Validating critical analytical variables of a multiplexed gene expression assay measuring tumor inflammation designed to predict response to anti-PD1 therapy

Simina Popa[1], Sarah E. Church[1], Irena Pekker[1], Naeem Dowidar[1], Amy Sullivan[1], Celine Ngouenet[1], Carl Schaper, Xing Ren[1], Patrick Danaher[1], Sean Ferree[1], Brett Wallden[1]*

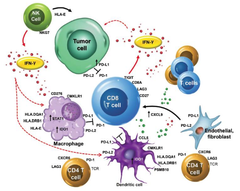
[1]NanoString Technologies, Seattle, WA USA

***Corresponding author** bwallden@nanostring.com

Abstract # 203

Background: The TIS is an investigational use RNA expression assay on the nCounter® Dx Analysis System that is being evaluated as a patient enrichment biomarker for response to anti-PD1 therapy. The development and analytical performance of the Tumor Inflammation Signature (TIS) assay have been described previously[1,2]. Here we describe the analytical validation of the RNA input range and analytical precision starting from RNA isolated from formalin fixed paraffin embedded (FFPE) tissue blocks.

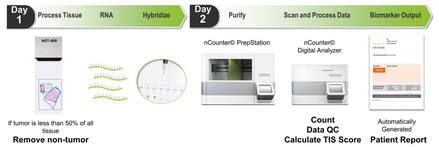
Overview of TIS Algorithm & Test



- The TIS 18-gene signature was derived by a cross-validated penalized regression for response to pembrolizumab in 220 patients from the KEYNOTE 012 (bladder, gastric, HNSCC, and triple-negative breast cancer [TNBC]) and KEYNOTE-028 (anal canal, biliary, colorectal, esophageal, and ovarian cancer) trials and was verified in an independent set of more than 200 patients from KEYNOTE-028 [1,2].
- TIS measures gene expression counts of 18 biomarker and 10 normalizer genes.
- Counts of RNA are used to derive a score. The score is converted to a biomarker category, which is developed for clinical decisions to treat with anti-PD1 therapy.
- Biomarker selection is expected to enrich for responders to anti-PD1 therapy.
- TIS is being developed for decentralized testing across CLIA laboratories
- The precision of the TIS score and biomarker category, defined as the closeness of agreement of repeated measurements of the same sample, were evaluated across multiple laboratory sites, reagent lots, and instruments.
- The accuracy (closeness of agreement) of the TIS biomarker category was evaluated at, below and above the recommended RNA input range relative to the recommended RNA mass.

Tumor Inflammation Signature (TIS) Assay Workflow

- H&E slides are reviewed by a board-certified Pathologist to identify the tumor.
- Unstained slides are deparaffinized and adjacent normal/non-tumor tissue is macro-dissected if it comprises <50% of the total tissue area.
- RNA is manually extracted from tumor with a GMP-manufactured RNA isolation kit.
- RNA is analyzed using the NanoString nCounter® Dx Analysis System.
- Both TIS Score and biomarker category can be output.
- Designed to enable ≤3 day turnaround time from sample receipt to results reporting.



$$\textit{Determine TIS Score for each sample } X \textit{ by } TIS\ Score(X) = \sum_j a_j X_j$$

Data Quality Controls for Each Assay Run and Sample

Quality Control	Control Description	Applies to
Aberrant Counts	Equimolar pool of all 28 synthetic RNA targets	Every Run
Linearity	Alien targets and their control probes	Every Sample
Background	No target negative control probes	Every Sample
Signal	Signature-specific probes and endogenous targets	Every Sample

Cancer Types Tested

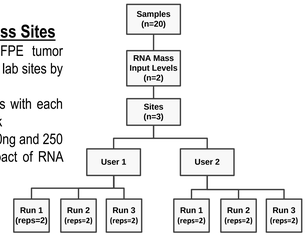
Each of the three studies presented in the results section of this poster included RNA derived from FFPE tissue samples from the 11 solid tumor types below.

Tumor Types			
Anal	Endometrial	Neuroendocrine	Thyroid
Biliary	Esophageal	Salivary	Vulvar
Cervical	Mesothelioma	Small Cell Lung	

Multisite Precision

Design for Precision Across Sites

- RNA from a panel of 20 FFPE tumor blocks tested at 3 independent lab sites by 2 lab users per site.
- 3 runs per user over ≥20 days with each user performing 1 run per week
- All testing performed at both 50ng and 250 ng RNA input to evaluate impact of RNA input on precision



Results for Precision Across Sites

- The 20 tissues span the majority of the expected range (4-10) of TIS Score values (Figure 1).
- The variance components analysis estimated an SD from site of ≤0.091 score units (<2% of total score range).
- Biomarker classification concordance between sites was 100% at the recommended mass input of 250 ng (lower 95% CI of 98.9%).
- Variance components analysis showed negligible site-to-site variance or operator-to-operator variance (<1.6% of the score range).

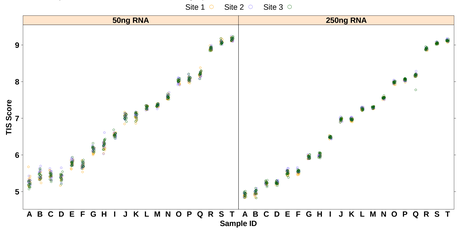


Figure 1. Multi-Site precision of TIS assay across 20 tissue samples: Plots of 36 RNA replicates tested across three sites for each of the 20 FFPE samples. Samples are colored by site.

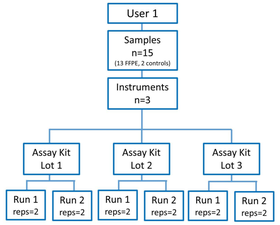
RNA Mass (ng)	Agreement between Site-Pairs [95% CI]			Average Between Sites
	Site 1 vs. Site 2	Site 1 vs. Site 3	Site 2 vs. Site 3	
50	**95.3%** [88.7,100]	**94.3%** [86.2,100]	**94.7%** [86.7,100]	**94.8%** [87.0,100]
250	**100%** [98.4,100]	**100%** [98.4,100]	**100%** [98.4,100]	**100.0%** [98.9,100]

Table 1. Observed Agreement Between Site Pairs. 95% Confidence intervals are shown in parentheses.

Reagent Lot and Instrument Variability

Design for Precision Across Lots and Instruments

- 13 pooled RNA samples generated from archived FFPE tumor tissue.
- One negative and one positive synthetic clinical control
- RNA tested across 3 instruments using 3 reagent kit lots by a single user at a single site



Results for Precision Across Lots and Instruments

- The 13 tissue and 2 clinical control samples are representative of the full range of TIS score values (Figure 2A and 2B). The variance components analysis estimated a total SD of 0.03 TIS Score units (<1% of total TIS Score range).
- Biomarker group classification agreement between lots was estimated as 100% (lower 95% CI of 95.3%). The same agreement rate was observed across instruments.

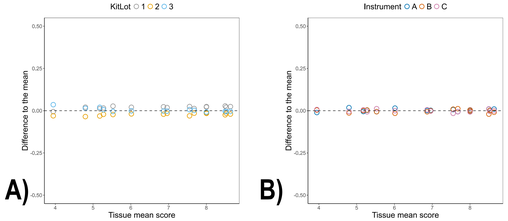
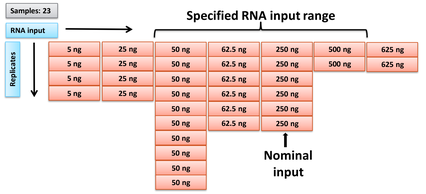


Figure 2 A). Precision of TIS assay across lots. Score difference of individual kit lot vs. score mean per sample. Points colored by kit lot. **B) Precision of TIS assay across Instruments.** Score difference of individual instrument vs. score mean per sample. Points colored by instrument.

RNA Input

Design for Accuracy Across the RNA Input Range

- The specified input range for the instructions for use is 50 ng – 500 ng. 23 FFPE tumor RNA samples were tested repeatedly between 5 ng and 625 ng RNA Input using two reagent lots.
- Observed agreement of classification at varying RNA inputs was determined by comparison to the nominal level of 250ng



Results for Accuracy Across the RNA Input Range

- Biomarker group classification concordance at 50ng of RNA relative to 250ng was estimated as 93.7% (lower 95% CI of 87.9%).
- Data Quality Controls maintain concordance >90% even below the minimum RNA input range (Table 2).

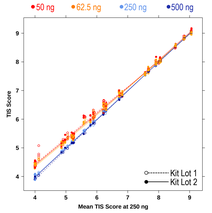


Figure 4: Impact of RNA input mass on Score: Data represent the average of replicates at 50, 62.5, 250, or 500 ng of RNA input plotted against the average of the replicates at 250 ng. Points are colored by the RNA mass.

RNA Mass (ng)	Quality Control Pass Rate	Agreement rate Compared to 250ng [95% CI]
5	10.9%	100% [95.1,100]
25	78.3%	98.2% [93.9,100]
50	96.5%	93.7% [87.9,100]
62.5	100%	95.7% [90.6,100]
250	100%	100% [97.3,100]
500	100%	100% [96.0,100]
625	100%	100% [96.0,100]

Table 2. Quality control pass rate and biomarker concordance rate at each RNA mass input.

Summary

The TIS biomarker category results are highly concordant across site, instrument, assay lot, and RNA input mass.

Result (n)	Agreement Rate (lower 95% CI)
Concordance Across Sites at 250 ng (n=20)	100% (98.9%)
Concordance Across Sites at 50 ng (n=20)	94.8% (87.0%)
Concordance Across Reagent Lots at 250 ng (n=13)	100% (95.3%)
Concordance Across Instruments at 250 ng (n=13)	100% (95.3%)
Concordance at RNA Input of 50 ng relative to 250 ng (n=23)	93.7% (87.9%)

Table 3. Impact of site, lot, instrument, and RNA Mass on biomarker categorical agreement rate: Data represent the average (and lower 95% CI) concordance relative to the average score for the same biological sample.

Conclusions

The NanoString TIS assay is a robust test, which profiles immune-related gene expression across multiple cancer types. The NanoString TIS assay is well suited for decentralized clinical testing with a turnaround time of 3 days or less (from sample receipt to test result).

- Quality controls included in each sample lane and each run are designed to protect against incorrect test results.
- The NanoString TIS assay score has high precision across multiple sites and users with a standard deviation representing less than 2% of the entire score range.
- The analytical performance of the NanoString TIS assay demonstrates it can generate highly accurate and reproducible test results across sites, instrument, reagent kit lot, and the specified range of RNA mass.

Citations

(1) Wallden B, et al. Development and analytical performance of a molecular diagnostic for anti-PD1 response on the nCounter® Dx Analysis System. J Clin Oncol 34, 2016 (suppl; abstr 3034)

(2) Ayers M, *et al.* 2017. IFN-γ–related mRNA profile predicts clinical response to PD-1 blockade. JCI 127(8):2930–2940.

Acknowledgements:

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Footnote: The NanoString TIS assay has not been FDA cleared or approved to identify patients for anti-PD1 treatment. In the United States, the TIS assay is For Investigational Use Only. The performance characteristics of this product have not been established.

Disclosures: S Popa, SE Church, I Pekker, N Dowidar, A Sullivan, C Ngouenet, X Ren, P Danaher, S Ferree, and B Wallden disclosed that they are employees of and shareholders in NanoString Technologies Inc. C Schaper discloses that he is a consultant for NanoString Technologies, Inc.