Investigating the Human Immune Response with Myriad RBM's TruCulture® System and the NanoString® nCounter® Autoimmune Profiling Panel.

Introduction

Autoimmune Disease is a blanket term used to describe almost 80 unique illnesses that include multiple sclerosis, systemic lupus erythematosus, type 1 diabetes, psoriasis, and rheumatoid arthritis. There is growing evidence showing that inflammation caused by the breakdown of immune checkpoints is a key mechanism in the progression of autoimmunity. Additionally, therapy induced autoimmunity is increasingly being observed as more patients receive checkpoint blockade therapy to activate a suppressed immune response in cancer. Research tools that allow for the study and characterization of autoimmune biology and development of gene signatures that predict an autoimmunity flare or response to a given therapy will be valuable assets for the research community.

A recurring challenge in studying the human immune system is correlating in vitro results to in vivo responses, including modeling the complex and variable pathways of immune activation. This application note describes a protocol demonstrating the feasibility of using Myriad RBM's TruCulture whole blood collection and culture system for secreted protein profiling in conjunction with NanoString's nCounter gene expression analysis platform.

Myriad RBM's TruCulture is an in vitro model system for characterizing the in vivo stimulation and response of circulating immune cells. These immune-phenotyping studies are important for basic research as a tool to characterize immune regulation and dysregulation, as well as for pharmacodynamics studies in drug development to understand drug dosing, safety, and efficacy. TruCulture combines whole blood collection and culture with immunomodulatory agents in a closed system, allowing for retention of all immune cells including mononuclear cells, granulocytes, and platelets along with circulating soluble factors.

TruCulture can be used to investigate immune cell activation under a variety of different culture conditions and/or stimuli and the closed system does not require extensive sample manipulation, specialized equipment or technical expertise; the procedure can be performed at the site of sample collection. Because it retains the cell pellet and culture supernatant, the TruCulture system enables both gene expression profiling and protein secretion analysis.

NanoString's nCounter technology enables gene expression profiling using direct counting of individual RNA transcripts from purified RNA or lysate from fresh frozen tissue, FFPE, PBMCs, whole blood, serum, and other biofluids without enzymatic reactions. RNA is hybridized to oligonucleotides labeled with optical barcodes to enable direct, next-day quantification via digital counting of each barcode. The system can measure up to 800 transcripts over a six-fold $\log_{10}$ dynamic range with high accuracy and reproducibility because it does not rely on enzymatic reactions which can introduce bias. As a result, the nCounter platform is well suited for translational research where disease biomarkers discovered on the bench can be translated into a diagnostic assay in the clinic.

NanoString has developed the nCounter Autoimmune Profiling Gene Expression Panel specifically for the fields of autoimmunity and chronic inflammation. This panel covers 770 human genes encompassing 35 pathways and processes involved in immune system dysfunction such as Treg differentiation and Type I Interferon signaling. This panel includes gene pathways involved in over 30 approved and investigational therapies for autoimmune disease. In addition, the cell profiling feature included in the panel allows for the relative quantification of 14 different immune cell types[1].

The TruCulture-NanoString Solution:

Combining the TruCulture system with nCounter gene expression profiling enables the study of circulating immune responses through rapid and easy collection, stimulation, and subsequent profiling of leukocytes from healthy or diseased donors. Here, we describe transcriptional and proteomic profiling of blood from healthy donors stimulated with a cytokine or pathogen associated molecule. The stimulants chosen activate the same immune pathways that are often dysregulated in autoimmunity: Tumor necrosis factor alpha (TNFα) is a pluripotent cytokine which broadly stimulates acute immune responses and staphylococcal enterotoxin B (SEB) is a potent superantigen capable of antigen-independent stimulation of T cells and antigen presenting cells. This strategy of using TruCulture along with nCounter analysis can be easily adapted to the study of clinical samples from relevant pathological conditions, such as autoimmune disease.

Figure 1. The NanoString nCounter Autoimmune profiling panel covers 770 human genes encompassing 35 pathways and processes associated with autoimmune disease such as inflammatory signaling and tissue stress and damage. The panel includes gene pathways involved in over 30 approved and investigational therapies for autoimmune disease.

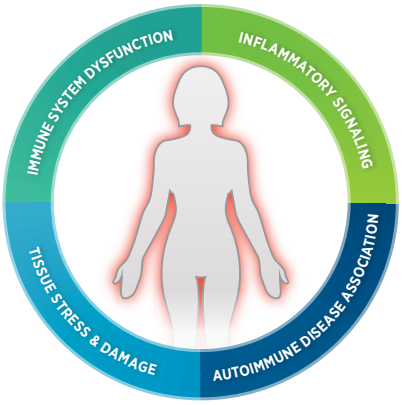
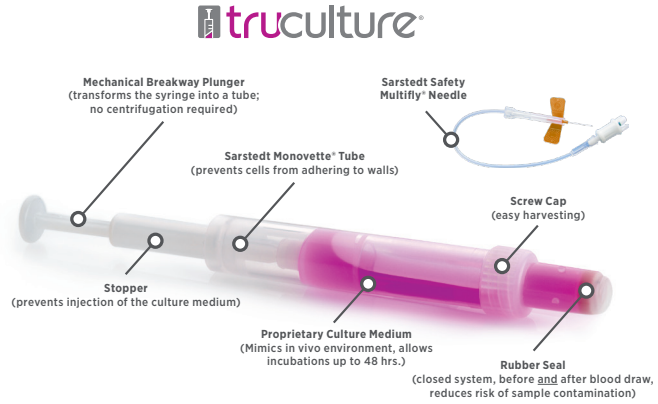


Figure 2. TruCulture System



Methods:

This section describes a workflow for purifying total RNA from TruCulture tubes for analysis with the nCounter Gene Expression Assay. Note that modifications may be necessary depending on deviations in blood volume, sample handling, or RNA purification methodology. A step-by-step reference guide can be found online at **nanostring.com/truculture**.

For this study, 1 mL of blood from each of eight healthy donors was collected in single or duplicate (13 samples in total per treatment) in TruCulture tubes containing the following stimulation: Medium only (no stimulation; Myriad RBM Catalog No. 782-001086), SEB (Myriad RBM Catalog No. 782-001087), and TNFα (Myriad RBM Catalog No. 782-001295). Immediately following blood draw, TruCulture tubes were transferred to a heat block and incubated at 37°C for 24 hours. Following incubation, the supernatant was separated from the cell layer using the Seraplas valve. The supernatants were removed, and secreted proteins analyzed using Myriad RBM's OptiMAP panel (which profiles for IFNγ, IL-2, IL-13, IL-17, TNFα, IL-6, IL-1β, IL-12p70, IL-23, IL-10, GM-CSF, IL-8, and CXCL5 (ENA-78)). After removal of the Seraplas valve, the cell layer was collected and transferred to a 15 mL conical tube containing 3 mL of RNAlater™. The cell samples stored in RNAlater were kept at 4°C until RNA was extracted by pelleting the cells, removing the supernatant, and processing with the Ambion RiboPure (Thermo) kit according to manufacturer's instructions. RNA concentration and purity were assessed by Nanodrop UV spectroscopy and adjusted to a final concentration of 20 ng/µl by dilution with molecular biology grade water as necessary. Eight microliters of each RNA sample (between 40 to 100 ng of total RNA) was hybridized with the nCounter Autoimmune Profiling panel codeset for 16 hours at 65°C. Note that more RNA (up to several hundred nanograms) may be added to the hybridization to detect low expressing genes. Automated processing and data collection were performed using the nCounter MAX system (prep station and digital analyzer). Gene expression data analysis was performed using the nSolver 4.0 software package with the Advanced Analysis module. The Autoimmune Profiling panel includes six positive controls for hybridization, which monitor systemic variability in pipetting and hybridization processing. Also included in the panel are 20 housekeeping genes, which are used by default in the nSolver software to normalize data for variations in RNA input and RNA integrity. The Advanced Analysis module can be used to conduct differential expression analysis, pathway analysis, cell type profiling, and data visualization.

Results:

Table 1 details the total RNA yield and RNA purity from each TruCulture tube. No effect on RNA yield or purity was observed due to the different TruCulture stimulants. The purity of the RNA isolated from TruCulture samples is comparable to traditional cell culture samples and each TruCulture cell sample yielded RNA of sufficient quantity and quality for nCounter analysis.

Table 1. Total RNA yield and purity of extractions from the equivalent of 1 mL whole blood culture in the TruCulture system.

Treatment no. of Replicate Tubes		Total RNA Yield (ng)		A260/A280	
		Mean	Range (min-max)	Mean	Range (min-max)
Null	13	1004.1	433.2-2649.0	1.73	1.50-1.95
SEB	13	824.7	487.4-1788.6	1.73	1.47-1.89
TNFa	13	991.0	352.1-4193.9	1.70	1.33-1.97

The TruCulture system is designed to minimize technical variability of whole blood culture. RNA analysis of human leukocytes is often hampered not only by the inherent variability between individuals but also by the variability introduced by extensive laboratory manipulations needed for in vitro cultures. By culturing whole human blood in the TruCulture system, these potential variables are minimized. This ability of TruCulture to minimize baseline differences in the immune response allows for pathway expression analysis that may have gone unnoticed. Figure 3 demonstrates consistent nCounter gene expression analysis from replicate TruCulture tubes.

Figure 3. Transcript counts from replicates of TNFα-treated TruCulture tubes from a single donor, generated using the NanoString nCounter Autoimmune Profiling panel, illustrating the high reproducibility of the NanoString platform.

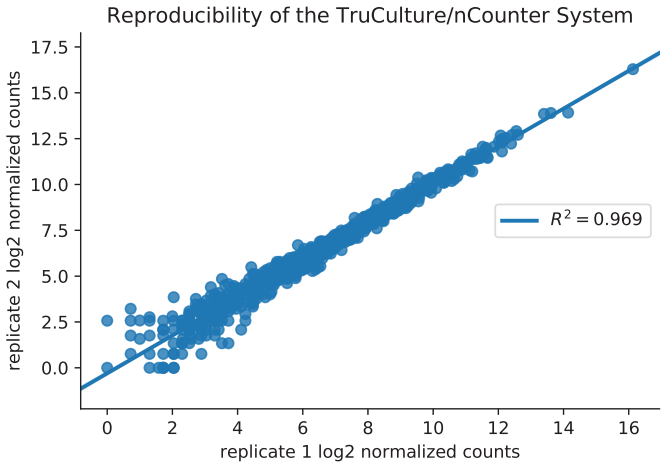
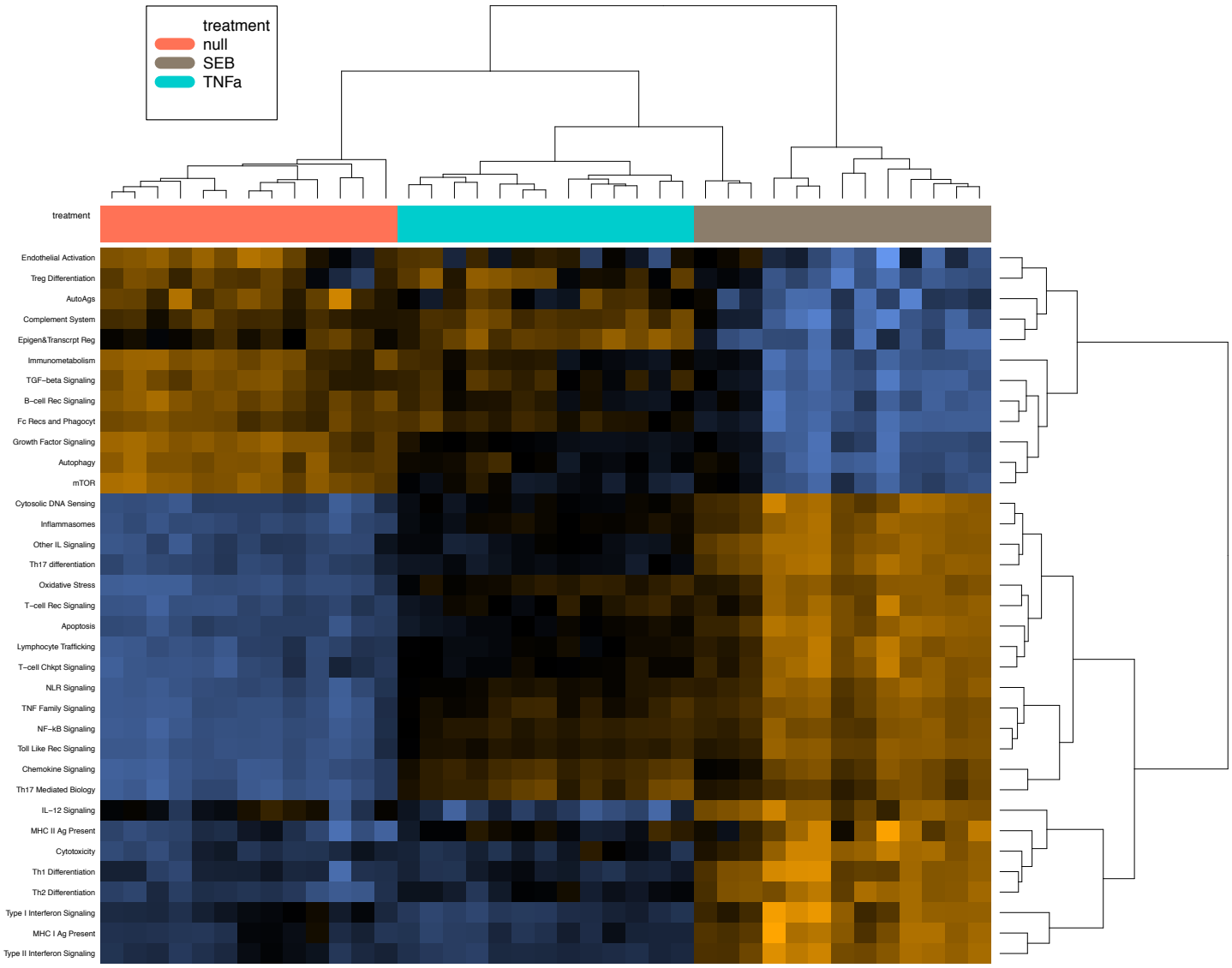


Figure 4 shows a gene expression pathway heatmap of data generated using the NanoString Autoimmune Profiling panel organized by unsupervised clustering. Orange and blue colors indicate relative up and down regulation, respectively, of a given pathway within the sample set. SEB and TNFα treatments produce distinct pathway expression profiles compared to null, and samples are clustered based on the biological responses to each treatment condition. Upon SEB stimulation, several regulatory pathways such as mTOR, TGF-β, and Treg are downregulated.

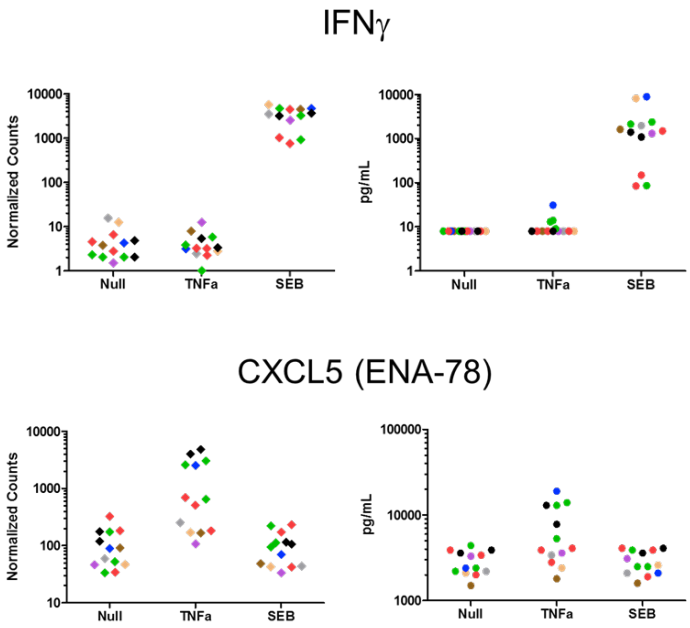
Interestingly, samples stimulated with TNFα show a mixed expression response. Notably, while SEB stimulation led to upregulation in mostly inflammatory (type 1 and 2 interferon signaling, inflammasome) and T cell activation pathways (Th1, 2, and 17 differentiation, IL-12, MHC Class I and Class II Antigen Presentation), these pathways are downregulated in TNFα stimulated samples. This correlates to the known mechanisms of SEB action, as the SEB endotoxin mimics MHC/TCR dependent T cell activation.

Figure 4. Gene expression pathway heatmap of data generated using the nCounter Autoimmune Profiling Panel, organized by unsupervised clustering.



For analysis of secreted proteins, TruCulture supernatants were analyzed using Myriad RBM's OptiMAP panel, a 13-analyte panel developed using Myriad RBM's Multi-Analyte Profiling platform. Figure 5 shows representative OptiMAP and NanoString results for two proteins and their respective transcripts: IFN-γ and CXCL5 (ENA-78). There is a clear correlation between the transcript counts (left panels) and the concentration of protein (right panels). Whole blood samples stimulated with TNFα induced high expression of CXCL5 RNA that correlates with an increased concentration of the secreted protein. Similarly, samples stimulated with SEB show high expression of IFN-γ RNA and this is reflected in the increased IFN-γ protein measured in the supernatant. In general, the concentration of expressed protein measured by the OptiMAP panel correlated well with the respective transcript counts measured with the nCounter Autoimmune Profiling Panel, suggesting that the two methods corroborate each other and can be used in conjunction to profile immune responses.

Figure 5. Normalized transcript counts (left panels) generated with the nCounter Autoimmune Profiling Panel and supernatant concentration (in pg/1mL whole blood) of secreted protein (right panels) generated with Myriad RBM's OptiMAP panel from whole blood stimulated with either null (medium only), TNFα, or SEB. Each subject is designated by a different color.



Conclusions:

Recent publications have shown the utility of the TruCulture system paired with nCounter gene expression analysis to describe the human immune response to immunostimulatory agents including Toll-like receptor (TLR) agonists and microorganisms[2,3]. This application note describes an easy to use, reproducible workflow for studying immune responses from whole blood using the Myriad RBM TruCulture system and NanoString gene expression analysis with the nCounter Autoimmune Profiling panel. The results showed excellent technical reproducibility and correlation between mRNA and protein. With minimal hands-on time, whole blood can be collected, treated, and processed to identify immune signatures for different stimuli. This culture and profiling system will have applications to a variety of research fields, including immunology, autoimmune disease and immuno-oncology.

References:

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