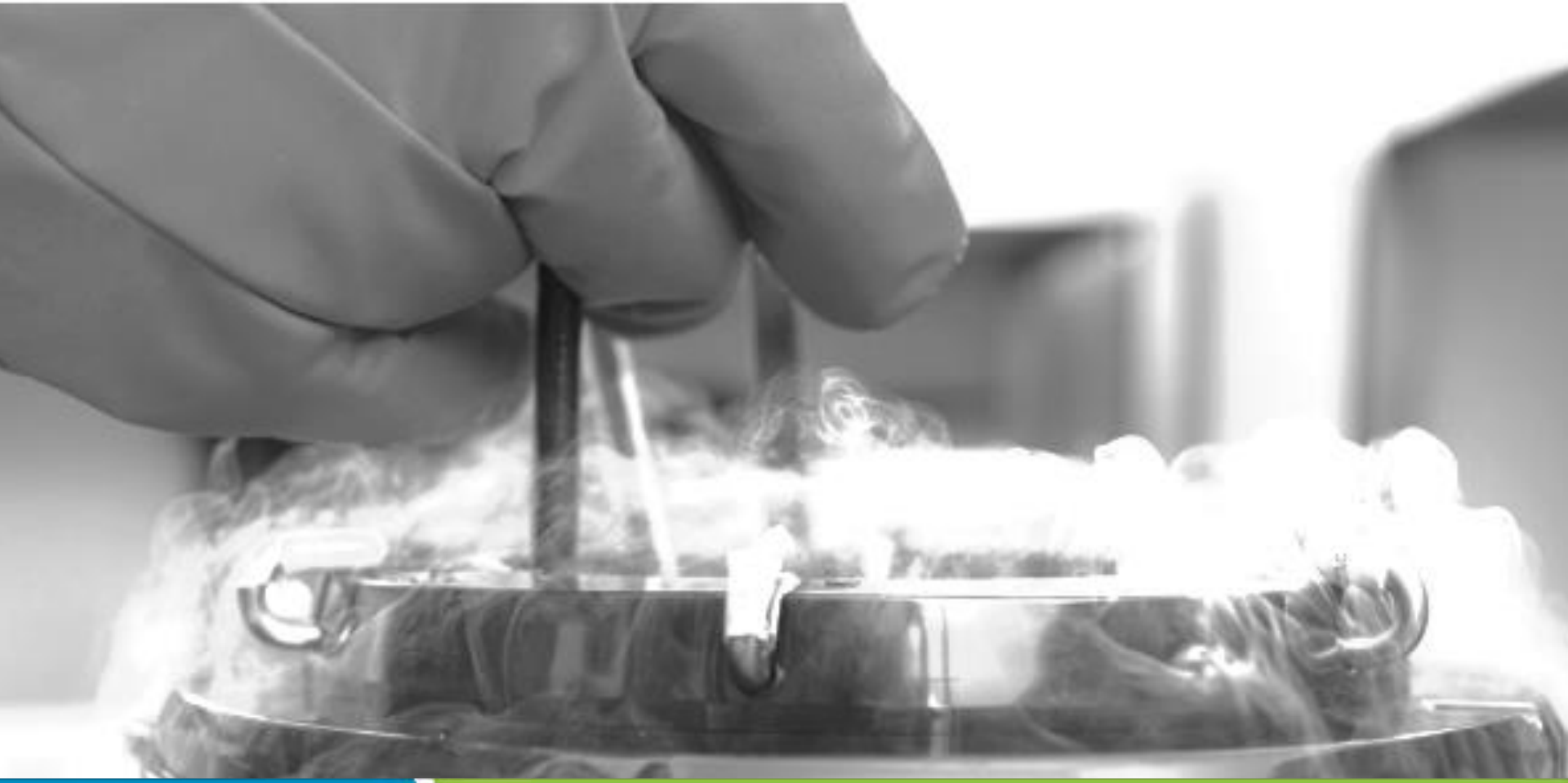

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Mastering Cellular Cryopreservation

The basics of cellular cryopreservation for research & clinical use

Your presenter

Suzanne Badoux
Field Application Specialist

- MSc in Medical Biology from the University of Lausanne, Switzerland
- Product support and training at BI Europe

- suzanne@bioind.com
- support@bioind.com

Webinar agenda

- Cryopreservation Basics and Limitations
- Choosing a Cryopreservation Medium
- Troubleshooting
- Safety Tips and Considerations

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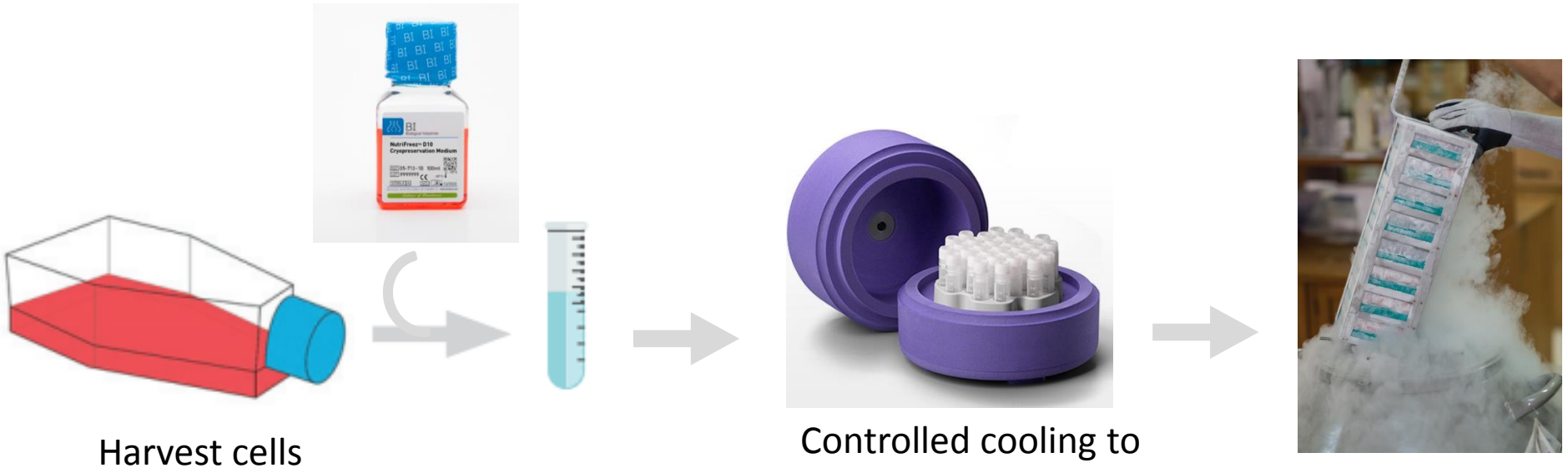
Cellular Cryopreservation Basics

The purpose of cryopreservation is to **store cells indefinitely** by **halting the cell's metabolism with ultralow temperatures.**

The freeze-thaw process is **stressful to all cells and tissues**. Therefore, effective techniques were developed to prevent cell death and damage.



Harvest cells

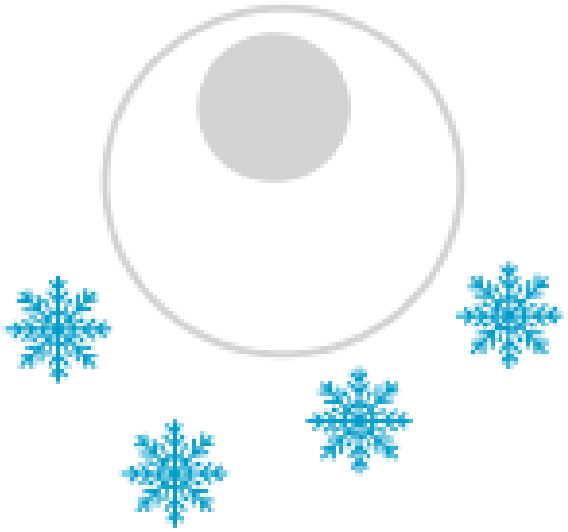
Controlled cooling to -80°C

Ultralow storage temperatures suspend all molecular processes and prevents free radical generation that negatively effects cryopreserved cultures (Baust J. , 2007; Baust, Corwin, Van Buskirk, & Baust, 2015).

Intracellular ice

Mechanical damage when thawing

Extracellular ice



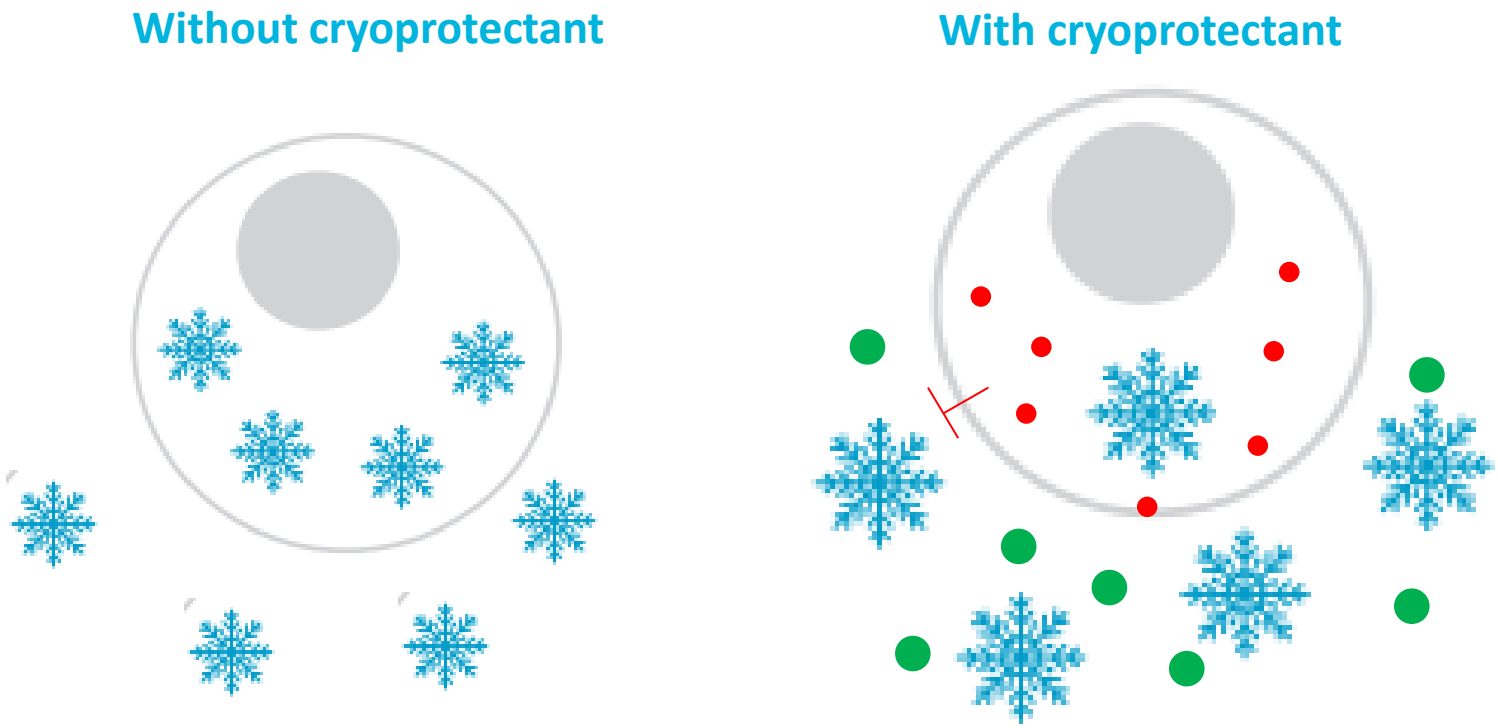
Water migrate out:

- Dehydration
- Shrinkage
- Cell death

- Using appropriate **cryoprotective agents**
- Controlling the **cooling rate**
- Maintaining appropriate storage temperatures
- Controlling the rewarming rate

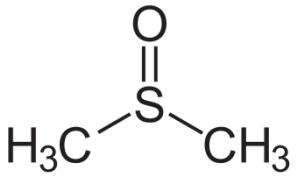
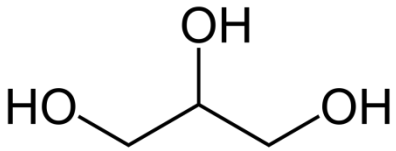
Cryoprotectants are compounds that protect cells from intracellular ice formation.

DMSO, glycerol, ethylene glycol, and propylene glycol are all permeating cryoprotectants.



Their mechanism of action involves entering the cell freely and replacing water, lowering the amount of ice formed, and acting as a secondary solvent for salts (Lovelock, 1953; Pegg, 1984).

Which Cryoprotectant?

DMSO	Glycerol
- DMSO being more common for mammalian cells. - Used at a concentration of max 10%.	- Most commonly used for preservation of microorganisms, red blood cells and spermatozoa (Jang, et al., 2017).
	

While these agents protect cells during the slow freezing process they can also cause cell toxicity, especially at room temperature.

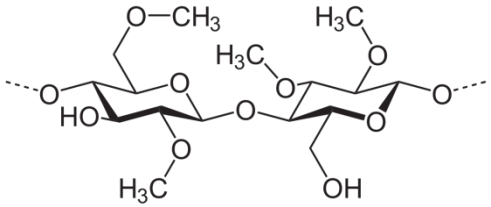
In standard home brew solution FBS is used to reduce the cytotoxic effect.

FBS advantages	FBS disadvantages
- May help to protect cells	- Not a cryoprotective agent - Undefined, contains growth factors, hormones - Increases variability - Not recommended for cell banking, clinical applications - Increases risk of contamination - Fluctuating costs

Animal component free replacement?

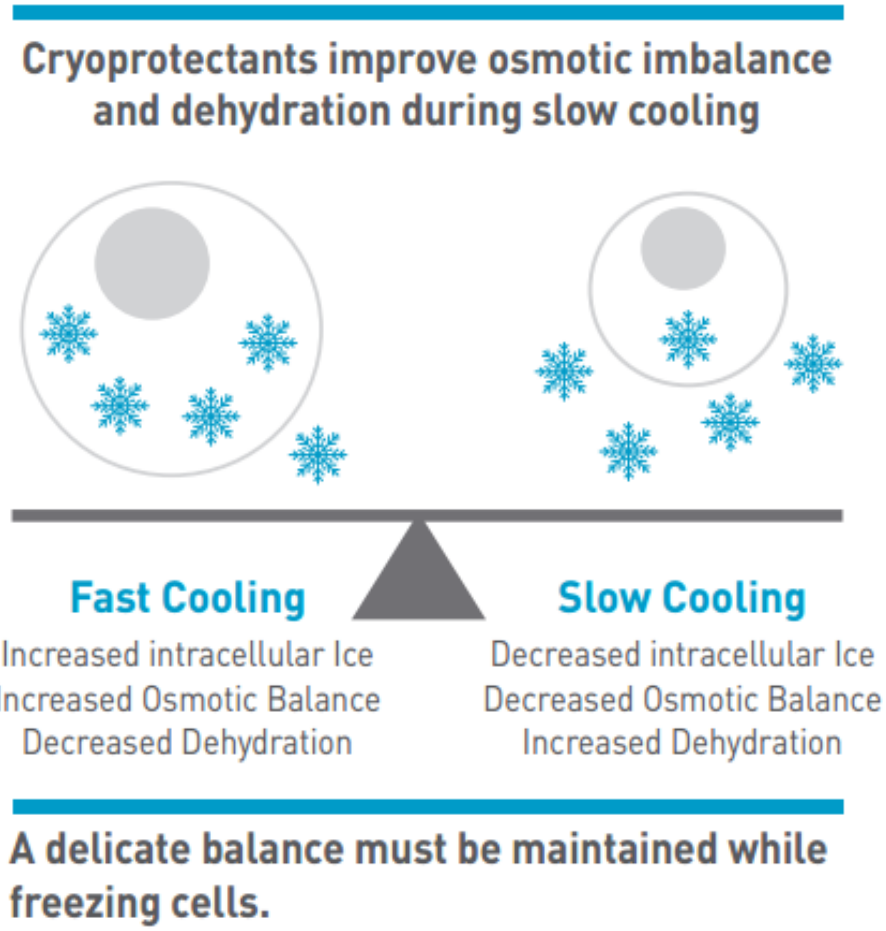
Methylcellulose has been suggested as a protective agent in cell cryopreservation and is used as suitable replacement for FBS.

- ✓ Chemically defined
- ✓ Protective

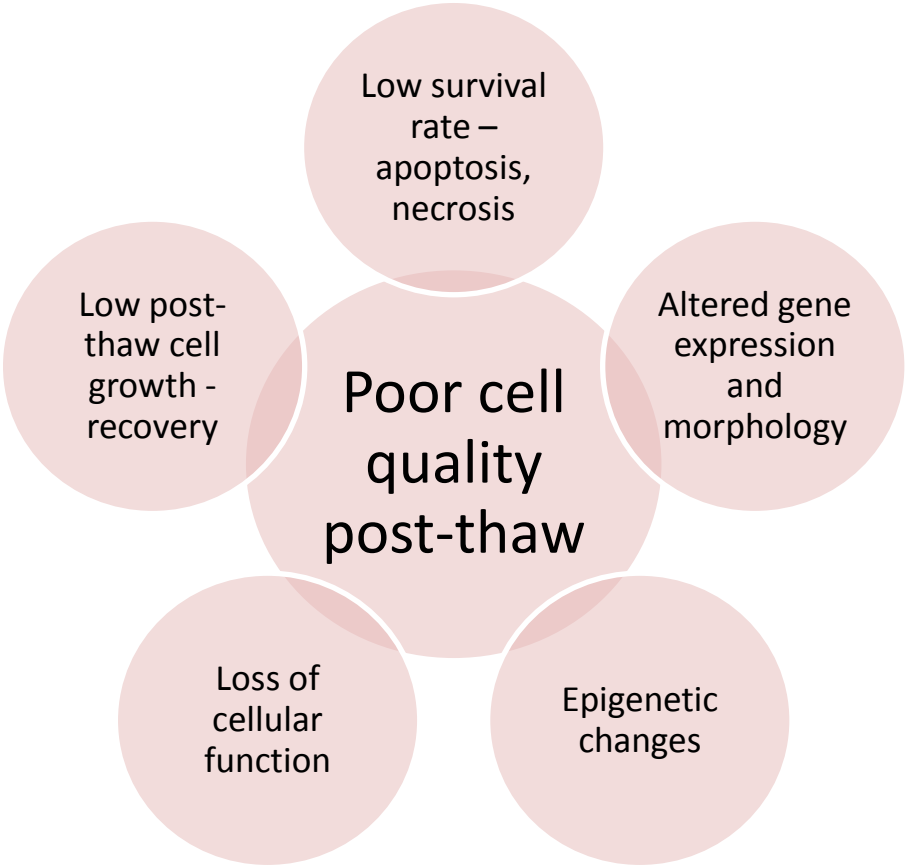


Mizrahi A, Moore GE, Appl Microbiol. 1970 Jun; 19(6):906-10
Merchant DJ, Hellman KB, Schneider H, Muirhead EE.

During the freezing process ice forms in and out the cell and this is very much dependent on the **cooling rate**.



While optimized cryopreservation protocols and published formulations exist for most areas of research and medicine, **technical issues persist**.



Baust J., 2007; Van Buskirk, 2007; Baust, Corwin, Van Buskirk, & Baust, 2015; Allegrucci & Young,

Controlled Rate Freezing Methods

	LN_2 (Controlled rate freezers)	Step-down freezing
Principal	Controlled rate freezers may be programed for precise and active cooling protocols so that cells are frozen typically at -1°C/min with liquid nitrogen	The **more traditional approach** of step-down freezing uses a specialized freezing container that is designed to cool cells at -1°C/min in -80°C freezers (e.g. Mr. Frosty).
Advantages	- Actively monitored temperature - Some controlled rate freezers do not require any consumable cryogens.	- Does not require any special appliance - Cost effective
Requirements	- A controlled rate freezer - Appropriate storage vials - Specialized freezing solution	- Cryovials are - Specialized freezing solution - -80°C freezer
Long term storage	Liquid nitrogen or mechanical storage as long as it is below -135°C.	Liquide nitrogen

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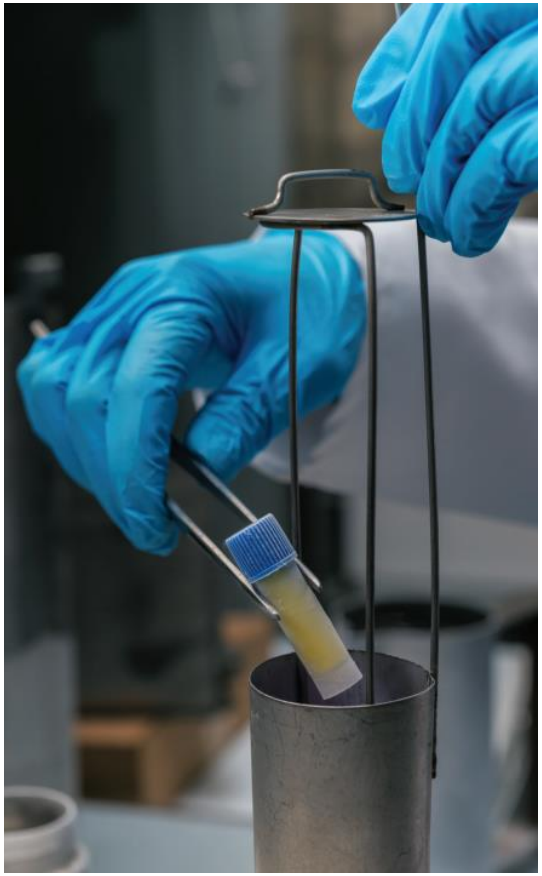
Choosing a Cryopreservation Media

What are my priorities and what are my cell needs?

- Required recovery rate?
- Required survival rate?
- Cryopreservation medium composition?
- Serum-free or xeno-free requirements?
- Which protocol and method do I want to use?
- Advantages of commercial media vs home brew?



CAPTURE TIME

NutriFreez™D10 Cryopreservation Medium

Ready-to-use, animal component-free, chemically defined, protein-free freezing medium

NutriFreez™ D10 Cryopreservation Medium

- ✓ Chemically defined and animal component-free
- ✓ Ready-to-use, stored at 4°C
- ✓ Manufactured under cGMP conditions
- ✓ FDA Drug Master File (DMF) submitted
- ✓ Contains: Methylcellulose and 10% DMSO

Cat. No.	Size
05-713-1A	500 mL
05-713-1B	100 mL
05-713-1C	20 mL
05-713-1D	10 mL
05-713-1E	50 mL

Submitted

FDA

DMF

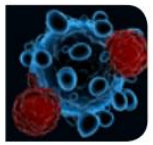
CE

CERTIFIED QMS ISO 9001 2015

CERTIFIED QMS ISO 13485 2016

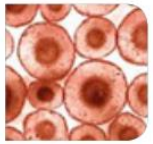
cGMP Manufacturing Facility

Cell Types and Applications

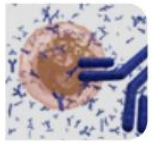


Sensitive Cells
(ex.T cells, beta cells)

Neurons, Astrocytes



Peripheral Blood
Mononuclear Cells (PBMCs)

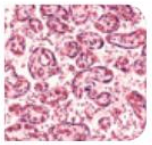


Hybridomas

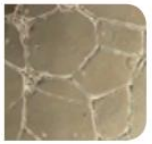
Human Pluripotent Stem
Cells (hPSC*)

- Embryonic Stem Cells (ES)
- Induced Pluripotent Stem Cells (iPS)

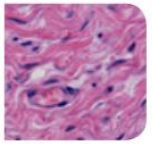
* Clumps or single cells in both feeder-dependent and feeder-free culture



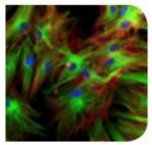
Cord Blood Cells



Human Endothelial
Cells (EC)

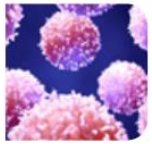


CHO Cells



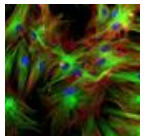
Mesenchymal Stem Cells
(MSCs) from various sources:

- Bone Marrow (BM-MSC)
- Adipose Tissue (AT-MSC)
- Umbilical Cord Tissue (UC-MSC)
- Dental Pulp Tissue (DP-hMSC)



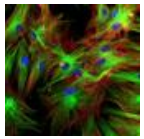
Multiple mammalian cell
lines, adherent and in
suspension, ≥90% recovery:

MRC-5, HEK-293, HepG2, HeLa, BSC-1, BGM, 3T3, MA-10, BHK-21, B16-F10, MA-10



Validation for human mesenchymal stem cells

NutriFreez™ D10 versus serum free cryopreservation products containing 10% DMSO



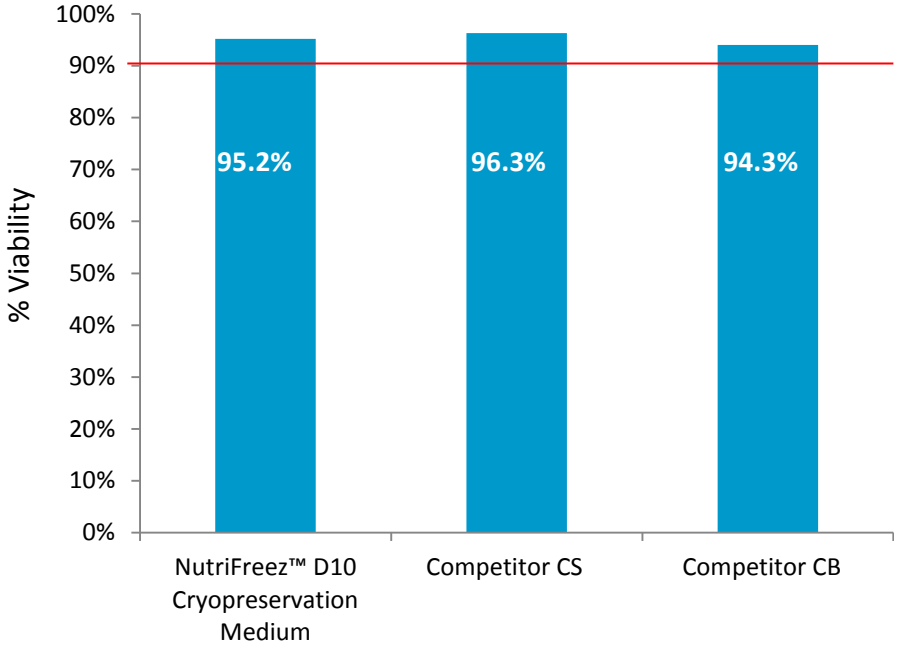
In house data

BI Biological Industries

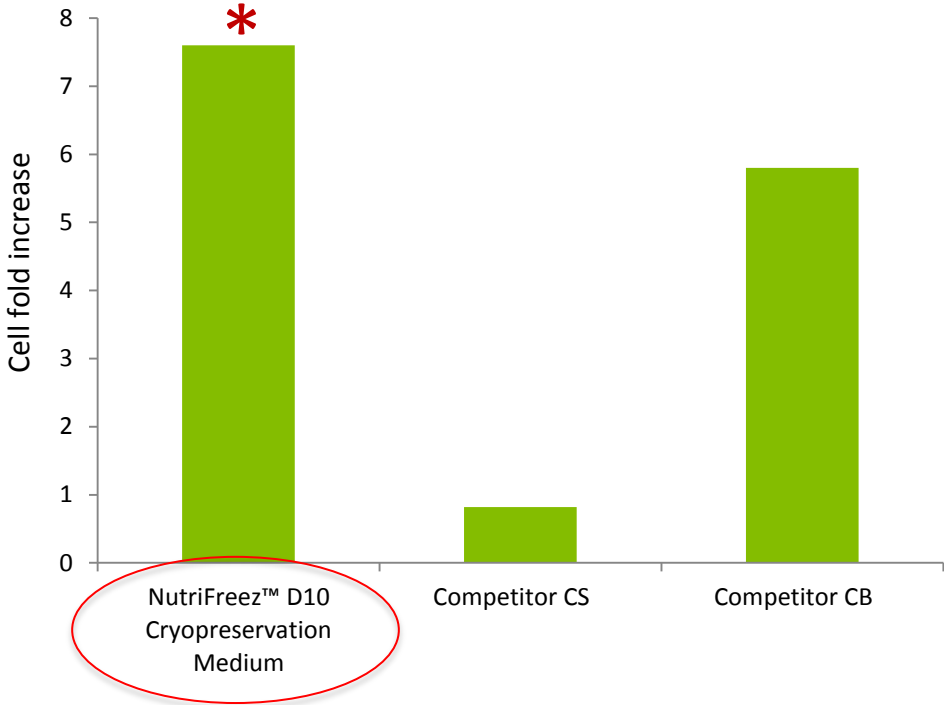
≥ 94% Viability
directly post-thaw

Superior Recovery
3 days post-thaw

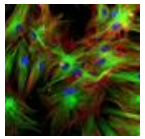
A



B



All Serum-Free Freezing Products yield greater than 94% viability (A**) however, hMSC-BM cells cryopreserved with NutriFreez™ D10 exhibited superior recovery** (B) after 3 days of growth compared to competitors, while keeping normal cell morphology (C)

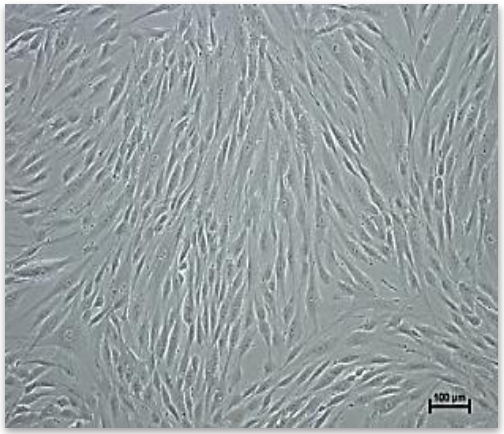
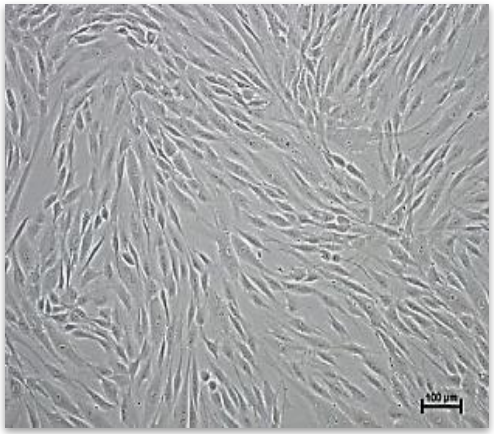


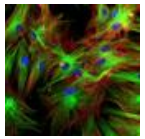
In house data

BI Biological Industries

Maximum Cell Proliferation with Normal Morphology of BM-hMSC

3 days Post-Thaw

NutriFreez™ D10 Cryopreservation Medium	Cryostor® CS10	STEM-CELLBANKER®
10% DMSO	10% DMSO	10%DMSO
		
38,000 cells/cm² Normal morphology	~4,000 cells/cm² Abnormal morphology	29,000 cells/cm² Normal morphology



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Evaluation of Different Cryopreservation Agents for Mesenchymal Stem Cell as Final Study Product

Mahmoud Salkhordeh[1], Yuan Tan[1], Lauralyn McIntyre[2,3], Duncan J. Stewart[1,3], Shirley H.J. Mei[1]

[1] Regenerative Medicine Program, Ottawa Hospital Research Institute, Ottawa, Ontario, Canada; [2] Clinical Epidemiology Program, Ottawa Hospital Research Institute, Ottawa, Canada; [3] Department of Medicine, University of Ottawa, Ottawa, Canada

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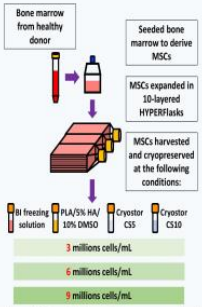
INTRODUCTION

Mesenchymal stem cells (MSCs) have been shown to exert important immunomodulatory effects in both acute and chronic diseases. In acute inflammatory conditions such as septic shock, immunomodulatory cell therapy must be administered within hours of diagnosis; therefore a cryopreserved, allogeneic cell product that can be thawed prior to infusion to the patient is best suited for this purpose. The objective of this study is to determine an optimal cryopreservant that can preserve the viability of MSCs after thawing. We systematically tested different cryopreservants and evaluated key cell product parameters to compare the relative performance merits of post-thawed MSC products.

METHODS

MSCs were cryopreserved in four different cryopreservant solutions at concentrations of 3 x 10^6, 6 x 10^6, or 9 x 10^6 cells/mL for at least 3 weeks in liquid nitrogen prior to experimentation. A final cell concentration of 3 x 10^6 cells/mL was achieved with no dilution (from 3 x 10^6 cells/mL), 1:1 dilution (from 6 x 10^6 cells/mL), or 1:2 dilution (from 9 x 10^6 cells/mL) using PLA/5% HA as diluting solution.



The size and granularity of cells post-thaw were analyzed by flow cytometry. Post-thawed cells were characterized for MSC surface markers. Cell viability was measured at 0, 2, 4, and 6 hours post thaw with Trypan blue exclusion method and Annexin V/Propidium iodide (AV/PI) analysis by flow cytometry.

Funding was provided by Ontario Institute Regenerative Medicine and the Stem Cell Network

RESULTS

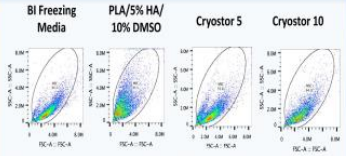


Figure 1. Comparison of size and granularity immediately post-thaw from MSCs cryopreserved in BI freezing media, PLA/5% HA/10% DMSO, CryoStor 5 or CryoStor 10 . MSCs were cryopreserved at 3 million cells/mL.

RESULTS

Table 1. Comparison of MSC surface markers post thaw from four different cryopreservation agents. N=3, shown as mean ± SEM.

	BI Freezing Media	PLA/5% HA/ 10% DMSO	CryoStor 5	CryoStor 10
CD73	98 ± 0.7	99 ± 1.06	99 ± 0.99	98 ± 1.10
CD90	98 ± 1.21	98 ± 1.68	98 ± 0.32	99 ± 0.76
CD105	89 ± 2.21	90 ± 2.48	94 ± 2.56	85 ± 2.25
CD14	0.11 ± 0.14	0.06 ± 0.08	0.08 ± 0.03	0.04 ± 0.07
CD19	0.00 ± 0.01	0.05 ± 0.08	0.08 ± 0.03	0.02 ± 0.01
CD34	0.03 ± 0.03	0.10 ± 0.07	0.06 ± 0.03	0.01 ± 0.01
CD45	0.04 ± 0.07	0.03 ± 0.05	0.08 ± 0.01	0.02 ± 0.02
HLA-DR	0.08 ± 0.09	0.01 ± 0.01	0.07 ± 0.03	0.05 ± 0.08

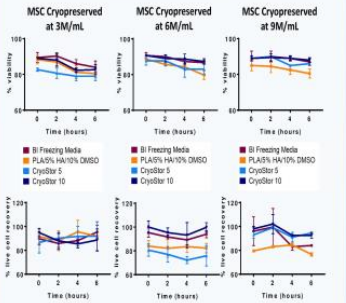


Figure 2. Comparison of cell viability and recovery by Trypan blue exclusion at 0, 2, 4 and 6 hrs post thaw from MSCs cryopreserved in BI freezing media, PLA/5% HA/10% DMSO, CryoStor 5 or CryoStor 10. N=2-3, Error bars indicate SEM.

RESULTS

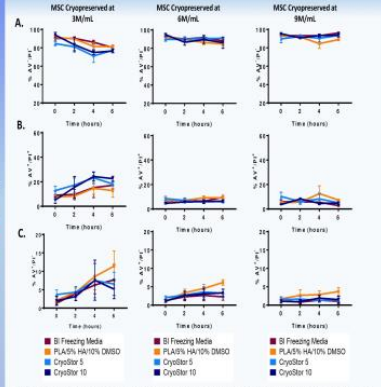


Figure 3. Comparison of (A) viable, (B) dead and (C) apoptotic cells by Annexin V/PI staining and flow cytometry analysis at 0, 2, 4 and 6 hrs post thaw from MSCs cryopreserved in BI freezing media, PLA/5% HA/10% DMSO, CryoStor 5 or CryoStor 10. N=2-3, Error bars indicated SEM.

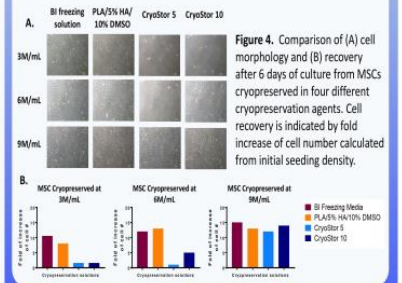


Figure 4. Comparison of (A) cell morphology and (B) recovery after 6 days of culture from MSCs cryopreserved in four different cryopreservation agents. Cell recovery is indicated by fold increase of cell number calculated from initial seeding density.

CONCLUSION

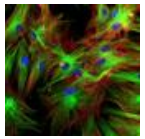
MSCs can be cryopreserved at concentrations up to 9 x 10^6 cells/mL in all four tested cryopreservant solutions. Cells cryopreserved in BI freezing media exhibit the best post-thaw viability, followed by PLA/5% HA/10% DMSO. Dilutions of total percentage of cryopreservant resulted in improved viability of MSCs post thaw up to 6 hours as evidenced by better cell recovery after 6 days of culture.

First in-human **clinical trial** evaluating allogeneic mesenchymal stem/stromal cells in septic shock patients.

Name: **Cellular Immunotherapy for Septic Shock (CISS2), Phase 2**

Conditions: Septic Shock, Sepsis, Pathologic Processes, Shock, Infection, Systemic Inflammatory Response Syndrome, Inflammation

ISCT 2018



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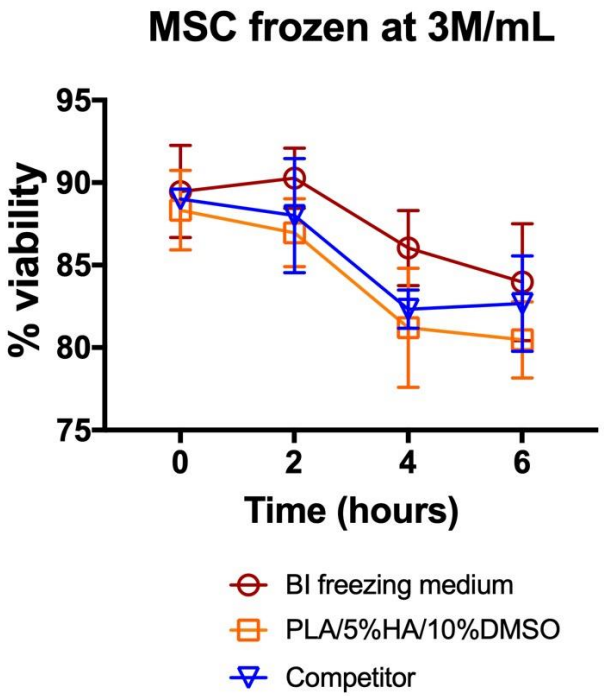
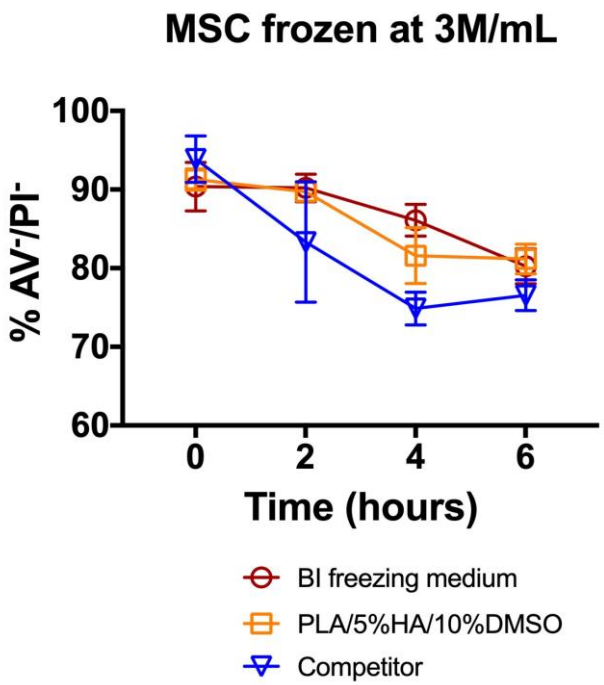
Viability via Trypan Blue exclusion



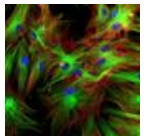
Viability via Annexin V/PI staining FACS analysis



* PLA-Plasma Lysate
**HA-Human Albumin/5% HA/10

Allogeneic mesenchymal stem cells exhibit **superior cell viability over home-brew and competitor** (direct post-thaw)

* Data Acknowledgment: Prof. Shirley H.J. Mei and research team Yuan Tan and Mahmoud Salkhordeh, Regenerative Medicine Program, Ottawa Hospital Research Institute (Ottawa, Canada).



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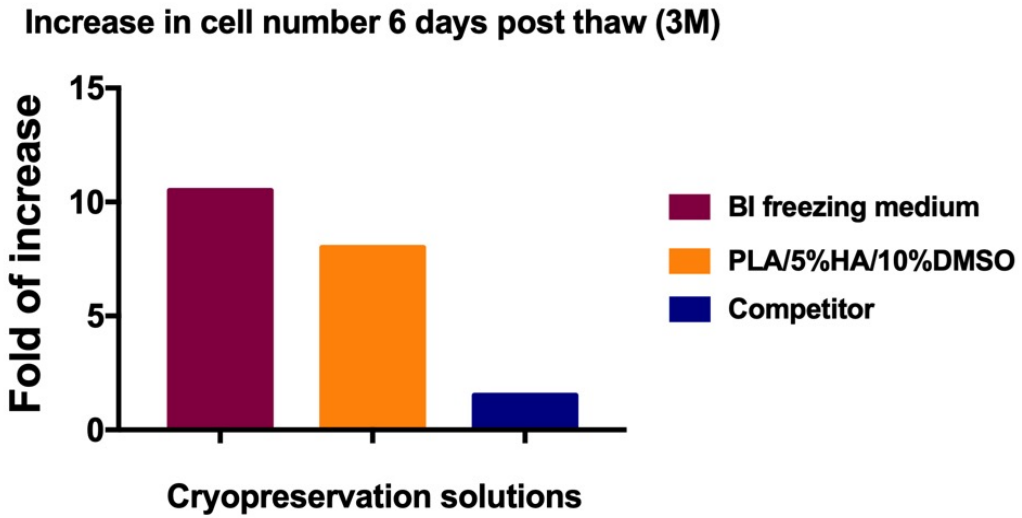
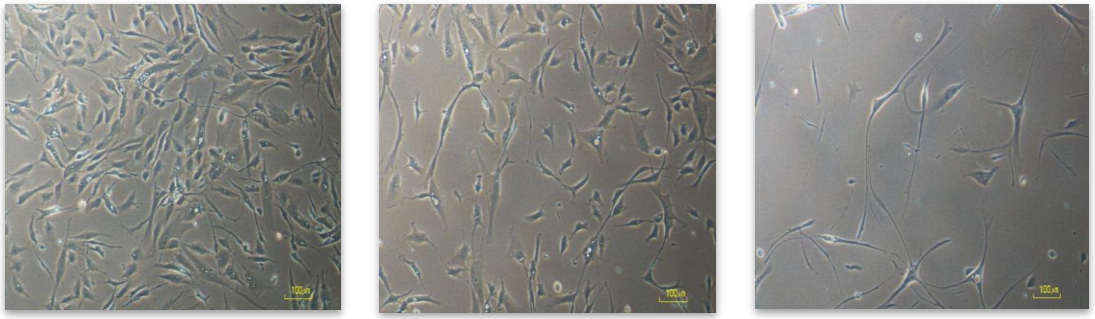
Recovery- 6 days post-thaw



Allogeneic MSC **exhibit superior recovery over competitor and home-brew freezing solutions*.**

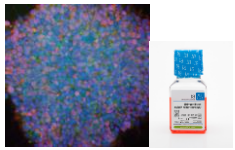
Morphology - 6 days post-thaw

NutriFreez™ D10 **PLA/5% HA/10% DMSO** **Competitor10%DMSO**



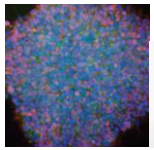
Allogeneic MSC **show normal morphology after post-thaw recovery*.**

* Data Acknowledgment: Prof. Shirley H.J. Mei and research team Yuan Tan and Mahmoud Salkhordeh, Regenerative Medicine Program, Ottawa Hospital Research Institute (Ottawa, Canada).



Validation for human embryonic stem cells

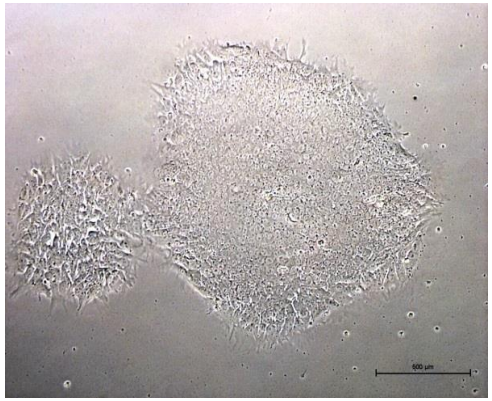
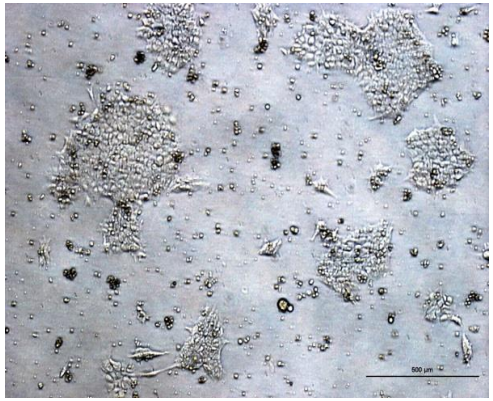
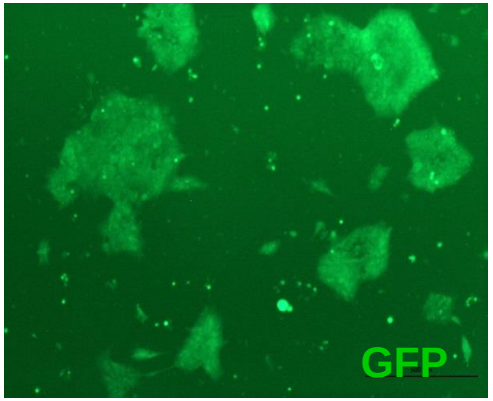
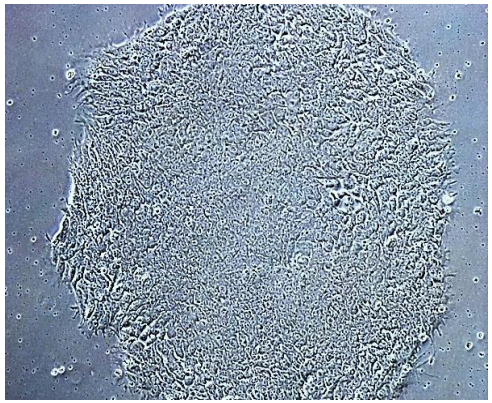
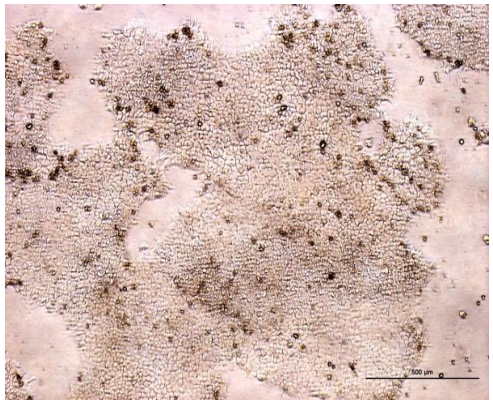
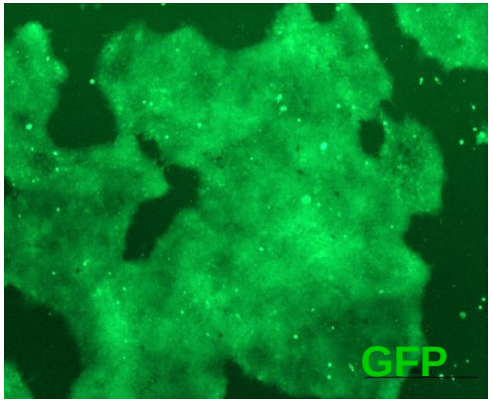
NutriFreez™ D10 versus serum free cryopreservation products containing 10% DMSO



In house data

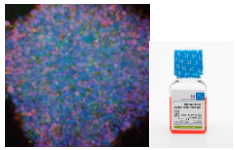
BI Biological Industries

Recovery 1, 2 and 4 days post-thaw

	H1 hESC	BGO1V/hOG (hESC)	BGO1V/hOG Oct4-GFP reporter
Day 1 Post- thaw			
Day 2-4 Post- thaw			

Representative results (x200) of colony morphology

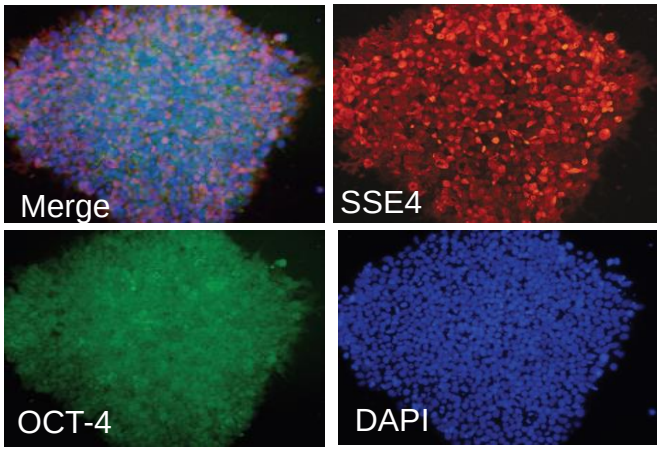
hESC show excellent recovery via morphology and attachment 1, 2 and 4 days post thaw after cryopreservation in NutriFreez™ D10.



hESC H1 post –thaw pluripotency surface marker expression

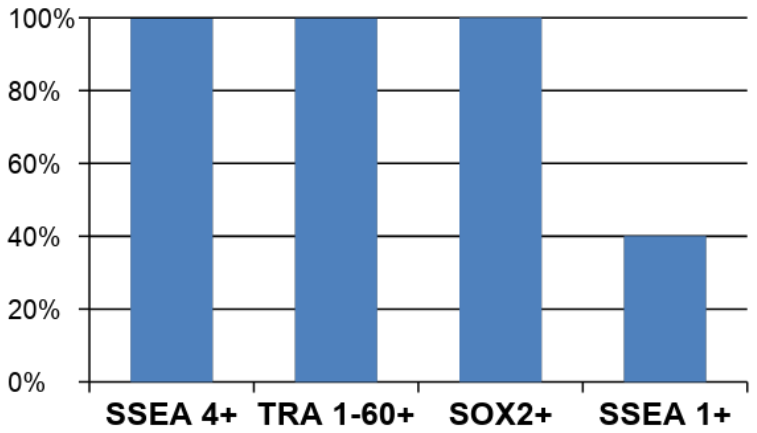
A

by Immunofluorescence

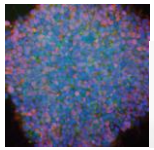


B

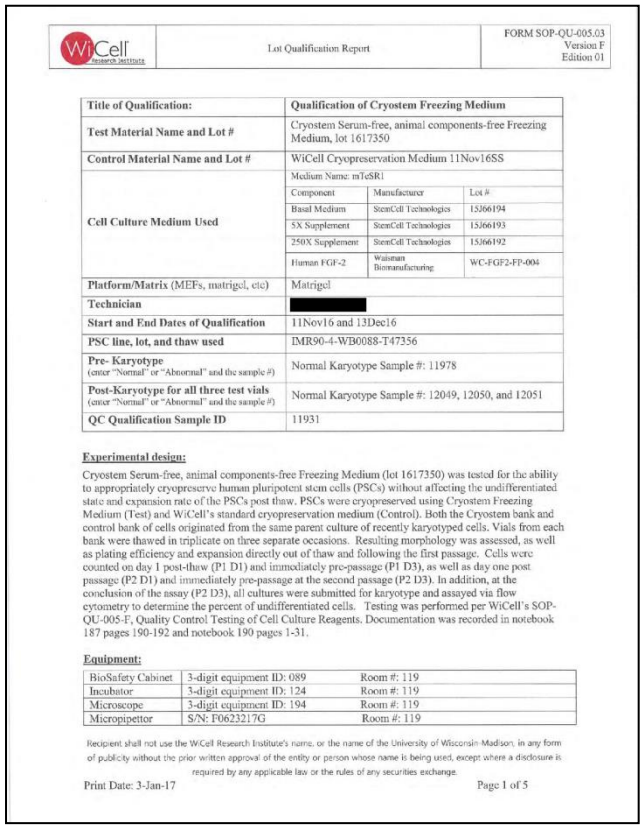
by FACS Analysis



hESC (H1 hESC), post-thaw demonstrate excellent cell morphology over time with normal expression level of pluripotency hESC surface markers (A, B).



"Human Pluripotent Stem Cells cryopreserved with NutriFreez™ D10 had no effect on cell proliferation, differentiation, morphology or karyotype"*



WiCell Research Institute | Lot Qualification Report | FORM SOP-QU-005.03 Version F Edition 01

Title of Qualification:	Qualification of Cryostem Freezing Medium
Test Material Name and Lot #	Cryostem Serum-free, animal components-free Freezing Medium, lot 1617350
Control Material Name and Lot #	WiCell Cryopreservation Medium 11Nov16SS
Cell Culture Medium Used	Medium Name: mTeSR1 Component / Manufacturer / Lot # Basal Medium / StemCell Technologies / 15J66194 5X Supplement / StemCell Technologies / 15J66193 250X Supplement / StemCell Technologies / 15J66192 Human FGF-2 / Waisman Biomanufacturing / WC-FGF2-FP-004
Platform/Matrix (MEFs, matrigel, etc)	Matrigel
Technician	[redacted]
Start and End Dates of Qualification	11Nov16 and 13Dec16
PSC line, lot, and thaw used	IMR90-4-WB0088-T47356
Pre- Karyotype (enter "Normal" or "Abnormal" and the sample #)	Normal Karyotype Sample #: 11978
Post-Karyotype for all three test vials (enter "Normal" or "Abnormal" and the sample #)	Normal Karyotype Sample #: 12049, 12050, and 12051
QC Qualification Sample ID	11931

Experimental design:

Cryostem Serum-free, animal components-free Freezing Medium (lot 1617350) was tested for the ability to appropriately cryopreserve human pluripotent stem cells (PSCs) without affecting the undifferentiated state and expansion rate of the PSCs post thaw. PSCs were cryopreserved using Cryostem Freezing Medium (Test) and WiCell's standard cryopreservation medium (Control). Both the Cryostem bank and control bank of cells originated from the same parent culture of recently karyotyped cells. Vials from each bank were thawed in triplicate on three separate occasions. Resulting morphology was assessed, as well as plating efficiency and expansion directly out of thaw and following the first passage. Cells were counted on day 1 post-thaw (P1 D1) and immediately pre-passage (P1 D3), as well as day one post passage (P2 D1) and immediately pre-passage at the second passage (P2 D3). In addition, at the conclusion of the assay (P2 D3), all cultures were submitted for karyotype and assayed via flow cytometry to determine the percent of undifferentiated cells. Testing was performed per WiCell's SOP-QU-005-F, Quality Control Testing of Cell Culture Reagents. Documentation was recorded in notebook 187 pages 190-192 and notebook 190 pages 1-31.

Equipment:

BioSafety Cabinet	3-digit equipment ID: 089	Room #: 119
Incubator	3-digit equipment ID: 124	Room #: 119
Microscope	3-digit equipment ID: 194	Room #: 119
Micropipettor	S/N: F0623217G	Room #: 119

Recipient shall not use the WiCell Research Institute's name, or the name of the University of Wisconsin-Madison, in any form of publicity without the prior written approval of the entity or person whose name is being used, except where a disclosure is required by any applicable law or the rules of any securities exchange.

Print Date: 3-Jan-17 Page 1 of 5

Qualification test conducted by WiCell, one of the leading hESC cell banks in the USA.

WiCell tested the ability of NutriFreez™ D10 Cryopreservation Medium to appropriate cryopreserve pluripotent stem cells (PSC's) without affecting the undifferentiated state and the expansion rate of PCS's post thaw.

- Please note that this test was conducted under the product brand name CryoStem™. The NutriFreez™ brand name replaces CryoStem™ and is the same formulation depicted here in this study

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Safety Tips and Considerations

Troubleshooting: viability issues after cryopreservation

Issue	Suggestions
Toxic Cryoprotectant	Use commercially available and defined media according to manufacturers' instructions. Remove cryoprotectant promptly after thaw. Do not allow cells to remain at room temperature in cryoprotectant media.
Improper Cooling Rate	Use a gradual cooling rate of -1°C/min. To attain this rate, use a thermally insulated freezing container or a controlled rate freezer.
Post-Freeze Temperature Flux	Maintain the cryogenic temperature of cell vials after they reach <-130°C. Keep cells on dry ice when transporting and make sure liquid nitrogen tanks are filled properly.
Improper Thawing Rate	Cells must be thawed quickly. Use a 37°C water bath or dry thawer to thaw vials.
Incorrect Cell Density	Freeze and plate cells at the appropriate density for the cell type. Cell lines should have published density requirements. Typical density per frozen vial is $1x10^6$ - $10x10^6$ cells/ml. Testing for optimal freeze-thaw density may be necessary.

Cryopreservation Tips

01

» Label your vials (cell, passage, lot #, date, your name) with a marker that will withstand alcohol and liquid N2.
» Printed cryolabels also work well.
» Keep the records online as well as a hardcopy.

02

» Follow your cryopreservation protocol carefully.
» Work quickly and step by step.
» Add the freezing media gradually
» Do not allow freezing media to return to room temperature.

03

» Cells may be frozen as aggregates or single cells. However, greater cell survival may be accomplished by dissociation into single cells prior to freeze-thaw.

04

» It is best to cryopreserve when cells are at their maximum growth rate (log phase).
» Cryopreservation at ~80% confluence should work best!

05

» Mycoplasma testing is recommended before freezing.

06

» Follow institutional regulation and guidelines for all hPSC work.

07

» **DO NOT** sacrifice sterility and personal safety for speed!

"Slow freezing, quick thawing is the way for optimal cell recovery“

Dr. Oren Ben Yosef, BI Technical Support

THANK YOU

support@bioind.com

www.bioind.com

To learn more, download our cryopreservation guide including detailed protocols and tips!