HyCryo-STEM shows equal or improved performance to industry standard for cryopreservation of stem cells

HyClone™ HyCryo-STEM cryopreservation medium is designed to freeze cells traditionally sensitive to the cryopreservation process, such as neural progenitor or stem cells and embryonic stem cells (Fig 1). The product has a DMSO-based formulation and is chemically defined and serum-free to ensure lot-to-lot consistency and to provide control over the entire stem cell workflow. Provided at a 2× concentration, the HyCryo-STEM freeze medium is added to cells suspended in their own conditioned growth medium to minimize osmotic shock during cryopreservation. In this study, we demonstrate that the post-thaw recovery rate of pluripotent stem cells and neural progenitor and stem cells is either equivalent to or better than the industry standards. In addition, HyCryo-STEM medium also helps maintain the stemness of these cells, providing healthy and stable stocks of stem cells for downstream applications.

Fig 1. HyCryo-STEM cryopreservation medium for stem cells.

Introduction

Cryopreservation is a critical step in the cell culture workflow, preventing genetic drift and allowing for long-term storage and transportation of cells. During cryopreservation, cells are exposed to a number of stress factors including osmotic imbalance, damaging free radicals, and dormant metabolic activities. Certain cell types, such as pluripotent stem cells and other progenitor cells can be very sensitive to cryopreservation, resulting in low recovery of viable cells or changes in stem cell properties upon thaw. Standard cryopreservation medium formulations, especially the home-brew freeze cocktails, do not always adequately protect stem cells from preservation-induced stress. HyCryo-STEM cryopreservation medium is designed with a formulation that mitigates underlying causes of preservation-related stem cell death and maintains their differentiation potential (Fig 2).

Materials and methods

Cell culture

Mouse cortical stem cells were cultured as a monolayer on poly-D-lysine/laminin-coated tissue culture dishes in DMEM/F-12 supplemented with N2, stable glutamine dipeptide, EGF, and bFGF. Neurosphere cultures of mouse or rat cortical stem cells were conducted in suspension on low-binding dishes in the same growth medium. Fresh growth factors were added every 1–2 d and the medium replaced every 2–3 d for all cultures until passage. At log growth phase, cells were harvested using HyQTase™ cell detachment solution. Cryopreservation and further differentiation were conducted.

The human induced pluripotent stem (iPS) and embryonic stem (ES) cells were cultured on feeder layers of irradiated mouse embryonic fibroblasts (MEFs). The iPS and ES cells were grown in DMEM/F-12 supplemented with 20% serum replacement, stable glutamine dipeptide, non-essential amino acids, β-mercaptoethanol, and bFGF. Anti-apoptotic reagent was used for the initial 48 h upon seeding. Thereafter, growth medium was replaced every day until next passage. Cells were harvested for cryopreservation when 60% to 90% confluency was reached and > 50% of the colonies had tight borders.

Cryopreservation

Before harvesting cells, the spent growth medium was collected from culture and clarified by filtration (0.2 µm polyethersulfone [PES] filter). Cells were harvested when in log phase growth as described above. Thereafter, the cells were resuspended in their own conditioned growth medium after centrifugation. Equal volume of the 2× formulation of HyCryo-STEM was slowly added to the resuspended cells, a few drops at a time with gentle mixing. The resulting cell suspension (~ 1×10^6 cell/mL) was divided into in 1 mL aliquots in cryotubes before being transferred to a cryocontainer and placed in a -80°C freezer overnight. The cells were stored in liquid nitrogen until thaw.

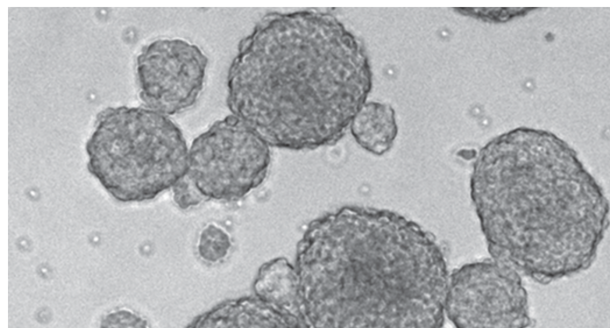

Fig 2. Post-thaw neurosphere culture of rat cortical stem cells.

Upon thaw, cells were quickly incubated in a 37°C water bath, and removed before all the ice had melted. The cells were rinsed with basal growth medium (without the growth supplements) to remove the cryopreservation reagent. Hereafter, the cells were resuspended in the complete growth medium and either used for viability assay or for further growth. To assess viability, the trypan blue exclusion assay was used. Cells were counted in 0.2% trypan blue solution using a hemacytometer.

Colorimetric metabolic assay

Post-thaw growth curves of the cells were generated based on the metabolic conversion of a tetrazolium dye at appropriate time points. The absorbance or optical density at 490 nm (OD 490) was measured spectrophotometrically. Initial seed density used was an equal number of viable cells per well, based on viability assay results upon thaw. For post-thaw growth curves, ES cells were grown on matrix-coated plates to minimize background signal from mouse embryonic feeder (MEF) cell layer.

Differentiation

Post-thaw, rat and mouse cortical stem cells were allowed to recover for 2–3 d in low-binding plates. After forming small neurospheres, the cells were plated onto poly-D-lysine/laminin-coated tissue culture dishes in the appropriate differentiation medium. Neuronal differentiation medium consisted of basal medium, serum, and stable glutamine dipeptide. Oligodendrocyte differentiation medium consisted of basal medium, serum, stable glutamine dipeptide, and tri-iodothyronine. Astrocyte differentiation medium consisted of DMEM, N2, stable glutamine dipeptide, and 1% fetal bovine serum (FBS). After 5 and 10 d, for mouse and rat cells, respectively, the cells were fixed and stained for immunocytochemistry with appropriate cell type markers.

Post thaw, human iPS and ES cells were allowed to recover for one passage on MEF cells as described in Section Cell culture. Cells were cultured on feeder-free, matrix-coated plates in DMEM/F-12 supplemented with N2, serum substitute, stable glutamine dipeptide, non-essential amino acids, β-mercaptoethanol, BSA, penicillin, and streptomycin. Growth factor bFGF and an anti-apoptotic reagent were used for 2-3 d before appropriate induction factors were added to the medium. For ectoderm, 100 ng/mL Noggin was supplemented for two weeks. For endoderm, 100 ng/mL activin A was supplemented for two weeks. For mesoderm, 50 ng/mL activin A and 50 ng/mL BMP-4 were supplemented for one week before medium without the induction factors were used for an additional week. Two weeks following the induction of differentiation, the cells were fixed and stained for immunocytochemistry with appropriate cell type markers.

Immunocytochemistry

All cells were fixed for staining using 4% paraformaldehyde and blocked with 5% goat serum in Dulbecco's PBS with 0.1% Triton™-X100 (except O4 marker, which required blocking solution without Triton-X100). All primary antibodies were incubated overnight at 4°C in blocking solution. Secondary antibodies in blocking solution were incubated for 1–2 h at room temperature. Finally, all cells were stained with Hoechst to mark nuclei. Immunofluorescence was acquired and analyzed using the ArrayScan™ reader with Target Activation Bioapplication software (Cellomics). Quantification is based on the average fluorescence intensity per object for a minimum of 40 000 cells.

Nestin expression was used to determine the proliferating qualities of the cortical progenitor cells. Following differentiation, the neurons were stained for β3-tubulin, the oligodendrocytes for O4, and the astrocytes for GFAP. The pluripotent state of human iPS and ES cells were assessed by staining for pluripotent marker Oct4. The cells differentiated into the three germ layers were identified by β3-tubulin for ectoderm, FOXA2 for endoderm, and smooth muscle actin (SMA) for mesoderm.

Results

Cryopreservation of neural progenitor and stem cells

Upon freeze and thaw of the neural progenitor cells, cell viability is measured. When compared with the industry standard cryopreservation medium containing 10% DMSO, cells recovered from HyCryo-STEM demonstrate a greater than 100% improvement in viability for mouse cortical stem cells and a greater than 20% improvement for rat cortical stem cells (Fig 3A). Neural progenitor cells recovered from the HyCryo-STEM medium also show normal growth rate as measured by the colorimetric metabolic assay (Fig 1B), indicating healthy metabolic state of the viable cells. The performance of HyCryo-STEM is not limited to the adherent culture. The neurospheres in suspension culture demonstrate similar degree of improvement over the standard method in post-thaw cell recovery (Fig 4).

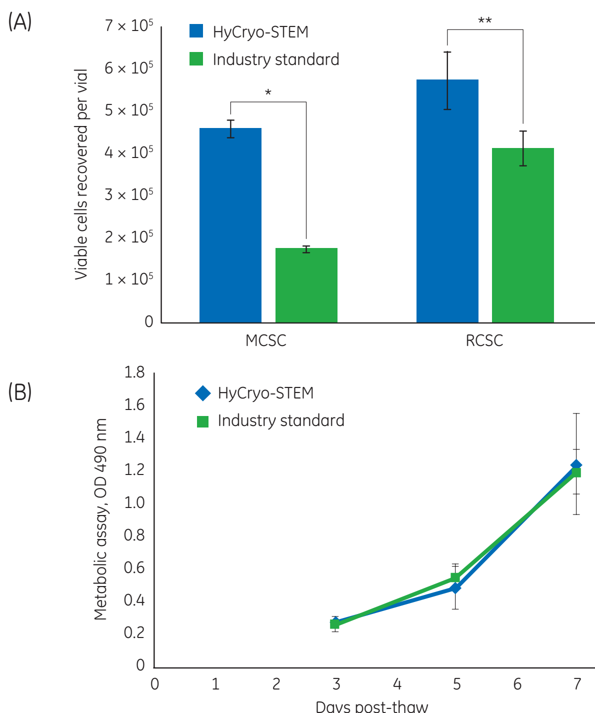

Fig 3. HyCryo-STEM has greater recovery of viable neural progenitor cells compared with industry standard cryopreservation medium (growth medium containing 10% DMSO). (A) Mouse cortical stem cells (MCSCs) are grown in adherent culture and rat cortical stem cells (RCSCs) are grown in neurosphere culture prior to freeze. Trypan blue exclusion assay is performed for cell viability assessment post-thaw (*p value $< 1 \times 10^{-7}$, **p value < 0.01). (B) Post-thaw growth curves of rat cortical stem cells are generated using colorimetric metabolic assay.

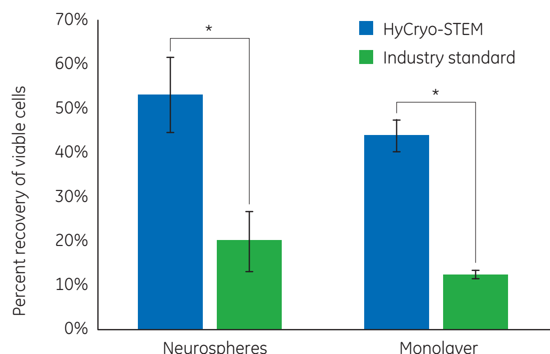

Fig 4. HyCryo-STEM displays similar advantages over the industry standard cryopreservation medium (growth media containing 10% DMSO) for both monolayer and neurosphere culture conditions. Mouse cortical stem cells are either cultured as an adherent monolayer or are allowed to form neurospheres prior to freeze. Trypan blue exclusion assay is performed post-thaw for cell viability assessment (*p values $< 1 \times 10^{-4}$).

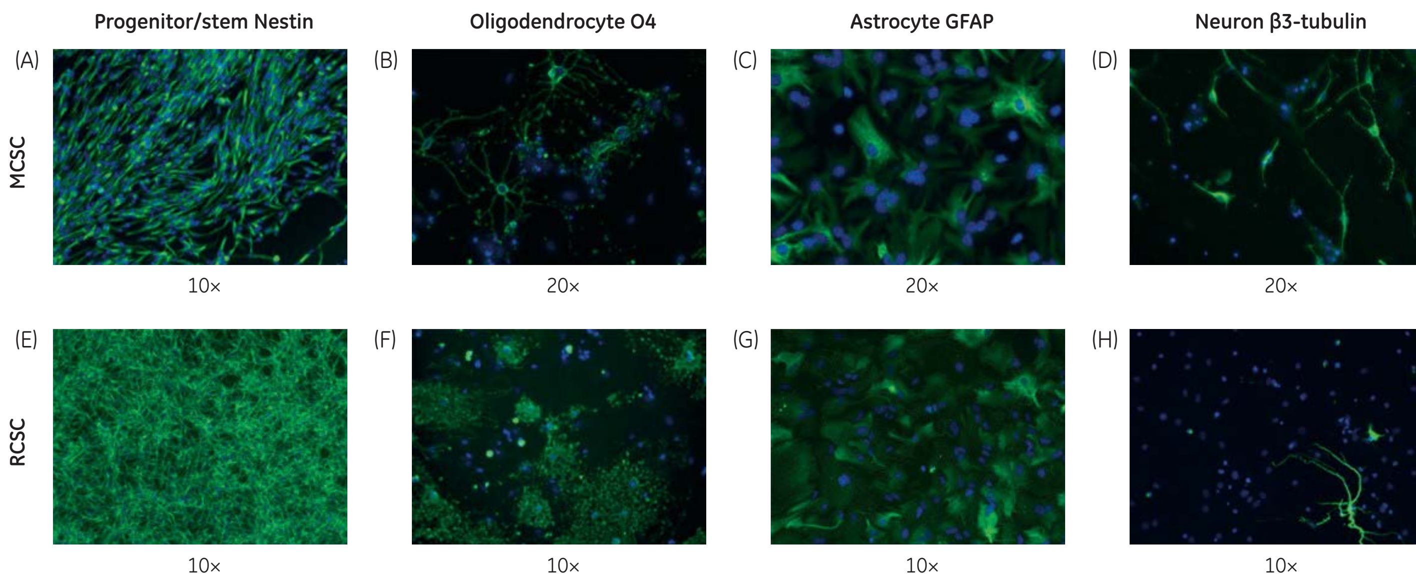

Fig 5. HyCryo-STEM maintains tripotency of neural progenitor cells post-thaw. Mouse cortical stem cells (MCSCs) or rat cortical stem cells (RCSCs) are grown as a monolayer in adherent culture post-thaw from HyCryo-STEM. (A, E) The MCSCs and RCSCs are immunostained with the progenitor cell marker, Nestin (green). Following directed differentiation, (B, F) oligodendrocytes are stained with O4 marker (green), (C, G) astrocytes with GFAP marker (green), and (D, H) neurons with β3-tubulin marker (green). All cells are counter-stained with Hoechst (blue).

In addition to preserving the viability of neural progenitor cells, HyCryo-STEM also maintains their stemness, as demonstrated by the immunocytostaining of the progenitor cell marker Nestin for both mouse and rat cortical stem cells post-thaw (Fig 5A and E).

Furthermore, mouse and rat cortical stem cells are able to differentiate into neurons (Fig 5D and H), astrocytes (Fig 5C and G) and oligodendrocytes (Fig 5B and F).

Taken together, our study shows that HyCryo-STEM is an excellent choice for the cryopreservation of neural progenitor cells.

Cryopreservation of human pluripotent stem cells

Human ES and iPS cells were preserved in HyCryo-STEM cryopreservation media. After they were thawed from liquid nitrogen, the cells were assessed for viability and plated onto the feeder layers for passaging. Viable recovery from HyCryo-STEM is measured for both human iPS and ES cells in comparison with those from an industry standard freeze medium (a cocktail of 25% serum replacement and 10% DMSO). HyCryo-STEM allows for recovery of viable human pluripotent stem cells similar to that of the industry standard cryopreservation media (Fig 6A). The human ES cells recovered from HyCryo-STEM grow at a similar rate to those recovered from the industry standard cryopreservation medium (Fig 6B).

Human iPS cells and ES cells recovered from HyCryo-STEM display healthy colony morphology with tight borders (data not shown). Their pluripotency is maintained during cryopreservation in HyCryo-STEM medium as demonstrated by the post-thaw immunocytostaining of the pluripotency marker Oct4 (Fig 7A and E). After a single passage recovery from HyCryo-STEM, human iPS and ES cells were induced to differentiate into all three germ layers as shown by ectoderm marker β3-tubulin (Fig 7B and F), mesoderm marker SMA (Fig 7C and G), and endoderm marker FOXA2 (Fig 7D and H). The differentiation efficiency is not altered compared with cells recovered from industry standard cryopreservation medium (data not shown).

In summary, HyCryo-STEM freeze medium is an excellent serum-free and chemically defined alternative for preserving human pluripotent stem cells.

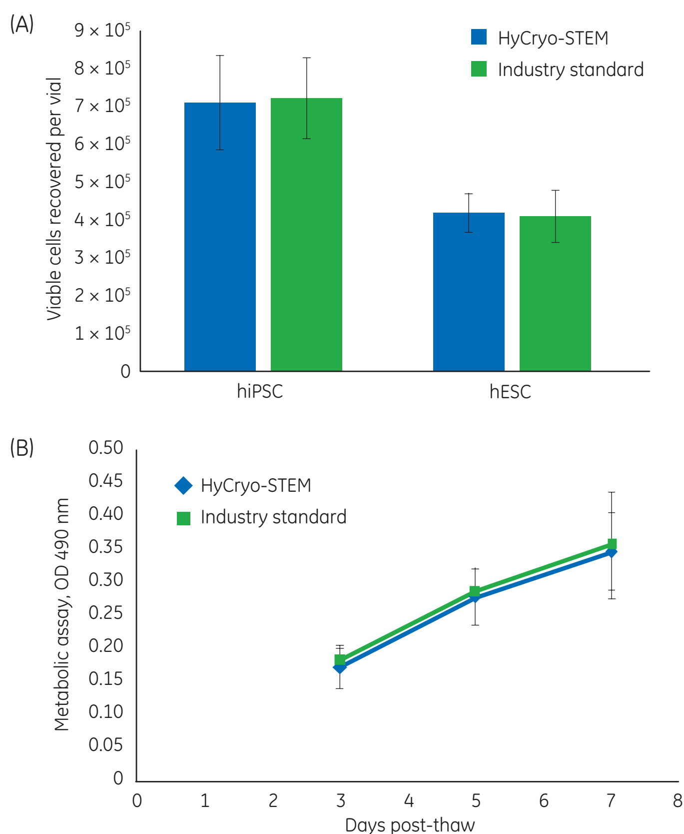

Fig 6. HyCryo-STEM achieves similar recovery of viable human pluripotent stem cells compared to the industry standard cryopreservation media (a cocktail of 25% serum replacement and 10% DMSO). (A) Trypan blue exclusion assay is performed post-thaw to assess viability of human iPSC and ESC. (B) Post-thaw growth curves of human ESC are generated with colorimetric metabolic assay.

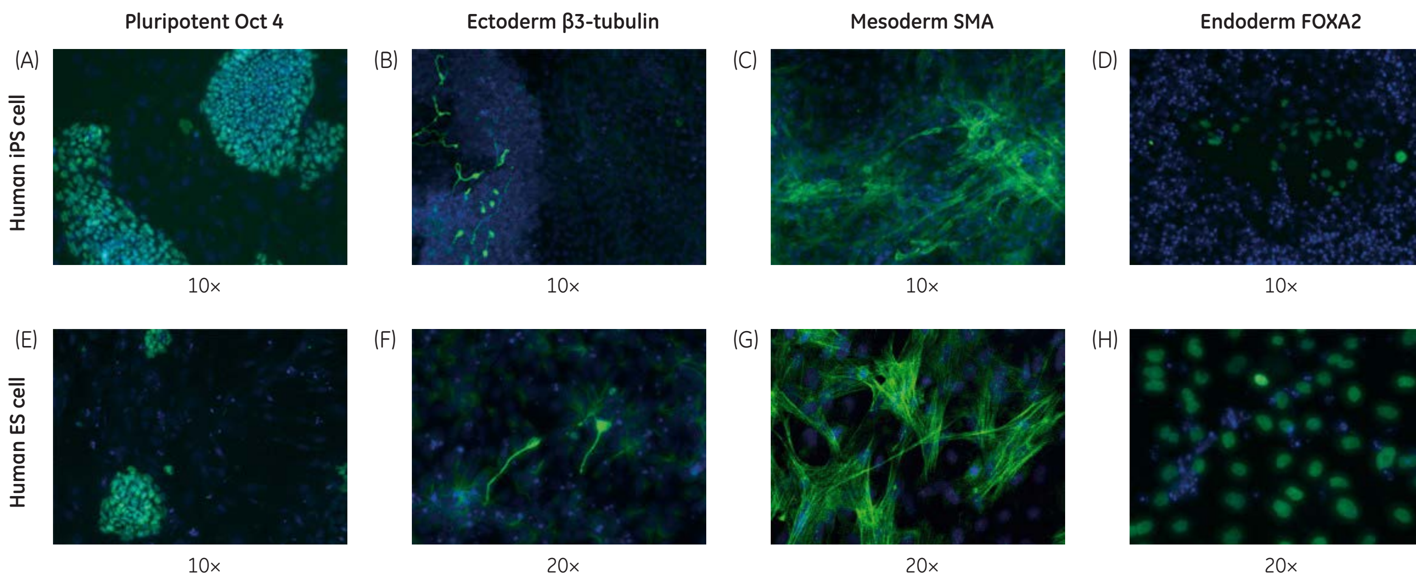

Fig 7. HyCryo-STEM maintains differentiation potential of human pluripotent stem cells post-thaw. Human iPS and ES cells are grown on MEFs post- thaw from HyCryo-STEM. (A, E) Pluripotency is evaluated by immunocytostaining of Oct4 (green). Following differentiation, each of the germ layers is stained. Ectoderm (B, F) is stained with β3-tubulin marker (green), mesoderm (C, G) with SMA marker (green), and endoderm (D, H) with FOXA2 marker (green). All cells are counter-stained with Hoechst (blue).

Conclusions

The formulation of HyCryo-STEM cryopreservation medium minimizes osmotic shock in stem cells during cryopreservation and allows for rapid restoration of their metabolic activities upon thaw. HyCryo-STEM is an excellent choice for cryopreservation of neural progenitor or stem cells as demonstrated by improved cell viability over standard cryopreservation media and by the maintenance of tripotency of the neural progenitor cells.

HyCryo-STEM is a serum-free and chemically defined alternative for cryopreservation of human pluripotent stem cells as evidenced both through maintenance of cell viability as well as unaltered ability to differentiate into all three germ layers.

Ordering information

Product	Description	Product code	VWR product code
HyCryo-STEM 2×	Cryopreservation medium for stem cells	SR30002	89399-934
HyQTase solution	Cell detachment solution	SV30030	82013-746
DMEM/F-12	With 2.50 mM L-glutamine, 15 mM HEPES	SH30023	16777-133
Fetal bovine serum	Characterized, US origin	SH30071	16777-014

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