TECH NOTE

Simultaneous Profiling of Proteins and RNA with 3D Biology™ Technology: Assay Validation and Immuno-Oncology Applications

Introduction

The NanoString 3D Biology Technology enables profiling of RNA and protein up to 800-plex from a single sample. To develop the nCounter® Vantage 3D™ RNA:Protein Immune Cell Profiling Assay for research use, NanoString worked with BioLegend to source antibodies with a high level of quality and validation for immunology applications. This Tech Note describes the processes employed by BioLegend to validate antibodies against key immuno-oncology targets and NanoString to further validate 30-plex protein expression profiling assays in combination with 770-plex RNA expression analysis for use on the nCounter Analysis System.

NanoString Protein Detection

The nCounter system uses molecular "barcodes" and single molecule imaging to detect and count hundreds of unique transcripts in a single reaction. Each color-coded barcode is attached to a single target-specific probe corresponding to a nucleic acid of interest. Mixed together with controls, they form a multiplexed CodeSet (Figure 1). In the area of immuno-oncology, NanoString developed the PanCancer Immune Profiling panel to measure expression of 770 RNA designed

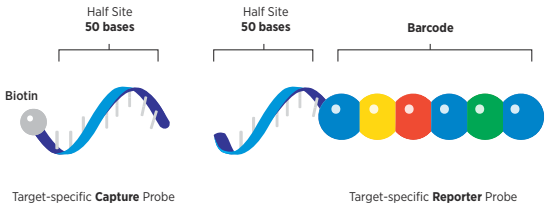


Figure 1. Illustration of the NanoString proprietary optical barcoding technology.

to profile the human immune response in all cancer types. To quantitate protein expression levels in a multiplex format, NanoString's proprietary digital barcoding technology is utilized. Multiplex antibody cocktails specific to proteins of interest are labeled with unique synthetic DNA oligonucleotides. Each DNA oligonucleotide is recognized by a unique Reporter probe that contains a fluorescent barcode (Figure 2). After sample preparation and probe hybridization, samples are loaded on the nCounter system for data collection. Barcodes are counted and tabulated for each target protein and this analysis can be combined with nucleic acid profiling from a single sample.

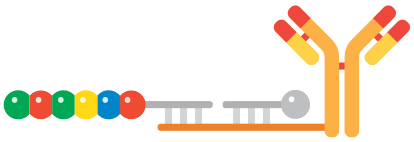
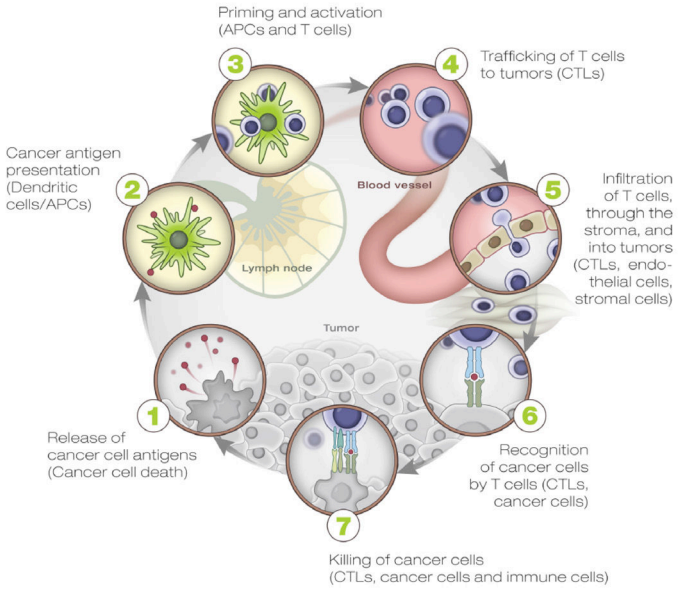


Figure 2. Illustration NanoString protein detection chemistry.

Content Development

The targets included in the Vantage 3D RNA:Protein Immune Cell Profiling Assay were selected to map to key stages of the Cancer Immunity Cycle described by Chen and Mehlman (2013, Figure 3).

This assay includes 30-plex profiling of immune cell surface markers. Development of this assay began by sourcing well characterized primary antibodies against these targets of interest for inclusion in the NanoString Assay and BioLegend was selected for the content and workflow compatibility in the Vantage 3D Protein Immune Cell Profiling Panel.



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Vantage 3D Protein Immune Cell Profiling Content	
Stage of Cancer Immunity Cycle	**Associated Proteins**
[2] Antigen presentation	CD4, CD40, CD40L
[3] Priming and activation	PD-1, PD-L1, PD-L2, IL2R, NCAM, GITR, OX40, CD27, CD28, CD-127, CD137
[4-5] Trafficking and infiltration	CD9
[6-7] Recognition of and killing cancer cells	PD-1, PD-L1, PD-L2, BTLA, HLA-DRA
Immune modulation	PD-1, ICOS, KIR3DL1, NKp46, CTLA-4, CD3E, CD8A, CD14, CD19, CD33, CD68, CD163, CD45RO, NT5E

Figure 3. The Vantage 3D Protein Immune Cell Profiling Assay profiles 30 key cell surface markers utilizing BioLegend antibodies.

BioLegend Validation

BioLegend's antibody validation process involves testing and qualification at all stages of development, including hybridoma clone verification, applications testing, and quality control. Extensive work is done to verify the quality of the hybridoma and the antibodies that are produced from that hybridoma. If the hybridomas are poor producers or produce poor quality antibodies, they will undergo subcloning and reworking to find a suitable subclone. All hybridomas are tested for mycoplasma and only mycoplasma-free hybridomas go on to produce antibodies for finished goods. For monoclonal antibodies (not IgM), antibody purity is always >95% and guaranteed to be low in aggregates. Additional testing includes verification of the isotype by ELISA.

Once an antibody reaches the application testing stage, clone specifications need to be met to advance the product. The specifications for flow cytometry testing, which is most relevant for the Vantage 3D™ Protein Immune Cell Profiling panel, involves titration of the antibody and staining on positive and negative cell populations to confirm specificity (Figure 4).

Antibodies are also tested against other antibody clones for that antigen, as well as blocking with purified antibody to further confirm specificity. Additional testing involve functional assays as required (Figure 5). Only antibodies that meet the specification ranges are qualified to become products. Further quality control includes application-specific post-bottle testing and customer feedback and satisfaction monitoring.

NanoString Validation

The NanoString specific antibody validation process begins with incoming QC to confirm concentration and purity, followed by barcoding, and purification. Incoming antibodies are labeled with a unique DNA oligonucleotide for detection by NanoString optical barcodes. The antibodies are then concentrated and purified by size exclusion. DNA and protein gels are used to identify and collect DNA-labeled antibody prior to analytical and functional testing.

The labeled antibodies next undergo analytical testing. This process begins with validation in cell line transfection models to ensure the labeling process did not affect the ability of the antibody to recognize its target epitope (Figure 6A). Next the antibodies are utilized in titration studies to determine performance and compare data generated by NanoString quantification to the mean fluorescence intensity profiled by flow cytometry in the case of the Vantage 3D Protein Immune Cell Profiling Panel (Figure 6B).

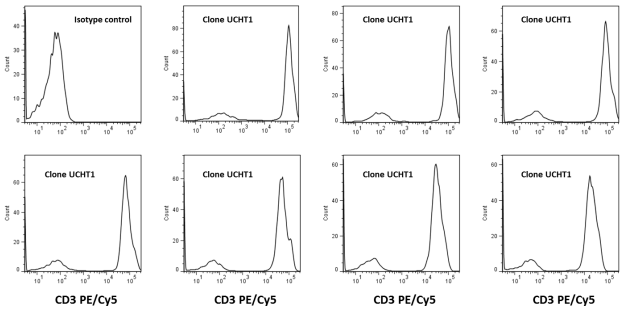

Figure 4. CD3 PE/Cy5 titration on normal human peripheral blood lymphocytes ranging from 1.0 - 0.015 µg/test. Isotype control and clone UCHT1 are shown as indicated.

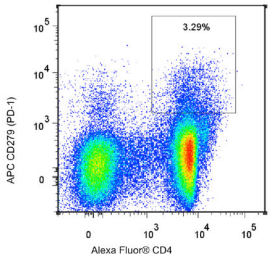

Figure 5A. Dot plot shows flow cytometry application verifying upregulation of mouse CD271 (PD-1) in CD4 T cells in a mouse melanoma cancer model.

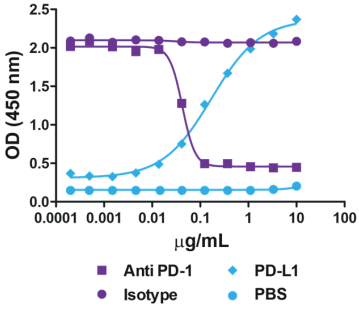

Figure 5B. Line graph shows blocking effect of anti-human PD-1. The antibody inhibits the binding of PD-1 and PD-L1.

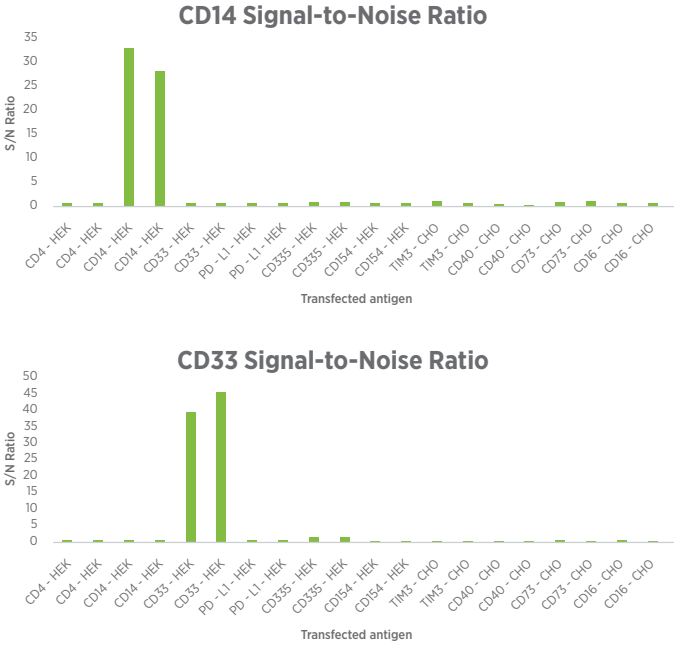

Figure 6A. Example of analytical validation using expression models for labeled antibodies for CD14 and CD33. NanoString signal is only detected when the appropriate protein is expressed.

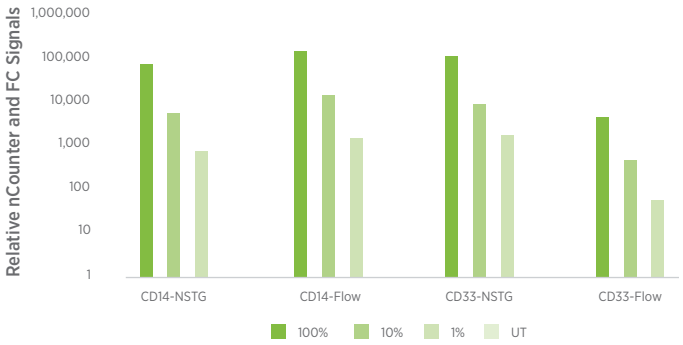

Figure 6B. This is followed by titration experiments comparing NanosString "counts" to the mean fluorescence intensitiy quantitated by flow cytometry. Antibodies that do not pass this functional validation are not included in NanoString assays.

Next, NanoString compares each antibody in a single-plex format to the multiplex assay format, and data for the Vantage 3D Protein Immune Cell Profiling panel is shown here (Figure 7). Any antibodies that do not result in the same digital quantification in both assay formats are not included in the final multiplex antibody cocktail.

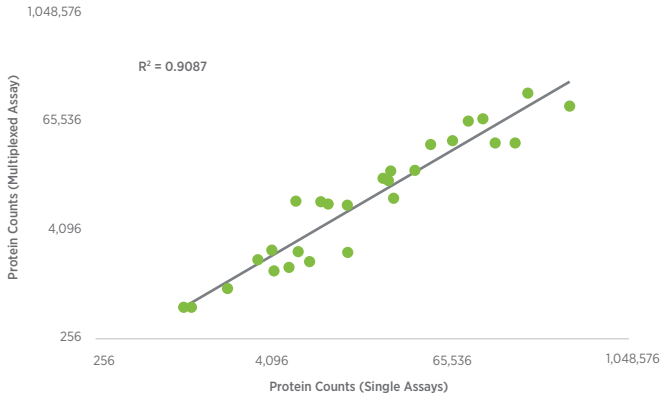

Figure 7. Digital counts of protein expression in single-plex assays versus 30-plex assay.

Finally, functional validation is performed on biological samples. In the case of the Vantage 3D™ Protein Immune Cell Profiling Panel, NanoString quantification is again compared to the mean fluorescence intensity profiled by flow cytometry. This functional validation ensures the protein quantification in digital counts maps to the expression profile generated by flow cytometry (Figure 8). This experiment also highlights the importance of profiling both RNA and protein simultaneously as the two do not always correlate, as is shown in the case of CD45 and CD8a.

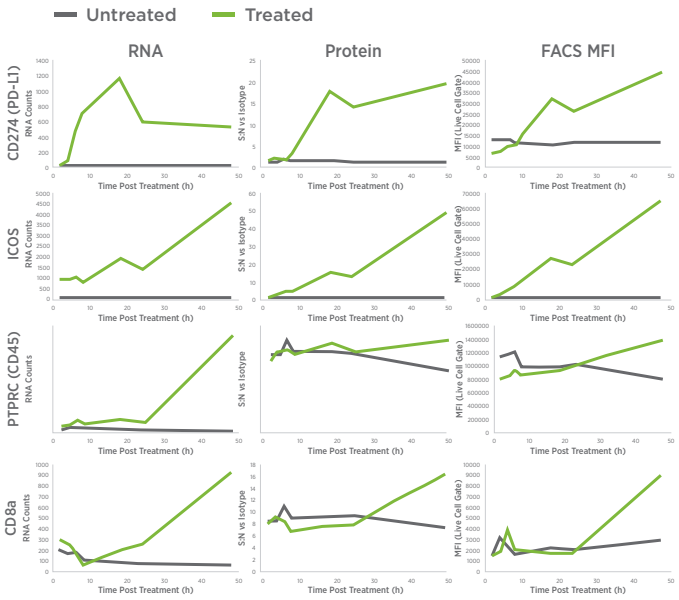

Figure 8. PBMC were stimulated with PMA and profiled by flow cytometry for protein analysis and the Vantage 3D RNA:Protein Immune Cell Profiling Assay for simultaneous RNA and protein analysis (input of 50,000 PBMC). Data from both platforms showed high correlation. The addition of RNA profiling from the same sample on the nCounter platform also highlights key concordance and disconcordance between the two analytes.

3D Flow™ Analysis

3D Flow Analysis integrates NanoString Immune Cell Assays with flow cytometry cell sorting. To streamline this workflow, cells are co-stained in a single 30+ antibody stain containing both fluorescently-tagged sorting antibodies and oligo-tagged antibodies for subsequent NanoString analysis. To ensure the multiplexed nature of this stain step does not result in interference between antibodies, BioLegend antibody-conjugated panels were designed to minimize interactions between fluorescently-tagged and oligo-tagged antibodies (Figure 9).

Product	Product Description	Catalog Number
BioLegend: Human CD4 Treg Surface ID Panel	Flow cytometry antibody panel to identify or sort human Treg cells, Contains: anti-CD127, anti-CD25, anti-CD3, anti-CD4 and live/dead stain	BioLegend CAT#362251
BioLegend: Human Activated CD8 T Cell ID Panel	Flow cytometry antibody panel to identify or sort activated human CD8 T cells, Contains: anti-CD25, anti-CD3, anti-CD8a, anti-CD45RO and live/dead stain	BioLegend CAT#362252

Figure 9. BioLegend antibody panels designed for compatibility with 3D Flow Analysis of Tregs and activated CD8 T cells.

To test these panels, cells stained with only the BioLegend panels were compared to cells co-stained with the BioLegend panel and NanoString antibodies. Histograms from analytical flow cytometry of these two cell populations were overlaid, minimal shifts in the profiles of these cells across each sorting antibody show there is not significant interference due to antibody co-stain (Figure 10).

A similar comparison between sorting-only stain and co-stain using analytical flow cytometry can be extended to test additional, custom antibody panels, demonstrating there is no antibody interference when using new sorting antibodies. This allows 3D Flow Analysis to fit into sorting workflows already in place with antibodies currently used to sort cells of interest.

Conclusion

NanoString and BioLegend work to provide the highest quality reagents for immune applications. The work described in this Tech Note ensures both the specificity and accuracy of data when running high-plex, multi-omic NanoString 3D Biology assays. This work provides confidence that changes observed are due to differences biology and not technical variation.

Learn More

To learn more about NanoString 3D Biology Technology and available pre-validated protein assays visit:

http://3d.nanostring.com

For more on NanoString 3D Flow Analysis, visit:

http://nanostring.com/scientific-content/immunology-inflammation

To learn more about BioLegend and the validated antibodies available for immunology applications visit:

http://biolegend.com/qc
http://Biolegend.com/reproducibility
http://biolegend.com/nanostring_validation

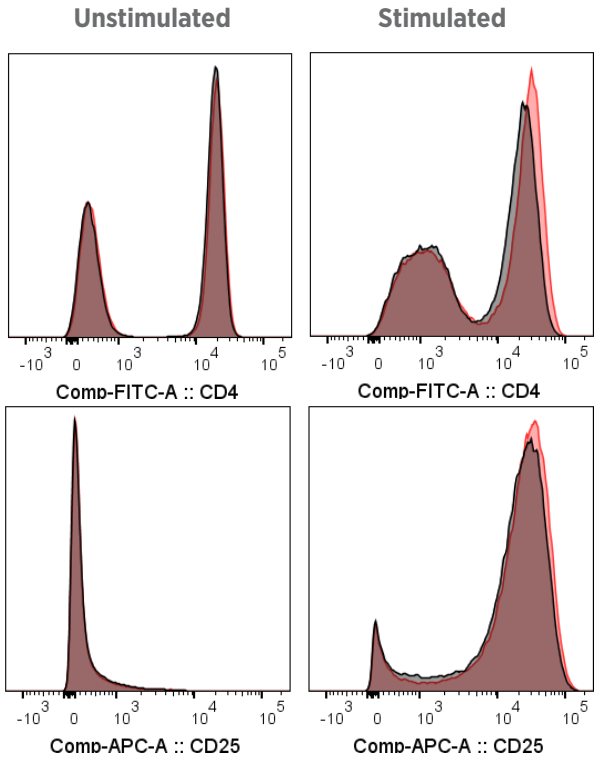

Figure 10. Flow cytometry histograms of cells stained with either BioLegend only (grey) or both BioLegend plus NanoString conjugated antibodies (red).

For more information, visit nanostring.com

NanoString Technologies, Inc.
530 Fairview Avenue North
Seattle, Washington 98109
T (888) 358-6266
F (206) 378-6288
nanostring.com
info@nanostring.com

Sales Contacts
United States **us.sales@nanostring.com**
EMEA: **europe.sales@nanostring.com**
Asia Pacific & Japan **apac.sales@nanostring.com**
Other Regions **info@nanostring.com**

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