Peripheral Blood Mononuclear Cell Isolation

Application Note

CORNING

Content originally prepared for ThermoGenesis by Christy Kim, Jon Ellis, Stephen Truong, John Perea, Zelenia Contreras, Jillian Miller, and Philip Coelho

Introduction

Peripheral blood mononuclear cells (PBMCs) are valuable for both clinical and research applications. Isolating pure populations of PBMCs from whole blood traditionally requires sample dilution and use of a density gradient medium to deplete red blood cells (RBC), granulocytes (GRN) and platelets (PLT).[1] This open, manual process involves a high risk of contamination. In addition, selective loss of specific populations of lymphocytes[2,3] and phenotypic discrepancies have been associated with the use of density gradient media.[4-6] Further, this method involves multiple tedious steps that are dependent upon highly skilled laboratory personnel, making the process cost-ineffective and standardization very difficult.[7] To be compliant with current good manufacturing practices (cGMP), manufacturers of cellular therapies must find alternative methods of PBMC isolation that are user-independent, reproducible, and closed to ensure sterility.

PBMC Protocol using the Corning® X-LAB® System

The Corning X-LAB System is a functionally closed, sedimentation-based system that reliably and reproducibly isolates PBMCs without the need for density gradient media or manual transfer steps. The X-LAB System features fully customizable protocols that can process 40 to 240 mL of source material and isolate mononuclear cells (MNC) in a user-defined harvest volume between 3 to 40 mL in just 35 minutes. The PBMC Protocol using the X-LAB System automates MNC isolation by compartmentalizing RBC/GRN, MNC, and plasma/PLT fractions using highly sensitive infrared sensors to ensure reproducibility of the manufacturing process.

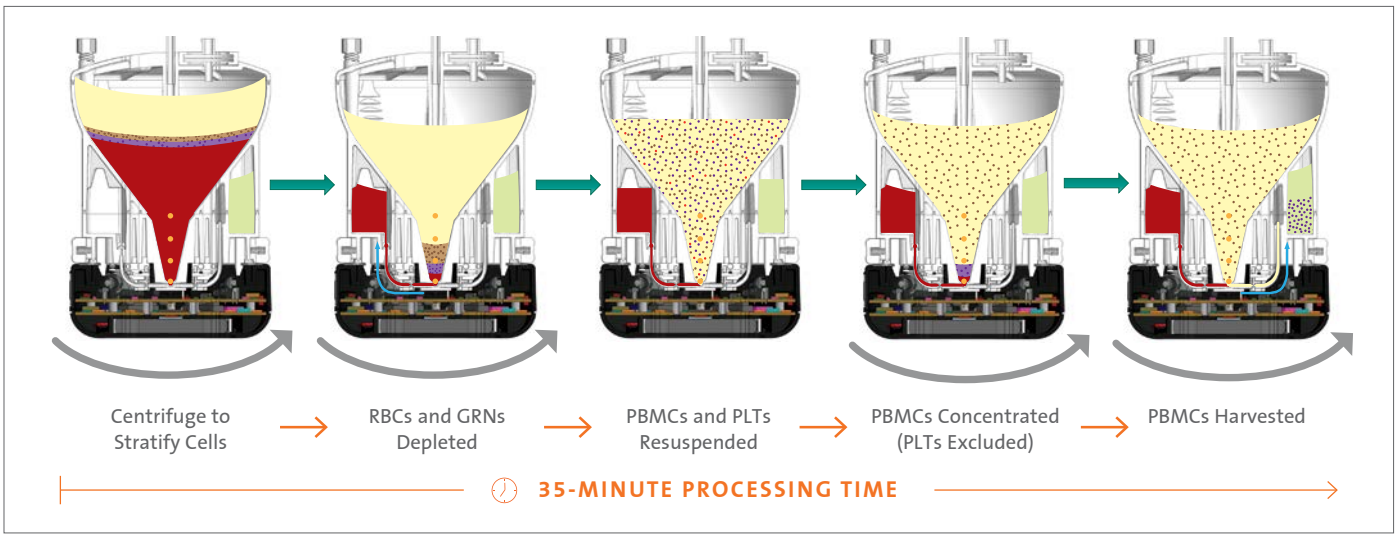
Methods and Materials

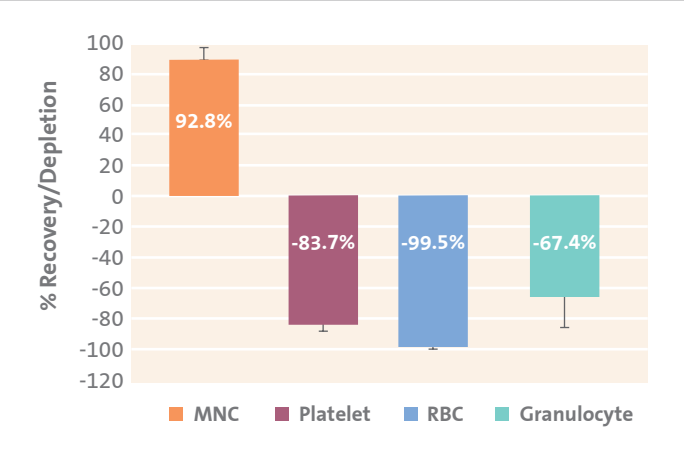
To evaluate the performance of the PBMC Protocol, 23 X-LAB Cartridges were loaded with peripheral blood (mean volume 148.8 ± 2.0 mL) less than 24 hour post-collection. Cartridges were then mated with their pre-programmed X-LAB Control Modules and placed in a 750 mL swinging bucket centrifuge.

The automated centrifugation protocol involved:

1. Centrifugation at 2000 x g for 20 min. to sediment the bulk RBC/GRN fraction.
2. Centrifugation at 50 x g for 5 min. for depletion of the bulk RBC/GRN fraction.
3. Centrifugation at 1000 x g for 5 min. to sediment residual RBC/GRNs.
4. Centrifugation at 50 x g for 1 min. for further depletion of residual RBC/GRNs*.
5. Centrifugation at 1000 x g for 1 min. to sediment the MNCs, leaving the platelets suspended.
6. Centrifugation at 50 x g for 2 min. to harvest the purified MNC fraction.

*Cartridges were then removed from the centrifuge, briefly agitated to resuspend MNCs and PLTs in the main chamber, and returned to the centrifuge.





	Pre-processing		Post-processing	
	Hematocrit	CD45⁺ Viability	Hematocrit	CD45⁺ Viability
Average	39.0%	97.8%	2.5%	96.7%
SD	2.9%	1.0%	0.4%	1.3%

Results and Discussion

The X-LAB PBMC protocol generated MNC recoveries of 92.8±4.8% while efficiently depleting PLTs (83.7±3.3%), RBCs (99.5±0.1%), and GRNs (67.4±19.6%). Average post-processing CD45⁺ cell viabilities were 96.7% with a 15.6-fold hematocrit reduction.

Conclusions

The PBMC Protocol using the Corning X-LAB System overcomes the limitations of traditional density gradient separation by providing an automated, closed system that isolates MNCs with high recoveries, viability and purity, and that is designed to meet user cGMP needs.

Efficient depletion of unwanted cellular fractions is essential for downstream assays and applications. For instance, in positive magnetic activated cell selection (MACS) of CD34⁺ cells, high RBC, GRN, and PLT contamination have been shown to significantly reduce the purity and yield of CD34⁺ cells due to nonspecific binding and sequestering of cells of interest in clumps and clots.[8-10] Further, if the isolated MNCs are to be cryopreserved, RBC contamination impairs MNC function following thawing, as RBCs are prone to lysis.[11,12]

The adoption of the Corning X-LAB PBMC Protocol reduces process variability while optimizing the recovery of physiologically relevant cell populations, providing performance, consistency, and may be suitable for clinical applications.

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Corning® X-WASH® System for Closed, Sterile PBMC Washing After Thaw

Application Note

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Introduction

In recent years, cell therapies relying on immune cells have become more prominent. Many of the workflows for this type of therapy involve isolating PMBCs from whole blood. To simplify the process, initial work is performed in acquiring a large population of PBMCs which are cryopreserved for future processing. Unfortunately, the cryopreservation process can impact cell viability due to ice formation during freezing[1]. Cryoprotective agents (CPAs), such as dimethyl sulfoxide (DMSO), are often added to freezing media in order to reduce ice formation and increase cell survival. However, DMSO itself can be cytotoxic, therefore, its removal post-thaw is often recommended[2]. In this article, we demonstrate how the Corning X-WASH system can be utilized to reduce DMSO concentrations, exchange buffer, and concentrate cells in a sterile, closed system. Additionally, the X-WASH system provides flexibility in that it allows the user to balance DMSO reduction with cell recovery, which can be negatively impacted by washing cells[3]. Here, we compare peripheral blood mononuclear cell (PBMC) recovery and viability from two commonly used cryopreservation formulations in association with two DMSO reduction protocols.

Materials and Methods

PBMCs were isolated from fresh, whole blood containing Anticoagulant Citrate Dextrose Solution A from healthy donors using the Corning X-LAB® System as previously described in Corning X-LAB System for Closed, Sterile PBMC Recovery (Corning App Note CLS-AN-632). PBMCs were equally divided between 90% fetal bovine serum (FBS) (Corning Cat. No. 35-010-CV) containing 10% DMSO (Corning Cat. No. 25-950-CQC), or a protein-free freeze media with 5% DMSO (BioLife Solutions Cat. No. 205102). Cells were frozen overnight at -70°C using a Corning CoolCell® FTS30 freezing container (Corning Cat. No. 432006) and then transferred to the vapor phase of liquid nitrogen for at least 24 hours.

On the day of thaw, RPMI 1640 (Corning Cat. No. 15-040-CM) supplemented with 10% FBS and 2 mM L-glutamine (Corning Cat. No. 25-005-CI) was pre-warmed to 37°C. PBMCs were thawed into 200 mL of pre-warmed media and added to an X-WASH cartridge for processing. A 1 mL sample was taken prior to centrifugation to determine the initial cell count using a Beckman Coulter DxH 520 analyzer. Cells from each CPA formulation were processed via one of two protocols; centrifugation and immediate harvest in a volume of approximately 4 mL (dilution) or centrifugation followed by a 200 mL media exchange prior to harvest in a volume of approximately 4 mL (1X wash). Cells were collected and the harvest chamber was washed with 4 mL of phosphate buffered saline (Corning Cat. No. 21-040-CV). PBMCs were assessed for recovery and viability. Additionally, the supernatant was collected and DMSO concentration was quantified via ultra-performance liquid chromatography (ULPC).

Results and Discussion

Two different DMSO reduction protocols were utilized to compare PBMC recovery from both CPA formulations tested. Figure 1 shows cell recovery of conditions tested from 3 separate donors. A two-way ANOVA found statistically significant, higher recovery with 10% DMSO versus 5% DMSO ($p<0.05$). This is not surprising due to the cryoprotective role that DMSO plays. Additionally, we also saw higher cell recovery when the dilution wash protocol was utilized as compared to the 1X wash protocol ($p<0.01$). The results showed a statistically significant difference in cell viability between CPAs ($p<0.05$) but not wash protocols (Figure 2). The lower viability with the 10% DMSO solution could be attributed to the cytotoxic effect of DMSO itself. Finally, Figure 3 shows DMSO concentrations in final cell product. Dilution of the frozen PBMCs resulted in DMSO concentrations of less than 500 parts per million (ppm) when a 10% DMSO concentration was used and averaged 230 ppm when a 5% DMSO concentration was used. The addition of a 200 mL buffer exchange further diluted the DMSO concentration to less than 3 ppm for 10% DMSO and less than 2 ppm for 5% DMSO (with several of the samples being below the 1 ppm detection limit of the instrument).

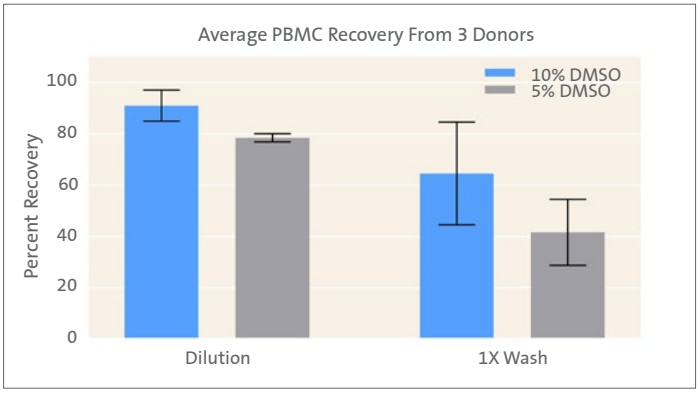

Figure 1. Average PBMC recovery from 3 donors. PBMC recovery from the Corning X-WASH system with dilution or 1X wash protocol. Data is average with standard deviation from 3 donors.

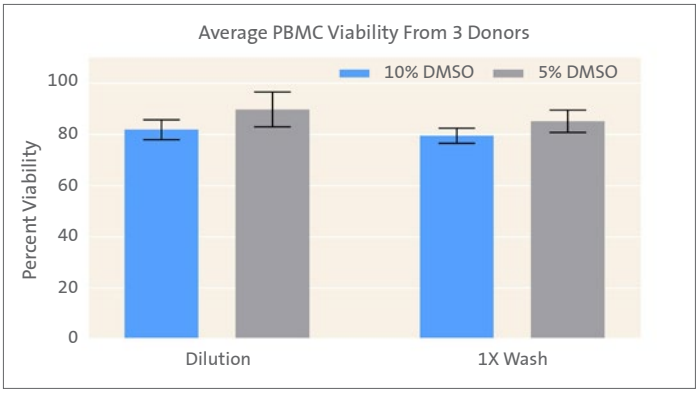

Figure 2. Average PBMC viability after Corning X-WASH system recovery. Average PBMC viability using X-WASH after dilution or 1X wash protocol. Data is average with standard deviation from 3 donors.

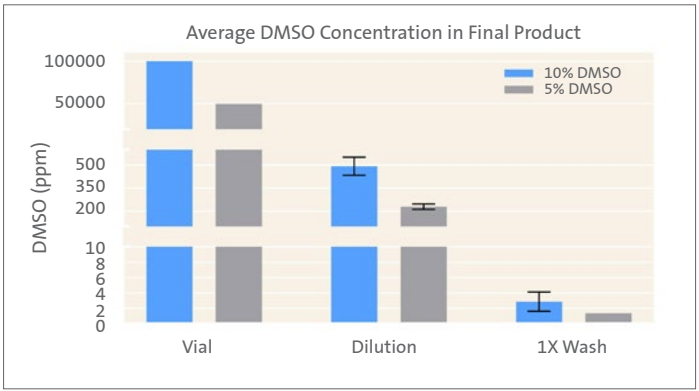

Figure 3. Final DMSO concentration. Average DMSO concentration in final product after dilution or 1X wash protocol. Data is average from 3 independent experiments with the exception of 1X wash in which multiple samples were below the 1 ppm detection limits of the instrument.

Conclusions

In order to enable PBMC cryopreservation in a research or cell therapy workflow, process optimization is essential. It is necessary to reduce unwanted components such as DMSO as much as possible, while at the same time maximizing cell recovery. Importantly, DMSO reduction and maximizing cell recovery may not be independent. If cell recovery is the priority, a singular, large volume dilution might be ideal, as increased washing can result in less recovery. Alternatively, if reducing the cryoprotectant as much as possible is the priority, further dilution can be achieved by adding more washes or starting with a lower cryoprotectant concentration. With the Corning X-WASH system, the user can determine what criteria are more pertinent for their application.

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Corning® X-WASH® System for DMSO Reduction of Cryopreserved Human Mesenchymal Stem Cells

Application Note

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Introduction

With more cell-based therapies going through clinical trials, there is an increasing need for more robust tools to simplify workflows. Cryopreservation is a necessary part of workflows for both autologous and allogeneic therapies[1]. The ability to cryopreserve cells for cell therapy increases the potential range of administration, shelf life and time for safety testing to occur[2]. Cryoprotectants, such as dimethyl sulphoxide (DMSO), are often added to freezing media in order to reduce ice crystal formation and increase cell survival post-thaw. However, DMSO itself can be cytotoxic so it is necessary to reduce its final concentration as much as possible[3]. In this article, we demonstrate how the Corning X-WASH can reduce the concentration of DMSO used in cryopreserved cells through a semi-automated, closed system. Using the Corning X-WASH, we were able to achieve a significant reduction in DMSO concentration of cryopreserved bone marrow-derived human mesenchymal stem cells (hMSC), while maintaining high cell recovery, viability, and multipotency.

Materials and Methods

Bone marrow-derived hMSCs (RoosterBio MSC-1M-5XF) were scaled up in RoosterNourish™-MSC-XF (RoosterBio KT-016) per vendor recommendations. Cells were harvested from a Corning CellSTACK® 10-chamber vessel (Corning 3270) with TrypLE™ Express (Gibco 12604021) and frozen into 50 mL Corning Cryopreservation Bags (Corning 91-200-88). Approximately 70 million cells were processed into each bag containing 10 mL of a 90% fetal bovine serum (FBS) (Corning 35-010-CV) and 10% DMSO (Corning 25-950-CQC).

On the day of thaw, wash buffer was prepared and warmed to 37°C. Wash buffer consisted of phosphate buffered saline (PBS) (Corning 21-040-CM) supplemented with 2% human serum albumin (Baxter 2G0012) and 5% glucose (Tecknova G0550). hMSCs were thawed into 200 mL of wash buffer and added to an X-WASH cartridge for processing. A 1 mL sample was taken prior to centrifugation to determine the starting cell count. Cells were processed via one centrifugation step at 300 xg for 5 minutes followed by a buffer exchange with 200 mL fresh wash buffer. X-WASH cartridges were then processed to harvest cells. An overview of the workflow is shown in Figure 1. One milliliter from the supernatant was collected to analyze final DMSO concentration via ultra-performance liquid chromatography (ULPC). Cells were collected from harvest chamber which was then washed with an additional 4 mL of PBS to ensure complete recovery. Cells were re-plated into a T-75 flask at 10,000 cells per cm^2 to ensure typical cell morphology and multipotency.

To assess multipotency, hMSCs were harvested after 72 hours of culture with Accutase® (Corning 25-058-CI). Cells were stained using a Human MSC Analysis Kit (BD Biosciences 562245) per vendor's protocol. Once stained, marker expression was assessed using MACSQuant® Analyzer 10 (Miltenyi Biotec).

Results and Discussion

Here we demonstrated greater than 70% recovery of hMSCs following a 200 mL wash after thaw (Figure 2). Additionally, viability was maintained above 93% for all three runs (Figure 3). MSCs were re-plated in order to observe morphology and assess marker expression. Typical morphology was observed after 72 hours of growth (Figure 4). The International Society for Cellular Gene Therapy (ISCT) has defined the minimal criteria for hMSC quality as expressing >95% of CD105, CD73, and CD90, and lack of expression (<2%) of typical hematopoietic markers CD45, CD34, CD14 or CD11b, CD79a or CD19, and HLA-DR surface molecules[3].

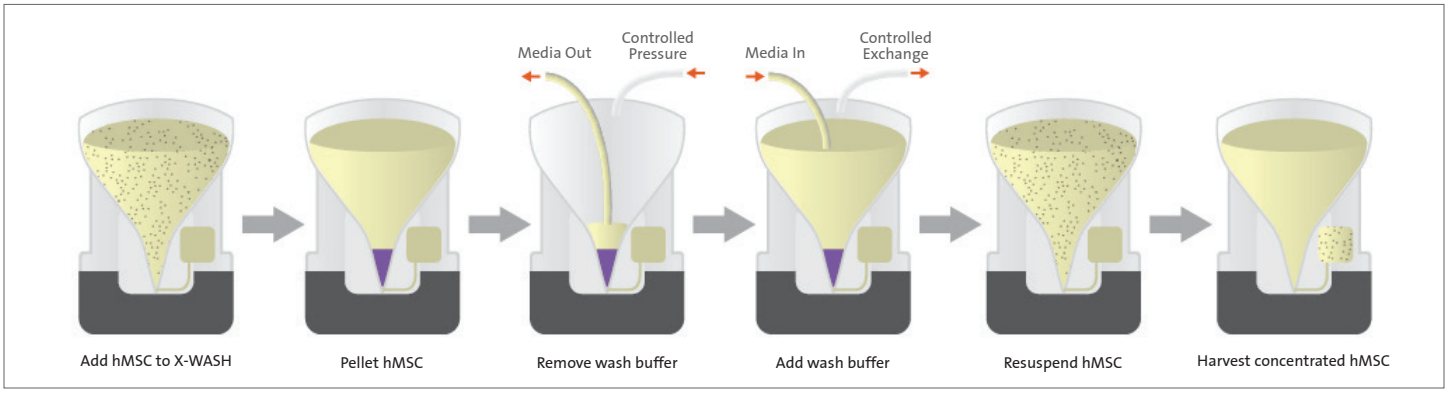

Figure 1. hMSC Corning X-WASH system workflow.

Figure 5 shows greater than 99% expression for positive markers CD105, CD73, and CD90, and less than 0.5% expression of negative markers CD45, CD34, CD11b, CD19, and HLA-DR. Lastly, ULPC analysis showed that the final DMSO concentration present was reduced by at least 400-fold, when a 200 mL dilution followed by an additional 200 mL wash was utilized (Figure 6).

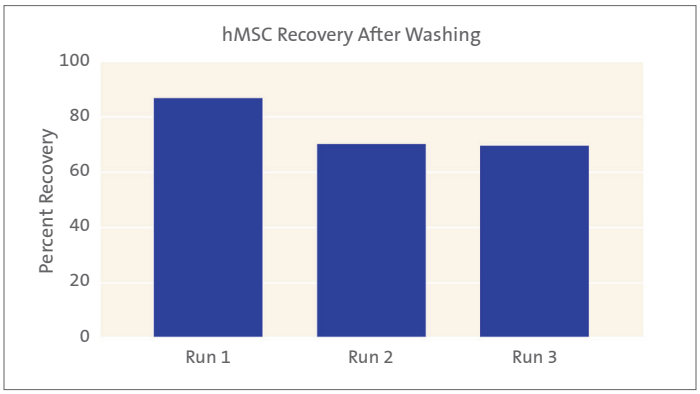

Figure 2. hMSC recovery after washing. hMSC recovery after washing using the Corning X-WASH system. Data is from 3 independent runs.

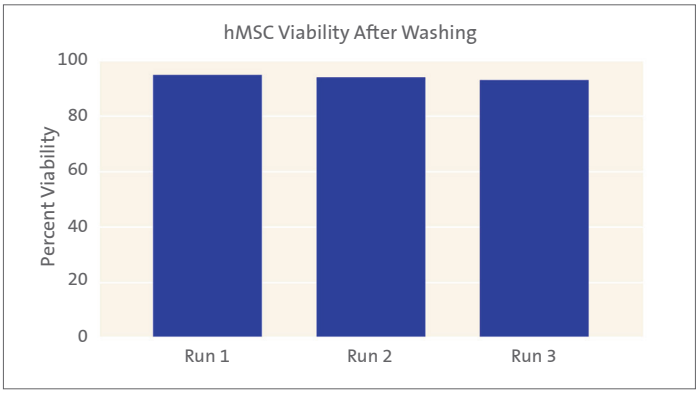

Figure 3. hMSC viability after washing. hMSC viability after washing using the Corning X-WASH system. Data is from 3 independent runs.

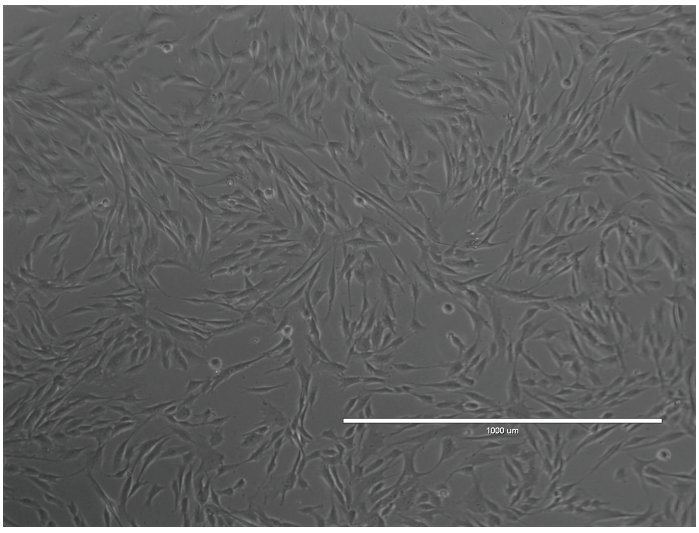

Figure 4. Typical hMSC morphology. Representative photomicrograph of hMSCs 72 hours after washing with the Corning X-WASH system. 4X objective.

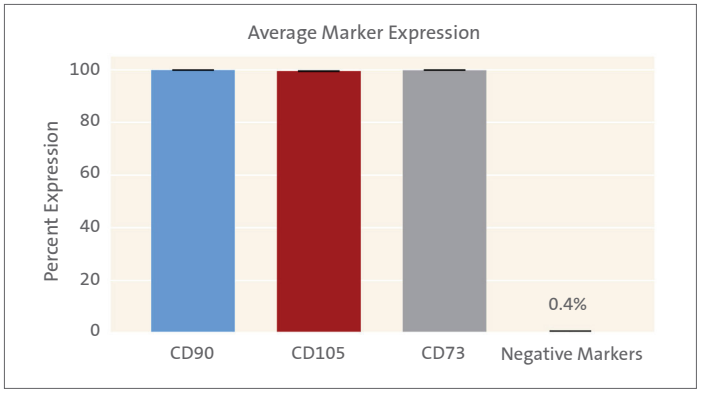

Figure 5. hMSC multipotency. Average marker expression of hMSC after Corning X-WASH system processing with standard deviation. N=3.

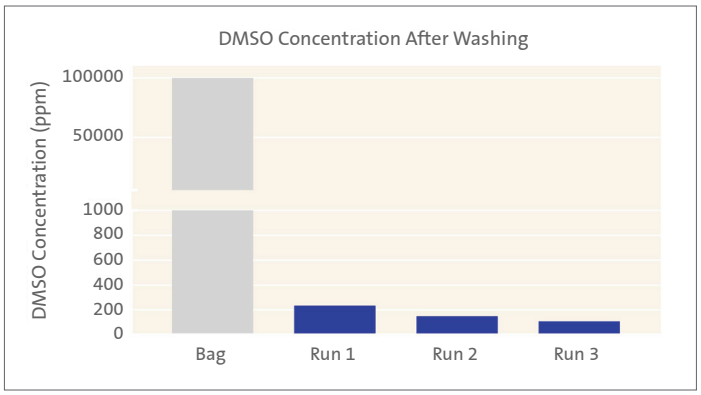

Figure 6. Final DMSO concentration after washing. DMSO concentration in the final product after 200 mL dilution followed by 200 mL wash. Data is from 3 independent runs.

Conclusions

In order to address the growing demand for cell-based therapies, optimization of cryopreservation and cell recovery is essential. With some hMSC therapies projecting as many as 1 billion cells per dose, it will be essential to have high recovery and viability post-cryopreservation[4]. High hMSC recovery and viability must be maintained while minimizing any undesired components from manufacturing. The Corning X-WASH system allows for reduction of DMSO and other reagents from cell products. More importantly, the Corning X-WASH allows cell processing and collection in a sterile and closed system.

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DMSO Removal and CD3⁺ Cell Selection from Cryopreserved Apheresis Products Using Novel BACS Technology

Content originally prepared for ThermoGenesis by Dalip Sethi[1], Jillian Miller[1], Zelenia Contreras[2], John Perea[2], and Stephen Truong[2]
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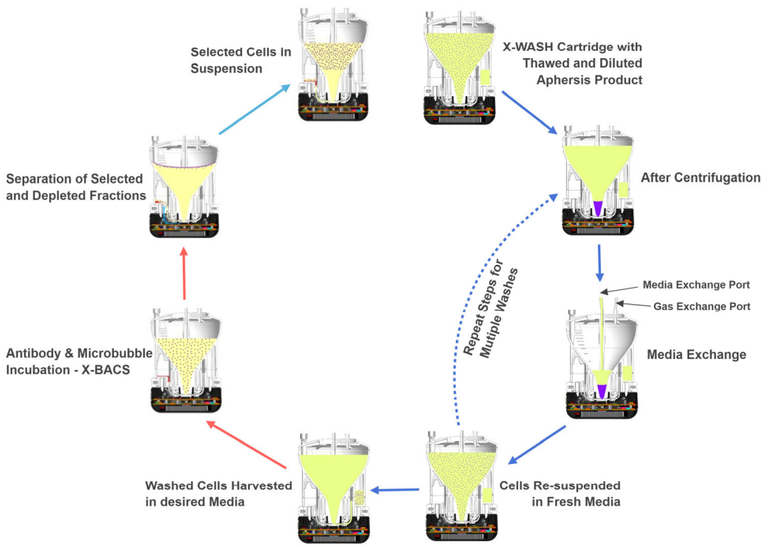
Introduction

Cancer therapy is a complex and growing field of medicine. With the advent of adoptive immunotherapy, coupled with excellent clinical outcomes in CD19⁺ hematologic malignancies, CAR-T cell therapy has created intense interest within the medical and scientific communities. Although genetic-modification of immune cells is one of the most advanced therapies, the manufacturing process still relies on traditional cell processing techniques. We have previously developed a quick and easy method to remove DMSO from thawed apheresis products and have now coupled this technology with the selection of CD3⁺ T-cells using ThermoGenesis' technology, Buoyancy-Activated Cell Sorting (X-BACS™) process. DMSO removal was successfully achieved using the X-WASH® System.

Process Flow

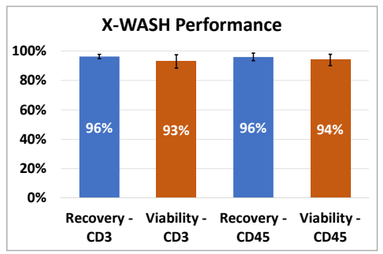


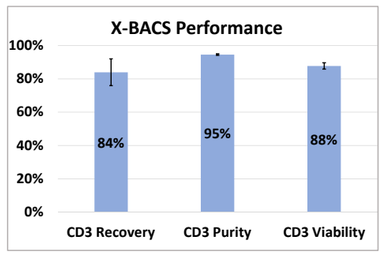
Materials and Methods

Healthy adult cryopreserved apheresis samples were thawed at 37°C and diluted with thaw-wash media (2.5% HSA, 5% Dextran, and DNase I in saline). The sample was transferred to an X-WASH Disposable Cartridge. Post-centrifugation, waste media was removed using positive pressure, and new media was added using controlled negative pressure. Washed cells were transferred to a new Disposable Cartridge and incubated with anti-CD3 antibody followed by incubation with microbubble reagent. The target and non-target fractions were separated using centrifugation. CD3⁺ cells were also studied for expansion using CD3/CD28 and IL-2 activation.

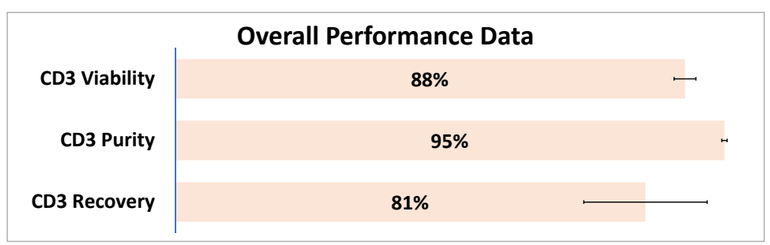
Results and Discussion

The DMSO removal from cryopreserved samples and CD3⁺ cell isolation was accomplished in two phases. Each phase was studied for recovery efficiency and cell viability.

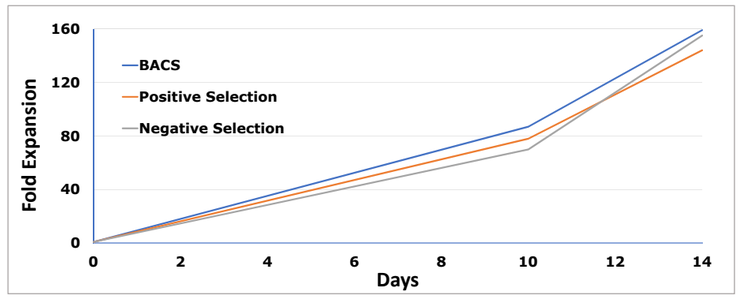




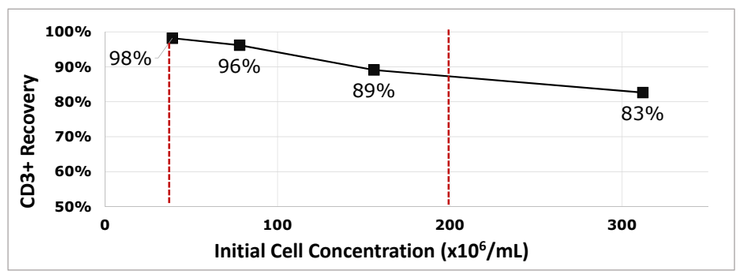
Overall recovery was calculated by multiplying values obtained in X-WASH and X-BACS steps. A mean CD3⁺ cell recovery of 81% was observed (n=3).



In a separate experiment, the expansion of CD3⁺ cells isolated using X-BACS was equivalent to CD3⁺ cells isolated using technologies from other manufacturers. Viable CD3⁺ cells were quantified over 14 days using flow cytometry.



To assess the consistency of CD3⁺ cell recoveries using the X-BACS process, the antibody and microbubble concentrations were kept constant while cell concentrations were increased. The results indicate only a marginal drop in CD3⁺ cell recovery within the normally expected dose range (30 to 200 x 10^6/mL).



Conclusions

DMSO removal using the X-WASH System resulted in cell recoveries of greater than 90% with no significant loss of viability. A mean CD3⁺ cell recovery of 84% was accomplished using the X-BACS process with high purity (>94%). In brief, we have developed an efficient method for removing DMSO and isolating CD3⁺ T-cells from cryopreserved apheresis samples that can be used for CAR-T cell manufacturing.

Quick and Easy Method for Removal of DMSO from Thawed Cell Products

Content originally prepared for ThermoGenesis by Dalip Sethi[1], Jon Ellis[2], Zelenia Contreras[2], John Perea[2], Stephen Truong[2], Jillian Miller[1]*
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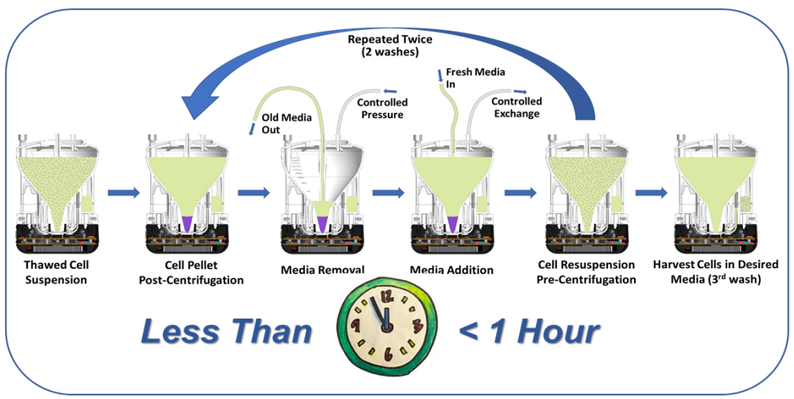
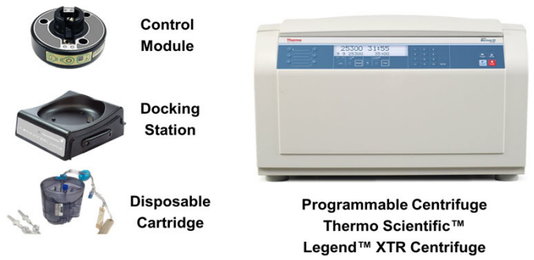
Introduction

Cell products, such as Hematopoietic Stem Cells (HSCs), have been cryopreserved and stored for decades using the cryoprotectant dimethyl sulfoxide (DMSO) at temperatures below -135°C. Although the cell products have demonstrated excellent post thaw viability, DMSO is considered a potential cause of infusion related adverse events.[1,2] Therefore, removal of DMSO and cell lysis products by washing the cell product after thawing may reduce the severity of some transplant related complications.[3,4] We have developed a quick and easy method to wash thawed apheresis products using the X-WASH™ System. Utilizing this protocol, nucleated cell recoveries were greater than 85% with no significant loss of cell viability. The entire process took less than one (1) hour.

Process Outline



Materials and Methods



Programmable Centrifuge
Thermo Scientific™
Legend™ XTR Centrifuge

Healthy adult cryopreserved Leukopak (Quarter collection) were purchased from HemaCare. Samples (n=3) were thawed as per supplier instructions. Briefly, the cryo-bag was removed from liquid nitrogen storage and immediately placed into a 37°C water bath without figure 8 motion or flicking. Thaw-wash media (2.5% HSA, 5% Dextran, and 20 µg/mL DNase I in saline) was added to the thawed products, and the sample was transferred into a transfer bag (optional step). The sample was transferred to the X-WASH Disposable Cartridge. Post-Centrifugation, waste media was removed using positive pressure, and new media was added using controlled negative pressure. The final centrifugation cycle harvested the cell pellet into the harvest chamber, which was pre-filled with media prior to centrifugation.

Results and Discussion

The reduction in DMSO in the washed product was calculated based on volume reduction. With the assumption of 10% DMSO in the starting material and even distribution of DMSO in cells and buffer. Actual DMSO quantification will be conducted in future experiments.

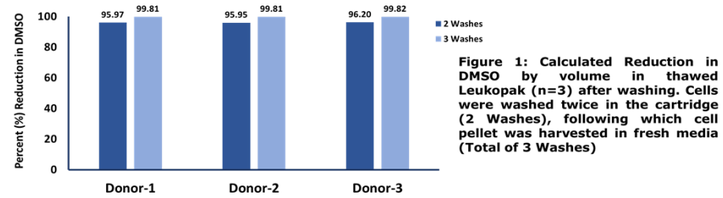


Figure 1: Calculated Reduction in DMSO by volume in thawed Leukopak (n=3) after washing. Cells were washed twice in the cartridge (2 Washes), following which cell pellet was harvested in fresh media (Total of 3 Washes)

$CD45^+$ cells (total and viable) and $CD3^+$ cells (total and viable) were quantified in pre-wash and post-wash fractions. The mean (SD) recoveries for the total and viable $CD45^+$ cells were 96.0% (2.6%) and 93.7% (3.7%). The mean (SD) recoveries for the total and viable $CD3^+$ cells were 96.3% (1.5%) and 92.4% (5.2%).

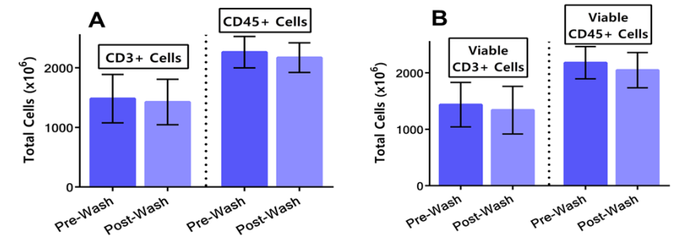


Figure 2: CD45+ and CD3+ Cells (Total (A) & Viable (B)) in Pre-Wash and Post-Wash fractions (n=3).

Conclusions

In conclusion, we have developed a fast and efficient method to remove DMSO from thawed cellular products. The method resulted in cell recoveries of greater than 90% with no significant loss of viability. The method is easy to implement in a standard laboratory.

References

1. Zambelli, et al. (1998) Clinical toxicity of cryopreserved circulating progenitor cells infusion. Anticancer Res. 18(6B); 4705-4708.
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