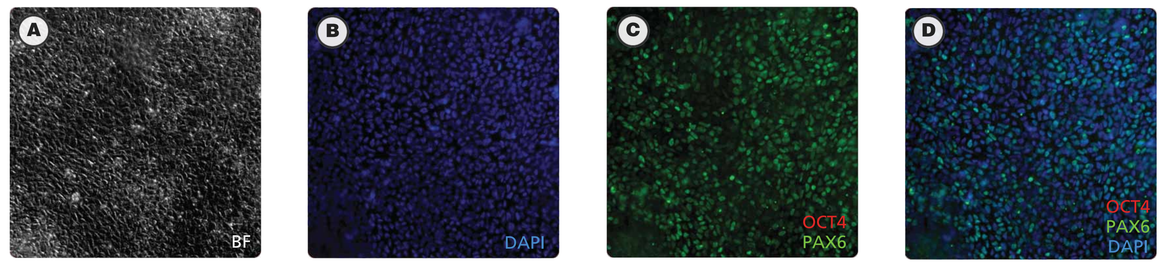


FIGURE 5: Neural induction of hiPSC cultures derived using a xeno-free workflow
Undifferentiated hiPSC colonies were harvested and plated as a monolayer in STEMdiff™ Neural Induction Medium. Cells were fixed after 7 days in culture and imaged using either **A)** brightfield (BF) or **B)** DAPI (blue), **C)** OCT4 (red) and PAX6 (green). Representative images show the absence of OCT4 expression and presence of PAX6 expression. These data indicate cells grown using a XF workflow retain the ability to differentiate to neural progenitor cells.

Summary

- Newborn foreskin fibroblasts can be reprogrammed using a completely xeno-free (XF) workflow and retain high quality hiPSC colony morphology and high expansion rates with ReLeSR™
- hiPSC cultures grown using XF workflow maintain high expression of hiPSC markers (>90%) and retain a normal karyotype
- hiPSC cultures grown using XF workflow retain downstream differentiation capability