NanoString PanCancer IO 360™ Gene Expression Panel
Best Practices Guide for FFPE Samples

The PanCancer IO 360 panel is a 770 gene CodeSet that is designed for profiling tumor biopsies and characterizing gene expression patterns associated with the tumor, the immune response, and the microenvironment which shape tumor-immune interactions. The panel contains a number of gene signatures which describe key aspects of immuno-oncology to aid in sample characterization. Among these signatures is the Tumor Inflammation Signature (Ayers *et al.* JCI, 2017) which measures pre-existing, peripherally suppressed adaptive immune responses in the tumor. The Tumor Inflammation Signature (TIS) in this panel is for Research Use Only (RUO). In the clinical trial setting, the Tumor Inflammation Signature assay is being studied as an Investigational Use Only (IUO) device. This Best Practices guide is intended to give practical guidance for using the panel to ensure the best quality data possible.

Slide Preparation

Slide Storage

- It is recommended to store cut slides in a desiccated environment.

Shipping Slides

- Place each FFPE slide mounted tissue section in a separate slot within a slide box.
- Include a sample manifest of the slides being shipped.
- To prevent slide breakage during transportation, a thin sheet of the bubble wrap or paper may be placed inside the slide box to prevent any collision within the box.
- Secure the box with tape or rubber bands to prevent the box from opening.
- The FFPE slide mounted tissue sections are shipped at ambient room temperature (21°C ± 5°C).
- Mark the shipment to be fragile.

Tissue Processing

The panel was developed for use on unstained FFPE slides 4–10 µm thick from archived tumor tissue excision or small biopsies (such as core needle).

The panel can be run with RNA extracted from whole tissue sections from FFPE slides (i.e. not macrodissected). NanoString has demonstrated that the Tumor Inflammation Signature can be calculated from this material in most circumstances. However, the following procedure provides the most rigorous sample processing guidance NanoString has developed to obtain accurate Tumor Inflammation Signature scores.

- The protocol has been tested using unstained FFPE slides 4–10 µm thick from archived tumor tissue excision or small biopsies.
- It is beneficial if FFPE tissue is assessed by a pathologist, lab technician and/or using digital pathology for an H&E slide from the same FFPE block to:
  - Measure the percent of viable tumor surface area (not including necrosis, fibrosis or other dead tissue not containing viable nuclei).
  - Assess presence of non-tumor or normal tissue area containing lymphoid aggregates (e.g. benign lymph nodes, Peyer's patches or tissue-specific non-tumor related lymphatic structures).
- Based on histological assessment, samples with <50% viable tumor surface area (per total surface area) should be macrodissected to remove non-tumor tissue. Similarly, non-tumor or normal tissue area containing anatomical non-contiguous lymphoid structures should be removed by macrodissection prior to RNA extraction.
- Recommended minimum tissue input (for 5 µm slides) is 48 mm^2. When eluted in 30 µl, this will result in a final RNA concentration of 10 ng/µl of RNA and permit 50 ng assay input approximately 95% of the time. **Increased tissue input improves likelihood of obtaining RNA concentrations recommended for input.** See table below for minimum slide input recommendations.

Tumor Surface Area (mm^2)	Minimum Recommended Number of Slides (assuming 5 µm** tissue)
2-4	12
5-7	8
8–15	6
16–23	3
24–47	2
>48	1

**slide input should be adjusted based on thickness of the tissue

RNA Extraction

- NanoString does not recommend a specific RNA extraction kit, but does recommend kits with less than or equal to 30 µl elution volume. Elution volumes greater than 30 µl will result in dilute RNA. Collecting the eluate and passing it through the column again can also be performed to obtain increased concentration.
- NanoString recommends that the customer perform the Proteinase K digestion step for 2–3 hours. RNA previously extracted but not treated with Proteinase K can still be used in the assay, however, contaminants from the preservation method have the potential to decrease assay performance.
- While a DNase step is often not required, it is **highly recommended** to perform a DNase step for samples used with this panel in order to remove genomic DNA contamination that may cause overestimation of RNA concentration, potentially leading to reduced detection of low-expressing genes.
- Recommended RNA QCs measured by spectrophotometer:
    - Concentration: ≥10 ng/µl
    - Purity: A260/A280 ratio between 1.7 and 2.3
    - Please contact a NanoString representative for additional suggested QCs for other RNA quantification platforms.

Concentrating RNA

- If RNA is dilute (<20 ng/µl), investigators can choose to concentrate the RNA to increase the amount that can be used in the hybridization reaction. This step is not required, but can result in higher counts.
- NanoString suggests the Norgen RNA Clean-Up and Concentration Micro-Elute Kit or the Zymo Research RNA Clean and Concentrator 5. If using the Zymo kit it is recommended to make the following modification:
    - The elution volume is reduced to 6 µl.
    - 1 µl is then used for quantitation, and 5 µl are used in the hybridization reaction.

Panel Standard

The PanCancer IO 360™ panel includes a PanCancer IO 360™ Panel Standard containing a pool of synthetic DNA oligonucleotides that correspond to the target sequence of each of the 770 unique probe targets in the panel. This allows for normalization for possible user, instrument, and lot-to-lot variation, which is critical when comparing data from studies run at different times.

NanoString recommends that one lane of each cartridge be reserved to run Panel Standard to ensure the highest fidelity data. However, at the user's discretion, the use of the Panel Standard may be reduced to fewer lanes total per experiment.

> **NOTE**: Users should take extreme care to avoid contaminating samples, reagents or master mix with the Panel Standard, as this will produce unusable data. Change tips before and after dispensing the Panel Standard.

Use of the PanCancer IO 360™ Panel Standard:

1. The PanCancer IO 360™ Panel Standard is supplied at a concentrated stock solution in 4.5 µL and stored at less than -20° C. Remove one tube of PanCancer IO 360™ Panel Standard stock solution and thaw completely at room temperature.
2. Briefly vortex, then spin down.
3. Dilute by adding 33 µL TE, pH 8.0, or nuclease-free water directly to the stock solution.
4. Mix well by vortexing at least 10 seconds, then spin down.
5. Use 5 µL of the dilute PanCancer IO 360™ Panel Standard in the hybridization reaction, following the nCounter XT Gene Expression Assay manual, MAN-10023.
6. The diluted PanCancer IO 360™ Panel Standard sample should not be re-frozen. If additional Panel Standards need to be run in the future, prepare a fresh dilution.

PanCancer IO 360™ Panel Assay

- Recommended minimum RNA input is 50 ng*.
- Hybridization time from 15–24 hours, longer hybridization times can generate higher counts for low-input samples.

> **NOTE**: *If the recommended amount of RNA is not available, it may be possible to use the low-input amplification process to enrich the input material. Please speak with your NanoString representative for additional information.

nCounter® FLEX or MAX Prep Station and Digital Analyzer Settings

- Prep Station should be run using high sensitivity.
- Default Digital Analyzer scan is 280 FOVs; it is recommended to scan with 555 FOVs.

Processing Samples and Imaging on the SPRINT System

Follow the nCounter® SPRINT Profiler manual for instructions to run the sprint system.

Data Processing and Analysis

Panel Standard and Data QC

- The Panel Standard can be used for normalization to as low as 100 counts per gene.
- Normalization of data to Panel Standard can be performed in nSolver using the ratio function.
    - It is recommended to normalize data to Panel Standard as it is used in the experiment (per cartridge or per experiment). For example if Panel Standard is run on each cartridge, the 11 samples on the same cartridge would be normalized using the ratio function to the 1 lane of Panel Standard on the same cartridge.

Signature Analysis

- For signature analysis, NanoString provides an IO 360 Data Analysis Service. For further information please contact your local representative or support using the email list below.

For additional help or information, please contact us at DAS@nanostring.com