Transition to GMP, chemically-defined, xeno-free cryopreservation media increases post-thaw functionality of clinically-relevant cell banks

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BioLife Solutions® BIOPRESERVATION TOOLS FOR CELLS, TISSUES, AND ORGANS

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ABSTRACT

Cryopreservation is an essential element for the generation of master and working banks for cell therapy and regenerative medicine applications. As these products are scaled-up for commercialization, cryopreservation protocols established early in preclinical development must be amended to comply with Good Manufacturing Practices that allow for large-scale production of cells for clinical use. Optimization of cryopreservation protocols for master and working cell banks prior to scale-up therefore ensures GMP compliance, acceptable viability, and a return-to-function of frozen stocks post-thaw. Using representative human embryonic stem cells (hESC) and Jurkat T-cells, our group demonstrates that utilization of a GMP-manufactured, serum-free, protein-free, animal-origin-free, cryopreservation media (CryoStor®) resulted in a significant increase in viable cell recovery and functional cell recovery as determined using dye exclusion assays and functional indicators for up to 48 hours post-thaw. The intracellular-like media also reduced the DMSO concentration required to achieve acceptable post-thaw viable recovery. The process outlined in these studies provides a roadmap that can be rapidly and easily implemented to cryopreserve valuable cell therapy samples, and ensure desired viability and functional recovery of master and working cell banks following long-term frozen storage.

METHODS

Cell Culture: The Jurkat (Clone E6-1) human acute T-cell leukemia (ATCC, Manassas, VA) was cultured in RPMI 1640 (Lonza, Walkersville, MD) supplemented with 10% FBS (Atlas Biologicals, Fort Collins, CO). The ELF1 human Embryonic Stem Cell line (University of Washington, Seattle, WA) was cultured in an albumin-free, chemically defined, E8™ medium (ThermoFisher Scientific, Waltham, MA).

Cryopreservation: Jurkat and ELF1 cells were cryopreserved in the indicated cryomedia, placed in 1.2 or 2.0 ml cryovials, and cooled at 2-8°C for 10 min. Cryovials were frozen using either an isopropanol freezing device or controlled rate freezer, and then transferred to LN_2 storage for a minimum of 24 hr. Samples were thawed in a 37°C water bath, immediately resuspended in culture media (1:10 dilution), and then either plated on laminin-coated culture vessels (hESCs) or cultured in suspension (Jurkat).

Viability & Recovery: Structural cell viability was evaluated immediately post-thaw and at indicated time periods via membrane integrity using either the NucleoCounter NC-200 or NC-3000 imaging cytometer (ChemoMetec) and a Via-1 cassette.

Apoptosis: Post-cryopreservation caspase 3/7 activity was determined in Jurkat T-cells at the indicated time point using the NucView488™ fluorescent substrate and the NC-3000 equipped with the Flexicyte package. Results were normalized to non-frozen controls.

Cellular Glutathione: Cellular thiol content (glutathione) was determined using the VitaBright-48 bimane reagent and the NucleoCounter NC-3000.

Statistical Analysis: For all measurements, data represent the mean of 3-14 independent experiments and are normalized to pre-freeze conditions and 37°C controls as indicated. Groups were analyzed for statistical significance via two-way ANOVA with Tukey post-hoc comparisons and $p<0.05$ set *a priori*. Error bars represent standard error of the mean (SEM).

Abbreviations: Ctrl – culture control, CS5 – CryoStor® CS5, PLA – PlasmaLyte A, HSA – human serum albumin, GSH – glutathione, DMSO – dimethylsulfoxide, GMP – Good Manufacturing Practices

CONCLUSIONS

1. Measures of immediate post-thaw membrane integrity can vary over time and may not accurately reflect long-term cell viability following cryostorage. Additional measures of cellular function should be employed to more accurately assess cell health following cryopreservation.
2. The onset of cryopreservation-induced apoptosis can be accurately detected within hours post-thaw by the measurement of caspase 3/7 activity and DNA fragmentation. The presence of apoptotic indicators corresponds to the observed decline in long-term cell viability as determined by membrane integrity.
3. Altered cellular redox balance can be detected rapidly post-thaw and suggest oxidative stress following cryopreservation. Oxidative stress and decreased GSH levels may contribute to cryopreservation-induced cell death/damage.
4. Measures of post-thaw cell viability and functionality are highly dependent upon the cryomedia employed. Optimized CryoStor® cryomedia improves viable recovery versus traditional 'home-brew' cryomedia, reduces cryopreservation-induced apoptosis, and conveys more accuracy to membrane integrity as an indicator of cell viability.
5. Optimized CryoStor® media exhibits increased viable recovery and functionality post-thaw versus traditional 'home-brew' cryomedia. The effectiveness of CryoStor ® CS5 was superior to that of a non-GMP traditional 'home-brew' T-cell cryomedia composed of PlasmaLyte A/10% w/v Human Serum Albumin/5% v.v DMSO. Using CryoStor® media, Jurkat T-cells and ELF1 hESCs can be successfully cryopreserved following a traditional freezing protocol.
6. These data stress the need for multiple measures of cell viability post-thaw, and suggest that optimized CryoStor® media offers a more robust solution for the accurate evaluation of post-thaw viability from master and working cell banks using multiple measures of viability post-thaw.
7. hESCs can be expanded, cryopreserved, thawed and successfully returned to culture in a cell culture media and GMP-manufactured cryomedia that are chemically defined and do not contain serum or albumin. These conditions will be required to manufacture cellular therapies derived from pluripotent cells.
8. Intracellular-like, chemically defined, and xeno-free CryoStor® media manufactured under GMP can facilitate the rapid optimization of cryopreservation protocols for clinically-relevant master and working cell banks.

RESULTS

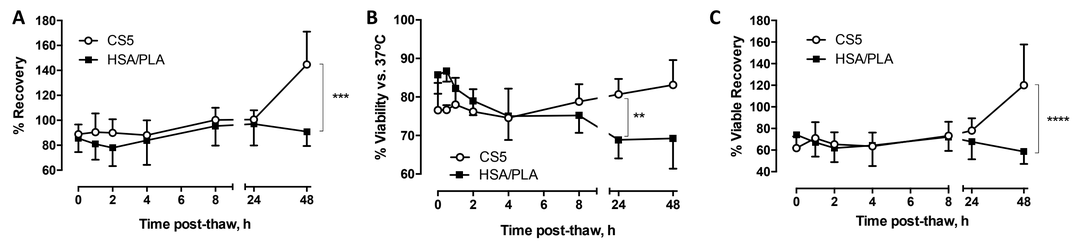


Figure 1. CryoStor® reduces post-cryopreservation cell loss and speeds cell expansion. Jurkat cells cryopreserved at a density of $5x10^6$/ml in either a traditional T-cell cryomedia composed of PlasmaLyte A supplemented with 10% human serum albumin and 5% DMSO (HSA/PLA) or GMP-manufactured CryoStor® media preformulated with 5% DMSO (CS5) were thawed in a 37°C water bath after LN_2 storage. Immediately post-thaw and at increasing intervals up to 48 hr, viability and cell number was determined using the Via-1 cassette and visualized by an automated NucleoCounter NC-3000 imaging cytometer. (**A**) Initial cell recovery/count was similar between CS5 and HSA/PLA but increased significantly in CS5 at 48 hr (***$p<0.001$). Jurkat cells cryopreserved in CS5 exhibited increased (**B**) viability (**$p<0.001$) at 24 hr and (**C**) viable recovery (****$p<0.0001$). Jurkat expansion was evident at 48 hr post-thaw following cryopreservation in CS5.

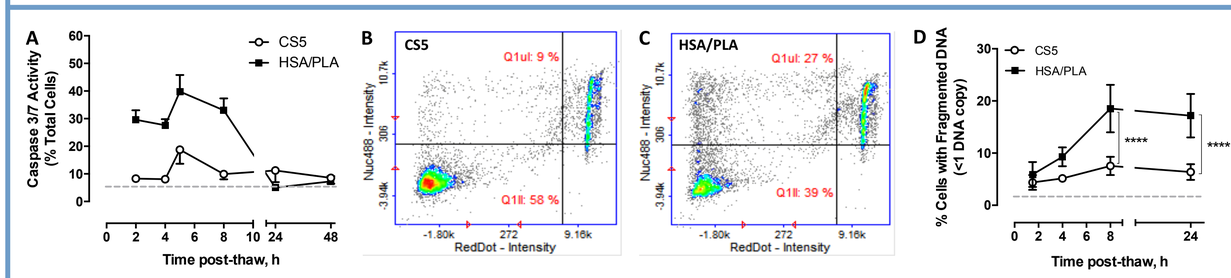


Figure 2. CryoStor® reduces cryopreservation-induced apoptosis. To determine the mechanism by which cryopreservation associates with a decline in cell viability at 24 hr, Jurkat cells cryopreserved in the indicated cryomedia were evaluated for caspase 3/7 activity at incremental time points. (**A**) Increased caspase 3/7 activity was evident 2 hr post-thaw in Jurkat cells cryopreserved in HSA/PLA that normalized following 24 hr of post-thaw culture. Caspase 3/7 activity was similar to non-frozen controls at all time points in Jurkat cells cryopreserved in CS5. Representative image cytometry dot-plot of fluorescence intensity of the caspase 3/7 fluorescence substrate (Nuc488™) and the necrotic indicator RedDot in Jurkat cells cryopreserved in (**B**) CS5 and (**C**) HSA/PLA. Fluorescence intensity was normalized to non-frozen controls as indicated. **(D)** Cell cycle analysis using DAPI revealed a significant increase in Jurkat cells exhibiting fragmented DNA when cryopreserved in HSA/PLA versus CS5, and correspond with the downstream effects of caspase 3/7 activity. Dashed lines in **(A)** and **(D)** represents caspase 3/7 activity and % cells with fragmented DNA in non-frozen controls, respectively. (****$p<0.0001$)

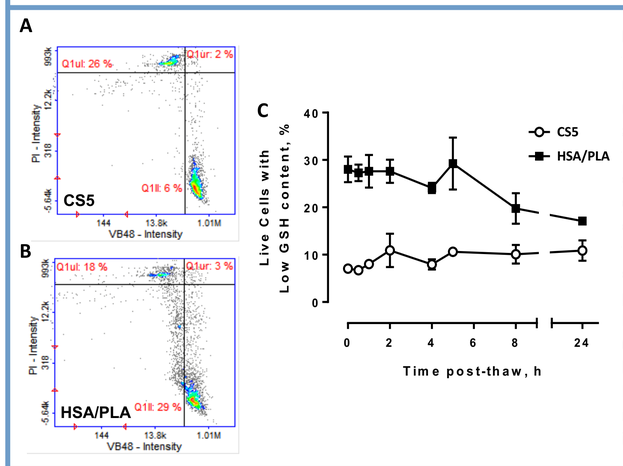
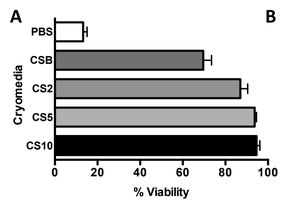
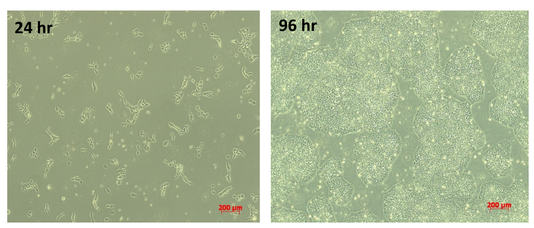


Figure 3. CryoStor® protects cellular redox balance. Glutathione (GSH) is the largest contributor to the cellular thiol pool and is essential for numerous oxidation/reduction reactions. An oxidative shift in the cellular redox balance results in the dimerization of two glutathione molecules and a general loss of cellular antioxidant capacity. Oxidative stress can be a trigger for apoptosis, secondary necrosis, and cellular dysfunction during environmental stress. To determine whether cryopreservation-induced cell death/damage associates with oxidative stress, Jurkat T-cells were evaluated immediately post-thaw and at increasing time intervals with the thiol-reactive fluorophore VB-48. Fluorescence dot-plots of VB-48 (reduced cellular thiol) versus Propidium Iodide (PI) (plasma membrane permeability) in Jurkat cells cryopreserved in (**A**) CS5 and **(B)** HSA/PLA. Fluorescence intensity was normalized to non-frozen controls as indicated. **(C)** Cryopreservation in HSA/PLA resulted in a large population of cells with low thiol (GSH) content versus non-frozen controls. Cryopreservation in HSA/PLA further required extended post-thaw culture to normalize cellular redox balance. In contrast, CS5 preserved cellular redox balance immediately post-thaw and throughout the 24 hr post-thaw culture.





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Parameter	
Viability [%]	91.6
Live cells [x 10^6]	3.8
Viable Recovery[%]	69.6
Yield/cm^2	152,000
Fold expansion	15.2

Figure 4. Optimized cryopreservation of a human Embryonic Stem Cell (hESC) working cell bank for preclinical development. The ELF1 hESC line was cryopreserved in CryoStor® cryomedia at increasing DMSO concentrations at a density of ~$1x10^6$ cells/ml in the presence of the ROCK1 inhibitor Y-27632. hESCs were frozen using an isopropanol freezing chamber to -80°C and then transferred to liquid phase LN_2 storage. hESCs were thawed in a 37°C water bath and then resuspended at a ratio of 1:9 in CGM. Immediately post-thaw, viability was assessed via membrane permeability using the NucleoCounter NC-100. (**A**) ELF1 hESCs exhibited elevated cell viability following cryostorage in CryoStor® media at all DMSO concentrations, compared to a PBS control, that was maximal using CS10 (10% DMSO). Post-thaw cell culture revealed improved long-term viability using CS10 (data not shown), which was chosen for subsequent cell manufacturing of a GMP working cell bank. hESCs were cryopreserved in CS10 and frozen using a LN_2 controlled rate freezer at a density of $5x10^6$ cells/ml to generate a working cell bank for preclinical development. Following LN_2 storage, hESCs were thawed as described previously and viability assessed using a 21 CFR Part 11-compliant NucleoCounter NC-200. hESCs were plated on laminin-coated culture dishes and expanded for 96 hr before harvest. (**B**) Microscopic evaluation of hESC morphology at 24 and 96 hr post-thaw reveals enhanced cluster formation and rapid expansion of cells cryopreserved in CS10. **(C)** Measures of viability, recovery, viability recovery, hESC yield, and fold expansion of hESCs after 96 hr of post-thaw culture. Optimized cryopreservation of hESCs using CS10 resulted in a GMP-compliant working cell bank that can be rapidly expanded for preclinical development.