NanoString PanCancer IO 360™ Panel Validation

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Performance validation of the NanoString PanCancer IO 360™ Gene Expression Panel for immuno-oncology gene signature generation

Immuno-Oncology is leading our understanding of the interactions between the immune system and cancer cells. Despite cancer immunotherapy demonstrating efficacy across many malignancies, most patients are yet to derive clinical benefit, and the need to further understand tumor-immune interactions for the development of personalized cancer immunotherapy remains.[1]

The complex interplay between cancer and the immune system is distinct to individual tumors, and the different interactions need to be evaluated simultaneously to navigate our understanding of phenotypes.[1–4] In our efforts to reconcile the need to personalize cancer immunotherapy, investigating immune-oncology gene signatures offers the opportunity to optimize current treatment and assess clinical value, identify predictive and actionable biomarkers, and guide novel drug development and companion diagnostics.

The NanoString Pan Cancer IO 360 Gene Expression Panel integrates multiple tumor and immune response parameters including 770 genes involved in immuno-oncology pathways and processes in the tumor, tumor microenvironment and immune activity, and generates 48 gene signatures that aid in the immunological characterization of samples for research purposes. Included in the gene signature algorithm is the Tumor Inflammation Signature (TIS), an 18-gene expression profile correlating distinct immune-related signatures with clinical benefits associated with Pembrolizumab-treated patients.[5] TIS measures pre-existing, peripherally suppressed adaptive immune responses, including antigen presentation, chemokine expression, cytotoxic activity, and adaptive immune resistance that are conserved across tumor types, and known to be associated with decreased PD-1/PD-L1 inhibitor efficacy, irrespective of the cancer's mutational burden.[6] A TIS score is generated through weighted linear combination of these gene expression values against stable housekeeping gene expression.[6]

This white paper presents NeoGenomics' analytical validation studies of the NanoString Pan Cancer IO 360 Gene Expression Panel, which can be used as a laboratory-developed test (LDT) or in a retrospective, exploratory manner.

Tumor Immunogenicity	Tumor Sensitivity to Immune Attack	Inhibitory Immune Mechanisms	Stromal Factors	Inhibitory Metabolism	Anti-Tumor Immune Activity	Inhibitory Immune Signaling	Immune Cell Population Abundance	
Antigen Processing Machinery	Apoptosis	IDO1 Gene Expression	Endothelial Cells	Glycolysis	Tumor Inflammation Signature (TIS)	CTLA4 Gene Expression	B Cells	NK CD56dim Cells
Antigen Presenting Machinery Expression Loss	Tumor Proliferation	PD-L1 Gene Expression	Stromal Tissue Abundance	Hypoxia	Cytotoxicity	IL10 Gene Expression	CD45+ Cells	Natural Killer Cell Abundance
Immunoproteasome	JAK-STAT Pathway Gene Expression Loss	B7-H3 Gene Expression			Interferon Gamma Signaling	Inflammatory Chemokines	CD8 T Cell	T Cells Abundance
MAGE Genes Expression		TGF-Beta Gene Expression			Interferon Signaling Response	Myeloid-Derived Inflammatory Signaling	Cytotoxic Cells	TH1 Cell (TBX21/T-bet) Expression
Loss of Mismatch Repair Gene Expression					Lymphoid Compartment Activity	PD-1 Gene Expression	Dendritic Cells	Treg (FOXP3 Expression)
Hypermutation					MHC Class II Antigen Presentation	PD-L2 Gene Expression	Exhausted CD8 Cell	
MSI Predictor					Myeloid Compartment Activity	TIGIT Gene Expression	Macrophage	
						ARG1 Gene Expression	Mast Cells	
						NOS2 Gene Expression	Neutrophils	

Tumor Foreignness		Tumor Microenvironment		Immune Activity	
Category	Human Genes	Category	Human Genes	Category	Human Genes
Release of Cancer Cell Antigens	74	Angiogenesis	40	T Cell Primary and Activation	151
Cell Cycling and Proliferation	54	Extracellular Matrix Remodeling	43	Killing of Cancer Cells	177
Tumor Intrinsic Factors	156	Collagens	6	Recognition of Cancer Cells by T Cells	103
Common Signaling Pathways	172	Metastasis	20	NK Cell Activity	28
Cancer Antigen Presentation	95	Immunometabolism	99	Myeloid Cell Activity	262
		Immune Cells Localization to Tumors	293		

The PanCancer IO 360 Panel is comprised of 770 genes involved in key human cellular pathways and processes.
Image: edited from NanoString Technologies® — Product Data Sheet nCounter® GX Human Inflammation Kit.

Study design

Performance specification of accuracy, precision, analytical specificity, and RNA input range of the PanCancer IO 360 panel were assessed in this study using a total of 40 Formalin Fixed Paraffin Embedded (FFPE) samples from solid tumor types, including melanoma; head and neck; colorectal; endometrial; NSCLC; ovarian; prostate; kidney and bladder. Specimen pathology review determined indication, specimen source, tumor percentage, and tumor surface area, and samples with less than 50% tumor were macrodissected for total RNA extraction and further evaluation. A single reference RNA provided by NanoString and a single IO 360 kit standard were included with clinical RNA samples on each run. TIS scores at NeoGenomics were calculated using the Tumor Inflammation Signature Algorithm App with data from clinical samples and the run-specific reference RNA, while a proprietary data analysis process was used to generate TIS scores at NanoString.

Specimen pathology review

- Indication
- Specimen source
- % viable tumor cellularity
- Tumor surface area
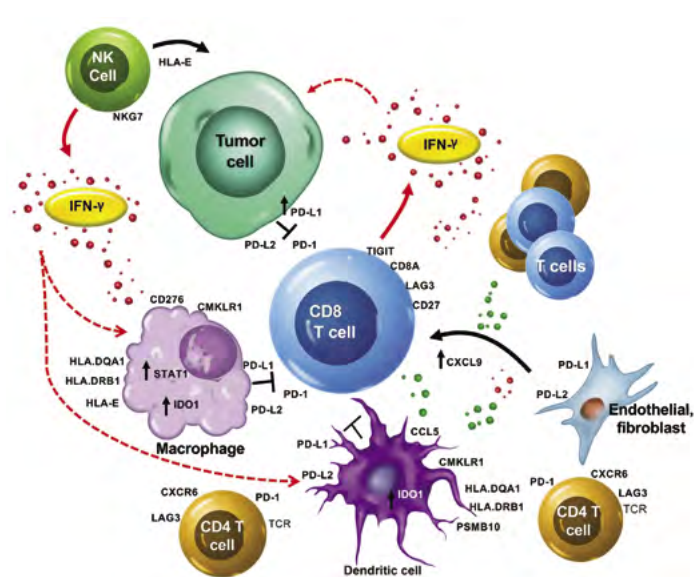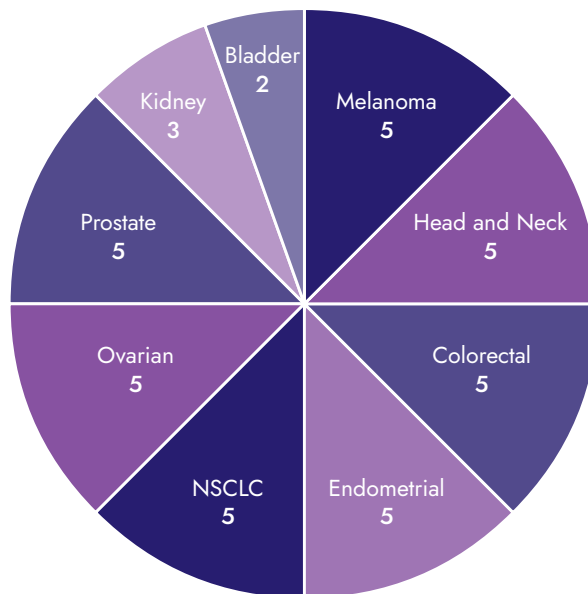 — Slides will be macrodissected if tumor % is <50%

Image: NanoString Technologies® Tumor Inflammation Signature.[5]

Validation samples by tumor type.

18-Gene Tumor Inflammation Signature					
CCL5	CD8A	STAT1	PD-L2/PDCD1LG2	HLA-DQA1	HLA-DRB1
CXCL9	CXCR6	TIGIT	PD-L1/CD274	HLA-E	CMKLR1
CD27	IDO1	LAG3	CD276	PSMB10	NKG7

18 genes of the host immune microenvironment involved in the PD-1/PD-L1 inhibitor pathway determine the Tumor Inflammation Signature (TIS).

Accuracy

Gene expression analysis was successful for all 770 gene targets included in the panel across all 40 samples, with valid internal and negative controls. Accuracy was demonstrated using concordance testing, comparing TIS scores generated from the PanCancer IO 360 panel at both NeoGenomics and NanoString and comparing those values. 38 samples passed NanoString's pre-defined QC threshold between 32-100 housekeeping gene geometric means and were used for concordance assessment and confirmation of accuracy. Similar TIS scores were obtained at NeoGenomics and NanoString for all 38 samples with an overall TIS score SD of 0.199. Differences were attributed to a varying number of counts for targets used in calculating the Prosigna score due to pre-analytic factors such as internal FFPE block variation, tissue specimen review, processing, and RNA isolation. These factors however, had no impact on overall TIS score generation for each sample tested, which led to 100% concordance of TIS scores.

	NeoGenomics vs. NanoString TIS Scores
Concordance % (n=38)	100%
Acceptance criteria	>95%

Repeatability and Reproducibility

For each pathology reviewed tissue sample, RNA was extracted and tested in duplicate to assess inter-FFPE block variation as well as the impact of tissue specimen review and processing on TIS scores. Two identical sets of samples were derived from two FFPE block specimens and reviewed independently by two separate pathologists. Reagent lot variability assessment was performed by testing four RNA samples using IO360 panel reagents of two different lots.

Repeatability was assessed in five samples that were tested in triplicates within a single run. The TIS score for each sample replicate was determined, and repeatability of the PanCancer IO360 panel evaluated by comparing the TIS scores between intra-run replicates of each sample. All three replicates of each sample gave similar TIS scores with an overall average TIS score SD of 0.07 and the results for all five samples matched the results previously obtained in the accuracy phase of this validation study.

Assay reproducibility was determined using five samples that were tested in five different runs under varying conditions, including the day of testing and varied operator and prep-station instrument. Similar TIS scores with an overall average TIS score SD of 0.11 were obtained. Varied conditions and independent pathology assessment of specimen did not contribute significantly to any observed variation. Rather, any differences in the TIS score values were attributed to differences in gene counts, including housekeeping gene counts due to pre-analytical factors such as FFPE-block variation and tissue specimen review and processing.

The acceptance criteria of ≥95% repeatability rate between TIS scores of intra-run replicates was achieved, and 100% repeatability and reproducibility rate of the PanCancer IO 360 Panel was determined.

	Test Results	Acceptability Criteria
Accuracy	100%	≥95%
Repeatability	100%	≥95%
Reproducibility	100%	≥95%

Sample ID	Pathologist	Tumor %	Tumor Surface Area (mm2)	Mean TIS Score	TIS Score SD
A	Pathologist -1	80	36	6.42	0.573
B	Pathologist -1	90	70	5.65	0.064
A	Pathologist -2	30	25	6.73	0.156
B	Pathologist -2	90	108	5.62	0.070

Impact of FFPE block variation and tissue specimen review/processing on PanCancer IO 360 testing and TIS score.

IO 360 Gene Expression Specificity	
Human validation samples (n=40)	All 770 gene targets expressed
Water samples (no –target) (n=4)	No cross reactivity
E. coli bacteria RNA samples (n=4)	No cross reactivity

Analytical specificity

To determine the level of signal that can be attributable to background noise and analytical specificity of the panel, the raw and normalized counts for all 770 targets included in the assay were analyzed on four no-target (water) and four bacterial (E. coli) RNA samples. For the water samples, mean raw counts were below 20 counts, and no quality control or normalization flags were encountered. Results showed zero cross-reactivity with bacterial RNA samples indicating high specificity of target probes to only human targets. Results supported that the PanCancer IO 360 panel was analytically specific in detecting human mRNA.

RNA input Testing

RNA input testing was performed by testing a single FFPE RNA sample across four RNA input levels within the assay specification range of 250ng, including 125ng, 62.5ng, and 50ng. Three replicates at each input level were assessed and all RNA samples were measured for quantity and quality using the Nanodrop 2000 and Agilent 2100 Bioanalyzer. TIS scores were successfully determined using the Tumor Inflammation Signature Algorithm App across all four RNA input levels. Correlations of input levels were excellent, with an average R^2 metric of 0.98352, and varying the input from 250ng to 50ng showed no impact on TIS score determination. The PanCancer IO 360 panel was, therefore, valid over a range of RNA inputs between 50ng-250ng. An RNA input of 250ng was optimal for testing, with an acceptable minimum of 50ng RNA.

RNA Input (ng)	Correlation Coefficient Relative to 250ng Input	Input Parameters for Testing at NeoGenomics
250	1.00000	Optimal
125	0.98524	Acceptable
62.5	0.97656	Acceptable
50	0.97230	Acceptable minimum

Correlation comparisons for various RNA input amounts.

About NeoGenomics Pharma Services

NeoGenomics' Pharma Services unifies several innovative companies' scientific and medical leadership under one leading brand, offering one of the most comprehensive laboratory services menu available for biomarker testing supporting oncology clinical trials globally. We provide our clients with an unparalleled level of expertise, service, flexibility, and scalability. Additionally, we offer alternative business models and solutions across the continuum of development from pre-clinical research and development through commercialization.

To learn more about NeoGenomics Pharma Services, visit us online at https://neogenomics.com/pharma-services.

NeoGenomics Pharma Services is your premiere research partner with NGS or other innovative services. Please contact NeoGenomics Pharma Services at 800.720.4363 or email at pharmaservices@neogenomics.com.

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