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nCounter® RNA : Protein Profiling

Exploring Immuno-oncology

Using nCounter® RNA : Protein Profiling Technology

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Translational Research | Immuno-oncology Research | RNA : Protein Profiling

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Exploring Immuno-oncology Using nCounter RNA:Protein Profiling Technology

Introduction

The ability of mutated cells to give rise to pathological 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 its periphery—as well as to robustly monitor changes in the immune response associated with potential therapeutic stimuli. Transcriptionally active genes and their respective protein products are important as molecular indicators of the physiological state of a cell population[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 report describes the first RNA:protein profiling panel that will enable researchers to create profiles of the human immune response to cancer types and accelerate the development of drugs, therapies, and predictive biomarker signatures for response to immunotherapeutic treatments. The nCounter RNA:Protein PanCancer Immune Profiling Panel (hereafter referred to as the nCounter RNA:Protein Immune Panel) is a highly multiplexed RNA and protein expression panel designed to quantitate 770 mRNAs and 30 proteins that fall into one or more of three functional categories:

1. Identification 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 immunotherapy, such as immune checkpoint regulation.
3. Identification of tumor-specific antigens, such as cancer-testis (CT) antigens.

Digital Measurement of Protein Expression

Specific hybridization of fluorescent barcodes to nucleic acid targets in solution is the foundation of NanoString's single-molecule detection technology[10]. 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 further refined the technique for commercial use. nCounter RNA:Protein profiling enables specific multiplexed detection of up to 30 protein targets in conjunction with up to 770 mRNA targets in a single assay, permitting quantitative assessment of both protein and nucleic acid biomarkers with limited sample input (**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.

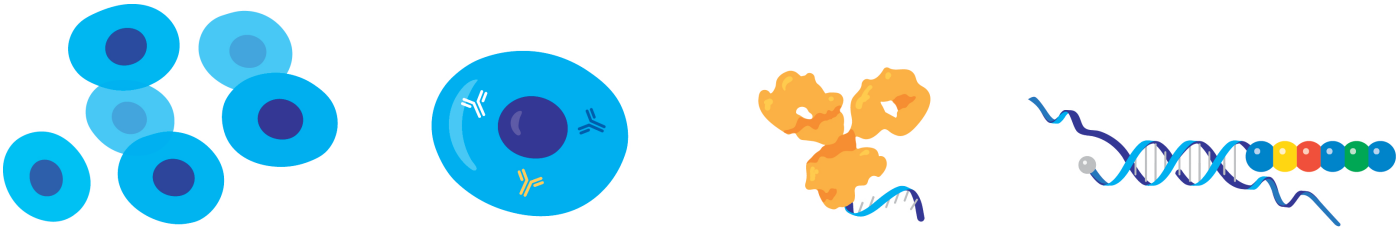


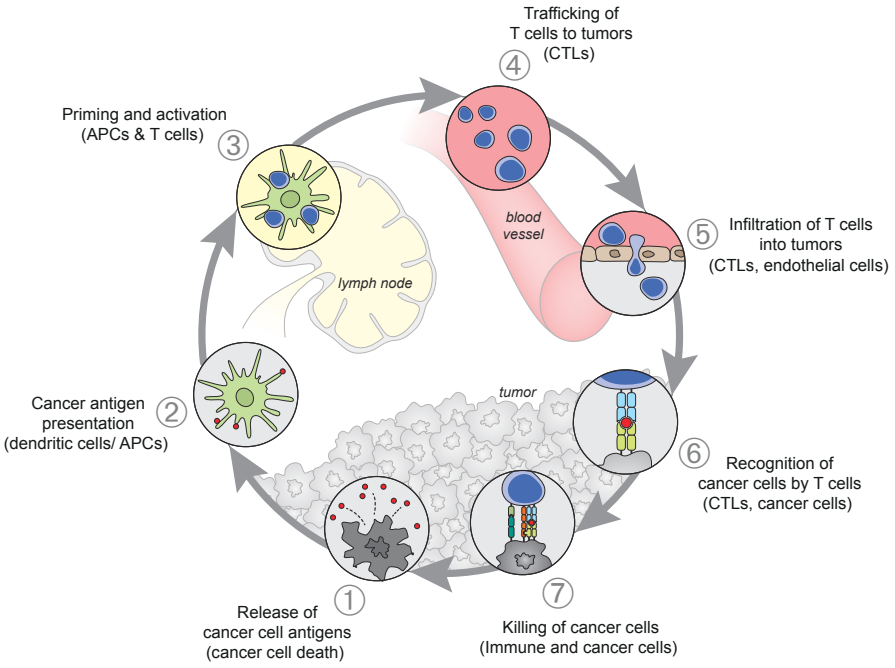
FIGURE 1 Illustration of protein detection and measurement using nCounter technology. Antibodies are covalently attached to unique DNA tags to enable their detection by nCounter probes. Although RNA samples are prepared separately, both fractions are combined in a single nCounter hybridization reaction during nCounter RNA:Protein profiling (see **APPENDIX A**).

Investigating the Cancer Immunity Cycle with nCounter RNA:Protein 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 RNA:Protein Immune Panel permits surveillance of these key regulatory elements via both gene expression and proteomic markers (**FIGURE 2** and **TABLE 1**). It has been shown that cancer therapy response rates may be improved by modulating the tumor microenvironment toward a state that is more supportive of immune function via several existing tools including cytokines, interleukins, interferons, and checkpoint regulators such as PD-1/PD-L1 and CTLA-4[14]. Effective modulation strategies require developing molecular signatures that can accurately assess biomarkers using a method that is practical for a clinical setting[15]. The nCounter RNA:Protein Immune Panel is an ideal starting point for the measurement of actionable molecules.

Identifying and observing discrete cellular populations within 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 periphery has been shown to be a significant and powerful predictor of patient survival[16,17]. By including markers demonstrated to specifically identify major immune cell populations within cancer samples (**APPENDIX B**), the nCounter RNA:Protein Panel can efficiently define both the immunological activity of these samples as well as identify changes in immune cell populations in response to external stimuli such as immunotherapeutic adjuvants.



Stage of Cancer Immunity Cycle	Proteins in the nCounter RNA:Protein Immune Panel
(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 proteins associated with each stage that are included in the nCounter RNA:Protein Immune Panel. Illustration used with permission and originally published in: Chen DS, Mellman I (2013) Oncology meets immunology: the cancer-immunity cycle. *Immunity* 39(1):1-10.

Simultaneous RNA and Protein Quantification in Multiplexed Format

The nCounter RNA:Protein assay workflow was adapted from standard flow cytometry techniques. Briefly, starting with a suspension of 250,000 cells, one fraction of 50,000 cells is removed and lysed for RNA detection using a typical nCounter gene expression protocol. The remaining 200,000 cells are stained with a 30-plex antibody cocktail (antibodies are attached to unique DNA tags), washed to remove unbound antibodies, and then lysed. Material from both RNA and protein preparations are combined for hybridization with nCounter probes and digital imaging.

Example data from this combined assay are provided in **FIGURE 3**, measuring expression of 30 proteins and 770 genes in the nCounter RNA:Protein Immune Panel. When gene and protein expression were measured separately, these counts were highly correlated with those from a combined assay (**FIGURE 3**; $R^2 > 0.99$). The combined assay was also highly reproducible ($R^2 > 0.99$; data not shown). Data were collected from a final input of just 10,000 cells for gene expression and ~500 cells for protein detection.

Correlation of nCounter Protein Measurements with Flow Cytometry

We compared the new nCounter RNA:Protein profiling assay to flow cytometry, a commonly used technology for measuring protein expression in limited samples. To characterize and validate nCounter RNA:Protein profiling, three different sample types (A, B, and C) were derived from HEK293T cells by simultaneously transfecting 10 different expression plasmids. The expression plasmids corresponded to protein targets detected by the nCounter RNA:Protein Immune Panel. Each sample type was derived by varying the amount of plasmid DNA for each of the targets transfected, thus creating variable expression of the 10 protein targets across these different samples.

Protein expression was measured in single-plex assays using flow cytometry and in a multiplexed assay using nCounter (**FIGURE 4**). The percentage of cells expressing each protein (in flow cytometry) and the digital counts of protein expression (nCounter) were compared between the samples. Fold change correlation was high between platforms ($R^2 = 0.94$ and 0.95).

Correlation of RNA and Protein Expression in Human PBMCs

To illustrate the performance of nCounter technology with relevant immune samples, NanoString assessed the correlation of mRNA and protein expression in induced vs. non-induced human PBMCs. PBMCs were induced with phytohaemagglutinin (PHA), a mitogen that triggers T lymphocyte cell division. Results from the highly multiplexed nCounter assay showed mRNA and protein expression levels were most highly upregulated for the alpha-chain of IL2 receptor (CD25) following PHA-stimulation (**FIGURE 5**), which has been previously observed[18] and is consistent with IL-2 dependent signaling. Activated PBMCs also exhibited an increase in inducible co-stimulatory (ICOS) CD278, CD40, and CD40 ligand (CD40L)—molecules that have been shown to be upregulated in various levels during T cell activation[19,20]. Observed changes in expression of these proteins were confirmed with flow cytometry (data not shown). Correlations between RNA and protein levels observed in induced vs. non-induced PBMCs via nCounter RNA:Protein profiling were consistent with previous measurements of expression correlation[21].

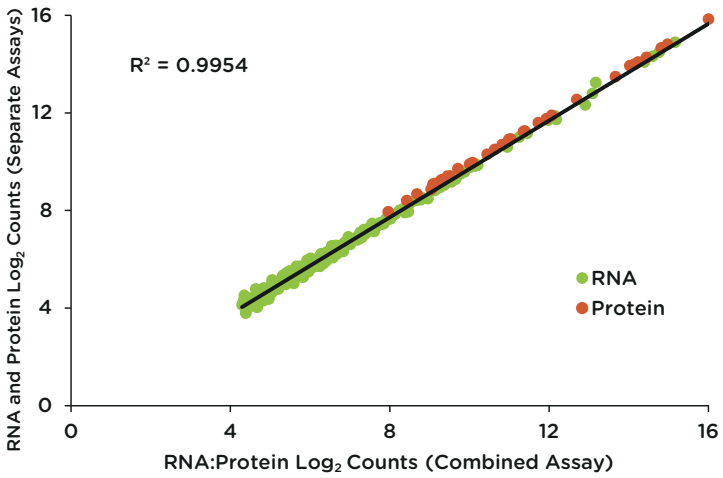


FIGURE 3 Correlations of signal for 770 genes and 30 proteins when run separately or in a combination assay. Transiently transfected HEK293T cells were used for the analysis. Assays for mRNA analysis and protein analysis were run in separate and combined nCounter assays. Data were normalized to either housekeeping genes (for mRNA) or to geometric mean of total signal in cartridge lane (for protein).

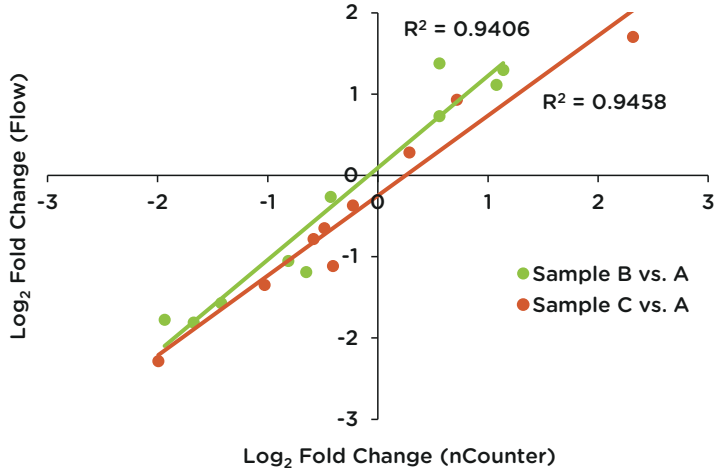


FIGURE 4 Correlations in protein signal fold change between flow cytometry and nCounter assay. Expression of 10 proteins in 3 cell lines was assayed by flow cytometry and nCounter. Sample A expressed all proteins at similar levels, while Samples B and C expressed proteins at variable levels. Fold change 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.

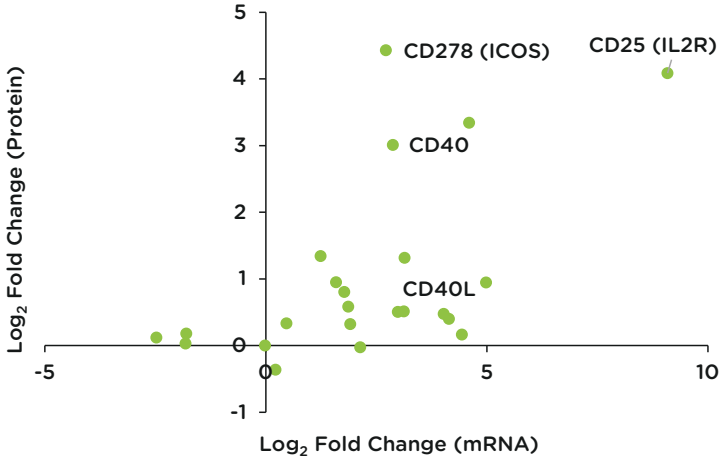


FIGURE 5 Direct correlations of fold change counts of mRNA and protein when analyzing induced vs. non-induced PBMCs with nCounter assay. Counts were measured for 30 targets of the nCounter RNA:Protein Immune Panel. Primary human PBMCs were induced for 48 hours with 10 µg/mL of PHA. Cells were stained for nCounter assay alongside non-induced, freshly thawed cells. Mean counts were averaged across 3 replicates.

Conclusion

For the first time, researchers can quickly assess the key regulatory molecules and pathways of cancer immunity, measuring RNA and protein expression in a single, simple-to-use assay. The nCounter RNA:Protein PanCancer Immune Profiling Panel permits sensitive and quantitative investigation of fundamental immunological processes at both the transcriptional and translational level using one platform, greatly reducing complexity in data analysis. Only a few thousand cells are required for read out, enabling analysis on precious clincial samples.

Data presented here demonstrate the reliability of nCounter technology when measuring protein expression in limited samples, whether measured as separate assays or in combination with RNA samples (**FIGURE 3**). The precision of nCounter RNA:Protein profiling technology and its concordance with the gold standard of flow cytometry is preserved (**FIGURE 3** and **FIGURE 4**). When applied to biological samples, nCounter RNA:Protein profiling offers information on both RNA and protein expression using the same digital platform (**FIGURE 5**). Such tools enable stratification of patients based on the expression profiles of immune-related genes and may facilitate the identification of better immunotherapeutic adjuvants, such as novel combinations of checkpoint inhibitors. Expression data from this panel has potential to promote a better understanding of the interactions between the host immune system and tumor microenvironment and to identify novel methods to predict and improve patient outcomes.

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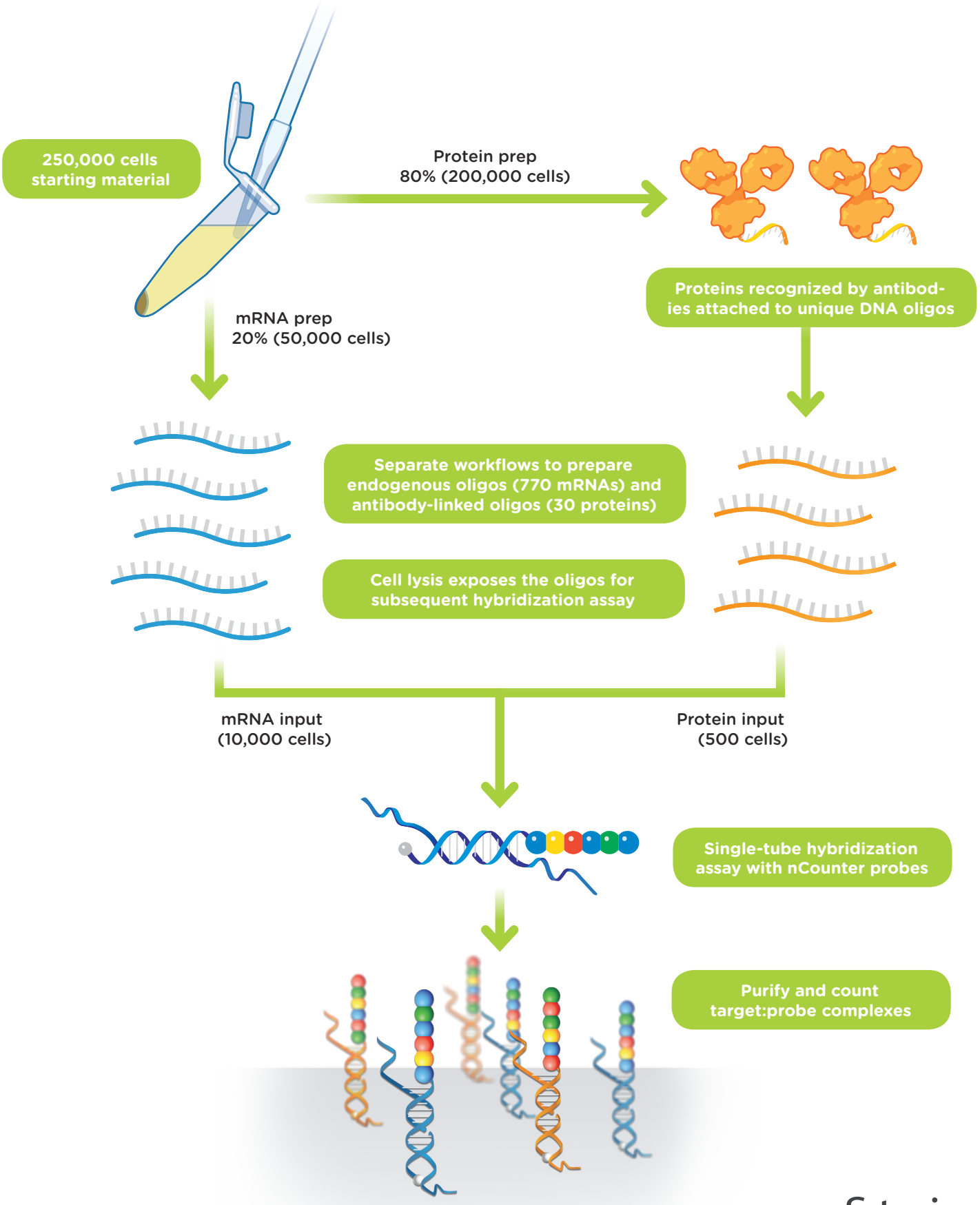
Appendix A: Workflow for RNA:Protein Profiling



Appendix B: Proteins Included in PanCancer Immune Profiling Panel

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)	Regulotory 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 primarly 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 reponses. 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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