Gene Expression Analysis of RBM's TruCulture® Samples with the NanoString® nCounter® Panel

Introduction

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 the TruCulture whole blood collection and culture system from Rules-Based Medicine (RBM), a Q² Solutions Company, in conjunction with NanoString's nCounter gene expression analysis platform.

RBM's TruCulture is an *in vitro* system *(Figure 1)* 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 can be used to investigate immune cell activation under a wide variety of 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 analysis of secreted proteins.

NanoString's nCounter technology enables gene expression profiling using direct counting of individual RNA transcripts 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 log10 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.

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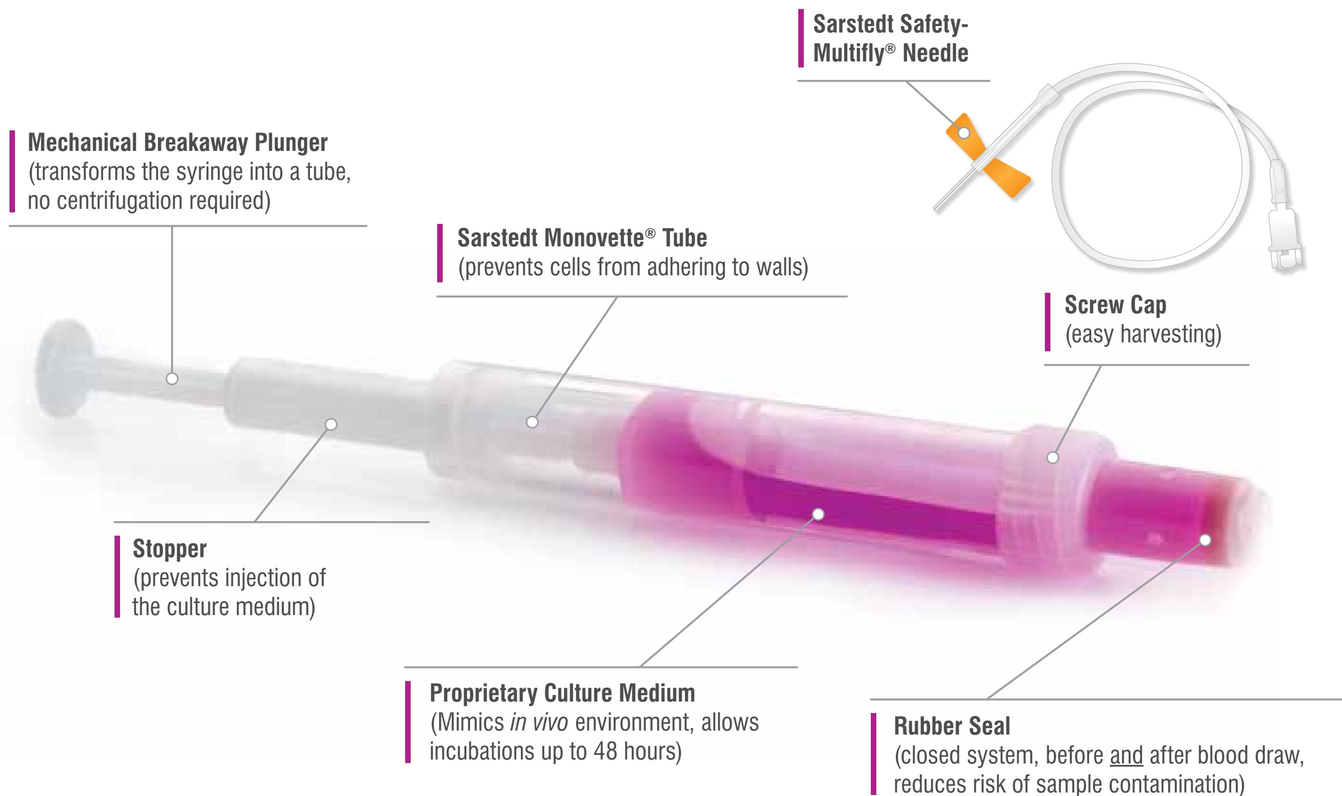
Figure 1: TruCulture system



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. 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.

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 RNA purification methodology. A step-by-step reference guide can be found online at rbm.q2labsolutions.com/products-services/truculture/.

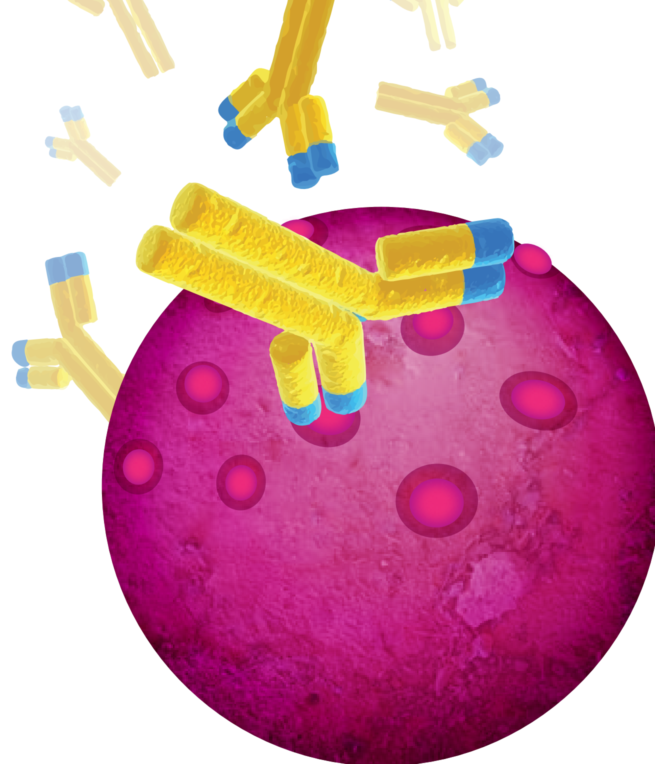
This application note describes transcriptional and proteomic profiling from TruCulture samples of healthy donors. The nCounter Autoimmune Profiling Gene Expression Panel developed by NanoString specifically for the fields of autoimmunity and chronic inflammation. This panel covers 770 human genes encompassing 35 pathways and processes involved in immune system function such as Treg differentiation and Type I Interferon signaling. For TruCulture, 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.

Protocol: From each of eight healthy donors, 1mL of whole blood was collected in single or duplicate (13 samples in total per treatment) TruCulture tubes containing the following stimulation: Null (medium only, no stimulation; RBM Catalog No. 782-001086), SEB (RBM Catalog No. 782-001087), and TNFα (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 RBM's OptiMAP panel (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.



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 2* demonstrates consistent nCounter gene expression analysis from replicate TruCulture tubes.

Table 1

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

Treatment number 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	1.5-1.95
SEB	13	824.7	487.4-1788.6	1.73	1.47-1.89
TNFα	13	991.0	352.1-4193.9	1.70	1.33-1.97

Figure 2

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 TruCulture and NanoString platforms.

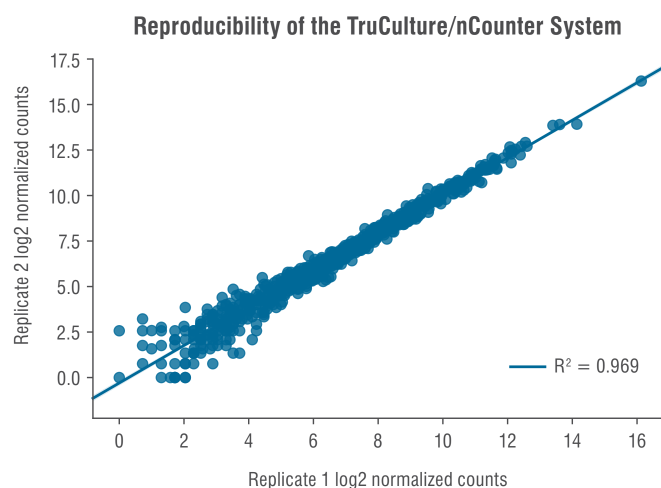
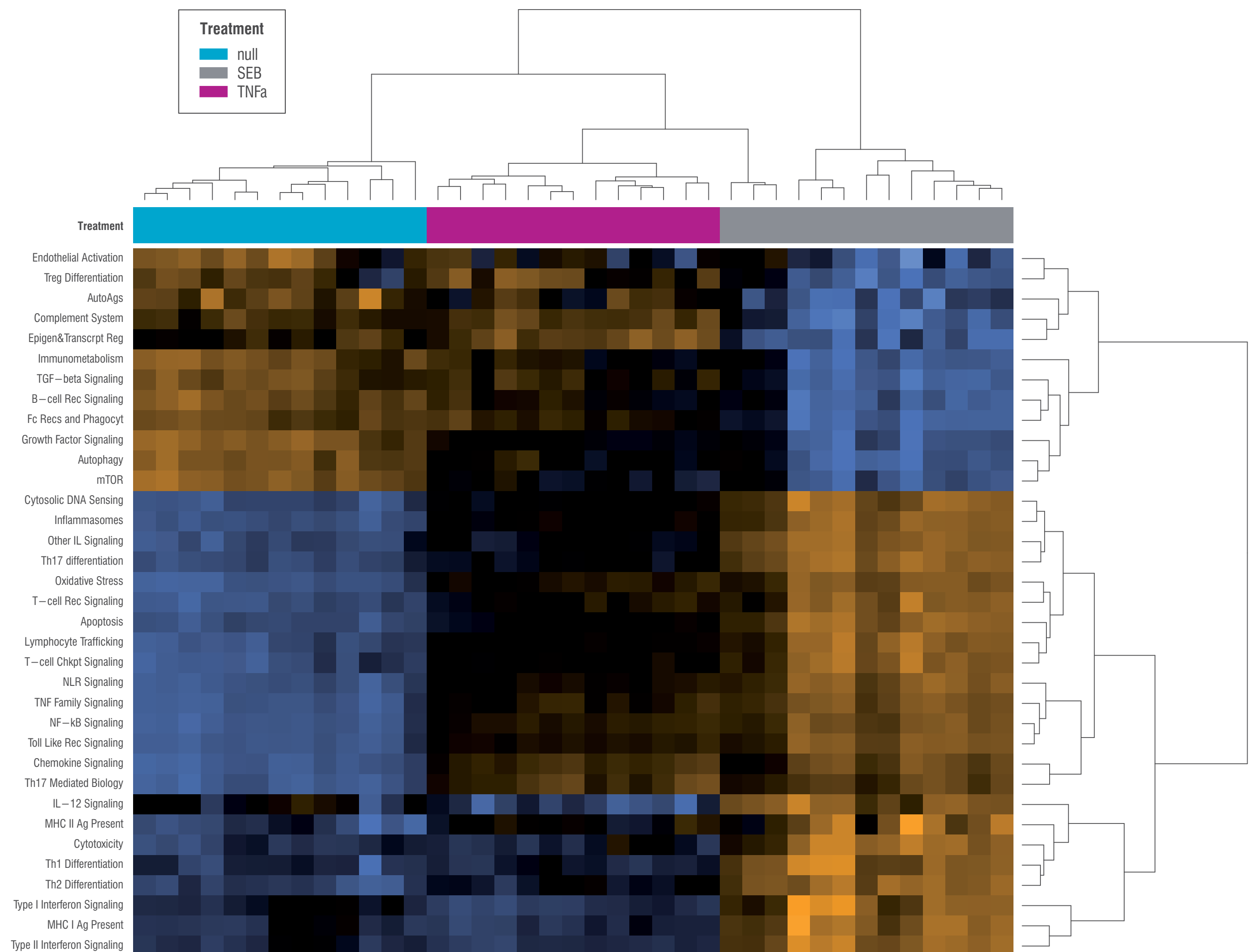


Figure 3 shows a gene expression pathway heatmap of data generated using the NanoString Autoimmune Profiling panel organized by unsupervised clustering. Orange and blue colors indicate relative up and down regulation, respectively, of a given pathway within the sample set. SEB and TNFα stimulations produce distinct pathway expression profiles compared to null, and samples are clustered based on the biological responses to each treatment condition. Upon SEB stimulation, several pathways such as mTOR, TGFβ, 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, class I and II 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 3

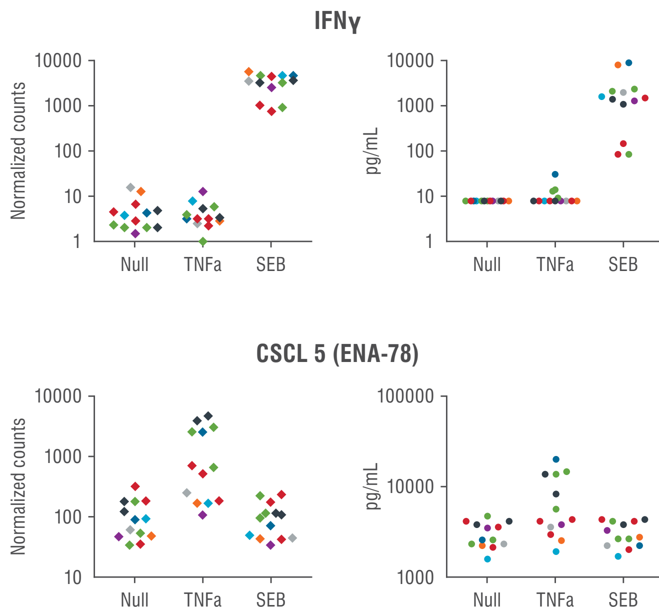
Gene expression pathway heatmap of data generated using the nCounter Autoimmune Profiling Panel, organized by unsupervised clustering.



For analysis of secreted proteins, TruCulture supernatants were processed using RBM's OptiMAP panel, a 13-analyte panel developed using RBM's Multi-Analyte Profiling platform. *Figure 4* shows representative OptiMAP and NanoString results for two proteins and their respective transcripts: IFNγ 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 had high expression of CXCL5 RNA that correlates with an increased concentration of the secreted protein. Similarly, samples stimulated with SEB show high expression of IFNγ RNA and this is reflected in the increased IFNγ 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 4

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



Conclusion

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 RBM TruCulture system and NanoString gene expression analysis with the nCounter assay 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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