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Discover the Freshness of Frozen PBMCs

An Assessment of Cryopreservation Impact on T Cell Activation

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Understanding the impact of cryopreservation on performance of PBMCs is critical. This study focuses on assessing functionality of PBMCs after cryopreservation in standard immune cell based assays. Here, we demonstrate that cryopreserved PBMCs are reliable, deliver reproducible data, and maintain functionality. Utilizing freshly isolated PBMCs directly from donation may not be as necessary as believed.

Introduction

Human peripheral blood mononuclear cells (PBMCs) consist of a mixed population of specialized immune cells. These immune cells (belonging to both lymphoid and myeloid lineages) play an important role in both the innate and adaptive immune systems and are critical in protecting the body from viral, bacterial, and parasitic infections. Upon interaction with various pathogens, cytokines, or chemokines, these cells can fully mature and activate into T cells, B cells, dendritic cells, and macrophages for example.

Isolated human PBMCs are utilized in various fields of research, including vaccine and drug development. Routinely, these cells are isolated from fresh leukopaks and frozen down for subsequent use in experimental testing. As PBMCs continue to see an increase in use in the testing of new biological therapeutic compounds, ensuring purified cells retain functionality after cryopreservation of either the leukopak or PBMCs is necessary. The ability to use cryopreserved cells and be assured of the same functionality as fresh cells allows more options for cell sources, reproducible results, and the ability to plan experiments with convenience. Understanding the impact of cryopreservation on functionality of cell types isolated from PBMCs will provide guidance on use of PBMCs from leukopaks.

This study focused on determining if PBMCs maintain function after cryopreservation in standard immune cell based assays.

Materials and Methods

Isolation of PBMCs from fresh and cryopreserved leukopaks

Apheresis material was collected from four individual donors. After collection, ½ the material was processed for isolation and the other ½ of the material was cryopreserved following a proprietary method. Fresh leukopak material was processed equivalently across all donors within 24 hours of collection and PBMCs were isolated following standard methods. Following PBMC isolation, cells were cryopreserved in liquid nitrogen (LN2) for future testing (these cells are referred to as freshly isolated/single cryopreserved PBMCs). After one week, cryopreserved apheresis material was thawed. Following thaw, PBMCs were isolated using standard isolation procedures. Cells were subsequently cryopreserved in LN2 for future assessment (these cells are referred to as dual cryopreserved PBMCs).

Assessment of PBMC yield and viability

To determine the impact of single and dual cryopreservation on PBMC viability and yield, PBMC samples were thawed in a 37°C water bath for 2 minutes, centrifuged for 15 minutes at 300xg and resuspended in media for counting. Cells were counted using trypan blue exclusion method and yield and viability were determined.

T cell proliferation assay

To determine the impact of single and dual cryopreservation on PBMC function, PBMC samples were thawed in a 37°C water bath for 2 minutes, centrifuged for 15 minutes at 300xg and resuspended in media for counting. Following counts, cells were resuspended at 10×10^6/mL and incubated with 5 µM CellTrace CFSE Cell Proliferation dye (Invitrogen, C34554) for 20 minutes. Cells were then incubated with 5x media volume to stop the CFSE reaction and remove unbound dye for 10 minutes. Cells were then centrifuged 300xg for 10 minutes and resuspended in X-Vivo® 15 Serum-free Hematopoietic Cell Medium (Lonza, 04-418Q) at 10×10^6/mL. After 5 minutes, cells were added to 24- or 48-well plates at 1×10^6/mL. Cells were then combined with either X-Vivo® 15 Media alone, X-Vivo® 15 Media + 5 ng/mL IL-2 (R&D Systems, 202-IL-500), or X-Vivo® 15 Media + 5 ng/mL IL-2 + 50 µL CD3/CD28 Dynabeads (ThermoFisher, 11131D). IL-2 was added to the media to maintain T cell health and allow for proliferation and growth after activation, it is not expected to result in T cell proliferation alone. CD3/CD28 Dynabeads were selected to provide prolonged T cell activation in culture without the need for media changes or replacement. Cells were incubated for 4 days at 37°C, 5% CO_2 to allow for T cell proliferation. After 4 days, cells were collected, stained with Live/Dead fixable violet dead cell dye (ThermoFisher, I34964) for 20 minutes and analyzed via FACS on the BD Canto II.

T cell activation as measured by cytokine production in the presence of IL-2

To determine the impact of single and dual cryopreservation on T cell generation of cytokines, PBMCs were thawed in a 37°C water bath for 2 minutes, centrifuged for 15 minutes at 300xg and counted. Following counts, cells were resuspended at 1×10^6/well in 24-well plates and incubated with either X-Vivo® 15 media alone, X-Vivo® 15 media + 5 ng/mL IL-2 (R&D Systems, 202-IL-500), or X-Vivo® 15 Media + 5 ng/mL IL-2 + 50 µL CD3/CD28 Dynabeads (ThermoFisher, 11131D) for four days. On days 0, 2, and 4, media was collected from each well and frozen for future cytokine analysis. After collection of all timepoints, samples were thawed, centrifuged at 10,000xg for 5 minutes at 4°C and assessed for IL-1β, IL-12, IL-4, IFNγ, TNFα, IL-10, and IL-6 cytokines using a 32×8 Simple Plex Cartridge (Protein Simple, SPCKE-PS-003426) and the ELLA automated cytokine instrument.

T cell activation without additional IL-2

To determine PBMC functionality as measured by cytokine production in the absence of IL-2, PBMCs were thawed in a 37°C water bath for 2min, centrifuged for 15 minutes at 300xg and counted. Following counts, cells were resuspended at 1×10^6/well in 24-well plates and incubated with either X-Vivo® 15 Media alone, or X-Vivo® 15 Media + 50 µL CD3/CD28 Dynabeads (ThermoFisher, 11131D) for four days. On days 0, 2, and 4, media was collected from each well and frozen for cytokine analysis. After collection of all timepoints, samples were thawed, centrifuged at 10,000xg for 5 minutes at 4°C and assessed for IL-2 and IFNγ cytokines using a 32×2 Simple Plex Cartridge (Protein Simple, SPCKC-PS-006011) and the ELLA automated cytokine instrument.

Results

PBMCs isolated from both fresh leukopaks and cryopreserved leukopaks retain high viability

PBMCs were isolated from fresh (within 24 hours of donation) or cryopreserved leukopaks. Following isolation, viability was measured via trypan blue and cells were subsequently cryopreserved. Cells were thawed after at least two weeks in cryopreservation and viability was again measured via trypan blue. Results indicate PBMCs isolated from fresh leukopaks had higher viability when measured pre-cryopreservation as compared with post-cryopreservation and when compared to the PBMCs isolated from cryopreserved leukopaks at both time points (Figure 1); however, both single and dual cryopreserved cells maintained viability above 90% and 85% respectively.

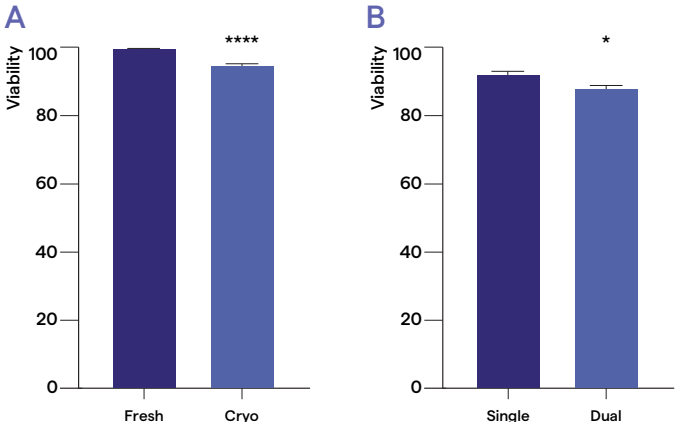

Figure 1.
Viability comparison immediately post-PBMC isolation from fresh or cryopreserved leukopaks (A) and post-PBMC cryopreservation of these fresh or cryopreserved leukopaks resulting in cells being cryopreserved once or twice P value is <0.0001 (B). Data is presented as the mean± standard error of the mean (SEM). Populations were analyzed by two-sided t-test P value is 0.0484.

T cell proliferative response is donor dependent but not altered by cryopreservation

Since both the fresh and cryopreserved leukopaks resulted in high viability post-PBMC cryopreservation, the next step was to assess the functional response of the cells. Cells were stained with CFSE dye as a means to measure downstream proliferation. After treating cells for four days with IL-2+Dynabeads, CFSE intensity was measured via FACS. To allow visual comparison, mean fluorescence intensity (MFI) of the dye dilution was assessed and a fold change was calculated compared to the negative control X-Vivo® 15 media only. Data demonstrated that IL-2 alone did not significantly increase T cell proliferation but cultures containing IL-2 and CD3/CD28 beads induced a strong proliferative response resulting in an average of 0.5 fold change decrease (Figure 2A). Furthermore, when data from all four donors tested were combined and assessed together, dual cryopreservation did not negatively impact T cell proliferation potential, meaning similar T cell proliferative responses were observed for both the single and dual cryopreservation samples. As expected, variations in T cell response were donor dependent. Overall, these data support the hypothesis that both single and dual cryopreservation samples display similar T cell proliferative responses.

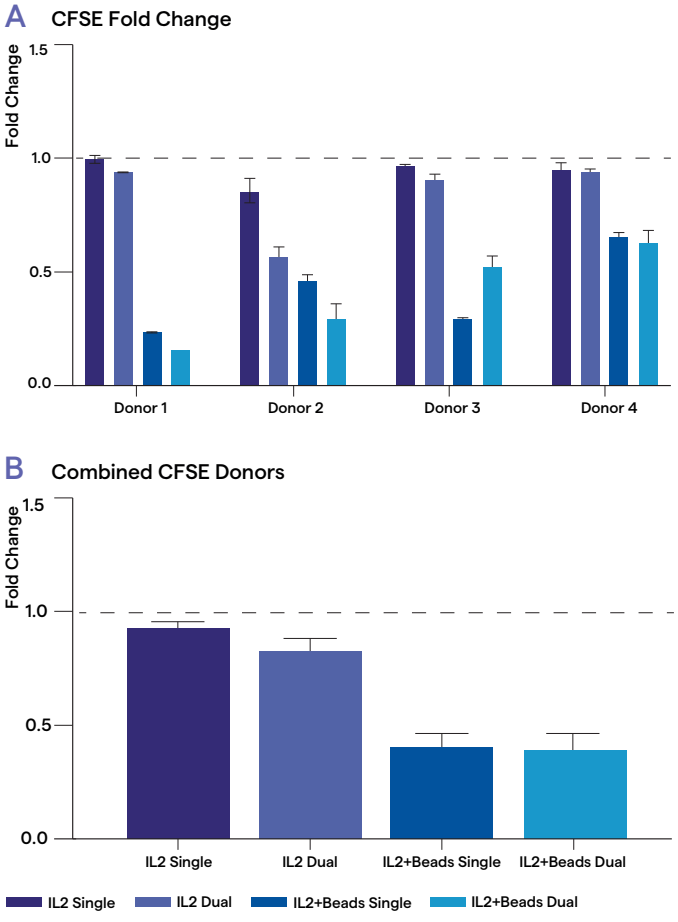

Figure 2.
Single and dual cryopreserved PBMCs were CFSE stained and cultured for for days with X-Vivo® 15, X-Vivo® 15 + IL-2, or X-Vivo® 15 + IL-2 + Dynabeads. Shown is the fold change decrease in CFSE staining compared to X-Vivo® 15 only treated PBMCs. (A) Individual donors, data is represented as mean± standard deviation (SD) (B) combined donors, data is represented as mean±SEM.

Cryopreservation does not change pro- and anti-inflammatory cytokines secretion profile

The next question was to investigate the relative difference between single and dual cryopreserved samples in their ability to be activated and secrete pro-inflammatory and anti-inflammatory cytokines. To test this, PBMCs were incubated with IL-2 and anti-CD3/CD28 DynaBeads for 7 days to allow for activation and cytokine secretion. Following incubation, cell supernatants from days 0, 2, and 7 were assessed for pro- (Figure 3) and anti-inflammatory (Figure 4) cytokines. As expected, cultures containing X-Vivo® 15 media alone did not activate and induce cytokine production for all donors and all time points assessed (Figures 3 and 4). By days 2 and 7, there was slight production of pro-inflammatory cytokines IL-1β and TNFα in the cultures containing IL-2 however this cytokine secretion was very minimal (Figure 3C and E). Following the pro-inflammatory trend, by days 2 and 7 there was slight production of the anti-inflammatory cytokine IL-4. However, this production was only seen in the cultures containing IL-2 and activated with beads (Figure 3D). Both pro-inflammatory and anti-inflammatory cytokine production was most prominent in cultures containing IL-2 and beads (Figures 3 and 4). The overall trend demonstrates that both anti- and pro-inflammatory cytokines were produced and secreted at measurable levels. Importantly, dual cryopreserved cells did not inhibit this cytokine release.

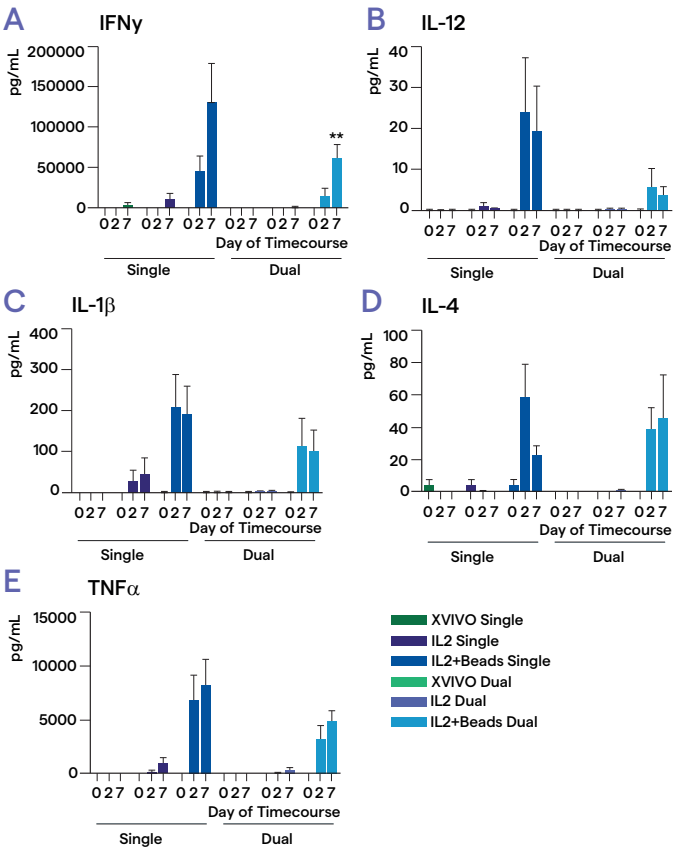

Figure 3.
Single and dual cryopreserved PBMCs were cultured with X-Vivo® 15, X-Vivo® 15 + IL-2, or X-Vivo® 15 + IL-2 + Dynabeads for 4 days. Following treatment, levels of pro-inflammatory cytokines IFNγ (A) IL-12 (B), IL-1β (C), IL-4 (D), TNFα (E) cytokine release was measured. This data is combined data from all donors. Data is represented as Mean±SEM, data was analyzed with 2-way ANOVA and Tukey's post test, only statistical differences between single and dual are shown * p<0.05, ** p<0.01, *** p<0.001, p<0.0001.

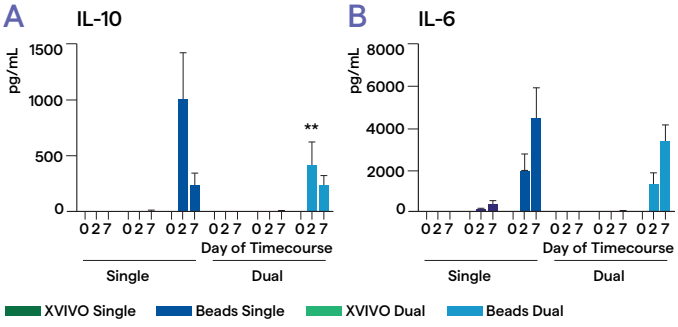

Figure 4.
Single or dual cryopreserved PBMCs were cultured for seven days with X-Vivo® 15, X-Vivo® 15 + IL-2, or X-Vivo® 15 + IL-2 + Dynabeads. Shown are the levels of anti-inflammatory cytokines IL-10 (A) and IL-6 (B) released following treatment This data is combined data from all donors. Data is represented as mean±SEM, data was analyzed with 2-way ANOVA and Tukey's post-test, only statistical differences between single and dual of the same condition are shown * p<0.05, ** p<0.01, *** p<0.001, p<0.0001.

IL-2 and IFNγ cytokine secretion is variable and dependent on donor but is not altered by cryopreservation

PBMCs were incubated with DynaBeads for 4 days in the absence of IL-2 to ensure the added IL-2 present in the previous experiment was not influencing the response and production of the other cytokines. Supernatants were harvested and assessed for IL-2 and IFNγ cytokine responses from days 0, 2, and 4. The three donors assessed demonstrated a variable IL-2 and IFNγ cytokine production response in dual cryopreserved cells compared to single cryopreserved cells (Figure 5). No significant difference was measured between IL-2 and IFNγ levels from single and dual cryopreserved PBMCs.

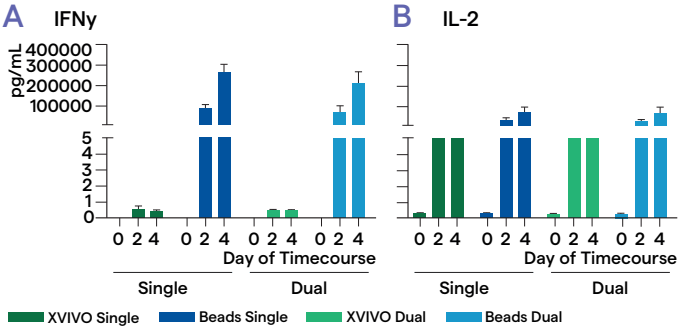

Figure 5.
Single and dual cyropreserved PBMCs were cultured with X-Vivo® 15 alone or X-Vivo® 15 with Dynabeads for 4 days. Shown are the cytokine levels of IL-2 (A) or IFNγ (B) released following treatment. This data is combined data from all donors. Data is represented as mean±SEM.

Discussion

Commonly, PBMCs are used during the drug development process in T cell proliferation assays to determine whether a compound or drug will elicit a T cell response to a foreign subsance. This T cell response can be measured by multiple ways *in vitro*, but standardly is assessed through proliferation to determine if an adverse drug reaction (ADR) will potentially occur *in vivo*. If a drug compound inappropriately binds to T cell receptors, it can result in T cell stimulation and activation associated clinically with delayed-type hypersensivity (Pichler *et. al.* 2015). Delayed-type hypersensitvity, as well as other ADRs are negative consequences that are screened for *in vitro;* thus ensuring PBMCs maintain the ability to

respond to stimulus is important for drug development. In addition to T cell interactions, immune cell signaling is important for compounds to elicit an immune response, which can also be measured through cytokine production following incubation of PBMCs with the compound of interest. For example, during a Th1 response, T cells secrete inflammatory cytokines to support cell-mediated immunity (IFNγ, IL-2, TNFα) while during a Th2 response, T cells secrete cytokines associated with a humoral-type immune response (IL-4, IL-5, IL-10), and lastly during a Th17 response, T cells produce pro-inflammatory cytokines to support responses against extracellular pathogens (IL-17, IL-21, IL-22) (Oosterhout & Motta, 2005, Brinkhoff *et. al.* 2018). Measuring the cytokines produced by PBMCs after exposure to a compound allows for potential determination of the type of T cell response (Th1 vs Th2 vs Th17). If a product causes a rapid release of unwanted pro-inflammatory cytokines, negative *in vivo* reactions such as anaphylaxis can occur (US FDA, 2014). Furthermore, ensuring dual cryopreservation does not negatively impact the ability of PBMCs to release cytokines and therefore appropriately respond to stimulus, is vital to ensure *in vitro* releavance.

PBMCs isolated from cryopreserved leukopaks had a lower viability than PBMCs isolated from fresh leukopaks. (Figure 1). While dual cryopreservation did decrease viability, the specifications were still well above industry standards (70% viability), therefore feasible for further downstream cell analyses. Other research has also demonstrated that cryopreservation minimally impacts yield and viability of PBMCs (Adriana et al. 2009) and does not impact functionality when cells are stored correctly (Angel *et al.* 2016). Further, previously published data from Lonza, indicated the PBMCs maintained similar phenotypes regardless of cryopreservation type, (Dunnick *et. al*, 2022). Other studies also confirm this finding as single cryopreservation did not impact cell subsets (Thyagarajan *et. al.* 2018) does not impact cell subsets.

The lack of impact on the phenotypes detected implies the cells should maintain similar levels of cytokine release profiles and T cell proliferative activity. To test whether the former statement was true, single and dual cryopreserved cells were exposed to CD3/CD28 Dynabeads to stimulate, activate, and elicit a T cell proliferative response. Following T cell activation, cells were measured for proliferation via CFSE dye dilution. Results indicated that dual cryopreservation of cells did not negatively impact T cell proliferation as both singly and dually cryopreserved cells had high levels of proliferation (Figure 2).

We further assesed the effects of single versus dual cryopreservation on cytokine production. Supernatants collected from days 0, 2, and 7 of PBMC culture with IL-2 and CD3/CD28 Dynabeads revealed detectable levels of both the classical anti-inflammatory and pro-inflammatory cytokine profiles (Figures 3 and 4). Cells that were cultured in IL-2 alone however lacked superlative cytokine production, indicating that the PBMCs required sufficient stimulation via IL-2 and activation via CD3/CD28 Dynabeads. To rule out the effect of exogenous IL-2 in the culture systems on other cytokine production, cells were cultured without exogenous IL-2 and IL-2 and IFNγ production was measured. Once again, the data revealed high levels of cytokine production from cells cultured with CD3/CD28 Dynabeads and no difference between the single or dual cryopreserved cell samples (Figure 5).

Our data indicate that ready to use Lonza PBMCs provide a functional product to detect T cell activation and proliferative response to stimulus and produce high levels of cytokines. Further, cryopreserving the leukopaks prior to PBMC isolation did not impact the isolation and proliferative potential of PBMCs. Thus frozen leukopaks can serve as another viable option from which PBMCs can be isolated.

Both fresh and cryopreserved leukopak types performed similarly in all assays tested, demonstrating that PBMCs isolated from both types of starting material are reliable, reproducible, and interchangeable. Processing samples directly from donation may not be as necessary as believed. As donor variation and *in vivo* relevance to a population becomes more important, there is need to increase the options of tools to choose from. Researchers now have the ability to obtain cells from donors of a certain race, medical history, or genetic predisposition based on the needs of the research. By providing researchers with the option to select fresh or cryopreserved leukopaks, as well as cryopreserved PBMCs, we provide an expanded offering of donors to meet research needs.

Knowledge that PBMCs isolated from fresh and cryopreserved leukopaks maintain function increases the confidence to use previously isolated frozen cells and generate experimental data with convenience.

References:

Adriana, W., Lin-Ye, S., Cynthia, W., Anne, S., Bruce, B., Raul, L., Dana, S., Patricia, D., Deborah, D., Eric, R., Nancy, R., Renee, J., Betty, B., Fran, K. M., Ruth, D., Elizabeth, M., & Terence, F. (2009). Optimization and Limitations of Use of Cryopreserved Peripheral Blood Mononuclear Cells for Functional and Phenotypic T-Cell Characterization. Clinical and Vaccine Immunology, 16(8), 1176–1186. https://doi.org/10.1128/CVI.00342-08

Angel, S., von Briesen, H., Oh, Y.-J., Baller, M. K., Zimmermann, H., & Germann, A. (2016). Toward Optimal Cryopreservation and Storage for Achievement of High Cell Recovery and Maintenance of Cell Viability and T Cell Functionality. Biopreservation and Biobanking, 14(6), 539–547. https://doi.org/10.1089/bio.2016.0046

Brinkhoff, A., Sieberichs, A., Engler, H., Dolff, S., Benson, S., Korth, J., Schedlowski, M., Kribben, A., Witzke, O., & Wilde, B. (2018). Pro-Inflammatory Th1 and Th17 Cells Are Suppressed During Human Experimental Endotoxemia Whereas Anti-Inflammatory IL-10 Producing T-Cells Are Unaffected. In Frontiers in Immunology (Vol. 9, p. 1133). https://www.frontiersin.org/article/10.3389/fimmu.2018.01133

Dunnick, K. Ngyuen,L, Menezes, A. Sharma A. (2022), Cryopreservation of Peripheral Blood Mononuclear Cells Does Not Alter Immune Cell Phenotype. Lonza Tech Note

Pichler, W. J., Adam, J., Watkins, S., Wuillemin, N., Yun, J., & Yerly, D. (2015). Drug Hypersensitivity: How Drugs Stimulate T Cells via Pharmacological Interaction with Immune Receptors. International Archives of Allergy and Immunology, 168(1), 13–24. https://doi.org/10.1159/000441280

Thyagarajan, B., Barcelo, H., Crimmins, E., Weir, D., Minnerath, S., Vivek, S., & Faul, J. (2018). Effect of delayed cell processing and cryopreservation on immunophenotyping in multicenter population studies. Journal of Immunological Methods, 463, 61–70. https://doi.org/https://doi.org/10.1016/j.jim.2018.09.007

van Oosterhout, A. J. M., & Motta, A. C. (2005). Th1/Th2 paradigm: not seeing the forest for the trees? European Respiratory Journal, 25(4), 591 LP – 593. https://doi.org/10.1183/09031936.05.00014105

US FDA, Guidance for Industry: Immunogenicity assessmen for therapeutic protein products August 2014, https://www.fda.gov/media/85017/download

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