

# Thermo Scientific HyCryo-STEM: A chemically defined and serum-free freeze media for stem cell cryopreservation

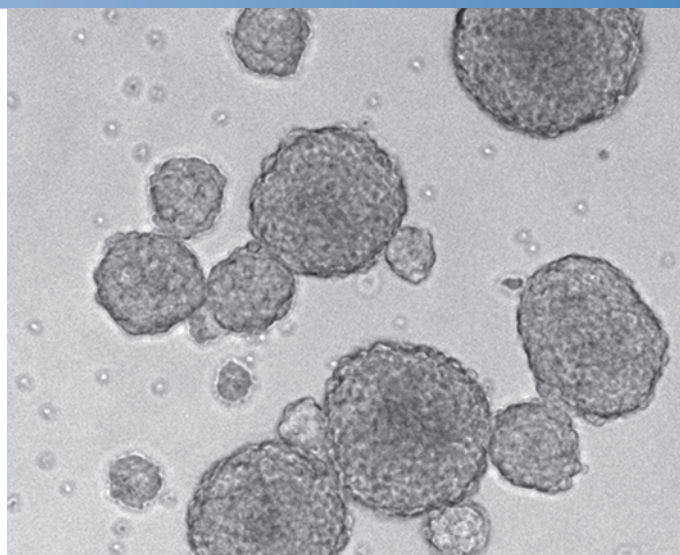
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## Key Words

HyCryo-STEM, cryopreservation, chemically defined and serum-free, embryonic stem cells, neural progenitor/stem cells

## Abstract

Thermo Scientific™ HyCryo-STEM™ cryopreservation media is designed to freeze cells that are traditionally sensitive to the cryopreservation process, such as neural progenitor/stem cells and embryonic stem cells. This DMSO-containing formulation is chemically-defined and serum-free. Provided at a 2x concentration, the HyCryo-STEM freeze media is added to cells suspended in their own conditioned growth media to minimize osmotic shock during cryopreservation. In this study, we demonstrate that the post-thaw recovery rate of pluripotent stem cells and neural progenitor/stem cells is either comparable to or better than the industry standards. In addition, HyCryo-STEM media also helps maintain the stemness of these cells, providing healthy and stable stocks of stem cells for downstream applications.



Post-thaw neurosphere culture of rat cortical 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 media

formulations, especially the home-brew freeze cocktails, do not adequately protect stem cells from preservation induced stress. Thermo Scientific HyCryo-STEM cryopreservation media is designed with an improved formulation that mitigates underlying causes of preservation-related stem cell death and maintains their differentiation potential. Its DMSO-based formulation is chemically-defined and serum-free. This ensures improved lot-to-lot consistency and provides better control over the entire stem cell workflow.



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## Materials

HyCryo-STEM, 2x cryopreservation media, Thermo Scientific HyClone Catalog #SR30002

DMEM/F-12, Thermo Scientific HyClone Catalog #SH30023

Fetal bovine serum, Thermo Scientific HyClone Catalog #SH30071

HyQtase™, Thermo Scientific HyClone Catalog #SV30030

Filtration unit, Thermo Scientific Nalgene Catalog #564-0020

CryoTube vials, Thermo Scientific Nunc Catalog #368632

Mr. Frosty™ container, Thermo Scientific Nalgene Catalog #5100-0001

15ml conical tubes, Fisher Scientific Catalog #05-539-12

50ml conical tubes, Fisher Scientific Catalog #05-539-8

Centrifuge, Thermo Scientific Sorvall Legend XTR centrifuge

Liquid nitrogen tank, Thermo Scientific CryoPlus 3

Spectrophotometer, Thermo Scientific Varioskan

Immunofluorescence image capture instrument and analysis software, Thermo Scientific Arrayscan

Centrifuge, Thermo Scientific Sorvall Legend XTR centrifuge

## 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 media. Fresh growth factors were added every 1-2 days and the media replaced every 2-3 days for all cultures until passage. At log growth phase, cells were harvested using HyQtase. 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,  $\beta$ -mercaptoethanol and bFGF. Anti-apoptotic reagent was used for the initial 48 hrs upon seeding. After that, growth media were replaced every day until next passage. Cells were harvested for cryopreservation when 60-90% confluency was reached and > 50% of the colonies had tight borders.

### Cryopreservation

Before harvesting cells, the spent growth media was collected from culture and clarified by filtration (0.2 $\mu$ m PES filter). Cells were harvested when in log phase growth as described above. Cells were then resuspended in their own conditioned growth media after centrifugation. Equal volume of the 2x formulation of HyCryo-STEM was slowly added, a few drops at a time, with gentle mixing to the resuspended cells. The resulting cell suspension (~1x10<sup>6</sup> cell/ml) was aliquoted in 1 ml per cryotube before being transferred to Mr. Frosty™ and placed in a -80°C freezer overnight. The cells were then stored in liquid

nitrogen until thaw. Upon thaw, cells were quickly incubated in a 37°C waterbath, and removed before all the ice had melted. The cells were rinsed with basal growth media (without the growth supplements) to remove the cryopreservation reagent. They were then resuspended in the complete growth media 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 taken using a Varioskan spectrophotometer. Initial seed density used was an equal number of viable cells per well, based on viability assay results upon thaw. For growth curves post-thaw, ES cells were grown on matrix-coated plates to minimize background signal from MEF feeder layer.

### Differentiation

Post-thaw, rat and mouse cortical stem cells were allowed to recover for 2-3 days 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 media. Neuronal differentiation media consisted of basal media, serum substitute and stable glutamine dipeptide.

Oligodendrocyte differentiation media consisted of basal media, serum substitute, stable glutamine dipeptide and tri-iodothyronine. Astrocyte differentiation media consisted of DMEM, N2, stable glutamine dipeptide and 1% FBS. After 5 or 10 days, 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 MEFs as described in cell culture section. Cells were then cultured on feeder-free, matrix-coated plates in DMEM/F-12 supplemented with N2, serum substitute, stable glutamine dipeptide, non-essential amino acids,  $\beta$ -mercaptoethanol, BSA, penicillin and streptomycin. Growth factor bFGF and an anti-apoptotic reagent were used for 2-3 days before appropriate induction factors were added to the media. For ectoderm, 100 ng/ml Noggin was supplemented for 2 weeks. For endoderm, 100ng/ml Activin A was supplemented for 2 weeks. For mesoderm, 50ng/ml Activin A and 50ng/ml BMP-4 were supplemented for 1 week and then media 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 hrs at room temperature. Finally, all cells were stained with Hoechst to mark nuclei. Immunofluorescence was acquired and analyzed using the Arrayscan with Target Activation Bioapplication. 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/stem cells. Following differentiation, the neurons were stained for  $\beta$ 3-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  $\beta$ 3-tubulin for ectoderm, FOXA2 for endoderm, and smooth muscle actin (SMA) for mesoderm.

## Results

### HyCryo-STEM is a superior choice for the cryopreservation of neural progenitor/stem cells

Upon freeze and thaw of the neural progenitor/stem cells, cell viability is measured. When compared to the industry standard cryopreservation media 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 (Figure 1A). Neural progenitor/stem cells recovered from the HyCryo-STEM media also show normal growth rate as measured by the colorimetric metabolic assay (Figure 1B), indicating healthy metabolic state of the viable cells. The superior 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 (Figure 2).

In addition to preserving the viability of neural progenitor/stem cells, HyCryo-STEM also maintains their stemness. This is demonstrated by the immunocytochemical staining of the progenitor/stem cell marker Nestin for both mouse and rat cortical stem cells post-thaw (Figure 3A, E). Furthermore, mouse and rat cortical stem cells are able to differentiate into neurons ( $\beta$ 3-tubulin staining; Figure 3D, H), astrocytes (GFAP staining; Figure 3C, G) and oligodendrocytes (O4 staining; Figure 3B, F). Taken together, our study shows that HyCryo-STEM is a superior choice for the cryopreservation of neural progenitor/stem cells.

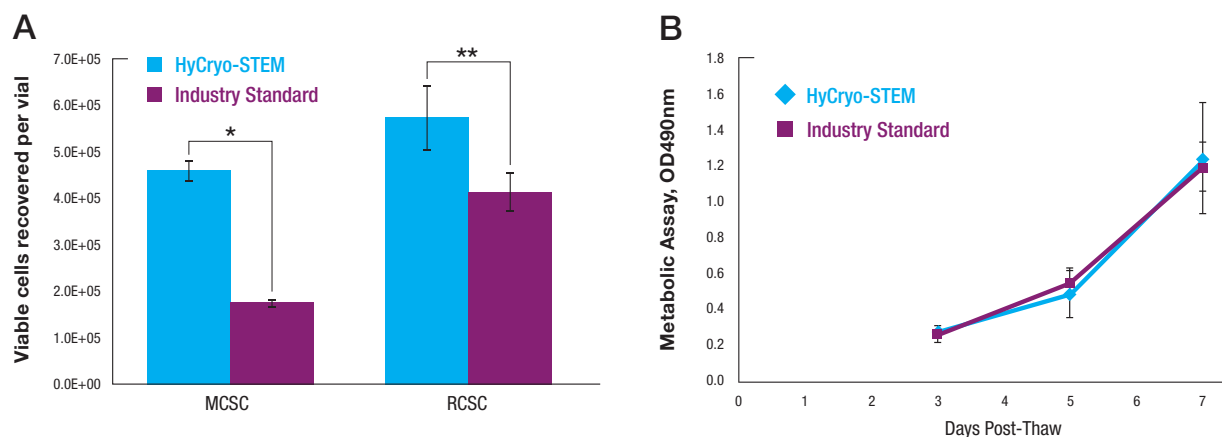


Figure 1. HyCryo-STEM has greater recovery of viable neural progenitor/stem cells compared to industry standard cryopreservation media (growth media containing 10% DMSO). (A) Mouse cortical stem cells (MCSCs) are grown in adherent culture and rat cortical stem cells (RCSCs) are grown in neurosphere culture prior to freeze. Trypan blue exclusion assay is performed for cell viability assessment post-thaw (\*p value < 1.0E-7, \*\* p value < 0.01). (B) Post-thaw growth curves of rat cortical stem cells are generated using colorimetric metabolic assay.

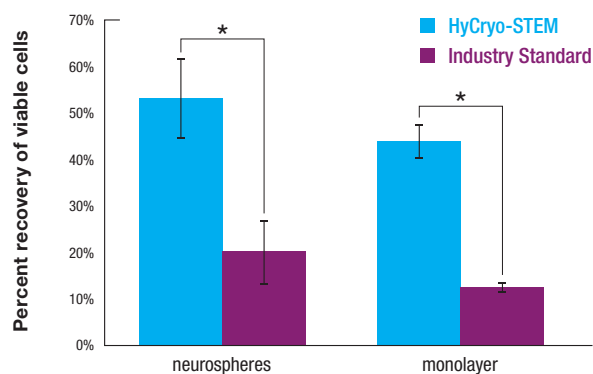


Figure 2. HyCryo-STEM displays similar advantages over the industry standard cryopreservation media (growth media containing 10% DMSO) for both monolayer and neurosphere culture conditions. Mouse cortical stem cells are either cultured as an adherent monolayer or are allowed to form neurospheres prior to freeze. Trypan blue exclusion assay is performed post-thaw for cell viability assessment. (\*p values < 1.0E-4)

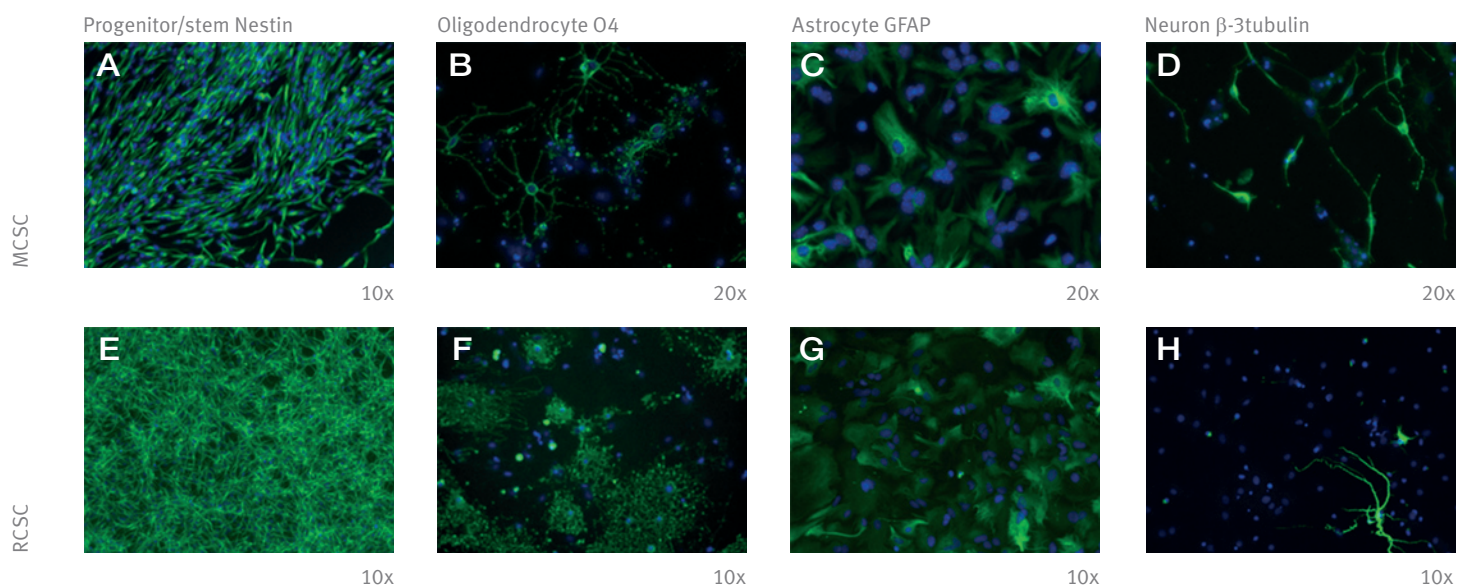


Figure 3. HyCryo-STEM maintains tripotency of neural progenitor/stem cells post-thaw. Mouse cortical stem cells (MCSCs) or rat cortical stem cells (RCSCs) are grown as a monolayer in adherent culture post-thaw from HyCryo-STEM. (A, E) The MCSCs and RCSCs are immunostained with the progenitor/stem cell marker, Nestin (green). Following directed differentiation, oligodendrocytes (B, F) are stained with O4 marker (green), astrocytes (C, G) with GFAP marker (green), and neurons (D, H) with  $\beta$ 3-tubulin marker (green). All cells are counter-stained with Hoechst (blue).



**HyCryo-STEM is an excellent serum-free alternative for cryopreservation of human pluripotent stem cells**

Human ES and iPS cells were preserved in HyCryo-STEM cryopreservation media. After they are thawed from liquid nitrogen, the cells are assessed for viability and are plated onto the feeder layers for passaging. Viable recovery from HyCryo-STEM is measured for both human iPS and ES cells in comparison to those from a standard freeze media (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 (Figure 4A). The human ES cells recovered from HyCryo-STEM grow at a similar rate to those recovered from the industry standard cryopreservation media (Figure 4B).

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 media as demonstrated by the post-thaw immunocyto staining of the pluripotency marker Oct4 (Figure 5A, E). After a single passage recovery from HyCryo-STEM, human iPS and ES cells are induced to differentiate into all three germ layers as shown by ectoderm marker  $\beta$ 3-tubulin (Figure 5B, F), mesoderm marker SMA (Figure 5C, G), and endoderm marker FOXA2 (Figure 5D, H). The differentiation efficiency is not altered compared to cells recovered from industry standard cryopreservation media (data not shown). Therefore, HyCryo-STEM freeze media is an excellent serum-free and chemically defined alternative for preserving human pluripotent stem cells.

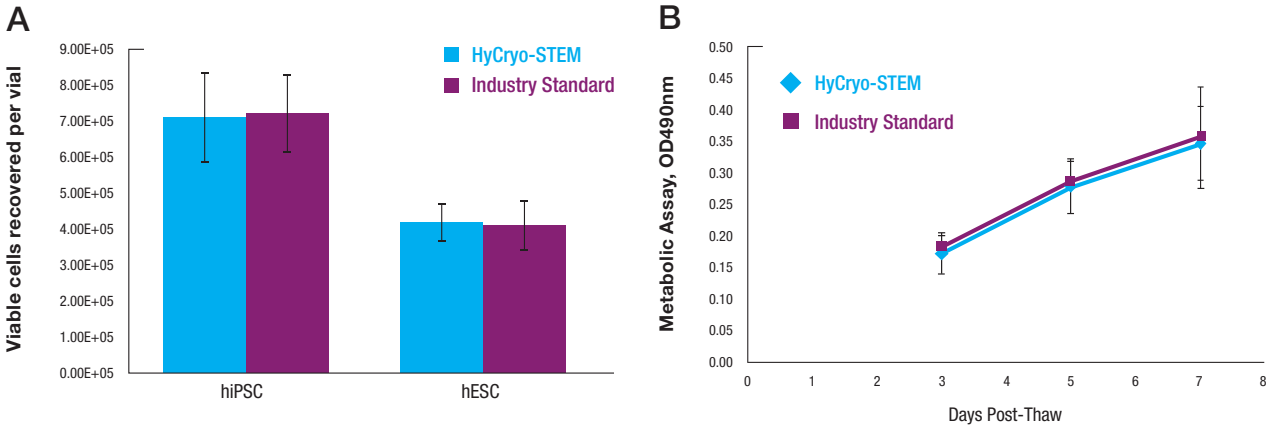


Figure 4. HyCryo-STEM achieves similar recovery of viable human pluripotent stem cells compared to the industry standard cryopreservation media (a cocktail of 25% serum replacement and 10% DMSO). (A) Trypan blue exclusion assay is performed post-thaw to assess viability of human iPSC and ESC. (B) Post-thaw growth curves of human ESC are generated with colorimetric metabolic assay.

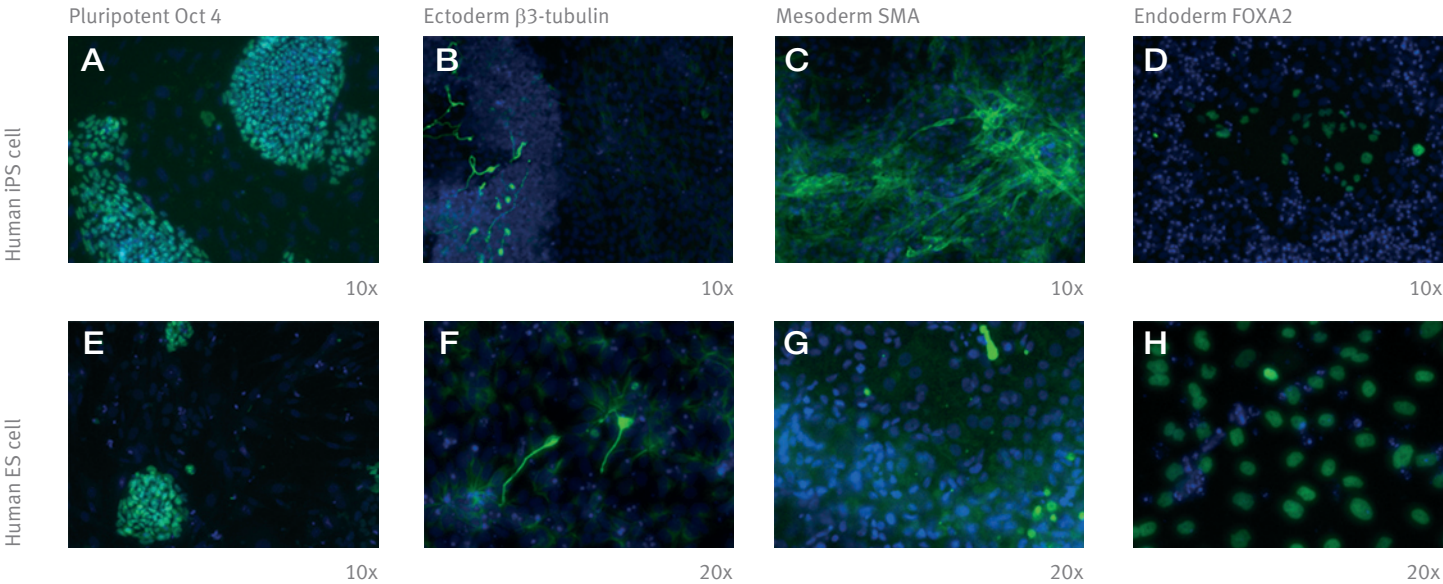


Figure 5. HyCryo-STEM maintains differentiation potential of human pluripotent stem cells post-thaw. Human iPS and ES cells are grown on MEFs post-thaw from HyCryo-STEM. (A, E) Pluripotency is evaluated by immunocyto staining of Oct4 (green). Following differentiation, each of the germ layers is stained. Ectoderm (B, F) is stained with  $\beta$ 3-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 improved formulation of HyCryo-STEM cryopreservation media minimizes osmotic shock in stem cells during cryopreservation and allows for rapid restoration of their metabolic activities upon thaw.
- HyCryo-STEM is a superior choice for cryopreservation of neural progenitor/stem cells as demonstrated by improved cell viability over standard cryopreservation media and by the maintenance of tripotency of the neural progenitor/stem cells.
- HyCryo-STEM is an excellent 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.

## Reference

*Thermo Scientific Nunc Solutions for Cell Culture and Growth brochure: BRLSPHYCRYO*

## Ordering Information

Product	Description	Size	Cat. No.
<b>HyCryo</b>	Cryopreservation media for general use	100 mls	SR30001.02
<b>HyCryo-STEM</b>	Cryopreservation media for stem cells	100 mls	SR30002.02

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