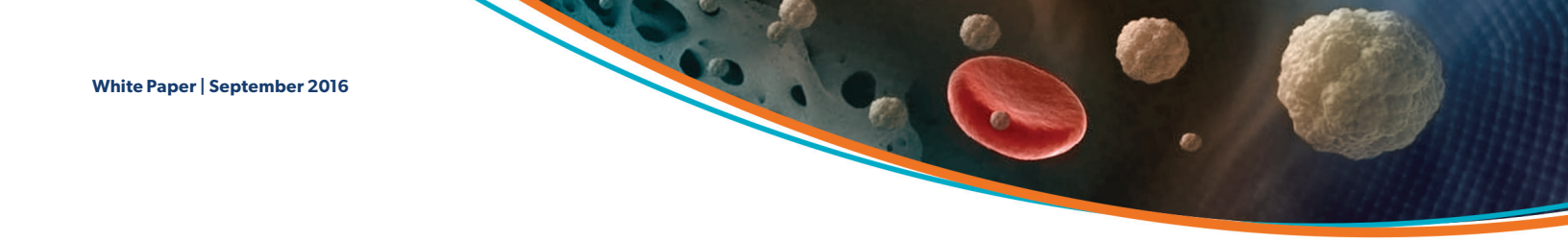

Cryopreservation of Primary Mammalian Cells

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Background

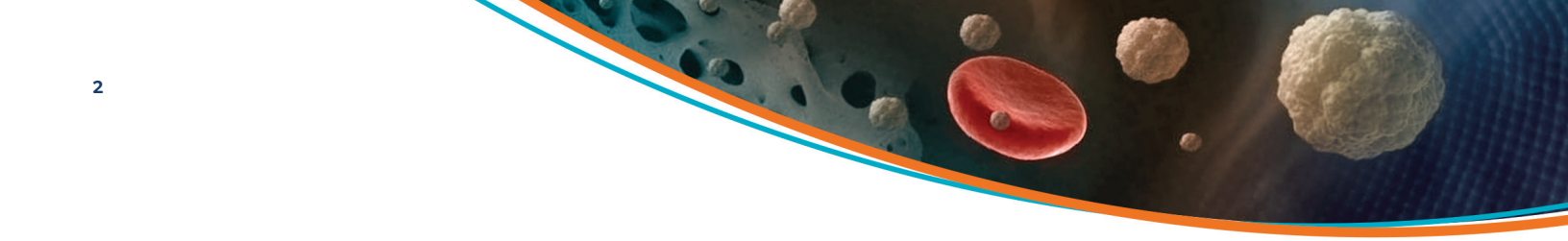
Ex vivo cell-based therapies for are increasingly being used as first line treatments for a variety of diseases. However, primary cells are difficult to maintain in cell culture, and often long term culture leads to loss of pluripotency and/or functionality, rendering the cells unsuitable for use in therapy. Cryopreservation of early passage primary cells in liquid nitrogen essentially freezes the cells in time, allowing them to be used months or years later with little to no loss of functionality and pluripotency. Thus careful cryopreservation offers a better solution than continuous culture for ex-vivo cell based therapies where several doses of therapy over weeks or months are required. This paper examines cryopreservation of primary cells with a focus on leukopak and T cell protocols.

Cryopreservation Protocols

Cryopreservation is used for long term storage of both primary and immortalized cell lines. With the latter, optimization of freeze / thaw protocols is less critical; any surviving cells can be grown indefinitely in cell culture to achieve the required total number of cells for a given experiment. Primary cells pose a different problem. Cryopreservation is necessary because primary cells often lose their functionality or pluripotency after prolonged growth in cell culture; after thawing cryopreserved primary cells it is likewise important to minimize length of time in cell culture prior to use. Thus optimizing the cryopreservation protocol for maximum viability and retention of functionality is essential for success.

A general cryopreservation protocol involves resuspending freshly isolated primary cells in a cocktail consisting on an isotonic electrolytic solution, a cryoprotectant, and a protein source, and then gradually reducing the temperature of the cells and solution to less than 150oC. Variations on media, protein source (serum or albumin) and cryoprotectant all influence the viability and functionality of the cells after thawing. In general, a cycle of cryopreservation and thawing is considered successful if cell viability is greater than 90%, cell count is within 10-15% of the number frozen, and (if applicable) cell populations are within 10% to 15% of the distribution seen in a freshly isolated sample (Ramos, 2014).

Cryopreservation presents challenges at both the freezing and thawing stages. The process of freezing leaves cells susceptible to damage on two fronts: the formation of intracellular ice crystals and the effects of increased solute concentration (solution effects). Successful cryopreservation protocols balance these two factors to result in minimal cell death. The appearance of intracellular ice crystals can be minimized by slow cooling the cells at a rate of -1oC per minute, using either a purpose-built slow cooling freezer rack or a rate-adjustable freezer. Slow cooling allows ice crystals to form in the extracellular space first, thus increasing the extracellular solute concentration and drawing water out of the cells to prevent formation of excessive ice crystals within the cells. Cellular dehydration, if unchecked, can in turn cause substantial damage to cells during the cryopreservation process due to the increased concentration of salt and other solutes. This can be alleviated by the addition of membrane-permeable cryoprotectants, the most common of which are dimethyl sulfoxide (DMSO) and glycerol. These function by reducing the salt concentration at a given temperature so that solution effects due to critical salt concentration occur at a lower temperature (ideally one low enough to prevent any lethal biochemical reactions due to high salt concentration) (McGann, 1978).



Rapid cooling is a less common method for cryopreservation, and relies on vitrification instead of dehydration of cells. Vitrification is carried out using high concentrations of non-membrane-permeable cryoprotectants that dehydrate the cell and flash freeze the remaining water with minimal crystallization, thereby minimizing damage from ice nucleation. Solution effects do not come into play because of the speed with which the cells are frozen. Hydroxy-ethyl-starch (HES), polyvinyl pyrrolidone (PVP) and Polyethylene oxide (PEO) are three common non-penetrating cryopreservation agents (McGann, 1978).

Cell death during the thawing process also occurs on two fronts: solvent concentration and ice recrystallization. The concentration of solvents used to prevent solution effects are cytotoxic at normal biological temperatures, and must therefore be added immediately prior to freezing and removed just after thawing or the recovered cell viability will be low. A second major cause of cell death during the cryopreservation and thawing process is ice recrystallization during thawing process; current research is investigating the use of ice recrystallization inhibitors such as polyvinyl alcohol or antifreeze glycoproteins to enhance the recovery rate of cryopreserved cells (Deller, 2016). Cryopreservation in liquid nitrogen has been shown to yield similar cell viability to cells cultured ex vivo if proper protocols are followed (Weinberg, 2009).

Cell Type Specific Challenges

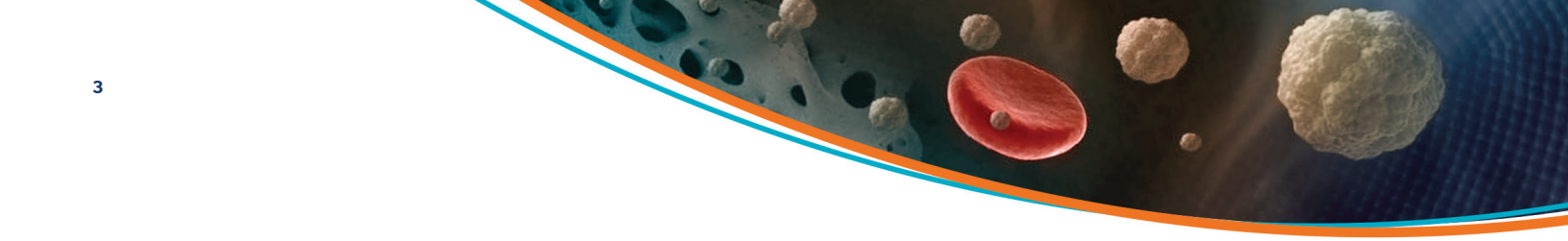
Although the basic protocol for cryopreservation of primary mammalian cells is similar among cell types, there are certain cell type-specific challenges that must be overcome for successful cryopreservation. These include the length of time in ex vivo culture as well as the addition of stimulants to promote growth of the cell type of interest as well as the type of medium used for cryopreservation (and addition of any factors to improve viability during the freezing process). Below we will examine strategies to optimize cryopreservation of (1) isolated T cells and (2) peripheral blood mononuclear cells (PBMCs), two important players in cell-based therapies.

Cryopreservation of T cells

T cell immunotherapies are an emerging field of cancer research. Such therapy can take the form of autologous tumor-reactive T cells or engineered chimeric antigen receptor (CAR) tumor reactive T cells. With the former, tumor-reactive CD8+ T cells are isolated from a patient and expanded ex vivo and then returned to the patient, often in concert with the cytokine IL-2. Research has shown that adoptive cell therapies work best when the transferred cells contain both mature cytotoxic T lymphocytes (CTLS) ready to kill cancer cells and a population of largely undifferentiated, multipotent cells that can continue to produce effector cells over time (Crompton, 2013). In long term culture, though, T cells begin to lose their multipotency and show signs of replicative senescence. It thus appears that a strategy involving rapid expansion of T cells followed by cryopreservation would best suit ex vivo T cell therapy.

A recent study of isolated murine CD8+ T-cells determined that cryopreserved and ex-vivo cultured murine T cells showed similar surface marker expression (CD44, CD62-L, CD69 and CD25), pro-inflammatory cytokine secretion patterns, and 3D scanning abilities. Both cryopreserved and fresh T cells were able to efficiently differentiate into effector T cells, as measured by surface marker expression. In addition, both cryopreserved and fresh cells were able to infiltrate and reduce tumor size by similar amounts (Nino, 2016).

Studies with human peripheral blood mononuclear cells have shown similar results with regard to the T cell compartment. A 2009 paper using human samples from HIV+ and HIV- donors determined that cellular proliferation and the proportion of CD4+ and CD8+ T cells was unchanged after storage in liquid nitrogen. When the cells were examined in more detail, however, fresh and cryopreserved T cells exhibited some differences in population dynamics. For example, the researchers found that certain cell surface markers – particularly CD45RO and CD62-L – appeared to be easily damaged during the cryopreservation process and thus skewed the analysis of post-thaw T cell populations. Likewise, T cell subsets correlating to activated and memory and regulatory T cells were underrepresented after thawing. Despite these slight changes in population distribution, T cell functionality was comparable between fresh and cryopreserved cells (Weinberg, 2009).



T cell cryopreservation protocol:

- Pellet cells by centrifugation for 10 minutes at 400xg.
- Resuspend at 2-5 x 10^6 per mL in appropriate cryopreservation medium (90% Fetal Calf Serum (FCS) or Human Serum Albumin (HSA) + 10% DMSO), or an appropriate commercial cryopreservation medium
- Transfer cells to cryogenic vials.
- Place in a -80 freezer in a freezing container that cools at a rate of 1oC per minute.
- After 48h transfer to liquid nitrogen storage.

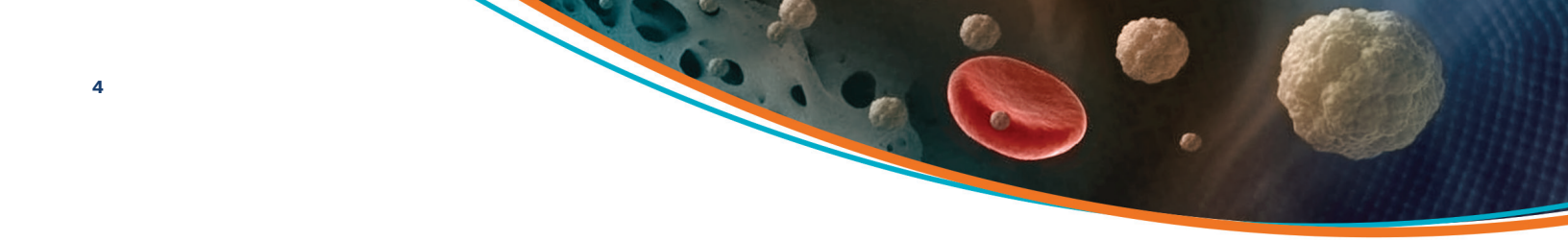
Cryopreservation of PBMCs (Leukopaks)

The PBMC population is made up of leukocytes (T cells, B cells, NK cells) and monocytes (which can differentiate into macrophages or dendritic cells). In contrast to T cells, which are generally isolated and grown in culture for a short time prior to freezing, PBMCs obtained via leukapheresis are processed and frozen as soon as possible after acquisition.

A number of studies have investigated the impact of cryopreservation on PBMCs in both healthy and diseased donors. In a 2007 study, researchers found that while the type of anticoagulant, PBMC isolation protocol, time to cryopreservation and cell shipping method all affected post-thaw viability, the largest decrease in viability, cell recovery and cell functionality was correlated with length of time between sample collection and cryopreservation. In all cases, as expected, a fresh sample yielded more cells than a cryopreserved sample; however it was shown that with prompt processing (less than 8 hours between collection and freezing) recovery of >94% was possible (Bull, 2007). Depending on the source and freezing protocol used, thawed PBMCs showed slight alteration in secretion of some cytokines including IFN-g, IL-6, IL-10, IL-12 and IL-13 (Axelsson, 2008; Mallone, 2007). Despite these background levels of cytokine expression, antigen-specific cytokine responses were still present and sometimes amplified, indicating that cellular functionality is retained following cryopreservation. PBMCs stored in liquid nitrogen have shown minimal loss of viability and function even after 15 months of storage (Weinberg, 2009).

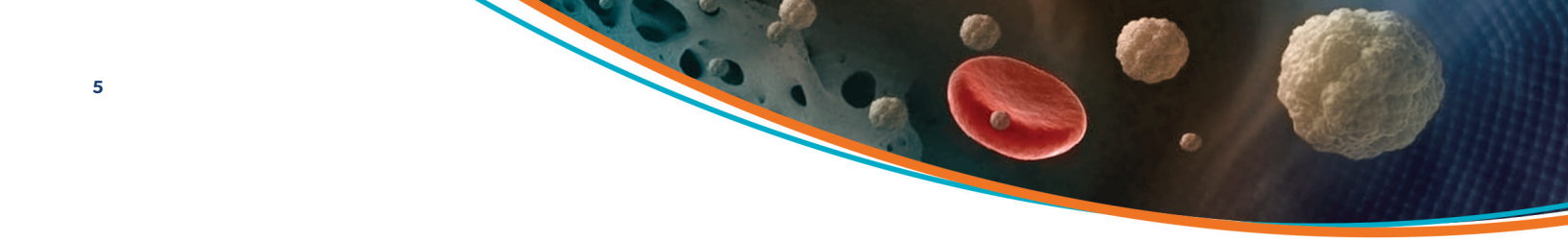
Cryopreservation of leukapheresis products (LP) offers researchers access to consistent and well characterized specimens that can reduce inter- and intra-experiment variability. In a study examining the processing of 280 leukopaks, researchers found that each leukopak produced an average of 326 vials vials (with 2x10^7 cells per vial). As a result of the abundant number of vials produced from the average leukopak, samples from the same donor can be used for many different experiments over months or years. Cryopreserved vials are also easily transferred between facilities (Garcia, 2014).

The cryopreservation protocol for leukopaks is identical to that of T cells listed above, save for the fact that the pelleted cells are resuspended at a density of 1-3x10^7 cells per milliliter. A freezing medium consisting of 90% Fetal Bovine Serum (FBS) and 10% DMSO is recommended (Ramos, 2014)



Summary

One of the major hurdles to ex vivo cell-based therapies is the fact that some types of primary cells lose their functionality and / or pluripotency when kept in long term culture. Cryopreservation of primary cells has the potential to enable long term studies and multi-dose therapies using cells that consistently exhibit the same functionality. Optimization of cryopreservation protocols generally results in >90% cell recovery, with time between sample acquisition and cryopreservation having the greatest affect on the percentage of cells recovered.



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