WHITEPAPER

Uncovering the Tumor-Immune System Interaction Using the nCounter® Vantage 3D™ RNA:Protein Immune Cell Profiling Assay

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Introduction

The ability of mutated cells to give rise to cancer relies upon their capability to interact with cells of the immune system and ultimately evade immune recognition, suppress immune activity, and persist in a chronically inflamed environment[1,2]. Many immune cell types are found in the tumor microenvironment, creating a complex milieu that affects the growth and evolution of cancerous cells[3-5]. Robust immune monitoring assays are essential for characterizing the immune status of cancer patients, and insights gained from studying the immune response in treated patients will help transition promising research into the clinic.

Tremendous growth in the understanding of the immune response to cancer is beginning to generate breakthroughs in cancer treatment[6]. However, our understanding of immuno-oncology is far from complete. There is a need for tools that better enable researchers to explore the complex relationship between the immune system and primary tumors — in both the tumor microenvironment and in peripheral blood — as well as to robustly monitor changes in the immune response associated with potential therapeutic approaches. Transcriptionally active genes and their respective protein products are important molecular indicators of the physiological state of cell populations[7]. As such, expression profiling of genomic and proteomic markers is critical for identifying relevant immune resistance mechanisms in the tumor microenvironment and developing predictive biomarkers for active immunotherapy[8,9].

This white paper describes RNA:Protein reagents for profiling the human immune response to tumors and for developing new therapeutic approaches and predictive biomarker signatures for immunotherapeutic treatment response. The nCounter® Vantage 3D™ RNA:Protein Immune Cell Profiling Assay is highly multiplexed and designed to quantitate mRNA and proteins using low levels of sample input. These assays detect 770 mRNAs and up to 30 proteins and are powered by 3D Biology™ technology for simultaneous detection of multiple analyte types.

Applications for these products include:

1. Identification and quantification of immune cells, such as those infiltrating a tumor or in a population of peripheral blood mononuclear cells (PBMCs).

2. Assessment of immunological function and response to immuno-modulation, such as checkpoint inhibitors

3. Identification and quantification of tumor-specific antigens, such as cancer-testis (CT) antigens.

4. Longitudinal monitoring of response to immune-modulating cytokine treatments.

A systems biology approach capable of capturing the functional behavior of multiple cell types that interact within the human immune system and tumor microenvironment is necessary to provide a comprehensive understanding of the complex mechanisms responsible for tumor immune responses and tolerance. Biomarkers will allow for optimization of potential immuno-oncology therapeutics providing mechanistic and patient selection information.

Digital Measurement of Gene and Protein Expression

Specific hybridization of fluorescent barcodes to nucleic acid targets in solution is the foundation of NanoString's single-molecule detection technology[10]. Over 1000 publications have shown this

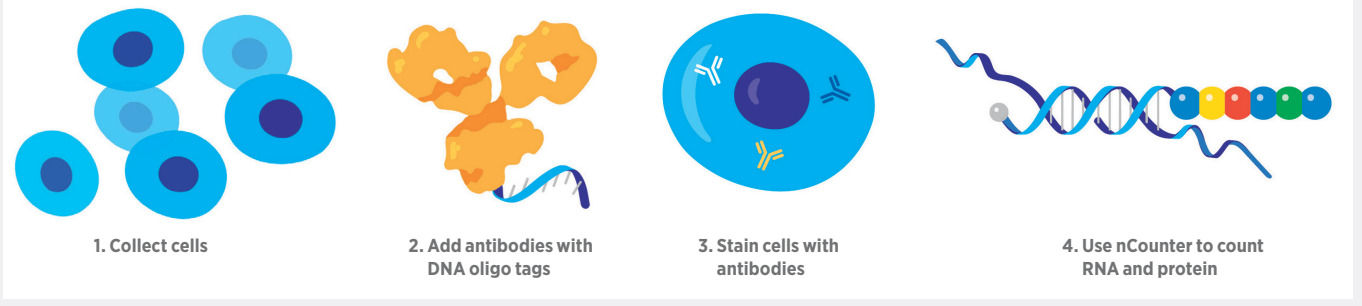


FIGURE 1: Illustration of mRNA and protein detection and measurement using nCounter technology. Antibodies are covalently attached to unique DNA tags to enable their detection by nCounter probes. RNA and protein fractions are combined in a single hybridization reaction for simultaneous RNA:Protein profiling.

technology is reliable for gene expression analysis. Recently, this technology was adapted by Ullal *et al.* (2014; Sci Transl Med) to enable detection of proteins via oligonucleotide-labeled antibodies (**FIGURE 1**)[11]. NanoString has further refined this technique by combining RNA and protein detection into a single assay, referred to as 3D Biology. RNA:Protein profiling enables specific, multiplexed detection of 30 protein targets in conjunction with up to 770 mRNA targets for quantitative assessments from limited samples (**APPENDIX A**). Additionally, as both protein and nucleic acid measurements are provided as digital nCounter® counts, data analysis and comparison of mRNA and protein expression is simplified.

Investigating the Cancer Immunity Cycle with nCounter Vantage 3D RNA:Protein Immune Cell Profiling

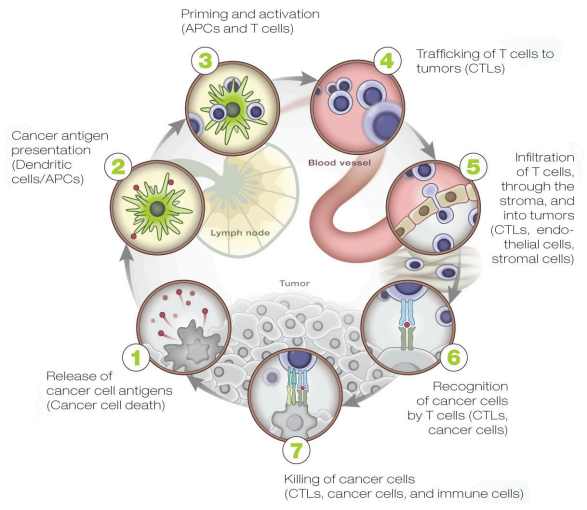
In their seminal review, Chen and Mellman describe a series of steps (**FIGURE 2**) that must be initiated and fostered for an anticancer immune response to effectively kill cancer cells, dubbing these steps the Cancer Immunity Cycle[12]. This cycle begins with the release of antigens by cancer cells. Released antigens are processed and then presented, which results in priming and activation of the adaptive immune response. Once activated, T cells traffic to the tumor, infiltrate, and recognize tumor cells, ultimately leading to their programmed destruction. When cancer cells are killed by invading T cells, additional antigens are released into the periphery and start the cycle anew.

Movement through this cycle is controlled by a key set of master regulators, collectively known as checkpoint regulators[13]. The nCounter Vantage 3D RNA:Protein Immune Cell Profiling Assay allows monitoring of these key regulatory elements via both gene expression and extracellular proteomic markers (**FIGURE 2**). Many of the steps of the cancer-immunity cycle are therapeutically exploitable as shown by the significant clinical benefit observed with checkpoint inhibitors (such as anti-PD-1/PD-L1 and CTLA-4)[14] in patients with melanoma and other solid tumors[15].

Identifying and measuring discrete cell populations within heterogeneous samples has been a focus of immunological study for decades. The classification and enumeration of immune cells via histopathological and flow cytometric analyses within tumor samples and in the peripheral blood has been shown to be a significant and powerful predictor of patient survival[16,17]. By including cell subset-specific surface markers as part of the assay (**APPENDIX B**), the nCounter Vantage 3D RNA:Protein Immune Cell Profiling Assay can efficiently measure both cell types and functions as well as follow their changes under therapeutic pressure.

Correlation of nCounter Protein Measurements with Flow Cytometry

We compared the digital protein counts to flow cytometry, a commonly used technology for measuring protein expression in cell suspensions. To characterize and validate the nCounter protein



Proteins in the nCounter Vantage 3D RNA:Protein Immune Cell Profiling Assay	
Stage of Cancer Immunity Cycle	Proteins in the nCounter Vantage 3D RNA:Protein Immune Cell Profiling Assay
(2) Antigen Presentation	CD4, CD40, CD154 (CD40L)
(3) Priming and Activation	CD279 (PD-1), CD274 (PD-L1), CD273 (PD-L2), CD25 (IL2R), CD56 (NCAM), CD357 (GITR), CD137 (OX40), CD27, CD28, CD127, CD137 (4-1BB)
(4-5) Trafficking and Infiltration	CD9
(6-7) Recognition and Killing of Cancer Cells	CD279 (PD-1), CD274 (PD-L1), CD273 (PD-L2), CD272 (BTLA), HLA-DRA
Immune Modulation	CD279 (PD-1), CD278 (ICOS), CD158e1 (KIR3DL1), CD335 (NKp46), CD152 (CTLA-4), CD3 (CD3E), CD8 (CD8A), CD14, CD19, CD33, CD68, CD163

FIGURE 2: Illustration of the Cancer Immunity Cycle and the associated proteins with each stage that are included in the nCounter Vantage 3D RNA:Protein Immune Cell Profiling Assay. Illustration used with permission and originally published in: Chen, D.S, Mellman, I. (2013), author update (2015) Oncology Meets Immunology: the Cancer-Immunity Cycle. Immunity 39(1):1-10.

detection technology, three different samples were produced by transfecting HEK293T cells with ten different expression plasmids that each correspond to proteins recognized by the nCounter Vantage 3D RNA:Protein Immune Cell Profiling Assay. Each sample was transfected with varying amounts of plasmid DNA, creating a mixed population of cells expressing the target proteins. Protein expression was measured in single-plex assays using flow cytometry and in a multiplexed assay using nCounter (**FIGURE 3**). The percentage of cells expressing each protein in flow cytometry and the digital counts of protein expression from the nCounter Analysis System were compared between the samples. Fold-change correlation was high between the two platforms (R_2 > 0.94).

Precise, Simultaneous Quantification in Multiplexed Format

In order to assess reproducibility and precision for the nCounter Vantage 3D RNA:Protein Immune Cell Profiling Assay, NanoString early access customers compared results across multiple users and sites (**FIGURE 4**). Gene and protein expression levels for 800 measurements were highly correlated in each example and highly reproducible.

Cell Profiling Application: Dynamic View of Leukocyte Differentiation

To illustrate the power of simultaneous RNA:Protein detection, time-course experiments for monocyte differentiation into macrophages were performed using a monocyte cell line THP1, phorbol myristate acetate (PMA) as an inducer of in vitro differentiation, and the nCounter Vantage 3D RNA:Protein Immune Cell Profiling Assay. When comparing routine gene and protein expression correlation at a single time-point (**FIGURE 5A**) with temporal expression profiles (**FIGURE 5B**), the digital counts vary by analyte type. When examining a single time-point, protein and gene expression levels appear to be concordant. While this correlation holds true for CD40 across all time point, CD4 and CD9 expression levels bifurcate at 48 hours and beyond, which may be evidence of differences between transcriptional and translational responses to PMA treatment.

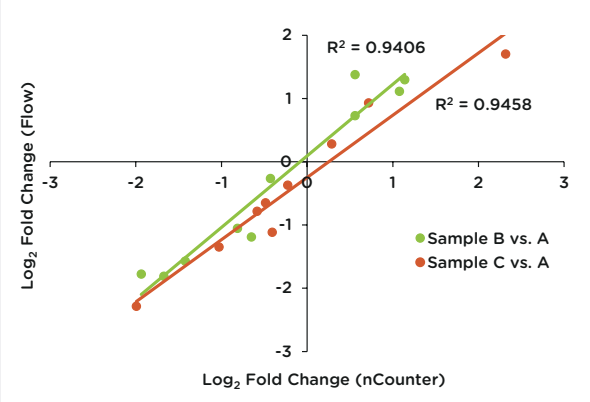


FIGURE 3: Correlations in protein signal fold-change between flow cytometry and nCounter assay. Ten proteins in three samples types with varying expression were assayed. Sample A expressed all proteins at similar levels, while Samples B and C expressed the same proteins at variable levels. Fold-change expression for Sample B vs. A (green) and Sample C vs. A (orange) are plotted. Flow cytometry data were averaged across 3 replicates; nCounter data were averaged across 6 replicates. CD127 data were omitted due to expression below the detection level in flow cytometry and low counts using nCounter.

Conclusion

For the first time, researchers can quickly assess the key regulatory molecules and pathways of cancer immunity, measuring gene and protein expression simultaneously in a single, simple-to-use assay. The nCounter Vantage 3D RNA:Protein Immune Cell Assays permit sensitive and quantitative investigation of fundamental immunological processes at both the transcriptional and translational level. Combining measurements onto one platform greatly reduces complexity in data analysis, and as few as cells are required for read out, permitting analysis on precious, clinically relevant samples.

The data presented here demonstrate the reliability of nCounter technology for measuring protein expression in limited samples. The precision of RNA:Protein profiling technology and its concordance with the gold standard of flow cytometry is preserved (**FIGURE 3)**. When applied to biological samples, RNA:Protein profiling offers information on both RNA and protein expression using the same digital platform and demonstrates precision for replicates generated by a single user and across several users (**FIGURE 4**). Expression data from these assays has the potential to promote a better understanding of the interactions between the host immune system and tumor microenvironment and to identify novel biomarkers allowing for optimization of potential immuno-oncology therapeutics providing a mechanistic and stratification information.

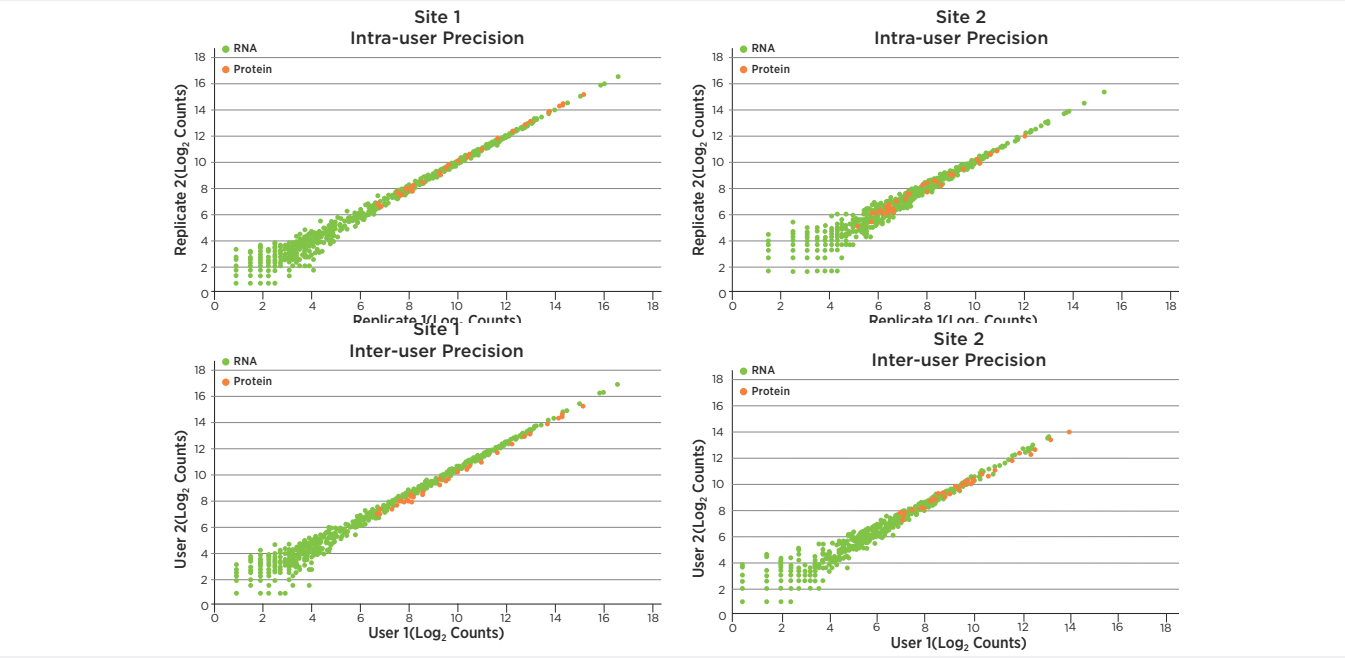


FIGURE 4: Precision intra-and inter-user data demonstrated for 800 RNA and protein measurements. Data were generated across two external sites with four different users to gauge precision and reproducibility for the assay. Site 1 used PBMCs to measure gene and protein expression between a single user (A) and two different users (C). Site 2 looked at CD3-stimulated T cells between a single user (B) and two different users (D).

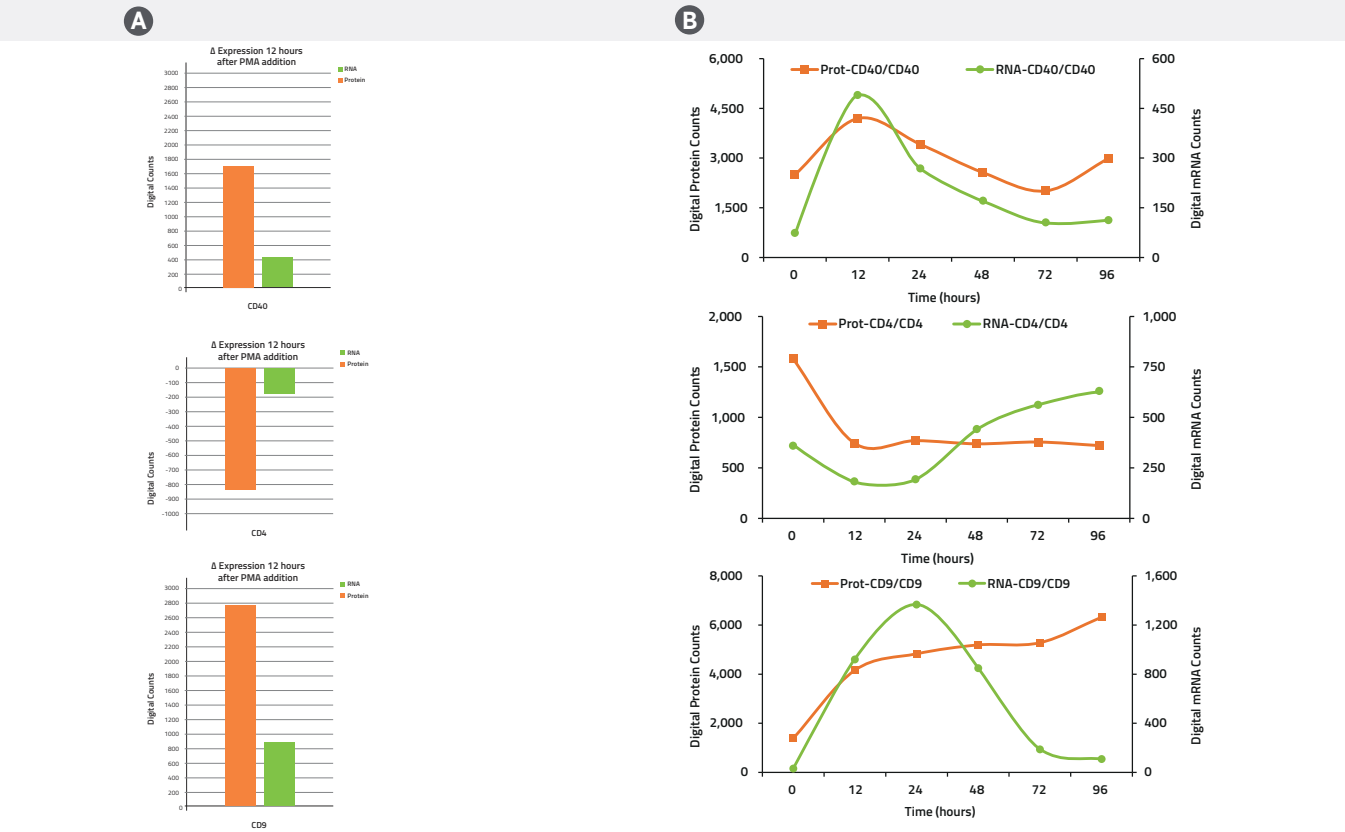


FIGURE 5: Comparison of static and dynamic expression illustrates relationships between gene and protein expression in monocytes. Monocytes (THP-1 cell lines) were stimulated with PMA for 12–96 hours to induce differentiation into macrophages. Data normalization was accomplished using ERCC controls and then using the geometric mean of proteins and genes. Protein background was determined by comparing to normalized data for IgG isotype controls. (A) Bar charts show differential expression of genes and proteins after 12 hours for each target. (B) Line graphs show time-course data from 0 to 96 hours, giving a deeper view of the changes in target co-expression.

Appendix A: Immune Cell Types and Genes Included in the nCounter Vantage 3D RNA:Protein Immune Cell Profiling Assay

	Cell Type			Description	Panel Genes
Adaptive Immune Response	B Cells			Perform several roles, including generating and presenting antibodies, cytokine production, and lymphoid tissue organization.	BLK, CD19 (CD19), CR2 (CD21), HLA-DOB, MS4A1 (CD20), TNFRSF17 (CD269)
	T Cells			Play a central role in immunity and distinguished from other lymphocytes (*e.g.*, B cells) by the presence of a T cell receptor (TCR) on the cell surface.	CD2, CD3E, CD3G, CD6
		Helper T Cells		A subset of CD3+CD4+ effector T cells that secrete cytokines with different activities.	ANP32B (APRIL), BATF, NUP107, CD28 (CD28), ICOS (CD278)
			T_H1	Produce IL-2 and IFNγ and promote cellular immunity by acting on CD8+ cytotoxic T cells, NK cells and macrophages.	CD38, CSF2 (GM-CSF), IFNG, IL12RB2, LTA, CTLA4 (CD152), TXB21, STAT4
			T_H2	Produce IL-4, IL-5 and IL-13 and promote humoral immunity by acting on B cells.	CXCR6 (CD186), GATA3, IL26, LAIR2 (CD306), PMCH, SMAD2, STAT6
			T_H17	Produce IL-17A, IL-17F, IL-21 and IL-22 and promote anti-microbial inflammation.	IL17A, IL17RA (CD217), RORC
			T_{FH}	Trigger the formation and maintenance of germinal centers through the expression of CD40L and the secretion of IL-21 and IL-4, thereby playing a critical role in mediating the selection and survival of B cells.	CXCL13, MAF, PDCD1 (CD279), BCL6
		Regulatory T Cells (T_{reg})		CD3+CD4+ T cells that inhibit effector B and T cells and play a central role in suppression of autoimmune responses.	FOXP3
		Memory T Cells	T_{cm} (central memory)	Educated T cells that rapidly respond to antigen. Central memory T cells express L-selectin and CCR7; they secrete IL-2, but not IFNγ or IL-4.	ATM, DOCK9, NEFL, REPS1, USP9Y
			T_{em} (effector memory)	Educated T cells that rapidly respond to antigen. Effector memory T cells do not express L-selectin or CCR7; they secrete effector cytokines like IFNγ and IL-4.	AKT3, CCR2 (CD192), EWSR1 (EWS), LTK, NFATC4
		Cytotoxic (CD8) T Cells		Effector T cells with cytotoxic granules that interact with target cells expressing cognate antigen and promote apoptosis of target cells.	CD8A (CD8), CD8B (CD8B), FLT3LG, GZMM (MET1), PRF1
		Gamma Delta T Cells (Tγδ)		Express surface antigen recognition complex type 2 and represent a small percentage of the peripheral T cell population. Functions span innate and adaptive immune responses, including direct cytolysis and establishment of memory phenotypes.	CD160, FEZ1, TARP (TCRG)
		Natural Killer (NK) Cells		Provide a rapid cytotoxic response to virally infected cells and tumors. These cells also play a role in the adaptive immune response by readily adjusting to the immediate environment and formulating antigen-specific immunological memory.	BCL2, FUT5, NCR1 (CD335), ZNF205
			$CD56_{bright}$	Constitute the majority of NK cells in secondary lymphoid tissues. Abundant cytokine producers and weakly cytotoxic before activation.	FOXJ1, MPPED1, PLA2G6, RRAD
			$CD56_{dim}$	Constitute the majority of NK cells in the periphery and are more cytotoxic than $CD56_{bright}$ cells.	GTF3C1, GZMB, IL21R (CD360)
Innate Immune Response	Dendritic Cells	Conventional (Myeloid) Dendritic Cells (DC)		Cells that process antigen material and present it on the cell surface to T cells, thereby acting as messengers between the innate and adaptive immune systems.	CCL13, CCL17, CCL22 (MDC), CD209 (CD209), HSD11B1
			iDC (immature)	Play a critical role in initiating tumor immunity. Tumor cells can exploit the functional roles of iDCs for tumor progression via release of soluble factors such as VEGF.	CD1A, CD1B, CD1E, F13A1, SYT17
			aDC (activated)	Promote the induction of the adaptive immune response by presenting captured antigen to naive T cells.	CCL1, EBI3, IDO1 (INDO), LAMP3 (CD208), OAS3
		Plasmacytoid Dendritic Cells (pDC)		Similar in appearance to plasma cells and share many characteristics with myeloid dendritic cells. These cells can produce high levels of IFNα.	IL3RA (CD123)
	Macrophages			Scavengers of dead or dying cells and cellular debris. Macrophages have roles in innate immunity by secreting pro-inflammatory and anti-inflammatory cytokines.	APOE, CCL7 (FIC), CD68, CHIT1, CXCL5, MARCO, MSR1 (CD204)
	Granulocytes	Mast Cells		Granulocytes that can influence tumor cell proliferation and invasion and promote organization of the tumor microenvironment by modulating the immune response.	CMA1, CTSG, KIT (CD117), MS4A2, PRG2, TPSAB1
		Neutrophils		Phagocytic granulocytes that act as first-responders and migrate towards a site of inflammation. Typically a hallmark of acute inflammation.	CSF3R (CD114), FPR2, MME (CD10)
		Eosinophils		White blood cells responsible for combating multicellular parasites and some types of infections.	CCR3 (CD193), IL5RA (CD125), PTGDR2 (CD294; GPR44), SMPD3, THBS1

Appendix B: Proteins Included in the nCounter Vantage 3D RNA:Protein Immune Cell Profiling Assay

Protein Name	Immunological Activity
CD3 (CD3E)	Member of the T cell receptor-CD3 complex, which plays an important role in coupling antigen recognition to several intracellular signal transduction pathways. The CD3 complex is found on the surface of all mature T cells.
CD4	Glycoprotein found on the surface of T helper cells, monocytes, macrophages, and dendritic cells. Acts as a co-receptor that amplifies TCR signaling via recruitment of Lck. It can also interact directly with MHC class II molecules on the surface of antigen-presenting cells.
CD8 (CD8A)	Cytotoxic T lymphocyte cell surface glycoprotein that mediates cell-cell interactions between MHC class I antigen-presenting cells and T cells.
CD9	Marker of naïve lymphocyte populations with roles in cell adhesion and migration.
CD14	Co-receptor expressed by macrophages, neutrophils, and dendritic cells that — along with TLR4 and MD-2 — is responsible for detection of pathogen-associated molecules such as lipopolysaccharide and lipoteichoic acid.
CD19	Expressed on the surface of dendritic cells and B cells, this surface molecule assembles with antigen receptors in order to decrease the threshold for antigen receptor-dependent stimulation.
CD25 (IL2R)	Expressed by lymphocytes, this receptor acts along with its ligand, IL2, to promote the differentiation of T cells into effector T cells and memory T cells after an antigen-mediated initial T cell response.
CD27	Member of TNF-receptor superfamily and required for the generation and long-term maintenance of T cell immunity via binding of its ligand, CD70. Known to play a role in regulating B cell activation and immunoglobulin synthesis.
CD28	Receptor for CD80 and CD86 proteins. Expressed on T cells that provide co-stimulatory signals required for T cell activation and survival.
CD33	Transmembrane receptor that binds sialic acids and a myeloid lineage cell marker.
CD40	Broadly expressed co-stimulatory protein found on antigen-presenting cells that is required for their activation, typically via binding of CD40L. This member of the TNF receptor superfamily is essential in mediating a variety of immune and inflammatory responses including T cell-dependent immunoglobulin class switching, memory B cell development, and germinal center formation.
CD45RO	Protein tyrosine phosphatase receptor specifically expressed in hematopoietic cells and shown to be an essential regulator of T cell and B cell antigen receptor signaling.
CD56 (NCAM)	Homophilic binding glycoprotein expressed on the surface of natural killer cells.
CD68	Glycoprotein expressed on monocytes and macrophages that binds to low density lipoprotein and serves as a marker of these lineages.
CD73 (NT5E)	Frequently used marker of lymphocyte differentiation. This protein catalyzes the conversion of AMP to adenosine.
CD127 (IL-7Ra)	Expressed on many immune cell types including B cells, T cells, monocytes, and dendritic cells, this receptor and its ligand, IL7, play a critical role in lymphocyte development.
CD134 (OX40)	Secondary costimulatory molecule expressed on activated antigen-presenting cells that plays a critical role in maintaining an immune response by enhancing the survival of T cells.
CD137 (4-1BB)	Expressed by activated T cells, dendritic cells, follicular dendritic cells, natural killer cells, granulocytes, and cells of blood vessel walls at sites of inflammation. Known to possess a co-stimulatory role in activated T cells, enhancing T cell proliferation and cytolytic activity.
CD152 (CTLA-4)	Regulatory protein expressed on the surface of helper T cells. Downregulates the immune response by sending an inhibitory signal to T cells.
CD154 (CD40L)	TNF superfamily protein primarily expressed on activated T cells. Binds to CD40 on antigen-presenting cells, often in a co-stimulatory role.
CD158e1 (KIR3DL1)	Transmembrane glycoprotein expressed by natural killer cells and subsets of T cells.
CD163	Receptor for the hemoglobin-haptoglobin complex that has been shown to mark cells of the monocyte and macrophage lineages.
CD272 (BTLA)	Induced during T cell activation, this regulatory protein acts to negatively regulate T cell immune responses. Ligand for TNFRSF14 (HVEM).
CD273 (PD-L2)	Co-stimulatory molecule essential for T cell proliferation and interferon-gamma production in a PDCD1-independent manner. Interaction with PDCD1 inhibits T cell proliferation by blocking cell cycle progression and cytokine production.
CD274 (PD-L1)	A regulatory protein ligand that modulates activation or inhibition of T cells, B cells, and myeloid cells. Reduces the proliferation of these CD8+ T cells via interaction with PD-1 and B7-1.
CD278 (ICOS)	Co-stimulatory molecule expressed on activated T cells that is thought to be particularly important for Th2 cell function.
CD279 (PD-1)	Promotes self-tolerance by stimulating apoptosis in antigen-specific T cells and reducing apoptosis in regulatory T cells.
CD335 (NKp46)	Triggering receptor expressed on the surface of activated natural killer cells.
CD357 (GITR)	Tumor necrosis factor receptor shown to have increased expression upon T cell activation and believed to play a role in immunological self-tolerance maintained by CD25+/CD4+ regulatory T cells.
HLA-DRA	Predominantly invariant HLA class II alpha chain paralog expressed on the surface of antigen-presenting cells including B lymphocytes, dendritic cells, and macrophages. It is the sole alpha chain for DRB1, DRB3, DRB4, and DRB5.

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