Effects of Sample Processing Procedures on PBMC Gene Expression

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Abstract

This study investigated the effects that sample processing had on PBMC gene expression profiles. Specifically, two vacutainer types were used in this study; sodium heparin CPT vacutainers and "standard" sodium heparin vacutainers as well as two freezing devices; CoolCell and programmable rate-controlled freezer. Biogen's clinical trials collect whole blood in sodium heparin CPT vacutainers and cryopreserve PBMCs using a CoolCell device because both are more convenient and economical for the site. The results revealed that when RNA from freshly isolated PBMCs from both vacutainer types were compared 21 DEGs were identified. Regardless of the vacutainer type, when comparing RNA from freshly isolated PBMCs to RNA from thawed cryopreserved cells using a CoolCell device ~50 DEGs were identified but only ~30 DEGs were found when RNA from freshly isolated PBMCs were compared to RNA from rate-control frozen PBMCs. Additional testing needs to be performed to better appreciate when to use each vacutainer. My results suggest that a rate-controlled freezer causes less DEGs than a CoolCell and that about 74% of the cryopreservation-related genes (17/23) were common regardless of vacutainer type or freezing device used. The data from this project will assist in the design of scientifically-based "Best Practices" needed for appropriate sample processing of PBMCs.

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Introduction

Hypothesis

The isolation of peripheral blood mononuclear cells (PBMCs) from venous blood for research and clinical analyses has become a common practice (Koncarevit, *et al*., 2014). Fan & Hedge (2005) has suggested that for clinical research, peripheral blood has been an appealing tissue type to collect as it exemplifies one of the most extensively used and easily obtained sample types in clinical settings. The collection of blood from donors is considered to be minimally invasive if standard clinical procedures are followed (Fan & Hegde, 2005). Isolating PBMCs from peripheral blood is ideal for assessing any changes in gene expression patterns and sample processing has been shown to affect gene expression (Debey *et al.*2004).

Recently, research by Lempicki *et al*. (2013) has shown that fresh PBMCs isolated by ficoll-density centrifugation from anti-coagulated whole blood collected in Acid Citrate Dextrose (ACD) vacutainers had a different gene expression pattern than PBMC aliquots that had been cryopreserved, stored and thawed. More specifically, this research group identified a total of 1,367 genes whose expression after 14 months of storage was affected (increased or decreased) >3 fold in PBMCs following isolation, cryopreservation and thawing as compared to freshly isolated PBMC aliquots that did not undergo cryopreservation. Sixty-six of these genes were shared among two or more major stress-related cellular pathways (stress responses, immune activation and

apoptosis/cell death).

Biogen's clinical trials currently uses Cell Preparation Tube (CPT) vacutainers containing the anti-coagulant, sodium heparin, as a means to isolate PBMCs from whole blood for biomarker discovery when multiple clinic trial sites are involved. Therefore, Biogen's Biobank is interested in determining the effect that the preservative, sodium heparin, has on gene expression of freshly isolated, cryopreserved and thawed PBMCs when whole blood is collected in a "standard" vacutainer that contains sodium heparin as compared to having whole blood collected in a CPT vacutainer that has sodium heparin inside. Additionally, this study will examine the effect that two different cryopreservation devices, e.g., programmable rate-controlled freezers and CoolCells have on PBMC gene expression. Both are commonly used to cryopreserve PBMCs. The rate-controlled freezers are commercially available programmable freezers that lower the temperature of PBMC-containing cryovials by -1^{o}C/minute until the temperature inside the vial reaches -150^{o}C. Once the cryovials reach -150^{o}C, the cryovials are transferred to long-term storage in freezers that maintain temperature at $\leq$-150^{o}C. Whereas, CoolCells are well-insulated commercial devices that require placing the PBMC-containing cryovials inside the device and then placing the device in a -80^{o}C freezer so that the cryovials containing the PBMCs decrease their temperature -1^{o}C/minute until it reaches -80^{o}C. Once the temperature inside the vials reach -80^{o}C while inside a CoolCell, which takes a minimum of 4 hours, the cryovials then need to be transferred to a freezer for long-term storage that maintains temperature at $\leq$-150^{o}C. The rate-controlled freezer is considerably more expensive than the CoolCell. As a result, Biogen typically has their clinical trial sites use the less expensive and smaller capacity CoolCell to cryopreserve

PBMCs. Therefore, it is important to determine if freezing PBMCs using a CoolCell device produces a different gene expression pattern than when a rate-controlled freezer is used.

For my project, I expect to see differences in the gene expression pattern between the freshly isolated PBMCs from the whole blood collected in a sodium heparin vacutainer versus freshly isolated PBMCs from sodium heparin CPT vacutainers. Furthermore, I expect fewer genes will be affected when freshly isolated PBMCs are compared to sister aliquots that undergo the freezing process in a rate-controlled freezer as compared to those that are placed inside a CoolCell device, all of which were stored in the freezer for 1 month before being thawed.

Background Information on Biobanks

Biobanks are responsible for the proper biospecimen handling, i.e., collecting, processing, inventorying, storing and shipping of biospecimens. As a result, Biobanks play a critical role in designing appropriate logistical biospecimen handling solutions that are necessary to answer basic science and clinical research questions that will ultimately improve patient care (Morente *et al*., 2011). Collecting significant numbers of biospecimens as well as the high quality data associated with each sample involves a considerable effort in planning. Advocating for and implementing Biobanks that operate based on scientifically based "Best Practices" would facilitate access to high quality biospecimens and related data for researchers. Therefore, this thesis will provide scientific information for establishing "Best Practices" for isolating, processing and cryopreserving human PBMCs using gene expression analysis.

Goals of Biogen's Biobank

Biogen's Biobank capabilities are evolving and the Biobank is striving to be "World Class" by 2017. A World-Class Biobank will help Biogen in reaching many of its strategic objectives. To be considered a World-Class Biobank will require focusing on 6 key imperatives: 1.) Operate a high quality Biobank, 2.) Expand services to include biospecimen processing and acquisition, 3.) Perform biospecimen-related research with a focus on biospecimen handling, 4.) Leverage technology to provide innovative operational solutions to the Biobank, 5.) Automate storage and implement high-throughput biospecimen processing and 6.) Expand and solidify information technology (IT) and operational systems integration across all Biobank locations.

Background Information on PBMCs

PBMCs are mononuclear white blood cells, e.g., Monocytes, T cells, B cells, and NK cells. PBMCs play a crucial role in the immune system as they recognize foreign bodies and help the body fight infection. PBMCs are used to evaluate different in vitro functional and phenotypic immunological assays such as enzyme-linked immunosorbent spot (ELISPOT) assays, proliferation assays, flow cytometry and cytometry by time-of-flight (CyTOF) determinations. In translational medicine, PBMCs can function as precursors for potential immunotherapy development and can be used for biomarker discovery. PBMC samples are an important biospecimen type collected by Biogen and are often collected, processed and cryopreserved at multiple clinical sites, which makes standardization a concern.

Ruitenberg *et al.* (2006) suggests that clinical researchers usually prefer to obtain whole blood from patients at pre-determined time points, collect and cryopreserve PBMCs and perform assays at a later time or after multiple biospecimens have been collected in an effort to batch samples. They also suggest that cryopreserving and evaluating biospecimens at a central laboratory is standard practice in order to minimize operator, site and temporal variability as well as to enhance the precision and accuracy of immunoassays. Ruitenberg *et al.* (2006) indicated that clinical investigators usually employ one of two methods to collect whole blood; 1). Using a vacutainer tube, or 2). Using a CPT vacutainer. Using a vacutainer tube means that samples are collected and shipped to the central laboratory to have PBMCs isolated using a ficoll density centrifugation method; whereas, using a CPT vacutainer generally means that upon collection the CPT vacutainers are centrifuged at the collection site and then shipped to the central laboratory for PBMC recovery and this separation technology offers processing versatility (Ruitenberg *et al., 2006).*

The last decade and a half has provided great advances in the knowledge of programmed cell death (apoptosis), flow cytometry methods and microarray gene expression analysis for PBMCs (Lempicki *et al*., 2013; Peng *et al*.2007; Germann *et al.* 2013). Studies taking advantage of propidium iodide viability staining used in flow cytometry have clearly shown that cryopreservation has a greater negative impact on PBMC quality than previously thought based on trypan blue cell staining (Lempicki *et al*., 2013; Peng *et al*.2007; Germann *et al.* 2013).

Gene Expression

Baechler *et al*. (2004) states that on a genomic scale, gene expression profiling has made extensive progress in identifying molecular pathways correlated with human malignancies as well as other various diseases. Previous studies that Baechler *et al*. (2004) investigated have largely depended upon using biopsied tissues from healthy volunteers and tumors from individuals affected with cancers. However, obtaining these types of samples can be difficult as these types of tissues are not always readily available for harvesting for all diseases; the procedure is invasive, uncomfortable to the patient and obtaining this type of material is costly (Baechler *et al*., 2004).

More recently the gene expression profiles of PBMCs were used to detect variations in expression patterns in healthy donors, with a goal of further characterizing underlying mechanisms of disease (Fan & Hegde, 2005). However, previous research has shown that gene expression was affected by issues related to the *ex vivo* handling of samples prior to the extraction of RNA (Baechler *et al*., 2004; Barnes *et al.* 2010; Debey *et al.*2004).

Whitney *et al*. (2003) found that experiments performed *in vivo* have delved into various facets of the physiological regulation of human gene expression programs, which includes identifying genes periodically expressed during various stages of the cell cycle, the response of human cells to different stimuli as well as the dissection of various signaling pathways. In a more clinical setting, gene expression profile patterns help to define the complexity of various biological processes associated with health as well as disease *in vivo* (Whitney *et al*., 2003). Gene expression profiling is helpful in predicting diseases as well as classifying biospecimens, determining the efficacy of drugs, toxicity

classification and prognosis of diseases (Fan & Hegde, 2005). Previous research has shown that the technical aspects of blood sampling, cell isolation, the techniques used for RNA isolation and various clinical aspects including length of time until data is analyzed and the temperature used during processing have an effect on gene expression patterns (Debey *et al*., 2004).

Previous Gene Expression Research

All of the information contained below was summarized from the work from a single reference, e.g., Lempicki et al., 2013.

Leidos Biomedical Research (formally SAIC-Frederick, Inc.) followed an IRB-approved protocol, in which whole blood was collected from 10 healthy donors in 2005. The whole blood was collected in vacutainers, which contained Acid Citrate Dextrose (ACD). Ficoll density-centrifugation was used to isolate PBMCs; the PBMCs were then washed, counted and the viability was determined using a light microscope along with a hemocytometer and trypan blue staining. Viability, apoptosis and necrosis were also determined using flow cytometry with propidium iodide, 7AAD and Annexin V staining, respectively, as a comparison.

Cells for storage were frozen at $10x10^6$ cells/ml using a cryococktail of 20% fetal calf serum + 7.5% dimethyl sulfoxide (DMSO) + 72.5% RPMI-1640 media. A rate-controlled freezer was used to cool all of the PBMC-containing vials to -150°C, once cooled to -150°C the sample vials were stored at three different storage conditions. The sample vials were sorted so that vials from each donor were administered to each of three storage conditions. The first condition was vapor liquid nitrogen storage at -150°C. At -

150°C, the samples were at a static storage state. The second condition mimics sample storage and temperature fluctuations associated with sample sorting and "sample cherry picking," a routine activity in Biobanks. Thus, in the second condition the samples were cycled 104 times between -150°C, -80°C and back to -150°C using a rate-controlled freezer. The rate-controlled freezer was programmed in the following way: the starting temperature was -150°C; the temperature was then increased +10°C/min until the temperature reached -80°C; once the temperature reached -80°C it held temperature at -80°C for 10 min; then the temperature was decreased at -30°C/min until it reached -150°C; once the temperature reached -150°C it held temperature at -150°C for 10 minutes. The temperature was finally held at -150°C until the vials were transferred to vapor phase LN2. The third and final condition was static storage of the PBMCs at -80°C.

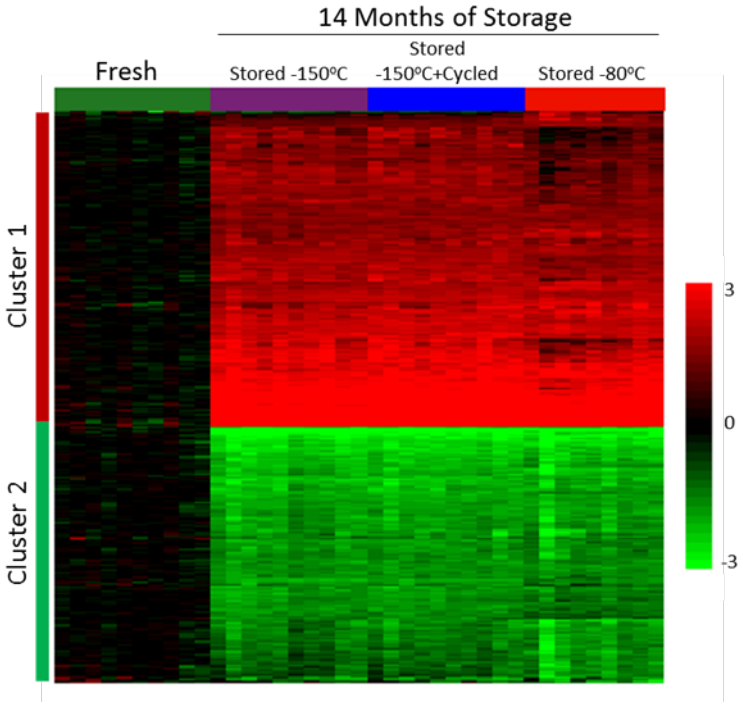
PBMCs were stored frozen for 14 months. After being stored for the desired amount of time, the samples were thawed rapidly, diluted in media, and counted using trypan blue exclusion for viability and cell recovery. Immediately after being counted, the cells were analyzed using flow cytometry for viability, apoptosis and necrosis. If it was determined that sufficient cells were available; an aliquot was placed in RLT lysis buffer for gene expression analysis.

Total RNA was extracted from cryopreserved PBMCs samples that were stored frozen for 14 months along with matching freshly isolated PBMC control samples. Microarray gene expression analysis was performed using the Affymetrix U133 2.0 Plus array in 2007 using the recommended manufacture's protocol at that time. Differentially regulated genes were defined as those that had an ANOVA $p<0.05$ and an absolute fold

change greater than five. Pathway analysis was conducted using DAVID and Ingenuity Pathway.

In Figure 1.1 below, the up-regulated genes (Clusters 1) were strongly associated with apoptosis and the MAP Kinase, JUN Kinase and p38 stress-activated Kinase pathways, respectively; whereas, Cell Death and Defense Response Pathways were similarly associated with both Clusters 1 and 2. Overall, there were 1,367 genes (in clusters 1 and 2) that showed 3-fold log difference.

Figure 1.1. Gene Expression Alterations by Cryopreservation Conditions.



Visual summary of 1,367 differentially expressed genes following cryopreservation. The expressed genes from PBMC samples stored for 14 months in the 3 storage conditions are presented in Cluster 1 (increased expression) and Cluster 2 (decreased expression) as related to freshly isolated PBMCs that have an absolute FC>3.

All of the information contained below was summarized from the work from a single reference, e.g., Ruitenberg *et al., 2006.*

Many previous studies have shown that several factors have an effect on functional T cell responses such as shipping, storage, age, cryopreservation and thawing of samples. Some of the same studies suggest that immunophenotyping, proliferation and functional assays should only be performed on fresh samples while other studies suggest that using frozen PBMCs is an acceptable option for functional assays. However, it is crucial that PBMCs are processed and handled in such a way as to not degrade the cells ability to have a response to activation stimuli. It is also crucial to ensure that enough viable PBMCs are collected during the isolation and cryopreservation process to be able to perform the desired studies.

Based on this information, Ruitenberg *et al.* decided to research whether or not samples processed using CPT vacutainers could be used as a replacement for ficoll density centrifugation since separating PBMCs using ficoll density centrifugation is a labor-intensive process. Researchers were interested in looking at blood samples from HIV seropositive patients. Typically, in HIV vaccine clinical trials the blood of HIV seropositive patients is collected at several different study sites, then the samples are shipped to a central laboratory where the samples undergo PBMC isolation and evaluation. For this study, two sites obtained whole blood from 19 seropositive HIV patients in both sodium heparin CPT vacutainers and sodium heparin vacutainers. At each collection site, the sodium heparin CPT vacutainers were centrifuged within 2 hours according to the manufacturers' specifications and inverted several times. The centrifuged sodium heparin CPT vacutainers and the unprocessed sodium heparin

vacutainers were then shipped at ambient temperature overnight to a central laboratory. Upon arrival the sodium heparin vacutainers were processed using ficoll density centrifugation and the processing of the sodium heparin CPT vacutainers was completed according to manufacturer's recommendations. The collected PBMCs from both vacutainer types were counted, cryopreserved with cryococktail and then stored frozen. The PBMCs were thawed and underwent cytokine flow cytometry (CFC) assays to determine the antigenic stimuli of the PBMCs.

Ruitenberg *et al.* found that with fresh blood samples, samples processed with ficoll density centrifugation had a significantly better viability compared to sodium heparin CPT processed PBMCs. But with cryopreserved samples the sodium heparin CPT processed PBMCs had a better viability than the ficoll density centrifugation processed PBMCs although the difference was not significant. Between the two methods of processing, the fresh and cryopreserved PBMCs recoveries were not significantly different. It was concluded that regardless of whether fresh samples or cryopreserved samples are used, sodium heparin CPT vacutainers are in fact a very effective method for obtaining functionally active PBMCs, at least for HIV seropositive patients. Overall, these results revealed that PBMCs prepared by either method actually perform equivalently meaning that using sodium heparin CPT vacutainers to collect PBMCs from blood may be a viable alternative to using ficoll density centrifugation, especially when collecting samples at various sites and shipping them to a central processing laboratory location.

All of the information contained below was summarized from the work from a single reference, e.g., Cosentino *et al., 2007.*

It is commonly accepted that PBMCs are fragile and that mishandling PBMCs during the cryopreservation and thawing process can jeopardize their function in comparison to fresh PBMCs. Published data has focused on how cryopreservation has impacted cell death. Specifically, ice crystal formation which is a phase change from liquid to crystalline solid as well as acute changes in osmolality which impacts cellular integrity can damage cells and decrease viable cell recovery. Based on this, freezing media and cryopreservatives were engineered to assist with reducing cell death and sustain viability during the process of freezing and thawing. Published data suggests more causes of cold-induced cell death such as cellular membrane damage, dehydration, and the formation of reactive oxygen species (ROS), all of which can have a negative effect on the quality of PBMCs. It is believed that necrotic events cause hypothermia and cryopreservation injury to cells. A major concern seems to be that apoptosis occurs when PBMCs are cryopreserved; this phenomenon is known as cryopreservation-induced delayed-onset cell death (CIDOCD).

The biological phenomenon known as apoptosis, results due to the activation of a genetically encoded cell death-signaling program. The activation of the program contributes to a form of cell death, which is differentiated from necrosis by a specific set of morphological features including chromatin condensation, membrane blebbing, loss of nuclear and cell volume and lastly a characteristic patterned fragmentation of nuclear DNA. Apoptosis results in the regulated elimination of cells at the same time as minimizing tissue damage without generating inflammation. At a molecular level, a receptor-linked or a DNA damage-induced death signal such as p53-inducible genes are involved in most apoptotic pathways. Furthermore, a signal transduction pathway exists

which involves mitochondrial-derived factors including cytochrome c release and ROS and it is under the control of regulatory factors such as intracellular Ca^{2+} ions, balance of antiapoptotic Bcl-2 and pro-apoptotic Bax proteins. Activated proteases known as caspases & DNA endonucleases are involved in the final, common apoptosis execution pathway. Cell freezing-cocktails minimize damage to cells due to ice crystal formation, but are not able to protect PBMCs from undergoing apoptosis caused by CIDOCD. CIDOCD has been reported to play a role in PBMC cryopreservation failure; however, there is limited understanding regarding the extent, timing and activation stressors.

Conclusion

Overall, the gene expression results from my thesis will offer insight into Biogen's goal of identifying a standardized way to process PBMCs for all of their clinical studies. My goal is to document that different PBMC isolation and processing procedures used in Biogen's basic and clinical research projects have an effect on the gene expression patterns of PBMCs, which is critical when these biospecimens are used in biomarker discovery.

Chapter II

Materials & Methods

Biogen has a volunteer donor program in which Biogen employees' donate whole blood for research purposes. There were 10 healthy donors that provided between 96 mLs – 106 mLs of whole blood for this thesis research study. Each individual donated whole blood approximately in equal amounts into 2 different vacutainers: sodium heparin CPT vacutainers and sodium heparin vacutainers. There were two cryopreservation methods used to viably preserve PBMCs: a CoolCell & a rate-controlled freezer, with each method preserving a multiple number of cryovials.

Each time an individual donated whole blood; the phlebotomist simultaneously collected both vacutainer types as required for this experiment. The whole blood was processed immediately after being collected and at least 4 aliquots of PBMCs were collected during the isolation process (please see Table 2.2 below for a summary of the specific steps used for the collection). RNA was isolated from the aliquots of biospecimens and later analyzed by gene expression to determine the specific cellular pathways that were affected.

By using the two vacutainer types and the two cryopreservation procedures, it begins to allow Biogen to better understand how these processing variables may affect gene expression profiles from PBMCs that are routinely used in biomarker discovery research.

Samples Uploaded into Biogen's Biobank's Watson Database

There were a total of 3820 samples produced for this project and all of them were entered into the Biobank's Watson Database so their chain of custody could be properly tracked. Each sample was uniquely labeled. Below in Table 2.1, the labeling scheme used in this thesis is summarized so that the samples could be tracked from the collection of whole blood through any specific processing that was performed, aliquot size stored and its intended use. The standard Sample ID label format used was: GX1.247802-1000-0. The Sample IDs are composed of 3 parts: **Study ID**, **Collection Event ID** and **Sequence Number**. The **Study ID** = GX1. The Study ID is the first 3 alpha numerics, e.g., GX1, which links/identifies the samples to Graduate Experiment 1. **Collection Event ID** = 247802. The Collection Event ID has 2 pieces of information embedded in it:

- The Donor ID is the first 4 digits 2478 02 that identifies the individual donating a specimen from Biogen's employee donor program.
- The last 2 digits, 247802, indicate which vacutainer type was used. 02 = sodium heparin CPT vacutainer; and 03 = sodium heparin vacutainer.

The **Sequence Number** is the last 5 digits of the Sample ID, -1000-0, that is preceded by a hyphen and that groups the first 4 digits together and separates the last digit by a second hyphen. The Sequence Number identifies a particular specimen material type that was collected as the "parent" material, e.g., 1000-0 (1st 8 mL Sodium Heparin CPT vacutainer); whereas, e.g., 4201-1 to 4201-5 (5 cryovials of cryopreserved PBMCs at $2x10^6$ cells/mL in RLT).

Table 2.1. Sample ID Assignments and Descriptions.

Sample ID	Description
GX1.247802-1000-0 -1100-0 -1200-0 -1300-0 -1400-0 -1500-0	Sodium Heparin CPT Vacutainers #1-6
GX1.247802-1600-0	Pooled Whole Blood in a 50 mL sterile conical tube
GX1.247802-2000-0 & -2100-0	Whole Blood CPT in RLT Aliquot #1 & #2
GX1.247802-2000-1 & -2100-1	RNA from Whole Blood CPT in RLT Aliquot #1 & #2
GX1.247802-2000-2 & -2100-2	QC Aliquot of RNA from Whole Blood CPT in RLT Aliquot #1 & #2
GX1.247802-2000-3 & -2100-3	Normalized RNA Aliquot from Whole Blood CPT in RLT Aliquot #1 &#2 for EA
GX1.247802-3000-0 & -3100-0	Fresh PBMCs in RLT Aliquot #1 & #2
GX1.247802-3000-1 & -3100-1	RNA from PBMCs in RLT Aliquot #1 & #2
GX1.247802-3000-2 & -3100-2	RNA QC Aliquot PBMCs in RLT Aliquot #1 & #2
GX1.247802-3000-3 & -3100-3	Normalized RNA Aliquot PBMCs in RLT Aliquot #1 & #2 for EA
GX1.247802-4000-0 -4100-0 -4200-0 -4300-0	1 mL aliquot of cryopreserved PBMCs at $5x10^6$ OR $10x10^6$ cells/mL, Using a CoolCell
GX1.247802-4001-0 to -4005-0 -4101-0 to -4105-0 -4201-0 to -4205-0 -4301-0 to -4305-0	Cryopreserved PBMCs at $2x10^6$ cells/mL in RLT, Using a CoolCell
GX1.247802-4001-1 to -4005-1 -4101-1 to -4105-1 -4201-1 to -4205-1 -4301-1 to -4305-1	RNA from Cryopreserved PBMCs at $2x10^6$ cells/mL in RLT, Using a CoolCell
GX1.247802-4001-2 to -4005-2 -4101-2 to -4105-2 -4201-2 to -4205-2 -4301-2 to -4305-2	QC Aliquot of RNA from Cryopreserved PBMCs at $2x10^6$ cells/mL in RLT, Using a CoolCell
GX1.247802-4001-3 to -4005-3 -4101-3 to -4105-3 -4201-3 to -4205-3 -4301-3 to -4305-3	Normalized Aliquot of RNA from Cryopreserved PBMCs at $2x10^6$ cells/mL in RLT for EA, Using a CoolCell
GX1.247802-5000-0 -5100-0 -5200-0 -5300-0	1 mL aliquot of cryopreserved PBMCs at $5x10^6$ OR $10x10^6$ cells/mL, Using a Rate-Controlled Freezer
GX1.247802-5001-0 to -5005-0 -5101-0 to -5105-0 -5201-0 to -5205-0 -5301-0 to -5305-0	Cryopreserved PBMCs at $2x10^6$ cells/mL in RLT, Using a Rate-Controlled Freezer
GX1.247802-5001-1 to -5005-1 -5101-1 to -5105-1 -5201-1 to -5205-1 -5301-1 to -5305-1	RNA from Cryopreserved PBMCs at $2x10^6$ cells/mL in RLT, Using a Rate-Controlled Freezer
GX1.247802-5001-2 to -5005-2 -5101-2 to -5105-2 -5201-2 to -5205-2 -5301-2 to -5305-2	QC Aliquot of RNA from Cryopreserved PBMCs at $2x10^6$ cells/mL in RLT, Using a Rate-Controlled Freezer
GX1.247802-5001-3 to -5005-3 -5101-3 to -5105-3 -5201-3 to -5205-3 -5301-3 to -5305-3	Normalized Aliquot of RNA from Cryopreserved PBMCs at $2x10^6$ cells/mL in RLT for EA, Using a Rate-Controlled Freezer

This table illustrates a typical set of Sample IDs and the Sample Description for all of the samples used in this research project obtained and processed from a single donor.

Preparing the Biosafety Cabinet

The blower in the Biosafety Cabinet was turned on for 15 minutes prior to use. After 15 minutes had elapsed, the inside and the front air grate of the Biosafety Cabinet were sprayed with Citrus II disinfectant. The Citrus II was wiped away using Kim wipes. Next the inside of the Biosafety Cabinet was sprayed with 70% Ethanol. The 70% Ethanol was wiped away using Kim wipes. The Biosafety Cabinet was then ready for use.

Setting the Acceleration & Deceleration Speeds on the Thermo Scientific Sorvall Legend XTR Centrifuge

The ACC/DEC key was pressed twice so that "Set deceleration" appeared on the display. The desired deceleration speed was selected by entering the appropriate number on the numeric pad. Number 0 is equivalent to off, number 1 is the slowest and number 9 is the fastest. The entry was confirmed by pressing ENTER. The ACC/DEC key was pressed once so that "Set acceleration" appeared on the display. The desired acceleration speed was selected by entering a 9 on the numeric pad. Number 0 is equivalent to off, number 1 is the slowest and number 9 is the fastest. The entry was confirmed by pressing ENTER.

Thawing Frozen Pre-Aliquoted Fetal Calf Serum (FCS) in the Water Bath

The water bath temperature was set at 37°C. The FCS aliquot was placed into the water bath for 5 minutes, within the 5 minute time interval the conical tube was inverted several times. After 5 minutes, the FCS aliquot was removed from the waterbath, the tube was sprayed with 70% ethanol and then the 70% ethanol was wiped off with a Kim wipe.

The FCS aliquot was inverted 10 times. If the FCS aliquot was not thawed, the above steps were repeated until it was thawed. Once the FCS was thawed, it was placed on wet ice or at 4°C until it was used. Once the FCS aliquot was thawed it was consumed that day, as it cannot be used at a later date and it cannot be re-frozen.

Estimating the Volume in a Vacutainer

Using a set of vacutainer tares containing measured water in 1 mL increments from 1 mL to 10 mLs, the volume of blood in each vacutainer was estimated.

Isolating PBMCs from Whole Blood in Sodium Heparin CPT vacutainers

The sodium heparin CPT vacutainers containing whole blood were stored in an upright position at room temperature until centrifugation. The sodium heparin CPT vacutainers were re-mixed by gently inverting the tubes 8 to 10 times before centrifugation.

Two aliquots of 300 µL of whole blood from each donor's vacutainer were taken and each aliquot was placed into a 15 mL conical tube per vacutainer type. The whole blood was centrifuged at 1400 x g for 5 minutes at 10°C. Using a 5 mL serological pipette, the supernatant was removed and the pellet was dislodged. The whole blood was washed in 5 mL of sterile PBS and centrifuged at 1400 x g for 5 minutes at 10°C. Using a 5 mL serological pipette, the supernatant was removed and the pellet was dispersed by tapping the tube against the technician's hand. Using a P-1000 pipette with a sterile plugged aerosol-free pipet tip, 600 µL of room temperature RLT lysis buffer were added

to each tube and then resuspended up pipetting up and down 20 times. The entire volume of each tube was placed into prelabeled cryovials and stored at –80°C.

The sodium heparin CPT vacutainers were centrifuged in a horizontal rotor (swing-out head) for 30 minutes at 1650 x g at room temperature with the acceleration speed set at 9 and the deceleration speed set at 2. After centrifugation, PBMCs and platelets were localized in a whitish layer just under the plasma layer and just above the CLEAR density gradient fluid layer. Being as delicate as possible, each blood tube was slowly inverted 5 times to homogenize the plasma and the PBMCs/platelets. Using a 5 mL serological pipette the homogenized plasma and PBMCs/platelets from each sodium heparin CPT vacutainer were aspirated and pooled into a pre-labeled 50mL conical tube. The sodium heparin CPT vacutainers were then properly discarded. The 50 mL conical tube was centrifuged for 15 minutes at 300 x g at room temperature with the acceleration speed set at 9 and the deceleration speed set at 6. Without disturbing the PBMC pellet, a 10 mL serological pipette was used to aspirate and discard as much plasma as possible. Using a 10 mL serological pipette, 10 mL of sterile RPMI-1640 were added to the 50 mL conical tube containing the PBMC pellet. Using the same 10 mL serological pipette, the PBMC pellet was resuspended by pipetting the RPMI-1640 up and down. Enough sterile RPMI-1640 was poured into the 50 mL conical tube to bring the total volume up to 50 mL. The 50 mL conical tube was centrifuged for 15 minutes at 300 x g at room temperature with the acceleration speed set at 9 and the deceleration speed set at 6. Using a 10 mL serological pipette, as much supernatant as possible was aspirated & discarded without disturbing the cell pellet. The PBMC pellet was resuspended by using a 10 mL serological pipette to add 10 mL of sterile RPMI-1640 to the 50 mL conical tube

containing the cell pellet. Using the same 10 mL serological pipette, the PBMC pellet was resuspended by pipetting the RPMI-1640 up and down. Enough sterile RPMI-1640 was poured into the 50 mL conical tube to bring the total volume up to 50 mL. The 50 mL conical tube was centrifuged for 10 minutes at 300 x g at room temperature with the acceleration speed set at 9 and the deceleration speed set at 6. Using a 10 mL serological pipette, as much supernatant as possible was aspirated & discarded without disturbing the cell pellet. Three 50 mL conical tubes containing 45 mL of sterile RPMI-1640 (90%) + 5 mL of FCS (10%) were created to use for subsequent wash & cell counting steps during the isolation. These 3 conical tubes were kept on wet ice until they were used. The PBMC cell pellet was resuspended in half of the 90% sterile RPMI-1640 + 10% FCS solution. Using a 5 mL serological pipette, the cell suspension was gently mixed by aspirating and expelling the liquid. The resuspension of the PBMC pellet was completed by adding the remaining half of the 90% sterile RPMI-1640 + 10% FCS solution. Using a 5 mL serological pipette, the PBMC suspension was gently mixed by aspirating and expelling the liquid. The PBMCs were then counted using a Vi-CELL. The total # of PBMCs in the cell suspension was calculated by using the following formula:

Total viable PBMCs / mL ($x10^6$) x Total mL of cell suspension

Two aliquots of fresh PBMCs at $2x10^6$ were taken and each aliquot was placed into a 15 mL conical tube. The fresh PBMCs were centrifuged at 1400 x g for 5 minutes at 10°C. Using a 5 mL serological pipette, the supernatant was removed and the pellet was dislodged. The fresh PBMCs were washed in 5 mL of sterile PBS and centrifuged at 1400 x g for 5 minutes at 10°C. Using a 5 mL serological pipette, the supernatant was removed and the pellet was dislodged. Using a P-1000 pipette with a sterile plugged

aerosol-free pipet tip, 600 µL of room temperature RLT lysis buffer were added to each tube and then resuspended by pipetting up and down 20 times. The entire volume of each tube was placed into prelabeled cryovials and stored at –80°C.

The remaining PBMC suspension was inverted 4-5 times, and using a 25 mL serological pipette it was approximately evenly split between two prelabeled 50 mL conical tubes, one tube was designated to use a Biocision CoolCell FTS30 Cell Freezing Container to freeze half of the remaining cryopreserved PBMCs while the other tube was designated to use a rate-controlled freezer to freeze the other half of the remaining cryopreserved PBMCs (see Cryopreservation of PBMCs section below).

Isolating PBMCs from Whole Blood in Sodium Heparin Vacutainers Using Ficoll-Density Gradients

Two vacutainers of whole blood containing sodium heparin were pooled into a sterile 50 mL conical tube. For example, if there were 2 vacutainers, the whole blood in both vacutainers was pooled into one 50 mL conical tube. If there were 4 vacutainers, the samples were pooled into two 50 mL conical tubes.

Two aliquots of 300 µL of whole blood were taken from each donor's vacutainer and each aliquot was placed into a 15 mL conical tube per vacutainer type. The whole blood was centrifuged at 1400 x g for 5 minutes at 10°C. Using a 5 mL serological pipette, the supernatant was removed and the pellet was dislodged. The whole blood was washed in 5 mL of sterile PBS and centrifuged at 1400 x g for 5 minutes at 10°C. Using a 5 mL serological pipette, the supernatant was removed and the pellet was dislodged. Using a P-1000 pipette with a sterile plugged aerosol-free pipet tip, 600 µL of room

temperature RLT lysis buffer were added to each tube and then resuspended by pipetting up and down 20 times. The entire volume of each tube was placed into prelabeled cryovials and stored at –80°C.

The 50 mL conical tubes containing the whole blood were centrifuged at 450 x g for 20 minutes with the acceleration set at 9 and the deceleration set at 9. After centrifugation, the plasma was separated from the cellular components, (e.g., RBCs, PBMCs and polymorphonuclear cells) and all of the cellular components were pelleted at the bottom of the tube. Without disturbing the PBMC/platelet layer, a 5 mL serological pipette was used to gently aspirate and discard the plasma. Enough sterile plain RPMI-1640 media was poured into each conical tube to bring the volume up to 35 mL. The pelleted cells were resuspended by gently & slowly rocking the tube 5-10 times. To another sterile 50 mL conical tube, 13 mL of Ficoll-Paque was added. NOTE: There should be one 50 mL conical tube filled with Ficoll-Paque per 50 mL conical tube filled with pelleted cells. The cell suspension was slowly and carefully poured over the Ficoll-Paque to prevent it from mixing with the diluted blood. A clear interface (the location where the Ficoll-Paque and the cell suspension met) was observed. This was repeated for each tube. The samples were centrifuged at 900 x g for 30 minutes with the acceleration set at 9 and the deceleration set at 2. The tubes were removed from the centrifuge, placed in a biosafety cabinet and their caps were removed. The clear media was aspirated-off the top and discarded using a sterile 10 mL serological pipette except for approximately 10 mLs of media that was directly above the PBMC/platelet layer. Using a 5 mL sterile serological pipette, the PBMC/platelet layer was carefully collected and placed into another 50 mL conical tube. NOTE: This was a 1:1 transfer meaning all of the

PBMC/platelet layers were NOT pooled together, each 50 mL conical tube containing a PBMC/platelet layer was transferred into its own 50 mL conical tube. Enough sterile RPMI-1640 media was added to each 50 mL conical tube containing the PBMC/platelet layer to bring the volume up to 50 mL. The PBMC/platelet suspension was then gently inverted 4-5 times. The PBMCs were pelleted by being centrifuged at 450 x g for 10 minutes with the acceleration set at 9 and the deceleration set at 2. Using a 50 mL serological pipette, the supernatant was aspirated and discarded. The PBMCs were resuspended in 10 mL of plain sterile RPMI-1640 media and the PBMC suspension was gently inverted 4-5 times. Using a 10 mL serological pipet, ALL of the washed PBMCs were pooled into a single 50 mL conical tube. The now empty 50 mL conical tubes, which had previously contained the washed PBMCs, were rinsed with 5 mL of plain sterile RPMI-1640 media. The rinsed RPMI-1640 media containing any residual PBMCs was transferred to the single 50 mL conical tube containing that pooled PBMC suspensions. Enough sterile plain RPMI-1640 media was poured into the single 50 mL conical tube to bring the volume up to 50 mL. The PBMC suspension was gently inverted 4-5 times. The PBMC suspensions were centrifuged at 450 x g for 10 minutes with the acceleration set at 9 and the deceleration set at 9. A 10 mL serological pipet was used to aspirate and discard the supernatant. Three 50 mL conical tubes containing 45 mL of sterile RPMI-1640 (90%) + 5mL of FCS (10%) were created to use for subsequent wash and cell counting steps during the isolation. The three 50 mL conical tubes were kept on wet ice until they were used. Each donor's PBMCs were separately resuspended in half of 90% sterile RPMI-1640 + 10% FCS. Using a 5 mL serological pipette, the cell suspension was gently mixed by aspirating and expelling the liquid 4 – 5 times. The

volume of each PBMC resuspension was brought up to 50 mL with the 90% sterile RPMI-1640 + 10% FCS solution. Using a 5mL serological pipette, the PBMC suspension was gently mixed by aspirating and expelling the liquid. The PBMCs were then counted using a Vi-CELL. The total # of PBMCs in the cell suspension was calculated by using the following formula:

Total viable cells / mL ($x10^6$) x Total mL of cell suspension

Two aliquots of fresh PBMCs at $2x10^6$ were taken and each aliquot was placed into a 15 mL conical tube. The fresh PBMCs were centrifuged at 1400 x g for 5 minutes at 10°C. Using a 5 mL serological pipette, the supernatant was removed and the pellet was dispersed by gently tapping the tube in the palm of the technician's hand. The PBMCs were washed in 5 mL of sterile PBS and centrifuged at 1400 x g for 5 minutes at 10°C. Using a 5 mL serological pipette, the supernatant was removed and the pellet was dispersed. Using a P-1000 pipette with a sterile plugged aerosol-free pipet tip, 600 µL of room temperature RLT lysis buffer were added to each tube and then resuspended by pipetting up and down 20 times. The entire volume of each tube was placed into prelabeled cryovials and stored at –80°C.

The remaining PBMC suspension was inverted 4-5 times, and using a 25 mL serological pipette it was approximately evenly split between two prelabeled 50 mL conical tubes, one tube was designated to use a Biocision CoolCell FTS30 Cell Freezing Container to freeze the cryopreserved PBMCs while the other tube was designated to use a rate-controlled freezer to freeze the cryopreserved PBMCs (see Cryopreservation of PBMCs section below).

Cryopreservation of PBMCs

All of the 50 mL conical tubes that had remaining PBMCs isolated from the sodium heparin CPT vacutainers and the sodium heparin vacutainers were centrifuged at 300 x g for 10 minutes at room temperature with the acceleration speed set at 9 and the deceleration speed set at 6. Without disturbing the PBMC pellet, a 10 mL serological pipette was used to aspirate and discard as much supernatant as possible. From this point on the remainder of protocol was performed on WET ICE. The conical tubes containing the PBMC pellets were placed on wet ice while the cryococktail solution was prepared. The following 3 calculations were used to determine the amount of cryococktail solution (72.5% RPMI-1640 + 20% FCS + 7.5% DMSO) required to achieve a desired cell concentration of EITHER $5x10^6$ OR $10x10^6$ cells/mL depending on the cell count:

1). Total amount in mL of cryococktail solution needed for $10x10^6$ cells/mL =

Total # of cells in cell suspension ÷ $5x10^6$ cells/mL or $10x10^6$ cells/mL

2). Total amount in mL of RPMI-1640 (72.5%) in cryococktail =

Total amount in mL of cryococktail solution needed for $5x10^6$ or $10x10^6$ cells/mL x 0.725

3). Total amount in mL of FCS (20%) in cryococktail =

Total amount in mL of cryococktail solution needed for $5x10^6$ or $10x10^6$ cells/mL x 0.20

4). Total amount in mL of DMSO (7.5%) in cryococktail =

Total amount in mL of cryococktail solution needed for $5x10^6$ or $10x10^6$ cells/mL x 0.075

The rate-controlled freezer was turned on to ensure it was running for about 30 minutes before it was used. This allowed the probe temperature in the rate-controlled freezer to reach +4^{o}C before the cryovials with the PBMCs that are to be cryopreserved were placed inside.

For each conical tube containing PBMCs to be frozen using a CoolCell, the cryococktail required to achieve a desired cell concentration of either 5x10^{6} cells/mL or 10x10^{6} cells/mL was made in a 50 mL conical tube. A 5 mL serological pipette was used to resuspend the PBMC pellet in half of the desired volume of cryococktail solution to achieve the desired cell concentration of either 5x10^{6} cells/mL or 10x10^{6} cells/mL. Using the same 5 mL serological pipette, the PBMC suspension was mixed by aspirating and expelling the liquid 5 times. For complete resuspension, a 5 mL serological pipette was used to add in the remaining half of the desired volume of cryococktail solution, which achieved a cell concentration of either 5x10^{6} cells/mL or 10x10^{6} cells/mL.

From the cryopreserved PBMCs at either 5x10^{6} cells/mL or 10x10^{6} cells/mL, 1 mL of cell suspension was aliquotted into as many prelabeled sterile cryovials as possible. The vials were quickly transferred from wet ice into a Biocision CoolCell FTS30 Cell Freezing Container. Any empty slot (not filled by a PBMC-containing sample) within the Biocision CoolCell FTS30 Cell Freezing Container were filled with 2 mL CoolCell Filler Vials to ensure a controlled freezing rate of -1°C/minute. The Biocision CoolCell FTS30 Cell Freezing Container containing samples was then placed into a -70^{o}C freezer overnight. The next day, the frozen cryovials containing PBMCs residing in the Biocision CoolCell FTS30 Cell Freezing Container were transferred to a -150^{o}C freezer.

For each conical tube containing cells designated to be frozen using a rate-controlled freezer, the cryococktail required to achieve a desired cell concentration of either $5x10^6$ cells/mL or $10x10^6$ cells/mL was made in a 50 mL conical tube. A 5 mL serological pipette was used to resuspend the cell pellet in half of the desired volume of cryococktail solution to achieve the desired cell concentration of either $5x10^6$ cells/mL or $10x10^6$ cells/mL. Using the same 5 mL serological pipette, the cell suspension was mixed by aspirating and expelling the liquid 5 times. For complete resuspension, a 5 mL serological pipette was used to add in the remaining half of the desired volume of cryococktail solution, which achieved a cell concentration of either $5x10^6$ cells/mL or $10x10^6$ cells/mL.

From the cryopreserved PBMCs at either $5x10^6$ cells/mL or $10x10^6$ cells/mL, 1 mL of cell suspension was aliquotted into as many prelabeled sterile cryovials as possible. The vials were quickly transferred from wet ice to a rate-controlled freezer.

Immediately after loading the PBMC-containing cryovials in the rate-controlled freezer, the operator selected the program titled "Biogen Biobank (BBK) Run" and then clicked on "Run."

BBK Run:

- Hold at $+4^oC$
- Ramp 1^oC/min until sample = -4^oC
- Ramp 25^oC/min until chamber = -40^oC
- Ramp 15^oC/min until chamber = -12^oC
- Ramp 1^oC/min until chamber = -40^oC
- Ramp 10^oC/min until chamber = -150^oC

- Hold at -150°C until samples are retrieved to move to long-term storage

At the completion of the rate-controlled freezing cycle, the cryovials were removed from the rate-controlled freezer and quickly transported in a Styrofoam container that had liquid nitrogen-soaked absorbent pads to maintain a ≤-150°C temperature until the PBMC samples were stored long-term in a -150°C freezer.

Thawing Cryopreserved PBMCs

Thawing cryopreserved PBMCs was done using the following rapid thaw procedure: The water temperature in a water bath was at 37ºC before beginning this process. Using a serological pipette, 10 mL of CHILLED RPMI-1640 media plus 10% FCS were aliquoted into a 15 mL conical tube. When thawing more than one vial, each vial was transferred to a separate 15 mL conical tube. A maximum of 4 vials were taken through this procedure at one time. Immediately after removing the vials from the freezer, they were placed in a container of dry ice so all of the vials were covered in dry ice for transportation to the laboratory where the 37ºC water bath was located. The frozen vials were placed in the 37ºC water bath. The water was not allowed to reach the collar of the vial to prevent contamination. Just before the last few ice crystals melted in the vial, the vial was removed from the water bath. A kimwipe was sprayed with 70% ethanol & the vial was wiped off with it before being opened. A 2 mL serological pipette was used to draw up the cells and media from the cryovial and then to transfer the cells and media into the 15 mL conical tube containing the RPMI-1640 media + 10% FCS. Using the same serological pipette, 1.5 mL of the cell suspension were aspirated from the top of the 15 mL conical tube, used to rinse the vial and transferred back into the 15 mL conical tube. The

cells were gently pipetted up and down twice to mix. The cells were pelleted by being centrifuged at 500 x g for 10 minutes at 10°C with the acceleration set at max and the deceleration set at max. Using a 5 mL serological pipette, the supernatant was removed. The cells were gently resuspended in 10 mL of CHILLED RPMI-1640 media + 10% FCS. The PBMCs were pelleted by being centrifuged at 500 x g for 10 minutes at 10°C with the acceleration set at 9 and the deceleration set at 9. Using a 5 mL serological pipette, the supernatant was removed. The PBMCs were gently resuspended in 5 mL of CHILLED RPMI 1640 media + 10% FCS. The PBMCs where then counted on a Vi-CELL.

Up to 5 aliquots of cells at $2x10^6$ were pipetted into their own 15 mL conical tube. The conical tubes were then centrifuged at 1400 x g for 5 minutes at 10°C. Using a 5 mL serological pipette, the supernatant was removed and the pellet was dispersed by gently tapping on the side of the conical tube with a finger. The cells were washed in 5 mL of sterile PBS and centrifuged at 1400 x g for 5 minutes at 10°C. Using a 5 mL serological pipette, the supernatant was removed and the pellet was dispersed by gently tapping on the side of the conical tube using a finger. Using a P-1000 pipette with a sterile plugged aerosol-free pipet tip, 600 µL of CHILLED RLT lysis buffer were added to each tube and then resuspended up pipetting up and down 20 times. The entire volume of each tube was placed into separate prelabeled cryovials and stored at –80°C.

Table 2.2. Summary of Whole Blood & PBMC Aliquots Taken During Whole Blood Processing.

Aliquots Taken at the Indicated Processing Steps	PBMC Isolation from Whole Blood Collected in Sodium Heparin Vacutainers	PBMC Isolation from Whole Blood Collected in Sodium Heparin CPT Vacutainers
Aliquot # 1 (x2)	Pool Whole Blood Containing the Same Preservative into 50 mL conical tubes Collect Whole Blood from Pool & Add to RLT Lysis Buffer	Collect Whole Blood from Sodium Heparin CPT Vacutainers & Add to RLT Lysis Buffer
	Centrifuge 450 x g for 20 minutes at RT Discard supernatant Dilute Pelleted Cells with 35 mLs of RPMI-1640 media & Gently Rock 5-10 times	Centrifuge Sodium Heparin CPT Vacutainers at 1,650 x g for 30 minutes (No Brake)
	Pour Diluted Cells over 13 mL Ficoll Centrifuge at 900 x g for 30 minutes at RT	
	Discard Supernatant Transfer PBMC layer to 50 mL conical tube	Gently Mix PBMCs & Plasma Collect & Pool into a 50 mL conical tube Centrifuge PBMCs & Plasma at 300 x g 15 minutes at RT
		Aspirate & Discard Plasma Add 10mLs RPMI-1640 media to Pelleted PBMCs & Resuspend
	QS RPMI-1640 media to 50 mLs (Wash #1) Centrifuge PBMCs at 450 x g for 10 minutes at RT Discard Supernatant Resuspend PBMCs in 10 mLs RPMI-1640 media	QS RPMI-1640 media to 50 mLs (Wash #1) Centrifuge PBMCs at 300 x g for 15 minutes at RT Discard Supernatant Resuspend PBMCs in 10 mLs RPMI-1640 media
	Pool ALL Washed Cells into ONE 50 mL Conical Tube Rinse the Now Empty Conical Tubes with 5 mL of RPMI-1640 & Transfer the RPMI-1640 into the 50 mL Conical Tube Containing PBMCs	
	QS RPMI-1640 media to 50 mLs (Wash #2) Centrifuge PBMCs at 450 x g for 10 minutes at RT Discard Supernatant Resuspend PBMCs in 90% RPMI-1640 & 10% FCS	QS RPMI-1640 media to 50 mLs (Wash #2) Centrifuge PBMCs at 300 x g for 10 minutes at RT Discard Supernatant Resuspend PBMCs in 90% RPMI-1640 & 10% FCS
Aliquot # 2 (x2)	Count PBMCs & Add $2x10^6$ PBMCs to RLT Lysis Buffer	Count PBMCs & Add $2x10^6$ PBMCs to RLT Lysis Buffer
	Centrifuge PBMCs at 300 x g for 10 minutes at RT Discard Supernatant Place PBMCs on Wet Ice Add appropriate amount of Cryococktail for $5x10^6$or $10x10^6$ PBMCs Freeze	Centrifuge PBMCs at 300 x g for 10 minutes at RT Discard Supernatant Place PBMCs on Wet Ice Add appropriate amount of Cryococktail for $5x10^6$or $10x10^6$ PBMCs Freeze
Aliquot #3 (x2)	After 1 month, Thaw Samples Count PBMCs Add $2x10^6$ PBMCs to RLT Lysis Buffer	After 1 month, Thaw Samples Count PBMCs Add $2x10^6$ PBMCs to RLT Lysis Buffer

This table compares the two PBMC isolation methods used and shows at what step the whole blood & PBMC aliquots were collected.

Culturing and Harvesting Mouse Fibroblast Cells

Flasks of adherent mouse M2-10B4 primary stromal fibroblast cells were grown in RPMI-1640 + 10% FCS complete medium to ~60% confluency as observed by light microscopy. The complete medium was aspirated from the cell monolayer by pipet and discarded. The cell monolayer was briefly rinsed once with 7 mL PBS and then the PBS was discarded. Five mL of trypsin-EDTA was added to the cell monolayer and the cells were allowed to incubate in a 37^0C/5% CO_2 incubator for 3 minutes. Following incubation, the flask was tapped smartly on the hard edge of the counter to disrupt the trypsinized cells. Cell detachment was verified by light microscopy. The trypsinized cells were resuspended in 30 mL of complete medium to stop the reaction, and the whole volume was transferred to a 50 mL conical centrifuge tube and pelleted by centrifugation at 300 x g for 10 minutes at room temperature. Following centrifugation, the supernatant was resuspended in 10 mL of PBS. Cells were counted manually with a 1:2 dilution of trypan blue on a hemocytometer. When desired, aliquots of either $0.5x10^6$ or $1x10^6$ cells were made and they were placed into a 15 mL conical tube with an additional 10 mL of sterile PBS.

Vi-CELL XR Beckman Coulter Cell Counter Operating Instructions

The Vi-CELL XR 2.04.004 software program opens by double clicking on the icon that is located on the home screen on the computer connected to the Vi-CELL. In order for the program to start, the user logs in with their user name and password. The Vi-CELL initializes itself, which takes a few minutes. Once the status of the Vi-CELL is "Idle," the Vi-CELL Concentration Control needs to be retrieved from the $+4^0$C refrigerator. An

aliquot of 1.5 mL of the Vi-CELL Concentration Control is placed into a Vi-CELL sample cup. The sample is placed onto any position on the Vi-CELL. The Autosampler queue icon is selected by double clicking on it.

A new screen appears and the "Log In Sample" icon is selected by double clicking on it. In the new "Log In Sample" window that appeared, the following information for the Concentration Control is entered:

- Position-Corresponding position was selected
- Sample ID-Conc. Control_Users Initials_Date_Lot # of Conc. Control
- Cell Type-Conc. Control
- Dilution Factor-1
- Date-Automatically filled in for user
- Time- Automatically filled in for user

The "OK" icon is selected, and then the "Start" queue icon is selected. When the Concentration Control sample is finished running, the Beckman Coulter Run Results screen appeared. The Total cells/mL is looked at and confirmed that it was within +/- 10% of the concentration (Total count/µL) provided by Beckman Coulter for that specific bottle of Concentration Control. If the Concentration Control was not within +/- 10% of the concentration (Total count/µL) provided by Beckman Coulter for that specific bottle of Concentration Control, the Concentration Control was rerun.

To process samples; 1mL of a PBMC suspension was added into a Vi-CELL sample cup, and the sample was placed onto any position on the Vi-CELL. The Autosampler queue icon was selected by double clicking on it.

In the new screen that appeared, the "Log In Sample" icon was double clicked on. In the new "Log In Sample" window that appeared, the following information for the PBMC suspension was filled out:

- Position-Corresponding position was selected
- Sample ID-Vacutainer Type_Date of Extraction_Users Initials_Date Run on Vi-CELL
- Cell Type-PBMC #1
- Dilution Factor-1
- Date-Automatically filled in for user
- Time- Automatically filled in for user

Once the sample was finished running, the Beckman Coulter Run Results screen appeared. Using the Total cells/mL, the total number of cells in the PBMC suspension was calculated using the following equation:

Total # of cells in cell suspension = Total # cells/mL x10^6 x Total mLs of cell suspension

Manually Counting Cells Using a Hemocytometer

In a 2.0 mL cryovial, 100 µL of cell suspension & 900 µL of Trypan Blue were added & then it was gently mixed by finger flicking. Using a P-20 pipette with a sterile plugged aerosol-free pipet tip, 10 µL of the cell suspension & trypan blue mixture were added into the hemocytometer. The microscope was turned on high and it was placed on the lowest power objective (e.g. PhL). The Field diaphragm control lever was closed and the hemocytometer was placed on the microscope. All of the viable PBMCs (non-viable cells stain blue, viable cells appear bright white with a halo around them) in each of the

four quadrants were manually counted. The total cell count for all four quadrants is approximately 100 cells. If more than 100 cells were counted, the cell suspension was further diluted and the cells were recounted. The total # of cells in the cell suspension was calculated using the following equation:

Total # of cells in cell suspension = (Total # of cells in ALL 4 quadrants/# of quadrants) x constant of 10,000 cells/mL x dilution factor of trypan blue x total volume in mL of cell suspension

RNA Extraction Protocols

Four RNA extraction protocols were tested while troubleshooting the DNA contamination issue. The kit initially used to isolate RNA from the original test samples did not contain a DNase treatment, which was Qiagen's RNeasy Mini Kit without a DNase treatment. This procedure was compared to Qiagen's AllPrep DNA/RNA Mini Kit, RNeasy Mini Kit with and without a DNase treatment and RNeasy Plus Mini Kit.

Isolating RNA from Whole Blood, PBMCs & Cryopreserved PBMCs Stored in RLT Lysis Buffer Using Qiagen's RNeasy Mini Kit

The Purification of Total RNA from Animal Cells using Spin Technology protocol from Qiagen's RNeasy Mini Kit was used to isolate RNA from the whole blood, fresh PBMCs & cryopreserved PBMCs stored in RLT lysis buffer. If samples were frozen they were left on the bench to thaw for 1 hour. If samples were being DNase treated, the DNase stock solution was either made or if it was previously made the DNase was removed from the +4°C refrigerator. Using a P-1000 pipette, the DNase stock

solution was prepared by dissolving the lyophilized DNase I (1500 Kunitz units) in 550 µL of RNase-free water. The DNase stock solution was then mixed gently by inverting the vial. The DNase stock solution was stored in the +4°C refrigerator for up to 1 month. Using an L-1200 XLS multi-channel pipette, each of the lysed samples was transferred into a QIAshredder spin column for homogenization purposes. The QIAshredder spin columns were then centrifuged for 2 minutes at maximum speed (21,130 x g). Using an L-1200 XLS multi-channel pipette, 600 µL of 70% ethanol was added to each homogenized lysate and using the same L-1200 XLS multi-channel pipette, the samples were aspirated and dispensed 5 times to mix. Using an L-1200 XLS multi-channel pipette, 700 µL of lysate was transferred into an RNeasy spin column and centrifuged by holding the short button down on the centrifuge for 20 seconds to ensure that the samples were centrifuged for 15 seconds at greater than or equal to 8000 x g. After centrifugation, the flow-through was discarded. If the sample volume exceeded 700 µl, successive aliquots in the same RNeasy spin column were centrifuged and the flow-through was discarded after each centrifugation.

If samples were DNase treated it was at this point and the following instructions were followed, if samples were not being DNase treated, the next paragraph of this protocol was proceeded to. Otherwise, using an L-1200 XLS multi-channel pipette, 350 µL of Buffer RW1 were added to each RNeasy spin column. The lid was gently closed and the RNeasy spin columns were centrifuged for 15 seconds at greater than or equal to 8000 x g to wash the spin column membrane. After centrifugation, the flow-through was discarded. Using a P-200 & a P-1000 pipette, a mastermix was created in which 10 µL of DNase stock solution and 70 µL Buffer RDD were added into a 1.5mL fliptop tube per

number of RNeasy spin columns. The DNase mixture was gently mixed by inverting the 1.5mL fliptop tube and it was centrifuged briefly to collect residual liquid from the sides of the tube. Using a P-200 pipette, 80 µL of the DNase mixture were added directly to each RNeasy spin column membrane. The RNeasy spin columns were left on the benchtop to incubate for 15 minutes. The lid was gently closed and RNeasy spin columns were centrifuged for 15 seconds at greater than or equal to 8000 x g to wash the spin column membrane. After centrifugation, the flow-through was discarded. Using an L-1200 XLS multi-channel pipette, an additional 350 µL of Buffer RW1 were added to each RNeasy spin column. The lid was gently closed and the RNeasy spin columns were centrifuged for 15 seconds at greater than or equal to 8000 x g as instructed by Qiagen to wash the spin column membrane. This protocol was continued with the first Buffer RPE wash step below.

Using an L-1200 XLS multi-channel pipette, 700 µL of Buffer RW1 were added to the RNeasy spin columns. The RNeasy spin columns were centrifuged by holding the short button down on the centrifuge for 20 seconds to ensure that the samples were centrifuged for 15 seconds at greater than or equal to 8000 x g. After centrifugation, the flow-through was discarded.

Using an L-1200 XLS multi-channel pipette, 500 µL of Buffer RPE was added to the RNeasy spin columns. The RNeasy spin columns were centrifuged by holding the short button down on the centrifuge for 20 seconds to ensure that the samples were centrifuged for 15 seconds at greater than or equal to 8000 x g. After centrifugation, the flow-through was discarded. Using an L-1200 XLS multi-channel pipette, an additional 500 µL of Buffer RPE was added to the RNeasy spin columns. The RNeasy spin columns

were centrifuged for 2 minutes at maximum speed (21,130 x g). After centrifugation the RNeasy spin columns were placed into a new 2 mL collection tube and the old collection tube containing the flow-through was discarded. The RNeasy spin columns were centrifuged for 1 minute at maximum speed (21,130 x g). The RNeasy spin columns were then placed into new 1.5 mL collection tubes and 30 µL of RNase-free water were added directly to the spin column membrane. The RNeasy spin columns were centrifuged for 1 minute at maximum speed (21,130 x g). After centrifugation, an additional 30 µL of RNase-free water were added directly to the spin column membrane. The RNeasy spin columns were centrifuged for 1 minute at maximum speed (21,130 x g). After centrifugation, the samples were placed on wet ice and a manual volume check was performed on each sample. Lastly, a 5 µL aliquot was taken from each sample to use to perform quantitation at a later date. The stock samples & the 5 µL aliquots were stored at -80^{o}C.

It is important to note that the 1st set of RNA samples were extracted using the RNeasy Mini Kit without a DNase treatment. Upon concluding the DNA contamination evaluation, it was determined that the replacement RNA samples would be extracted using the RNeasy Plus Mini Kit (see protocol below) since it successfully removes DNA with a gDNA eliminator column.

Isolating RNA from PBMCs & Cryopreserved PBMCs Stored in RLT Lysis Buffer Using Qiagen's AllPrep DNA/RNA Mini Kit

The Simultaneous Purification of Genomic DNA and Total RNA from Animal Cells protocol from Qiagen's AllPrep DNA/RNA Mini Kit was used to isolate RNA from

the whole blood, PBMCs & cryopreserved PBMCs stored in RLT lysis buffer. Using an L-1200 XLS multi-channel pipette, the lysate was pipetted directly into a QIAshredder spin column placed inside of a 2 mL collection tube, and the spin columns were centrifuged for 2 minutes at maximum speed (21,130 x g). Using an L-1200 XLS multi-channel pipette, 1 volume of 70% ethanol (600 µL) was added to the flow-through. The lysate/ethanol mixture was pipetted up and down using the same L-1200 XLS multi-channel pipette. An L-1200 XLS multi-channel pipette was used to pipette 700 µL of the lysate/ethanol mixture into the RNeasy spin columns placed inside of 2 mL collection tubes. The lids were closed and the RNeasy spin columns were centrifuged by holding the short button down on the centrifuge for 20 seconds to ensure that the samples were centrifuged for 15 seconds at greater than or equal to 8000 x g and then the flow-through was discarded. If the sample volume exceeded 700 µl, successive aliquots in the same RNeasy spin column were centrifuged and the flow-through was discarded after each centrifugation. Using an L-1200 XLS multi-channel pipette, 700 µL Buffer RW1 were added to each RNeasy spin column. The lids were closed and the RNeasy spin columns were centrifuged by holding the short button down on the centrifuge for 20 seconds to ensure that the samples were centrifuged for 15 seconds at greater than or equal to 8000 x g and then the flow-through was discarded. Using an L-1200 XLS multi-channel pipette, 500 µL Buffer RPE were added to each RNeasy spin column. The lids were closed and the RNeasy spin columns were centrifuged by holding the short button down on the centrifuge for 20 seconds to ensure that the samples were centrifuged for 15 seconds at greater than or equal to 8000 x g and then the flow-through was discarded. An additional 500 µL Buffer RPE were added to each RNeasy spin column using an L-1200 XLS

multi-channel pipette. The lids were closed and the RNeasy spin columns were centrifuged for 2 minutes at maximum speed (21,130 x g). The RNeasy spin columns were placed inside of new 2 mL collection tubes and the old collection tubes containing the flow-through were discarded. The RNeasy spin columns were centrifuged for 1 minute at maximum speed (21,130 x g). The RNeasy spin columns were placed inside of new 1.5 mL collection tubes and using a P-200 pipette, 30–50 µL of RNase-free water were added directly to the spin column membrane. The lids were closed the RNeasy spin columns were centrifuged for 1 minute at maximum speed (21,130 x g) to elute the RNA. Using a P-200 pipette, an additional 30–50 µL of RNase-free water were added to the RNeasy spin columns. The lids were closed the RNeasy spin columns were centrifuged for 1 minute at maximum speed (21,130 x g) to elute the RNA. After centrifugation, the samples were placed on wet ice and a manual volume check was performed on each sample. Lastly, a 5 µL aliquot was taken from each sample to use to perform quantitation at a later date. The stock samples & the 5 µL aliquots were stored at -80^{o}C.

Isolating RNA from PBMCs & Cryopreserved PBMCs Stored in RLT Lysis Buffer Using Qiagen's RNeasy Plus Mini Kit

The Purification of Total RNA from Animal Cells protocol from Qiagen's RNeasy Plus Mini Kit was used to isolate RNA from the PBMCs & cryopreserved PBMCs stored in RLT lysis buffer. Using an L-1200 XLS multi-channel pipette, the lysate was pipetted directly into a QIAshredder spin column placed inside of a 2 mL collection tube, and the spin columns were centrifuged for 2 minutes at maximum speed (21,130 x g). The homogenized lysate was then transferred to a gDNA Eliminator spin

column placed in a 2 mL collection tube. The gDNA Eliminator Columns were then centrifuged for 30 seconds at maximum speed (21,130 x g). The columns were discarded and the flow-through was saved. Using an L-1200 XLS multi-channel pipette, 1 volume (600 µL) of 70% ethanol was added to the flow-through, and the lysate mixture was pipetted up and down several times to mix it. Up to 700 µL of the sample were transferred using an L-1200 XLS multi-channel pipette into an RNeasy spin column placed in a 2 mL collection tube. The lids were closed and the RNeasy spin columns were centrifuged by holding the short button down on the centrifuge for 20 seconds to ensure that the samples were centrifuged for 15 seconds at greater than or equal to 8000 x g and then the flow-through was discarded. If the sample volume exceeded 700 µl, successive aliquots in the same RNeasy spin column were centrifuged and the flow-through was discarded after each centrifugation. Using an L-1200 XLS multi-channel pipette, 700 µL Buffer RW1 were added to the RNeasy spin column. The lids were closed and the RNeasy spin columns were centrifuged by holding the short button down on the centrifuge for 20 seconds to ensure that the samples were centrifuged for 15 seconds at greater than or equal to 8000 x g and then the flow-through was discarded. Using an L-1200 XLS multi-channel pipette, 500 µL Buffer RPE were added to the RNeasy spin column. The lids were closed and the RNeasy spin columns were centrifuged by holding the short button down on the centrifuge for 20 seconds to ensure that the samples were centrifuged for 15 seconds at greater than or equal to 8000 x g and then the flow-through was discarded. Using an L-1200 XLS multi-channel pipette, an additional 500 µL Buffer RPE were added to the RNeasy spin column. The lids were closed and the RNeasy spin columns were centrifuged for 2 minutes at maximum speed (21,130 x g). The RNeasy

spin columns were placed inside a new 2 mL collection tube; the old collection tube containing the flow-through was discarded. The RNeasy spin columns were centrifuged for 1 minute at maximum speed (21,130 x g). The RNeasy spin columns were placed inside of new 1.5 mL collection tube and using a P-200 pipette, 30–50 µL of RNase-free water were added directly to the spin column membrane. The lids were closed the RNeasy spin columns were centrifuged for 1 minute at maximum speed (21,130 x g) to elute the RNA. Using a P-200 pipette, an additional 30–50 µL of RNase-free water were added to the RNeasy spin columns. The lids were closed and the RNeasy spin columns were centrifuged for 1 minute at maximum speed (21,130 x g) to elute the RNA. After centrifugation, the samples were placed on wet ice and a manual volume check was performed on each sample. Lastly, a 5 µL aliquot was taken from each sample to use to perform quantitation at a later date. The stock samples & the 5 µL aliquots were stored at -80^{o}C.

Post-RNA Extraction DNase Treatment

A TURBO DNA-free Kit was used to remove DNA from the RNA after the extraction process. A 0.1 volume of 10x TURBO DNase Buffer and 1 µL TURBO DNase were added to each tube of RNA. The tubes were mixed gently by finger flicking each tube 2-3 times. The samples were then incubated at 37°C for 30 minutes. A 0.1 volume of the resuspended DNase Inactivation Reagent was added to each sample and then gently mixed by finger flicking each tube 2-3 times. The samples were then incubated for 5 minutes at room temperature, during the incubation period the samples were mixed occasionally by finger flicking each tube 2–3 times to disperse the DNase

Inactivation Reagent. The samples were then centrifuged at 10,000 × g for 1.5 minutes then the RNA was transferred to a new pre-labeled tube via a pipette for storage at -80^{o}C.

Running DropPlate 96 S Plates on the Trinean DropSense 96 to Obtain a Concentration

Samples were placed on wet ice and allowed to thaw for 30 minutes. The appropriate number of DropPlate 96 S Plates were removed from their packaging. The following wells were used as blanks on plates; A1, A6, H8, H12, therefore 2 µL of RNase-free water were pipetted into those wells. Next 2 µL of sample were pipetted into an empty well; this step was repeated for all samples. The DropPlate 96 S Plates were run on the Trinean DropSense 96 using the appropriate program for unlabeled RNA samples.

Running RNA Nano Chips on Agilent Bioanalyzer to Obtain a RIN Score

Unopened RNA ladder was stored at -20°C, prepared ladder aliquots were stored at -70°C & all other reagents & reagent mixes were kept at 4°C when not in use to avoid poor results caused by reagent decomposition. The next few paragraphs detail how to set up the chip priming station, prepare the reagents needed to run the chips, how to load and run chips on the Bioanalyzer.

Replacing the syringe. The old syringe was unscrewed from the lid of the chip priming station. The old syringe was released from the clip and it was discarded. A new sterile syringe was removed from its packaging and it was inserted into the clip. Next the syringe was slid into the hole of the luer lock adapter and tightly screwed onto the chip priming station.

Adjusting the base plate. The latch on the chip priming station was pulled in order to open the chip priming station. Using a screwdriver, the screw located on the underside of the base plate was opened. The base plate was lifted, reinserted into position C and the screw was retightened.

Adjusting the syringe clip. The lever of the clip was released and slid up to the top position.

Preparing the RNA ladder. The ladder was removed from the -20°C freezer and it was placed on ice to thaw. Once thawed, it was spun down quickly and placed into an RNase-free vial. It was then heat denatured by being placed on a thermocycler set to 70°C for 2 minutes. Once denatured, the ladder was immediately placed on wet ice. Using a P-10 pipette, 5 µL aliquots were placed into 0.5 mL RNase-free vials. The aliquots of ladder not being used immediately were frozen at -70°C. The ladder being used immediately was placed on wet ice.

Preparing the gel. The gel was allowed to equilibrate to room temperature for 30 minutes. Using a P-1000 pipette, 550 µL of RNA gel matrix were pipetted into a spin column. The spin column was then centrifuged at 1500 x g for 10 minutes at room temperature. Using a P-200 pipette, 60 µL of the gel were aliquoted into 0.5 mL RNase-free microcentrifuge tubes. The filtered gel not being used immediately was stored at 4°C and consumed within 4 weeks. The filtered gel being used immediately was kept at room temperature.

Preparing the gel-dye mix. The RNA dye concentrate was allowed to equilibrate to room temperature for 30 minutes. The RNA dye concentrate was vortexed for 10 seconds and spun down quickly. Next 1 µL of RNA dye concentrate was added into the 65 µL filtered

gel aliquot. The solution was vortexed well. Then it was centrifuged at 13,000 x g for 10 minutes at room temperature. The prepared gel-dye mixture was used within 1 day.

Loading the gel-dye mix. A new RNA chip was placed on the chip priming station. Using a P-10 pipette, 9 µL of gel-dye mix were placed in the well-marked with a black circle containing a white letter G in it. The plunger was positioned at 1 mL and then the chip priming station was closed. The plunger was pushed down until it was held by the clip. After exactly 30 seconds the clip was released. After 5 seconds, the plunger was slowly pulled back to the 1 mL position. The chip priming station was opened and 9 µL of gel-dye mix were placed in the wells marked with a black letter G.

Loading the Marker. Using a P-10 pipette, 5 µL of RNA marker were pipetted into all 12 sample wells as well as the ladder well (this well was marked with a picture of a ladder).

Loading the Ladder & Samples. Using a P-10 pipette, 1 µL of prepared ladder was pipetted into the ladder well (this well was marked with a picture of a ladder). Up to 12 RNA samples were thawed on wet ice and using a P-10 pipette, 1 µL of each sample was aliquoted into 1 of 12 sample wells. Using a P-10 pipette, 1 µL of RNA marker was placed in any unused sample wells. The chip was then placed horizontally in the IKA vortexer and it was vortexed at 2400 rpm for 1 minute. The chip was then run in the Agilent 2100 Bioanalyzer instrument within 5 minutes of being vortexed using the Eukaryote Total RNA Nano Series II assay.

Sample Labeling, Hybridization and Scanning

Sample labeling and hybridization were carried out using the NuGEN Ovation Biotin systems as described in the NuGEN Ovation Biotin system for 96 well plates

(version 5). Washing, staining and scanning of the hybridized arrays was completed as described in the Eukaryotic Target Preparation protocol in the Affymetrix expression analysis technical manual (702064 rev 2) for GeneChip cartridge arrays using the GeneChip Array Station. In summary, 20 ng of PaxGene purified total RNA was annealed with 2 µL "first strand primer mix" (A1) at 65°C for 5 minutes and then it was chilled on ice. The annealed template was incubated at 48°C for 60 minutes with 6 µL of first strand master mix (A2 and A3) and then it was chilled to 4°C. To the completed first strand reaction, 10 µL of second strand master mix (B1 and B2) were added and the reaction was incubated at 37°C for 30 minutes, 75°C for 15 minutes, and then it was cooled to 4°C. To amplify the cDNA, 120 µL of SPIA master mix were added to the 20 µL of completed second strand reaction and the reaction was incubated at 48°C for 60 minutes. The amplified cDNA was purified using the 96 well dye terminator removal kit according to the manufacturer's recommendations. To fragment the purified and amplified cDNA, 5 µL of fragmentation buffer (F1) and enzyme mix (F2) were added to 25 µL of the amplified cDNA and the reaction was incubated at 50°C for 30 minutes and then chilled to 4°C. Biotin labeling of the fragmented cDNA was completed by the addition of 5 µL biotin labeling buffer (F3) and 2.5 µL of labeling enzyme mix (F4) followed by an incubation at 50°C for 30 minutes and then it was cooled to 4°C. The fragmented and labeled cDNA was then purified using the 96 well dye terminator removal kit according to the manufacturer's recommendations. 3 µg of fragmented labeled cDNA was resuspended in 300 µL 1X hybridization buffer containing 100 mM MES, 1 M [Na+], 20 mM EDTA, 0.01% Tween 20, 0.5 mg/mL Aceylated BSA, 0.1 mg/mL herring sperm DNA, control oligo B2, and control transcripts bioB 1.5 pM, bioC

5 pM, bioD 25 pM, and cre 100 pM, and hybridized to Human Genome U133 plus 2.0 GeneChip probe arrays according to manufacturer's protocol. The hybridized GeneChip probe arrays were washed and stained using Streptavidin-Phycoerythrinin and amplified with biotinylated anti-streptavidin GeneChip Fluidics Station 400 using an antibody amplification protocol. The GeneChip probe arrays were scanned using GeneArray Scanner 3000.

Quality Control of Gene Expression Data

Affymetrix scans were subject to quality control (QC) measures. These tests included a visual inspection of the distribution of raw signal intensities and an assessment of RNA degradation, relative log expression (RLE), and normalized unscaled standard error (NUSE). All sample scans passed these QC metrics and were included in the analysis.

The 60 CEL files were subjected to GC-content-based Robust Multi-array Average (GCRMA) normalization (version 2.20.0) (Irizarry, *et al*., 2003) & (Li & Hung Wong, 2001). Expression levels were log (base 2) transformed. Probe sets that exhibited an average normalized intensity > 4 across all samples and coefficient of variation > 10% were used in a Principal Component Analysis (PCA); there were 4,824 such probe sets. PCA results indicated two main groups of samples accounting for 28% of the data set variation in the first principal component. This variation was not due to the following data features: Donor ID, Freezing Device, or Vacutainer Type.

Careful inspection showed that the variation was based on whether the samples were from aliquot series "-1" (DNase treated post extraction) versus "-3" (DNase treated

during extraction and post extraction). It was confirmed that these groups of samples differed based on the number of times that the DNase treatment was performed. There were 32 DNA contaminated samples that had been DNase treated only once after extraction and the 28 replacement samples that had been DNase treated during the extraction process and after the extraction process.

The Combat Tool from Bioconductor's SVA software packages was used to correct for this technical artefact in the data (Leek, *et al*.). After Combat correction, 4,173 probe sets exhibited an average normalized intensity > 4 across all samples and a coefficient of variation > 10%. PCA on this set of normalized intensities did not show any clear, appreciable separation between the samples, artefact or otherwise. After the combat correction, there were no longer two main groups of samples in the PCA results and there wasn't any segregation based on vacutainer type or freezing device.

To identify genes that change due to freezing method or the type of vacutainer, a linear modeling approach was used to fit gene expression levels (Combat-corrected, log2 transformed) according to defined groups of samples and Bayesian posterior error analysis as implemented by Smyth (Bioconductor library limma, version 3.4.5) (Smyth, 2004). The following 7 Contrasts were calculated:

1.) Freshly isolated PBMCs collected in sodium heparin vacutainers vs Freshly isolated PBMCs collected in sodium heparin CPT vacutainers

2.) Freshly isolated PBMCs collected in sodium heparin vacutainers vs Thawed PBMCs collected in sodium heparin vacutainers and cryopreserved in a CoolCell device

3.) Freshly isolated PBMCs collected in sodium heparin vacutainers vs Thawed PBMCs collected in sodium heparin vacutainers and cryopreserved in a rate-controlled freezer

4.) Freshly isolated PBMCs collected in sodium heparin CPT vacutainers vs Thawed PBMCs collected in sodium heparin CPT vacutainers and cryopreserved in a CoolCell device

5.) Freshly isolated PBMCs collected in sodium heparin CPT vacutainers vs Thawed PBMCs collected in sodium heparin CPT vacutainers and cryopreserved in a rate-controlled freezer

6.) Thawed PBMCs collected in sodium heparin vacutainers and cryopreserved in a CoolCell device vs Thawed PBMCs collected in sodium heparin vacutainers and cryopreserved in a rate-controlled freezer

7.) Thawed PBMCs collected in sodium heparin CPT vacutainers and cryopreserved in a CoolCell device vs Thawed PBMCs collected in sodium heparin CPT vacutainers and cryopreserved in a rate-controlled freezer

Probe sets that exhibited a log-odds score (lods) greater than zero, absolute fold change >1.5, and average normalized intensity >4 were considered significantly different. All calculations and analyses were carried out using R (version 2.11.1) and Bioconductor computational tools (Gentleman, *et.al.*, 2005). As a sanity check, the data was also analyzed using only the "-3" aliquots and without any Combat correction. This approach was possible since there was an even distribution of sample representation across the "-1" and "-3" samples. There was a high amount of agreement between the two analysis approaches of using all samples versus just one set of aliquots.

Chapter III

Results

Whole Blood Collection and PBMC Isolation Yields

Between 96.5 mLs to 107.5 mLs of whole blood were collected in two different vacutainers from 10 healthy donors (5 males and 5 females) that participated in Biogen's IRB-reviewed employee biospecimen donor program. The donors were of various ethnicities and were between 29 and 55 years of age. Both types of vacutainers that were used contained sodium heparin. One vacutainer was the standard sodium heparin vacutainer made by Becton Dickinson (BD); whereas, the second vacutainer also manufactured by BD, contained sodium heparin and also a cell separation medium inside the vacutainer, this vacutainer was called a Cell Preparation Tube (CPT). Before the PBMCs were isolated, a representative whole blood aliquot was obtained from each vacutainer type and placed into RLT lysis buffer. This was done in an attempt to obtain a cellular sample that was as close to PBMCs in vivo as possible.

PBMCs were isolated from both types of vacutainers. Ficoll-density centrifugation was used to isolate PBMCs from the whole blood from the 6 sodium heparin vacutainers from each donor. Whereas, PBMCs were isolated directly from the 6 sodium heparin CPT vacutainers from each donor following centrifugation of the whole blood due to the cell separation medium and then the PBMCs were pooled to make a single sample. Once the PBMCs were isolated, they were counted using the Vi-CELL. The total PBMC yield per donor from the whole blood was between 18.62×10^6 –

84.28x10^6 PBMCs per vacutainer type as presented in Table 3.1. Eight of the donors (4 males and 4 females) had less blood collected in the Sodium Heparin CPT vacutainers but more PBMCs were isolated from these vacutainers than the Sodium Heparin vacutainers.

Table 3.1. PBMC Yield from Whole Blood Collected by Donor.

Subject	Age	Race	Gender	Vacutainer Type	Total Volume of all 6 Vacutainers (mL)	Total PBMC Count	Concentration of Frozen PBMCs (cells/mL)
2252	52	White	Male	Sodium Heparin CPT	48	37.73x10^6	5x10^6
				Sodium Heparin	55.5	22.05x10^6	10x10^6
2253	38	White	Male	Sodium Heparin CPT	48.5	55.86x10^6	10x10^6
				Sodium Heparin	53	53.90x10^6	10x10^6
2254	35	Asian	Male	Sodium Heparin CPT	46	57.33x10^6	10x10^6
				Sodium Heparin	50.5	46.06x10^6	10x10^6
2392	29	White	Male	Sodium Heparin CPT	49	34.79x10^6	10x10^6
				Sodium Heparin	57	31.36x10^6	10x10^6
2398	37	Hispanic	Male	Sodium Heparin CPT	46	43.61x10^6	10x10^6
				Sodium Heparin	55.5	62.72x10^6	10x10^6
2400	31	White	Female	Sodium Heparin CPT	48	69.09x10^6	10x10^6
				Sodium Heparin	55.5	45.08x10^6	10x10^6
2402	39	Black	Female	Sodium Heparin CPT	50	33.32x10^6	10x10^6
				Sodium Heparin	55	42.63x10^6	10x10^6
2407	55	White	Female	Sodium Heparin CPT	49.5	70.56x10^6	10x10^6
				Sodium Heparin	56	59.29x10^6	10x10^6
2409	47	Hispanic	Female	Sodium Heparin CPT	47.5	84.28x10^6	10x10^6
				Sodium Heparin	57.5	51.45x10^6	10x10^6
2478	34	White	Female	Sodium Heparin CPT	47.5	43.12x10^6	10x10^6
				Sodium Heparin	57.5	18.62x10^6	5x10^6

This table shows the yield of PBMCs based on the amount of whole blood obtained from each donor by vacutainer type. It also shows the metadata associated with each donor.

PBMC Aliquot Designation and Cryopreservation

From each donor, one 2x10^6 aliquot of freshly isolated PBMCs (non-frozen) was immediately added to RLT lysis buffer, the remaining cell suspension was split in half and each half was cryopreserved into aliquots of either 5x10^6 or 10x10^6 PBMCs/ml/cryovial, depending on the total number of cells available. One set of aliquots

was frozen using a CoolCell device while the second was frozen using a rate-controlled freezer. Regardless of the freezing methods, the cryopreserved PBMCs were stored frozen for 1 month in a -150°C mechanical freezer, then one aliquot from each freezing method was thawed, counted, aliquoted into $2x10^6$ PBMCs and placed into RLT lysis buffer. Cell recovery post thaw is presented in Table 3.2 for the PBMCs that were cryopreserved by the 2 freezing devices. Cell recovery varied greatly between donors and samples. The cell recovery ranged between 11.25% and 100% with a majority of the samples recovering less than half of the number of PBMCs that were cryopreserved based on the Vi-CELL counts.

Table 3.2. Summary of Donor IDs, Vacutainer Types, Custom IDs, Cell Counts & RIN Scores.

Subject	Vacutainer Type	Custom IDs	Total Cell Count at Time of Freezing	Total Cell Count at Time of Thawing	First RIN Score	Second RIN Score
2252	Sodium Heparin CPT	GX1.225202-3000-3	$2x10^6$	N/A	9.4	8.3
	Sodium Heparin CPT	GX1.225202-4101-1	$10x10^6$	$10x10^6$	2.6	8.3
	Sodium Heparin CPT	GX1.225202-5001-3	$10x10^6$	$3.68x10^6$	7.6	8.3
	Sodium Heparin	GX1.225203-3000-3	$2x10^6$	N/A	6.9	8.3
	Sodium Heparin	GX1.225203-4001-3	$5x10^6$	$1.92x10^6$	8.5	8.3
	Sodium Heparin	GX1.225203-5001-3	$5x10^6$	$1.98x10^6$	9.7	8.3
2253	Sodium Heparin CPT	GX1.225302-3000-3	$2x10^6$	N/A	6.9	8.3
	Sodium Heparin CPT	GX1.225302-4001-3	$10x10^6$	$4.68x10^6$	8.5	8.3
	Sodium Heparin CPT	GX1.225302-5101-1	$10x10^6$	$6.75x10^6$	7	8.3
	Sodium Heparin	GX1.225303-3000-3	$2x10^6$	N/A	8.4	8.3
	Sodium Heparin	GX1.225303-4001-3	$10x10^6$	$4.545x10^6$	6.4	8.3
	Sodium Heparin	GX1.225303-5001-3	$10x10^6$	$4.165x10^6$	7.9	8.3
2254	Sodium Heparin CPT	GX1.225402-3000-3	$2x10^6$	N/A	7.9	8.3
	Sodium Heparin CPT	GX1.225402-4001-3	$10x10^6$	$4.725x10^6$	8.5	8.3
	Sodium Heparin CPT	GX1.225402-5001-3	$10x10^6$	$5.715x10^6$	8	8.3
	Sodium Heparin	GX1.225403-3000-3	$2x10^6$	N/A	7.8	8.3
	Sodium Heparin	GX1.225403-4101-1	$10x10^6$	$4x10^6$	8.5	8.3
	Sodium Heparin	GX1.225403-5001-3	$10x10^6$	$4x10^6$	6.6	8.3
2392	Sodium Heparin CPT	GX1.239202-3000-3	$2x10^6$	N/A	9.7	8.3
	Sodium Heparin CPT	GX1.239202-4001-3	$10x10^6$	$4.23x10^6$	5.9	8.3
	Sodium Heparin CPT	GX1.239202-5001-3	$10x10^6$	$3.51x10^6$	8.5	8.3
	Sodium Heparin	GX1.239203-3100-1	$2x10^6$	N/A	6.8	8.3
	Sodium Heparin	GX1.239203-4001-3	$10x10^6$	$4x10^6$	8	8.3
	Sodium Heparin	GX1.239203-5101-1	$10x10^6$	$3.42x10^6$	6.6	8.3
2398	Sodium Heparin CPT	GX1.239802-3100-1	$2x10^6$	N/A	7.6	8.3
	Sodium Heparin CPT	GX1.239802-4101-1	$10x10^6$	$4.275x10^6$	7.9	8.3
	Sodium Heparin CPT	GX1.239802-5101-1	$10x10^6$	$5.13x10^6$	10	8.3
	Sodium Heparin	GX1.239803-3100-1	$2x10^6$	N/A	8.6	8.3
	Sodium Heparin	GX1.239803-4001-3	$10x10^6$	$3.96x10^6$	7.1	8.3
	Sodium Heparin	GX1.239803-5101-1	$10x10^6$	$4.365x10^6$	7.1	8.3
2400	Sodium Heparin CPT	GX1.240002-3000-3	$2x10^6$	N/A	7	8.3
	Sodium Heparin CPT	GX1.240002-4101-1	$10x10^6$	$3.285x10^6$	7.9	8.3
	Sodium Heparin CPT	GX1.240002-5001-3	$10x10^6$	$3.69x10^6$	5.4	8.3
	Sodium Heparin	GX1.240003-3100-1	$2x10^6$	N/A	6.7	8.3
	Sodium Heparin	GX1.240003-4001-3	$10x10^6$	$3.69x10^6$	5.2	8.3
	Sodium Heparin	GX1.240003-5001-3	$10x10^6$	$2.815x10^6$	5.9	8.3
2402	Sodium Heparin CPT	GX1.240202-3100-1	$2x10^6$	N/A	4.2	8.3
	Sodium Heparin CPT	GX1.240202-4001-3	$10x10^6$	$4.5x10^6$	8.7	8.3
	Sodium Heparin CPT	GX1.240202-5001-3	$10x10^6$	$2.915x10^6$	7.2	8.3
	Sodium Heparin	GX1.240203-3100-1	$2x10^6$	N/A	4.7	8.3
	Sodium Heparin	GX1.240203-4101-1	$10x10^6$	$1.125x10^6$	3.6	8.3
	Sodium Heparin	GX1.240203-5101-1	$10x10^6$	$2.7x10^6$	5.5	8.3
2407	Sodium Heparin CPT	GX1.240702-3100-1	$2x10^6$	N/A	5.2	8.3
	Sodium Heparin CPT	GX1.240702-4001-3	$10x10^6$	$3.105x10^6$	5.2	8.3
	Sodium Heparin CPT	GX1.240702-5101-1	$10x10^6$	$3.015x10^6$	4.6	8.3
	Sodium Heparin	GX1.240703-3100-1	$2x10^6$	N/A	5.7	8.3
	Sodium Heparin	GX1.240703-4001-3	$10x10^6$	$2.655x10^6$	6.4	8.3
	Sodium Heparin	GX1.240703-5101-1	$10x10^6$	$2.79x10^6$	4.9	8.3
2409	Sodium Heparin CPT	GX1.240902-3100-1	$2x10^6$	N/A	2.3	8.3
	Sodium Heparin CPT	GX1.240902-4101-1	$10x10^6$	$3.375x10^6$	4	8.3
	Sodium Heparin CPT	GX1.240902-5101-1	$10x10^6$	$3.15x10^6$	6.9	8.3
	Sodium Heparin	GX1.240903-3000-3	$2x10^6$	N/A	5.4	8.3
	Sodium Heparin	GX1.240903-4001-3	$10x10^6$	$4.815x10^6$	6.1	8.3
	Sodium Heparin	GX1.240903-5101-1	$10x10^6$	$4.50x10^6$	4.7	8.3
2478	Sodium Heparin CPT	GX1.247802-3100-1	$2x10^6$	N/A	4.2	8.3
	Sodium Heparin CPT	GX1.247802-4101-1	$10x10^6$	$2.0x10^6$	6	8.3
	Sodium Heparin CPT	GX1.247802-5101-1	$10x10^6$	$2.79x10^6$	6.3	8.3
	Sodium Heparin	GX1.247803-3100-1	$2x10^6$	N/A	3.8	8.3
	Sodium Heparin	GX1.247803-4001-3	$5x10^6$	$1.665x10^6$	7.9	8.3
	Sodium Heparin	GX1.247803-5001-3	$5x10^6$	$1.8x10^6$	5	8.3

This table shows in detail the 10 donors that were used, the two different vacutainer types, the custom ids of each sample, the cell count at the time the samples were cryopreserved and frozen, the cell count when the cryopreserved samples were thawed and counted, and the 2 RIN scores that were received during the two Bioanalyzer runs.

RNA Integrity Number (RIN) Score Quantification and Evaluation

After the various PBMC aliquots were placed into the RLT lysis buffer, RNA was isolated from the lysed PBMCs using the RNeasy Mini Kit. In order for the RNA to be run on the gene expression arrays, a RIN score of at least 7 was required. To ensure the RNA was of high quality, two rounds of quantitation were performed because in the first round of RIN evaluation only 45% (27/60) of the samples had a RIN $\geq$7, so samples were evaluated a second time in order to confirm these results. Likewise when the second round of RIN evaluation was performed, only 45% of the PBMC samples had a RIN $\geq$7 but for the most part, the samples in the first round of quantitation with a RIN $\geq$7 were not the same samples in the second round of quantitation with the RIN $\geq$7. Also there were considerable differences in the actual RIN score from when the sample was first tested till it was tested again. This lack of consistency as shown in Table 3.2 required further evaluation before the samples could be microarrayed.

Determining the Appropriate RNA Extraction Method and a Faulty Bioanalyzer

To explain the discrepancy in the two RIN scores, the possibility of DNA contamination of the RNA was considered. It is commonly known that DNA contamination can artificially inflate a RIN score and also cause the scoring to be inconsistent between evaluations. Originally when the 60 PBMC samples had their RNA extracted, the RNeasy Mini Kit was used. However, this kit did not have a DNase treatment step included in the process, which was thought to be the reason for the inconsistent and low RIN scores. Therefore, the mouse fibroblast cell line, M2-10B4, was expanded in culture. Mouse fibroblast cells were used since it is easy to extract high

quality RNA from them and it helped to eliminate human donor RNA recovery variability. Mouse fibroblast cells were harvested and four aliquots of $1x10^6$ mouse fibroblast cells were washed with PBS and placed into RLT lysis buffer. Each aliquot of mouse fibroblast cells had their RNA extracted using one of the following 4 commercially available RNA extraction kits: AllPrep DNA/RNA Mini Kit, RNeasy Mini Kit without a DNase Treatment, RNeasy Mini Kit with a DNase Treatment & RNeasy Plus Mini Kit. The RNA was quantitated and their RIN score was determined on the Biobank's Bioanalyzer. All 4 RNA extraction methods produced a significant amount of RNA from $1x10^6$ mouse fibroblast cells. However only the samples extracted from the RNeasy Mini Kit and RNeasy Plus Mini Kit produced samples that had electropherograms which produced quantifiable RIN scores. Both of these samples had a RIN score of 10 (electropherograms not shown). The RNA samples from the other 2 kits generated unreadable electropherograms, hence not producing a RIN score. The results can be seen in Table 3.3.

Table 3.3. RNA Extraction Method Comparison.

Sample	RNA Extraction Method	RNA Concentration (ng/ul)	RIN Score	Chip #
Mouse Cell Line	All-Prep	311.81	Unreadable	1
	RNeasy Mini DNase	551.94	Unreadable	1
	RNeasy Mini	450.59	10	2
	RNeasy Plus Mini	487.47	10	2

This table shows the RNA extraction method used, the RNA concentration, the RIN score & the Chip # the sample was run on for the mouse cell line.

Since the Biobank Bioanalyzer was not able to consistently determine a RIN score for most of the samples tested, a second Bioanalyzer was needed, so one was located, in the Molecular Biology Laboratory. Whole blood was collected in three sodium heparin vacutainers from a healthy volunteer that participated in the Biogen biospecimen donor program. The PBMCs were isolated by ficoll-density gradient, counted and then 1, 2, 4 and $10x10^6$ PBMCs were added to separate aliquots of RLT lysis buffer. These 4 PBMC samples were extracted using the RNeasy Plus Mini Kit and quantitated on the Trinean. The RNA concentration for the 4 concentrations of the PBMC was fairly linear. The RNA samples were evaluated on both Bioanalyzers. As Table 3.4 shows, the Biobank Bioanalyzer was not able to provide a RIN score for the 4 samples but the Molecular Biology Laboratory's Bioanalyzer was able to for all samples.

Table 3.4. RNA Concentration and Bioanalyzer RIN Comparison.

Human PBMC Number	RNA Concentration (ng/ul)	Biobank's Bioanalyzer	Molecular Lab's Bioanalyzer
1×10^6	14.76	Unreadable	5.70
2×10^6	27.53	Unreadable	8.80
4×10^6	51.55	Unreadable	9.90
10×10^6	154.41	Unreadable	10

This table shows the concentration & RIN scores from the Biobank's & Molecular Lab's Bioanalyzer for Human PBMC samples.

Third RIN Score Evaluation

Based on the above results, all of the original 60 PBMC samples were treated using DNase (post-extraction) to eliminate the DNA contamination issue, the samples then had their RIN scores calculated for a third and final time using the Molecular Biology Laboratory's Bioanalyzer. Table 3.5 shows the final RIN scores for all 60

PBMC samples being ≥7, which allows for the samples to qualify for microarray analysis.

Table 3.5. Results of the Post Extraction DNase Treatment.

Subject	Vacutainer Type	Custom IDs	Total Cell Count at Time of Freezing	Total Cell Count at Time of Thawing	First RIN Score	Second RIN Score	Final RIN Score
2252	Sodium Heparin CPT	GX1.225202-3000-3	$2x10^6$	N/A	9.4	8.3	9.4
	Sodium Heparin CPT	GX1.225202-4101-1	$10x10^6$	$10x10^6$	2.6	2.7	9.7
	Sodium Heparin CPT	GX1.225202-5001-3	$10x10^6$	$3.68x10^6$	7.6	7.8	9.3
	Sodium Heparin Vacutainer	GX1.225203-3000-3	$2x10^6$	N/A	6.9	3.4	9.6
	Sodium Heparin Vacutainer	GX1.225203-4001-3	$5x10^6$	$1.92x10^6$	8.5	7.4	9.1
	Sodium Heparin Vacutainer	GX1.225203-5001-3	$5x10^6$	$1.98x10^6$	9.7	9.2	9.5
2253	Sodium Heparin CPT	GX1.225302-3000-3	$2x10^6$	N/A	6.9	5.4	9.1
	Sodium Heparin CPT	GX1.225302-4001-3	$10x10^6$	$4.68x10^6$	8.5	8.6	9.1
	Sodium Heparin CPT	GX1.225302-5101-1	$10x10^6$	$6.75x10^6$	7.0	2.9	9.8
	Sodium Heparin Vacutainer	GX1.225303-3000-3	$2x10^6$	N/A	8.4	6.9	9.9
	Sodium Heparin Vacutainer	GX1.225303-4001-3	$10x10^6$	$4.545x10^6$	6.4	9.4	9.0
	Sodium Heparin Vacutainer	GX1.225303-5001-3	$10x10^6$	$4.165x10^6$	7.9	9.1	9.3
2254	Sodium Heparin CPT	GX1.225402-3000-3	$2x10^6$	N/A	7.9	5.1	9.3
	Sodium Heparin CPT	GX1.225402-4001-3	$10x10^6$	$4.725x10^6$	8.5	7.5	8.8
	Sodium Heparin CPT	GX1.225402-5001-3	$10x10^6$	$5.715x10^6$	8.0	9.1	9.1
	Sodium Heparin Vacutainer	GX1.225403-3000-3	$2x10^6$	N/A	7.8	5.6	9.4
	Sodium Heparin Vacutainer	GX1.225403-4101-1	$10x10^6$	$4x10^6$	8.5	2.3	9.1
	Sodium Heparin Vacutainer	GX1.225403-5001-3	$10x10^6$	$4x10^6$	6.6	8.6	9.0
2392	Sodium Heparin CPT	GX1.239202-3000-3	$2x10^6$	N/A	9.7	8.1	9.8
	Sodium Heparin CPT	GX1.239202-4001-3	$10x10^6$	$4.23x10^6$	5.9	10.0	7.6
	Sodium Heparin CPT	GX1.239202-5001-3	$10x10^6$	$3.51x10^6$	8.5	7.2	8.9
	Sodium Heparin Vacutainer	GX1.239203-3100-1	$2x10^6$	N/A	6.8	3.3	9.8
	Sodium Heparin Vacutainer	GX1.239203-4001-3	$10x10^6$	$4x10^6$	8.0	9.5	8.8
	Sodium Heparin Vacutainer	GX1.239203-5101-1	$10x10^6$	$3.42x10^6$	6.6	6.9	9.3
2398	Sodium Heparin CPT	GX1.239802-3100-1	$2x10^6$	N/A	7.6	4.2	8.4
	Sodium Heparin CPT	GX1.239802-4101-1	$10x10^6$	$4.275x10^6$	7.9	3.4	9.4
	Sodium Heparin CPT	GX1.239802-5101-1	$10x10^6$	$5.13x10^6$	10.0	3.9	9.8
	Sodium Heparin Vacutainer	GX1.239803-3100-1	$2x10^6$	N/A	8.6	6.9	9.9
	Sodium Heparin Vacutainer	GX1.239803-4001-3	$10x10^6$	$3.96x10^6$	7.1	9.7	8.6
	Sodium Heparin Vacutainer	GX1.239803-5101-1	$10x10^6$	$4.365x10^6$	7.1	3.5	9.5
2400	Sodium Heparin CPT	GX1.240002-3000-3	$2x10^6$	N/A	7.0	7.3	8.9
	Sodium Heparin CPT	GX1.240002-4101-1	$10x10^6$	$3.285x10^6$	7.9	4.1	8.6
	Sodium Heparin CPT	GX1.240002-5001-3	$10x10^6$	$3.69x10^6$	5.4	9.5	9.0
	Sodium Heparin Vacutainer	GX1.240003-3100-1	$2x10^6$	N/A	6.7	5.2	8.5
	Sodium Heparin Vacutainer	GX1.240003-4001-3	$10x10^6$	$3.69x10^6$	5.2	7.0	9.2
	Sodium Heparin Vacutainer	GX1.240003-5001-3	$10x10^6$	$2.815x10^6$	5.9	9.2	9.3
2402	Sodium Heparin CPT	GX1.240202-3100-1	$2x10^6$	N/A	4.2	3.0	9.9
	Sodium Heparin CPT	GX1.240202-4001-3	$10x10^6$	$4.5x10^6$	8.7	8.8	9.2
	Sodium Heparin CPT	GX1.240202-5001-3	$10x10^6$	$2.915x10^6$	7.2	8.9	9.4
	Sodium Heparin Vacutainer	GX1.240203-3100-1	$2x10^6$	N/A	4.7	3.0	10.0
	Sodium Heparin Vacutainer	GX1.240203-4101-1	$10x10^6$	$1.125x10^6$	3.6	3.1	7.4
	Sodium Heparin Vacutainer	GX1.240203-5101-1	$10x10^6$	$2.7x10^6$	5.5	4.0	9.8
2407	Sodium Heparin CPT	GX1.240702-3100-1	$2x10^6$	N/A	5.2	3.3	9.2
	Sodium Heparin CPT	GX1.240702-4001-3	$10x10^6$	$3.105x10^6$	5.2	9.4	8.9
	Sodium Heparin CPT	GX1.240702-5101-1	$10x10^6$	$3.015x10^6$	4.6	5.0	8.9
	Sodium Heparin Vacutainer	GX1.240703-3100-1	$2x10^6$	N/A	5.7	1.4	9.2
	Sodium Heparin Vacutainer	GX1.240703-4001-3	$10x10^6$	$2.655x10^6$	6.4	9.7	8.8
	Sodium Heparin Vacutainer	GX1.240703-5101-1	$10x10^6$	$2.79x10^6$	4.9	6.5	9.4
2409	Sodium Heparin CPT	GX1.240902-3100-1	$2x10^6$	N/A	2.3	3.7	10.0
	Sodium Heparin CPT	GX1.240902-4101-1	$10x10^6$	$3.375x10^6$	4.0	5.2	8.6
	Sodium Heparin CPT	GX1.240902-5101-1	$10x10^6$	$3.15x10^6$	6.9	4.5	9.4
	Sodium Heparin Vacutainer	GX1.240903-3000-3	$2x10^6$	N/A	5.4	8.3	9.5
	Sodium Heparin Vacutainer	GX1.240903-4001-3	$10x10^6$	$4.815x10^6$	6.1	9.3	8.9
	Sodium Heparin Vacutainer	GX1.240903-5101-1	$10x10^6$	$4.50x10^6$	4.7	3.2	9.7
2478	Sodium Heparin CPT	GX1.247802-3100-1	$2x10^6$	N/A	4.2	4.7	10.0
	Sodium Heparin CPT	GX1.247802-4101-1	$10x10^6$	$2.0x10^6$	6.0	3.3	8.9
	Sodium Heparin CPT	GX1.247802-5101-1	$10x10^6$	$2.79x10^6$	6.3	3.8	9.3
	Sodium Heparin Vacutainer	GX1.247803-3100-1	$2x10^6$	N/A	3.8	3.0	8.8
	Sodium Heparin Vacutainer	GX1.247803-4001-3	$5x10^6$	$1.665x10^6$	7.9	7.2	9.3
	Sodium Heparin Vacutainer	GX1.247803-5001-3	$5x10^6$	$1.8x10^6$	5.0	7.2	9.0

The column titled Final RIN Score shows the RIN score of each sample after the post extraction DNase treatment.

Analysis of Gene Expression Contrasts

There were 7 Contrasts focused on when analyzing the gene expression data. The 7 Contrasts are presented in Table 3.6, which also provides the number of Differentially Expressed Genes (DEGs) for each Contrast and a table designation that provides the Affy ID, Gene Name, Gene Title and Fold Change.

Table 3.6. Differentially Expressed Genes (DEGs) Present in Each Contrast.

Contrast Number	Contrast Description	Number of DEGs	List of Identified DEGs Located In:
1	Freshly isolated PBMCs collected in SH vacutainers vs Freshly isolated PBMCs collected in SH CPT vacutainers	21	Table 3.7
2	Freshly isolated PBMCs collected in SH vacutainers vs Thawed PBMCs collected in SH vacutainers and cryopreserved in a CoolCell device	50	Table 3.8
3	Freshly isolated PBMCs collected in SH vacutainers vs Thawed PBMCs collected in SH vacutainers and cryopreserved in a Rate-Controlled Freezer	23	Table 3.9
4	Freshly isolated PBMCs collected in SH CPT vacutainers vs Thawed PBMCs collected in SH CPT vacutainers and cryopreserved in a CoolCell device	48	Table 3.10
5	Freshly isolated PBMCs collected in SH CPT vacutainers vs Thawed PBMCs collected in SH CPT vacutainers and cryopreserved in a Rate-Controlled Freezer	29	Table 3.11
6	Thawed PBMCs collected in SH vacutainers and cryopreserved in a CoolCell device vs Thawed PBMCs collected in SH vacutainers and cryopreserved in a Rate-Controlled Freezer	0	N/A
7	Thawed PBMCs collected in SH CPT vacutainers and cryopreserved in a CoolCell device vs Thawed PBMCs collected in SH CPT vacutainers and cryopreserved in a Rate-Controlled Freezer	0	N/A

*SH=Sodium Heparin. This table shows all 7 Contrasts and the number of DEGs present in each Contrast.

The first Contrast was between the RNA extracted from PBMCs isolated from the whole blood collected in sodium heparin vacutainers from the 10 healthy donors and the RNA extracted from the PBMCs isolated from the whole blood collected in sodium heparin CPT vacutainers from the same 10 donors. As shown in Table 3.6, there were 21 DEGs identified between the PBMCs isolated from these 2 vacutainer types. Table 3.7 lists the 21 DEGs present in Contrast #1.

Table 3.7. List of 21 DEGs for Contrast #1.

Freshly isolated PBMCs collected in SH vacutainers vs Freshly isolated PBMCs collected in SH CPT vacutainers			
Affy Id	**Gene Name**	**Gene Title**	**Fold Change**
1554443_s_at	BEST1	bestrophin 1	-4.88
1554676_at	SRGN	serglycin	-6.97
201041_s_at	DUSP1	dual specificity phosphatase 1	-2.81
201464_x_at	JUN	jun proto-oncogene	-2.31
201465_s_at	JUN	jun proto-oncogene	-9.49
201466_s_at	JUN	jun proto-oncogene	-3.74
201502_s_at	NFKBIA	nuclear factor of kappa light polypeptide gene enhancer in B-cells inhibitor, alpha	-2.81
201531_at	ZFP36	ZFP36 ring finger protein	-1.75
201631_s_at	IER3	immediate early response 3	-2.78
201694_s_at	EGR1	early growth response 1	-3.61
205114_s_at	CCL3 /// CCL3L1 /// CCL3L3 /// LOC101060267	chemokine (C-C motif) ligand 3 /// chemokine (C-C motif) ligand 3-like 1 /// chemokine (C-C motif) ligand 3-like 3 /// c-C motif chemokine 3-like	-8.31
207113_s_at	TNF	tumor necrosis factor	-10.30
209189_at	FOS	FBJ murine osteosarcoma viral oncogene homolog	-7.31
209774_x_at	CXCL2	chemokine (C-X-C motif) ligand 2	-36.63
213281_at	JUN	jun proto-oncogene	-5.54
213524_s_at	G0S2	G0/G1switch 2	-10.48
223218_s_at	NFKBIZ	nuclear factor of kappa light polypeptide gene enhancer in B-cells inhibitor, zeta	-1.89
227404_s_at	EGR1	early growth response 1	-3.14
235102_x_at	SNORD3A /// SNORD3B-1 /// SNORD3B-2 /// SNORD3C /// SNORD3D	small nucleolar RNA, C/D box 3A /// small nucleolar RNA, C/D box 3B-1 /// small nucleolar RNA, C/D box 3B-2 /// small nucleolar RNA, C/D box 3C /// small nucleolar RNA, C/D box 3D	-56.35
236399_at	OTTHUMG00000038014 /// RP11-443B7.1	NULL /// NULL	-17.49
238893_at	LINC00936	long intergenic non-protein coding RNA 936	-2.67

This table lists the 21 DEGs for Contrast #1, by the Affy ID, Gene Name, Gene Title & Fold Change.

The second Contrast was between freshly isolated PBMCs collected in sodium heparin vacutainers and thawed PBMCs (also isolated from sodium heparin vacutainers) that had been cryopreserved in a CoolCell device. As presented in Table 3.6, there were

50 DEGs that were identified as being effected by cryopreserving the PBMCs in a CoolCell device, storing the PBMCs for 1 month at -150^{o}C and then thawing the PBMCs for analysis by microarray. Table 3.8 lists the 50 DEGs present in Contrast #2.

Table 3.8. List of 50 DEGs for Contrast #2.

Freshly isolated PBMCs collected in SH vacutainers vs Thawed PBMCs collected in SH vacutainers and cryopreserved in a CoolCell device			
Affy Id	**Gene Name**	**Gene Title**	**Fold Change**
1554892_a_at	MS4A3	membrane-spanning 4-domains, subfamily A, member 3 (hematopoietic cell-specific)	-127.04
200784_s_at	LRP1	low density lipoprotein receptor-related protein 1	2.06
202018_s_at	LTF	lactotransferrin	-462.45
203691_at	PI3	peptidase inhibitor 3, skin-derived	-105.29
204018_x_at	HBA1 /// HBA2	hemoglobin, alpha 1 /// hemoglobin, alpha 2	-629.09
204419_x_at	HBG1 /// HBG2	hemoglobin, gamma A /// hemoglobin, gamma G	-213.15
204505_s_at	DMTN	dematin actin binding protein	-5.64
204848_x_at	HBG1 /// HBG2	hemoglobin, gamma A /// hemoglobin, gamma G	-315.99
205033_s_at	DEFA1 /// DEFA1B /// DEFA3	defensin, alpha 1 /// defensin, alpha 1B /// defensin, alpha 3, neutrophil-specific	-3276.89
205513_at	TCN1	transcobalamin I (vitamin B12 binding protein, R binder family)	-154.81
205592_at	SLC4A1	solute carrier family 4, anion exchanger, member 1 (erythrocyte membrane protein band 3, Diego blood group)	-53.72
205624_at	CPA3	carboxypeptidase A3 (mast cell)	-58.47
205857_at	SLC18A2	solute carrier family 18 (vesicular monoamine), member 2	-2.39
206207_at	CLC	Charcot-Leyden crystal galectin	-1939.19
206676_at	CEACAM8	carcinoembryonic antigen-related cell adhesion molecule 8	-730.19
206834_at	HBD	hemoglobin, delta	-53.09
207269_at	DEFA4	defensin, alpha 4, corticostatin	-256.22
208304_at	CCR3	chemokine (C-C motif) receptor 3	-87.55
209116_x_at	HBB	hemoglobin, beta	-86.28
209369_at	ANXA3	annexin A3	-24.81
209395_at	CHI3L1	chitinase 3-like 1 (cartilage glycoprotein-39)	-68.34
209458_x_at	HBA1 /// HBA2	hemoglobin, alpha 1 /// hemoglobin, alpha 2	-689.3
209710_at	GATA2	GATA binding protein 2	-21.44
210244_at	CAMP	cathelicidin antimicrobial peptide	-11.92
210254_at	MS4A3	membrane-spanning 4-domains, subfamily A, member 3 (hematopoietic cell-specific)	-340.42
210517_s_at	AKAP12	A kinase (PRKA) anchor protein 12	-86.4
211696_x_at	HBB	hemoglobin, beta	-39.11
211699_x_at	HBA1 /// HBA2	hemoglobin, alpha 1 /// hemoglobin, alpha 2	-531
211745_x_at	HBA1 /// HBA2	hemoglobin, alpha 1 /// hemoglobin, alpha 2	-426.8
212182_at	LOC100996752 /// LOC101060261 /// NUDT4 /// NUDT4P1	uncharacterized LOC100996752 /// uncharacterized LOC101060261 /// nudix (nucleoside diphosphate linked moiety X)-type motif 4 /// nudix (nucleoside diphosphate linked moiety X)-type motif 4 pseudogene 1	-1.61
212531_at	LCN2	lipocalin 2	-178.8
212768_s_at	OLFM4	olfactomedin 4	-113.17
213515_x_at	HBG1 /// HBG2	hemoglobin, gamma A /// hemoglobin, gamma G	-106.83
214414_x_at	HBA1 /// HBA2	hemoglobin, alpha 1 /// hemoglobin, alpha 2	-12.44
217232_x_at	HBB	hemoglobin, beta	-79.45
217414_x_at	HBA1 /// HBA2	hemoglobin, alpha 1 /// hemoglobin, alpha 2	-403.4
220266_s_at	KLF4	Kruppel-like factor 4 (gut)	2.92
220751_s_at	FAXDC2	fatty acid hydroxylase domain containing 2	-12.75
223669_at	HEMGN	hemogen	-5.48
223670_s_at	HEMGN	hemogen	-11.01
227530_at	AKAP12	A kinase (PRKA) anchor protein 12	-127.85
229247_at	FBLN7	fibulin 7	-2.72
229551_x_at	ZNF367	zinc finger protein 367	-1.86
230381_at	C1orf186	chromosome 1 open reading frame 186	-23.15
230917_at	OTTHUMG00000176545 /// RP11-510J16.3	NULL /// NULL	1.78
234396_at	TRAV25 /// TRAV25	T cell receptor alpha variable 25 /// NULL	-6.14
234849_at	TRAV12-3 /// TRAV12-3	T cell receptor alpha variable 12-3 /// NULL	-2.07
238931_at	METTL16	methyltransferase like 16	-2.08
241015_at	---	---	-3.42
41469_at	PI3	peptidase inhibitor 3, skin-derived	-103.68

This table lists the 50 DEGs for Contrast #2, by the Affy ID, Gene Name, Gene Title & Fold Change.

Contrast #3 shows PBMC samples isolated from sodium heparin vacutainers that were cryopreserved with a rate-controlled freezer experienced slightly less than half that number of DEGs (23) than PBMC samples cryopreserved with a CoolCell. Table 3.9 shows the 23 DEGs present in Contrast #3.

Table 3.9. List of 23 DEGs for Contrast #3.

Freshly isolated PBMCs collected in SH vacutainers vs Thawed PBMCs collected in SH vacutainers and cryopreserved in a Rate-Controlled Freezer			
Affy Id	**Gene Name**	**Gene Title**	**Fold Change**
1554892_a_at	MS4A3	membrane-spanning 4-domains, subfamily A, member 3 (hematopoietic cell-specific)	-143.39
202018_s_at	LTF	lactotransferrin	-485.27
203691_at	PI3	peptidase inhibitor 3, skin-derived	-124.87
205033_s_at	DEFA1 /// DEFA1B /// DEFA3	defensin, alpha 1 /// defensin, alpha 1B /// defensin, alpha 3, neutrophil-specific	-2541.46
205513_at	TCN1	transcobalamin I (vitamin B12 binding protein, R binder family)	-169.94
205624_at	CPA3	carboxypeptidase A3 (mast cell)	-39.28
205857_at	SLC18A2	solute carrier family 18 (vesicular monoamine), member 2	-2.48
206207_at	CLC	Charcot-Leyden crystal galectin	-935.47
206676_at	CEACAM8	carcinoembryonic antigen-related cell adhesion molecule 8	-770.33
207269_at	DEFA4	defensin, alpha 4, corticostatin	-259.65
208304_at	CCR3	chemokine (C-C motif) receptor 3	-106.92
209369_at	ANXA3	annexin A3	-31.34
209395_at	CHI3L1	chitinase 3-like 1 (cartilage glycoprotein-39)	-62.54
209710_at	GATA2	GATA binding protein 2	-19.54
210244_at	CAMP	cathelicidin antimicrobial peptide	-11.99
210254_at	MS4A3	membrane-spanning 4-domains, subfamily A, member 3 (hematopoietic cell-specific)	-384.91
210517_s_at	AKAP12	A kinase (PRKA) anchor protein 12	-100.08
212531_at	LCN2	lipocalin 2	-107.11
212768_s_at	OLFM4	olfactomedin 4	-122.23
223670_s_at	HEMGN	hemogen	-12.21
227530_at	AKAP12	A kinase (PRKA) anchor protein 12	-146.52
230381_at	C1orf186	chromosome 1 open reading frame 186	-27.16
41469_at	PI3	peptidase inhibitor 3, skin-derived	-122.72

This table lists the 23 DEGs for Contrast #3, by the Affy ID, Gene Name, Gene Title & Fold Change.

A similar DEG response was seen when sodium heparin CPT vacutainers were used to isolate PBMCs. Table 3.11 shows that there were 48 DEGs affected when a

CoolCell (Contrast #4) was used to cryopreserve the PBMCs as compared to Table 3.12 which shows the 29 DEGs when the rate-controlled freezer was used (Contrast #5).

Table 3.10. List of 48 DEGs for Contrast #4.

Freshly isolated PBMCs collected in SH CPT vacutainers vs Thawed PBMCs collected in SH CPT vacutainers and cryopreserved in a CoolCell device			
Affy Id	**Gene Name**	**Gene Title**	**Fold Change**
1554892_a_at	MS4A3	membrane-spanning 4-domains, subfamily A, member 3 (hematopoietic cell-specific)	-39.23
202018_s_at	LTF	lactotransferrin	-149.84
203691_at	PI3	peptidase inhibitor 3, skin-derived	-26.53
204018_x_at	HBA1 /// HBA2	hemoglobin, alpha 1 /// hemoglobin, alpha 2	-280.28
204419_x_at	HBG1 /// HBG2	hemoglobin, gamma A /// hemoglobin, gamma G	-103.50
204848_x_at	HBG1 /// HBG2	hemoglobin, gamma A /// hemoglobin, gamma G	-138.43
205033_s_at	DEFA1 /// DEFA1B /// DEFA3	defensin, alpha 1 /// defensin, alpha 1B /// defensin, alpha 3, neutrophil-specific	-403.74
205101_at	CIITA	class II, major histocompatibility complex, transactivator	1.74
205513_at	TCN1	transcobalamin I (vitamin B12 binding protein, R binder family)	-41.86
205624_at	CPA3	carboxypeptidase A3 (mast cell)	-25.95
206207_at	CLC	Charcot-Leyden crystal galectin	-449.02
206676_at	CEACAM8	carcinoembryonic antigen-related cell adhesion molecule 8	-161.17
206834_at	HBD	hemoglobin, delta	-31.96
207269_at	DEFA4	defensin, alpha 4, corticostatin	-57.24
208304_at	CCR3	chemokine (C-C motif) receptor 3	-22.66
209116_x_at	HBB	hemoglobin, beta	-53.12
209458_x_at	HBA1 /// HBA2	hemoglobin, alpha 1 /// hemoglobin, alpha 2	-230.80
209710_at	GATA2	GATA binding protein 2	-9.46
209721_s_at	IFFO1	intermediate filament family orphan 1	1.90
210254_at	MS4A3	membrane-spanning 4-domains, subfamily A, member 3 (hematopoietic cell-specific)	-143.74
210517_s_at	AKAP12	A kinase (PRKA) anchor protein 12	-19.52
211696_x_at	HBB	hemoglobin, beta	-24.27
211699_x_at	HBA1 /// HBA2	hemoglobin, alpha 1 /// hemoglobin, alpha 2	-228.57
211745_x_at	HBA1 /// HBA2	hemoglobin, alpha 1 /// hemoglobin, alpha 2	-150.55
212225_at	EIF1	eukaryotic translation initiation factor 1	5.68
212531_at	LCN2	lipocalin 2	-34.70
212926_at	SMC5	structural maintenance of chromosomes 5	1.78
213515_x_at	HBG1 /// HBG2	hemoglobin, gamma A /// hemoglobin, gamma G	-64.76
213596_at	CASP4	caspase 4, apoptosis-related cysteine peptidase	5.90
213939_s_at	RUFY3	RUN and FYVE domain containing 3	2.17
214414_x_at	HBA1 /// HBA2	hemoglobin, alpha 1 /// hemoglobin, alpha 2	-7.82
215540_at	TRAJ45 /// TRAJ45 /// TRAV27 /// TRAV27	T cell receptor alpha joining 45 /// NULL /// T cell receptor alpha variable 27 /// NULL	-5.40
217046_s_at	AGER	advanced glycosylation end product-specific receptor	3.12
217232_x_at	HBB	hemoglobin, beta	-44.09
217414_x_at	HBA1 /// HBA2	hemoglobin, alpha 1 /// hemoglobin, alpha 2	-172.83
220384_at	NME8	NME/NM23 family member 8	2.01
220661_s_at	ZNF692	zinc finger protein 692	3.61
221428_s_at	TBL1XR1	transducin (beta)-like 1 X-linked receptor 1	-1.55
222529_at	SLC25A37	solute carrier family 25 (mitochondrial iron transporter), member 37	-5.08
222900_at	NRIP3	nuclear receptor interacting protein 3	-3.41
225172_at	CRAMP1L	Crm, cramped-like (Drosophila)	1.65
227530_at	AKAP12	A kinase (PRKA) anchor protein 12	-34.44
230381_at	C1orf186	chromosome 1 open reading frame 186	-5.73
233227_at	KIAA1109	KIAA1109	2.70
238561_s_at	UTP23	UTP23, small subunit (SSU) processome component, homolog (yeast)	-2.07
241525_at	LOC200772	uncharacterized LOC200772	8.47
39249_at	AQP3	aquaporin 3 (Gill blood group)	-3.43
41469_at	PI3	peptidase inhibitor 3, skin-derived	-21.89

This table lists the 48 DEGs for Contrast #4, by the Affy ID, Gene Name, Gene Title & Fold Change.

Table 3.11. List of 29 DEGs for Contrast #5.

Freshly isolated PBMCs collected in SH CPT vacutainers vs Thawed PBMCs collected in SH CPT vacutainers and cryopreserved in a Rate-Controlled Freezer			
Affy Id	**Gene Name**	**Gene Title**	**Fold Change**
1554892_a_at	MS4A3	membrane-spanning 4-domains, subfamily A, member 3 (hematopoietic cell-specific)	-59.59
202018_s_at	LTF	lactotransferrin	-304.22
203691_at	PI3	peptidase inhibitor 3, skin-derived	-46.06
205033_s_at	DEFA1 /// DEFA1B /// DEFA3	defensin, alpha 1 /// defensin, alpha 1B /// defensin, alpha 3, neutrophil-specific	-1818.53
205513_at	TCN1	transcobalamin I (vitamin B12 binding protein, R binder family)	-64.88
205624_at	CPA3	carboxypeptidase A3 (mast cell)	-63.71
206207_at	CLC	Charcot-Leyden crystal galectin	-544.99
206676_at	CEACAM8	carcinoembryonic antigen-related cell adhesion molecule 8	-332.05
206851_at	RNASE3	ribonuclease, RNase A family, 3	-8.99
207269_at	DEFA4	defensin, alpha 4, corticostatin	-104.44
208304_at	CCR3	chemokine (C-C motif) receptor 3	-30.59
209369_at	ANXA3	annexin A3	-24.32
209395_at	CHI3L1	chitinase 3-like 1 (cartilage glycoprotein-39)	-36.41
209710_at	GATA2	GATA binding protein 2	-11.58
210244_at	CAMP	cathelicidin antimicrobial peptide	-15.53
210254_at	MS4A3	membrane-spanning 4-domains, subfamily A, member 3 (hematopoietic cell-specific)	-185.27
210517_s_at	AKAP12	A kinase (PRKA) anchor protein 12	-28.41
212225_at	EIF1	eukaryotic translation initiation factor 1	6.10
212531_at	LCN2	lipocalin 2	-80.50
212768_s_at	OLFM4	olfactomedin 4	-40.08
212926_at	SMC5	structural maintenance of chromosomes 5	1.72
213939_s_at	RUFY3	RUN and FYVE domain containing 3	2.16
222040_at	HNRNPA1	heterogeneous nuclear ribonucleoprotein A1	4.01
222529_at	SLC25A37	solute carrier family 25 (mitochondrial iron transporter), member 37	-5.17
222900_at	NRIP3	nuclear receptor interacting protein 3	-3.22
227530_at	AKAP12	A kinase (PRKA) anchor protein 12	-49.76
230381_at	C1orf186	chromosome 1 open reading frame 186	-8.06
233227_at	KIAA1109	KIAA1109	3.40
41469_at	PI3	peptidase inhibitor 3, skin-derived	-37.80

This table lists the 29 DEGs for Contrast #5, by the Affy ID, Gene Name, Gene Title & Fold Change.

There were a total of 23 DEGs that were common between Contrast #2 and #3. This list is presented in Table 3.10.

Table 3.12. Comparison of DEGs Between Contrasts #2 & #3.

Comparison between Contrast #2 and Contrast #3.		
Affy Id	**Gene Name**	**Gene Title**
1554892_a_at	MS4A3	membrane-spanning 4-domains, subfamily A, member 3 (hematopoietic cell-specific)
202018_s_at	LTF	lactotransferrin
203691_at	PI3	peptidase inhibitor 3, skin-derived
205033_s_at	DEFA1 /// DEFA1B /// DEFA3	defensin, alpha 1 /// defensin, alpha 1B /// defensin, alpha 3, neutrophil-specific
205513_at	TCN1	transcobalamin I (vitamin B12 binding protein, R binder family)
205624_at	CPA3	carboxypeptidase A3 (mast cell)
205857_at	SLC18A2	solute carrier family 18 (vesicular monoamine), member 2
206207_at	CLC	Charcot-Leyden crystal galectin
206676_at	CEACAM8	carcinoembryonic antigen-related cell adhesion molecule 8
207269_at	DEFA4	defensin, alpha 4, corticostatin
208304_at	CCR3	chemokine (C-C motif) receptor 3
209369_at	ANXA3	annexin A3
209395_at	CHI3L1	chitinase 3-like 1 (cartilage glycoprotein-39)
209710_at	GATA2	GATA binding protein 2
210244_at	CAMP	cathelicidin antimicrobial peptide
210254_at	MS4A3	membrane-spanning 4-domains, subfamily A, member 3 (hematopoietic cell-specific)
210517_s_at	AKAP12	A kinase (PRKA) anchor protein 12
212531_at	LCN2	lipocalin 2
212768_s_at	OLFM4	olfactomedin 4
223670_s_at	HEMGN	hemogen
227530_at	AKAP12	A kinase (PRKA) anchor protein 12
230381_at	C1orf186	chromosome 1 open reading frame 186
41469_at	PI3	peptidase inhibitor 3, skin-derived

This table shows the common DEGS between Contrasts #2 & #3 by the Affy Id, Genes Name & Gene Title.

Interestingly, the same 23 DEGs that were common between Contrast #2 & Contrast #3 were also common when Contrast #4 was compared to Contrast #5, shown in Table 3.13.

Table 3.13. Comparison of DEGs Between Contrasts #4 & #5.

Comparison between Contrast #4 and Contrast #5.		
1554892_a_at	MS4A3	membrane-spanning 4-domains, subfamily A, member 3 (hematopoietic cell-specific)
202018_s_at	LTF	lactotransferrin
203691_at	PI3	peptidase inhibitor 3, skin-derived
205033_s_at	DEFA1 /// DEFA1B /// DEFA3	defensin, alpha 1 /// defensin, alpha 1B /// defensin, alpha 3, neutrophil-specific
205513_at	TCN1	transcobalamin I (vitamin B12 binding protein, R binder family)
205624_at	CPA3	carboxypeptidase A3 (mast cell)
206207_at	CLC	Charcot-Leyden crystal galectin
206676_at	CEACAM8	carcinoembryonic antigen-related cell adhesion molecule 8
207269_at	DEFA4	defensin, alpha 4, corticostatin
208304_at	CCR3	chemokine (C-C motif) receptor 3
209710_at	GATA2	GATA binding protein 2
210254_at	MS4A3	membrane-spanning 4-domains, subfamily A, member 3 (hematopoietic cell-specific)
210517_s_at	AKAP12	A kinase (PRKA) anchor protein 12
212225_at	EIF1	eukaryotic translation initiation factor 1
212531_at	LCN2	lipocalin 2
212926_at	SMC5	structural maintenance of chromosomes 5
213939_s_at	RUFY3	RUN and FYVE domain containing 3
222529_at	SLC25A37	solute carrier family 25 (mitochondrial iron transporter), member 37
222900_at	NRIP3	nuclear receptor interacting protein 3
227530_at	AKAP12	A kinase (PRKA) anchor protein 12
230381_at	C1orf186	chromosome 1 open reading frame 186
233227_at	KIAA1109	KIAA1109
41469_at	PI3	peptidase inhibitor 3, skin-derived

This table shows the common DEGS between Contrasts #4 & #5 by the Affy Id, Gene Name & Gene Title.

Surprisingly, then the freezing devices (rate-controlled freezer and CoolCell) were compared to each other by vacutainer type, there were no DEGs detected (Contrasts #6 and #7).

Chapter IV

Discussion

Hypothesis

Sodium heparin CPT vacutainers are what Biogen uses to collect whole blood for its multi-site clinical trials as a simpler and more consistent way to isolate PBMCs for biomarker discovery. The Biobank at Biogen is interested in looking at the gene expression profiles of isolated, cryopreserved and thawed PBMCs when whole blood is collected in a sodium heparin vacutainer compared to a sodium heparin CPT vacutainer. This study will also look at the effect on PBMC gene expression profiles when two different freezing devices are used: programmable rate-controlled freezers & CoolCells. Both devices are commonly used to cryopreserve PBMCs; however, the CoolCells are simpler to operate, less expensive, don't require liquid nitrogen to operate and they require less lab space compared to a rate-controlled freezer. As a result, Biogen has the clinical trial sites use CoolCells to cryopreserve the PBMCs. Based on this, it is important for Biogen to know if freezing PBMCs using a CoolCell device produces a different gene expression pattern than when a rate-controlled freezer is used, which is typically available in Biogen laboratories.

For my project, I expected to see differences in the gene expression patterns between the freshly isolated PBMCs from whole blood collected in sodium heparin vacutainer versus freshly isolated PBMCs from sodium heparin CPT vacutainers. Furthermore, I expected that fewer genes would be affected when freshly isolated

PBMCs were compared to sister aliquots that underwent the freezing process in a rate-controlled freezer as compared to those that were placed inside a CoolCell device, all of which were stored in the freezer for 1 month before being thawed.

PBMC Recovery

After cryopreserving PBMCs, typical cell recovery is > 80% of the total number of PBMCs initially cryopreserved. However, as seen in Table 3.2, it is apparent that the cell recovery for most of the cryopreserved PBMC samples is much less than 80%, the fresh PBMCs were immediately added to RLT lysis buffer so recovery determination was not relevant. The Vi-CELL was used to count all of the fresh cell aliquots and most of the cryopreserved cell aliquots used in this study since this machine is known to be accurate and it saves the user a lot of time compared to manually counting cells.

For Biogen's clinical trials, the Biobank was required to use the Vi-CELL to count the cells and also to manually count the cells using a hemocytometer. It was noticed that for the same sample the number of cells manually counted using a hemocytometer were much lower than the cell counts produced on the Vi-CELL. Due to the low cell recoveries for the samples in this thesis research study and other studies, it became apparent that the Vi-CELL was malfunctioning as it was counting more viable cells than there actually were. The malfunctioning ViCELL did have a negative effect on my thesis research study as I was using a lot less cells than were needed, so in the end much less RNA was produced than what was needed for downstream processing. More importantly when it was decided to use the backup samples to extract RNA, the backup

samples for 32 out of 60 samples were deemed unusable due to extremely low cell counts.

Results of the Whole Blood Aliquots

Initially when this experiment was designed, it was decided that two 50 μl aliquots of whole blood would be taken from each vacutainer as soon as the blood was drawn. The whole blood would then be pooled into two 300 μl aliquots according to donor id and vacutainer type, RLT lysis buffer would be added to each aliquot and the samples would be frozen at -80°C. These samples were to be a type of "in vivo like" control that would provide a baseline gene expression profile for both mononuclear and polymorphonuclear cells in the whole blood exposed to the preservative but without any processing, i.e., ficoll density centrifugation, centrifugation or washing steps, that could affect the PBMC gene expression. Unfortunately, RNA was not able to be extracted using the RNeasy Mini Kit from the whole blood samples collected directly from the 2 vacutainer types, i.e., Sodium Heparin and Sodium Heparin CPT. Qiagen did not recommend the RNeasy Mini Kit for the isolation of RNA from whole blood but since the RNeasy Mini Kit was used for all of the PBMC-containing samples, the extraction of whole blood was attempted for consistency. Hence this Contrast is not presented in Table 3.6 because it was not possible to generate these "in vivo like" control samples due to the incompatibly of the RNA extraction kit with the starting biological material, i.e., whole blood.

DNA Contamination Issues

The DNA contamination issue was the biggest set back as far as this thesis research study was concerned. This issue caused us to spend an entire month to troubleshoot. Over the course of the one-month period, 4 different RNA extraction kits were tested with the end goal of eliminating the DNA contamination issue. It quickly became apparent that the RNeasy Mini Plus kit was the best option since as it contains a genomic DNA eliminator column, which in turn produced the purist RNA.

While performing the DNA contamination experiments it was determined that the Bioanalyzer was malfunctioning. The issue was brought to our attention after RNA was extracted from the first set of cell aliquots, run on the Bioanalyzer twice to confirm that the RNA was of high quality and the RIN scores produced between the two runs were drastically different. Only 45% (27/60) of the RNA samples in the first Bioanalyzer run had a RIN $\geq$7, so samples were evaluated a second time. In the second Bioanalyzer run, only 45% of the RNA samples had a RIN $\geq$7. In most cases if a sample had a RIN $\geq$7 in the first run, it did not have a RIN $\geq$7 in the second run and overall there were considerable differences in the actual RIN scores of each sample between the first run and the second run. We initially thought that the discrepancies in the RIN scores could be due to DNA contamination so the Bioanalyzer was used for the DNA contamination evaluation.

An experiment was performed using the mouse fibroblast cell line, M2-10B4 in which the mouse cells had their RNA extracted using four different RNA extraction kits and then the samples were run on the Trinean and the Bioanalyzer.

The results showed that the RNA extracted using the RNeasy Mini Plus kit was very pure even though a RIN score was only determined for 2 of 4 samples. The fact that the two unreadable mouse RNA samples were run on the same Bioanalyzer chip that failed supported the suspicion that it was due to an equipment problem and not a specimen quality problem, even though the Biobank's Bioanalyzer had regular preventative maintenance. There were also RNA samples extracted from 10 PBMC samples isolated from the whole blood of healthy individuals that participated in the Biogen biospecimen donor program whose data was not shown. These additionally isolated human PBMC samples supported the conclusion that a DNase treatment was needed to improve the quality of the RNA and that the Biobank Bioanalyzer may be problematic.

Due to the inconclusive Bioanalyzer results, an additional experiment was performed using the mouse cells and PBMCs isolated from whole blood stored in sodium heparin vacutainers from Biogen's volunteer donor program. The second experiment was performed so that RNA samples could be created to run on the Biobank's Bioanalyzer and on the Molecular Lab's Bioanalyzer to determine if the Biobank's Bioanalyzer was functioning properly. The Biobank's Bioanalyzer did not call a RIN score for any of the samples, and even though the samples were extracted with the RNeasy Plus Mini kit the samples did not appear to be very pure which is unusual for this kit. The Molecular Lab's Bioanalyzer called a high RIN score for all of the samples and the samples appeared to be very pure. This experiment showed that even though the Biobank's Bioanalyzer had regular preventative maintenance, it was not operating correctly, it was a source of the inconsistent RIN scores that were obtained and it should not be used for any future

evaluation until a complete diagnostic evaluation is performed by a qualified technical representative.

Gene Expression Results of Vacutainer Types

I initially hypothesized that I would see differences in the gene expression patterns between freshly isolated PBMCs collected in sodium heparin vacutainers versus freshly isolated PBMCs collected in sodium heparin CPT vacutainers. One reason for my hypothesis is that sodium heparin vacutainers only contain sodium heparin while the sodium heparin CPT vacutainers contain sodium heparin and a cell separation medium. The second reason is that the sodium heparin vacutainers use ficoll density centrifugation to isolate the PBMCs from the whole blood while the sodium heparin CPT vacutainers simply use centrifugation.

The results of this thesis research study revealed that in Contrast #1, comparing freshly isolated PBMCs collected in sodium heparin vacutainers versus freshly isolated PBMCs collected in sodium heparin CPT vacutainers 21DEGs were identified. This number is much higher than I had anticipated given the fact that both vacutainer types contain the same preservative; however these results support my hypothesis. The 21 DEGs indicates that PBMCs isolated from these 2 vacutainer types do not have identical gene expression patterns. Even though both vacutainers contain sodium heparin, the sodium heparin CPT vacutainers have at least one known additional solution, i.e., cell separation medium, which may be responsible for the 21 DEGs that were identified but additional research is needed to address exactly what sample handling event was responsible. Likewise, looking at the 21 individual genes that are affected, it is not possible at this time to determine which of these DEGs are significant.

Ruitenberg *et al.* (2006) processed the same vacutainer types that were processed in this thesis research study; sodium heparin CPT vacutainers and sodium heparin vacutainers. Ruitenberg *et al.* (2006) results suggested that with fresh blood samples, samples processed with ficoll density centrifugation had a significantly better viability compared to sodium heparin CPT vacutainers processed PBMCs, but this was the opposite with cryopreserved samples as the sodium heparin CPT processed PBMCs had a better viability than the ficoll density centrifugation processed PBMCs although the difference was not significant. These researchers also concluded that regardless of the vacutainer type used, the fresh and cryopreserved PBMCs recoveries were not significantly different. It was concluded that regardless of whether fresh samples or cryopreserved samples are used, sodium heparin CPT vacutainers are in fact a very effective method for obtaining functionally active PBMCs, at least for HIV seropositive patients.

Ruitenberg *et al.* (2006) focused on cell viability and cell recovery, due to the Vi-CELL malfunctioning my cell viability and cell recoveries were affected so I am unable to make a comparison to their results. Also, Ruitenberg *et al.* (2006) concluded that sodium heparin CPT vacutainers were acceptable to use regardless of if the PBMCs were fresh or cryopreserved. My results revealed that 21 DEGs were identified when comparing fresh PBMCs between both vacutainer types. More research into the 21 DEGs as we are unable to conclude what significance of any these genes have.

Gene Expression Results of Freezing Methods

The results of this thesis research study revealed that zero genes were differentially expressed when comparing thawed PBMCs collected in sodium heparin vacutainers and cryopreserved in a CoolCell device versus thawed PBMCs collected in sodium heparin vacutainers and cryopreserved in a rate-controlled freezer. Similarly, zero genes were differentially expressed when comparing thawed PBMCs collected in sodium heparin CPT vacutainers and cryopreserved in a CoolCell device versus thawed PBMCs collected in sodium heparin CPT vacutainers and cryopreserved in a rate-controlled freezer. These results suggest that the gene expression profiles are not affected regardless if a rate-controlled freezer or a CoolCell device is used . It is important to note that the gene expression profiles were not affected when comparing the same vacutainer type with both freezing methods. We did not look at the following comparisons but we will look at them in the future:

1). Thawed PBMCs collected in sodium heparin CPT vacutainers and cryopreserved in a CoolCell device versus thawed PBMCs collected in sodium heparin vacutainers and cryopreserved in a CoolCell device.

2). Thawed PBMCs collected in sodium heparin CPT vacutainers and cryopreserved in a rate-controlled freezer versus thawed PBMCs collected in sodium heparin vacutainers and cryopreserved in a rate-controlled freezer.

3). Thawed PBMCs collected in sodium heparin CPT vacutainers and cryopreserved in a CoolCell device versus thawed PBMCs collected in sodium heparin vacutainers and cryopreserved in a rate-controlled freezer.

4). Thawed PBMCs collected in sodium heparin CPT vacutainers and cryopreserved in a rate-controlled freezer versus thawed PBMCs collected in sodium heparin vacutainers and cryopreserved in a CoolCell device.

These results were initially promising since using a CoolCell is less time consuming and less expensive to provide these to hundreds of clinical sites. This also suggests that the clinical trial sites using CoolCells will not be negatively affecting the gene expression profiles of the samples that they collect, which is especially critical when dealing with biomarker discovery.

However, in Contrast #2, comparing freshly isolated PBMCs collected in sodium heparin vacutainers versus thawed PBMCs collected in sodium heparin vacutainers and cryopreserved in a CoolCell device, 50 genes were differentially expressed. In Contrast #3, comparing freshly isolated PBMCs collected in sodium heparin vacutainers versus thawed PBMCs collected in sodium heparin vacutainers and cryopreserved in a rate-controlled freezer, 23 genes were differentially expressed. The same 23 DEGs (100% agreement) that occurred when a rate-controlled freezer was used to cryopreserve the PBMCs was part of the 50 DEGs that occurred when the CoolCell was used. This is interesting since both devices cool PBMCs at the same rate of -1^{o}C/minute. Also both Contrast #2 and #3 used PBMCs that were isolated from sodium heparin vacutainers and were processed identically except for the cryopreservation device. This shows how important it is not to perform assays with a mixture of freshly isolated and cryopreserved PBMC samples even though the PBMCs were collected and isolated from the same vacutainer type. Also researchers that need to use cryopreserved PBMCs from multiple studies need to check the sample annotation that resides in a biobank's inventory system

to ensure that the selected PBMCs were cryopreserved using the same freezing device to avoid the differences in possible DEGs.

Similarly in Contrast #4, comparing freshly isolated PBMCs collected in sodium heparin CPT vacutainers versus thawed PBMCs collected in sodium heparin CPT vacutainers and cryopreserved in a CoolCell device, 48 genes were differentially expressed. In Contrast #5, comparing freshly isolated PBMCs collected in sodium heparin CPT vacutainers versus thawed PBMCs collected in sodium heparin CPT vacutainers and cryopreserved in a rate-controlled freezer, 29 genes were differentially expressed. Again, there were fewer DEGs occurring with thawed cryopreserved PBMCs when a rate-controlled freezer was used than when a CoolCell was used.

When the DEGs obtained from Contrast #3 were compared to the DEGs obtained from Contrast #5, there was very good agreement between the 2 sets of PBMCs samples that both used the rate-controlled freezer. Of the 23 DEGs that occurred in Contrast #3, 21 (91% agreement) of these were identical to what occurred in Contrast #5, regardless of which vacutainer was used to collect the whole blood. However, there were no DEGs common between Contrast #1 and Contrasts #3 and #5, suggesting that the DEGs common to Contrasts #3 and #5 were related to the freezing or thawing process. In addition when all Contrasts #2 – #5 were compared, there were 17 of the 23 DEGs that were common (74% agreement). The importance of these DEGs needs further research but may be more cryopreservation relevant.

Lastly, when the freezing devices (rate-controlled freezer and CoolCell) were compared to each other by vacutainer type, there were no DEGs detected (Contrasts #6 and #7). This is in contrast to what was found when Contrasts #2 and #3 were compared

and Contrasts #4 and #5 were compared, which indicated that there were more DEGs identified when a CoolCell was used. It is important to stress that Contrasts #6 & #7 focus on comparing thawed cryopreserved PBMCs to thawed cryopreserved PBMCs while Contrasts #2-#5 focus on comparing freshly isolated PBMCs to thawed cryopreserved PBMCs which could explain the difference in results. This observation needs further investigation. In general, the largest changes in gene expression of the PBMCs occurred from freshly isolated PBMCs when compared between the 2 vacutainer types (Contrast #1).

Interestingly, Lempicki *et al.,* (2013), actually cultured some of their thawed cryopreserved PBMCs for 24 hours before analyzing the gene expression profiles. Lempicki *et al.,* (2013) gene expression analysis revealed that genes in the Cluster 1, 2, and 3, (these genes are involved in defense response), revealed similar gene expression profiles before and after the PBMCs were cultured for 24 hours while genes in cluster 4 and 5, (these genes are enriched and they are involved in general cell metabolism) revealed that the gene expression profiles could be rescued by culturing the PBMCs for 24 hours. Based on the results that Lempicki *et al.,* (2013) found it may be valuable for researchers to consider culturing cryopreserved PBMCs to rescue the gene expression profiles before running the samples on their desired assays. It may be possible that culturing the PBMCs and allowing the gene expression profiles to adjust could help decrease background or cryopreservation affects as compared to when the researchers thaw and run the PBMCs on their assays immediately. Of course, culturing PBMCs may also introduce DEGs that would need to be identified and properly accounted for when interpreting the data.

As part of this study I hypothesized that fewer genes would be affected when freshly isolated PBMCs were compared to sister aliquots that either underwent the freezing process in a rate-controlled freezer or a CoolCell device, all of which were stored for 1 month before being thawed. It seems as though regardless of the vacutainer type, when comparing PBMCs frozen using a CoolCell to Fresh cells about 50 differentially expressed genes are present. Again regardless of the vacutainer type, when comparing PBMCs frozen using a rate-controlled freezer to fresh cells close to 30 genes were differentially expressed. These comparisons suggest that my hypothesis was correct given the fact that that almost twice as many genes were expressed when comparing cells frozen in a CoolCell device to fresh cells versus cells frozen in a rate-controlled freezer to fresh cells.

Comparing Gene Expression in Lempicki *et al*. 2013

I compared all of the differentially expressed genes in my study to the ones expressed in Lempicki *et al*. 2013, and only 3 genes are shared. These 3 genes are LTF, DEFA1 & DEFA4, all of these genes are involved in the defense response. There were a few differences between the way Lempicki *et al*. 2013 and I handled/processed the samples, which could account for the difference in the gene expression profiles. Lempicki *et al*. 2013 froze their samples for 14 months before the gene expression arrays were run on the samples but I only froze the samples for 1 month due to the 9 month time constraint to complete this thesis. All of Lempicki *et al*. 2013 samples were frozen using a rate-controlled freezer whereas I used a CoolCell device and a rate-controlled freezer. Lempicki *et al*. 2013 used a different extraction kit, all of their samples were extracted

with RNeasy Mini Kit without a DNase treatment, this was the kit I initially used but it did not work well for me. Lastly, Lempicki *et al*. 2013 used Acid Citrate Dextrose vacutainers but I used sodium heparin CPT vacutainers and sodium heparin vacutainers because these two vacutainer types are used by Biogen.

Future Experiments

In order to confirm the gene expression data from this study, RT-PCR needs to be performed. Unfortunately, the RT-PCR is currently being performed but we do not have the results yet. However, the genes for the RT-PCR were chosen based on the gene expression results from Lempicki *et al.,* 2013. Therefore the RT-PCR will most likely need to be repeated using a different set of genes since only 3 genes expressed in this research overlapped with the gene expression results from Lempicki *et al.,* 2013.

An additional experiment was performed in which whole blood in CPT tubes was collected, the PBMCs were isolated and then the PBMCs were washed several times using four different wash buffers; PBS only, PBS + 10% FCS, RPMI-1640 only & RPMI-1640 + 10% FCS. Each of Biogen's clinical trials uses a different wash buffer so it is important to the company to determine if the wash buffers being used have an effect on the gene expression profiles. Also it is important to look at the cell viability using the different wash buffers since some media contain more nutrients for cells than others. The gene expression data was recently produced but it still needs to be analyzed.

Lastly, in the future we would like to perform a similar type of experiment but this time we would like to focus on the gene expression profiles of PBMCs when different cryococktails are used for cryopreservation. Each of Biogen's clinical trials uses

a different cryococktail so it is important to the company to determine if the cryococktails being used have an effect on the gene expression profiles. More importantly it would be beneficial for the company to find a cryococktail that minimally alters the gene expression profiles especially when using the samples for biomarker discovery. Some of the different cryococktails we are interested to test are: 1). 72.5% RPMI-1640, 20% FCS & 7.5% DMSO 2). 90% FCS & 10% DMSO.

Conclusions

Overall, the goal of this thesis research study was to provide scientific information using gene expression analysis to establish "Best Practices" for collecting, isolating, processing and cryopreserving human PBMCs. The results of this thesis research study suggest that using fresh cells is better than cryopreserving cells; however this is not typically always an option in the lab. If cryopreservation is needed, CoolCell devices and rate-controlled freezers are both solid options. But if the goal is to have the gene expression profile be as close to the gene expression profile of fresh cells as possible then a rate-control freezer should be used. Lastly, it seems as though sodium heparin CPT vacutainers and sodium heparin vacutainers are both acceptable choices when isolating PBMCs from whole blood. However, based on my gene expression data there are differences that can result and it will be important for the researcher to check the sample processing that was done to determine if it could affect her/his research especially when samples could be obtained across multiple studies whose sample processing is known to be different.

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