NanoString Breast Cancer 360™ Gene Expression Panel

Best Practices Guide

The Breast Cancer 360™ panel is a 776 gene CodeSet that is designed for profiling tumor biopsies and characterizing breast cancer specific gene expression patterns associated with the tumor, the immune response, and the microenvironment which impact tumor metastasis. The panel contains a number of gene signatures which describe key aspects of breast cancer biology and immune-oncology to aid in sample characterization. Among these signatures is the PAM50 Signature (Wallden *et al.* BMC Med Genomics, 2015) which classifies tumors into one of four molecular subtypes (Luminal A, Luminal B, HER2-Enriched, and Basal-like) that are associated with tumor biology and patient prognosis. The panel also includes the Tumor Inflammation Signature (Ayers *et al.* JCI, 2017) which measures pre-existing, peripherally suppressed adaptive immune responses in the tumor. The panel is intended for Research Use Only. 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

- NanoString recommends that cut slides be stored 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:

- Validated for unstained FFPE slides 4-10 µm thick from archived tumor tissue excision. (Compatibility with core needle and fine needle aspirates has not been validated at this time).
- 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).
        - For optimal PAM50 results, it is recommended that all samples should be macrodissected to remove non-tumor tissue.
    - The tumor cellularity percentage on the H&E stained slide must be ≥ 10%.

      NOTE: Tumor cellularity percentage refers to the percentage of viable tumor cells within the circled tumor area.
- Recommended minimum tissue input (for 5 µm slides) is 48 mm^2 to obtain a concentration of 10 ng/µl of RNA**. 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:

- The Roche hi-pure FFPET kit (SKU 06650775001) is recommended for RNA extraction. Other kits may be suitable but have not been evaluated for use with the Breast Cancer 360™ panel.
- It is recommended to perform the Proteinase K digestion step overnight for a total of 16-24 hours.
- It is important to include a DNase step during extraction to remove genomic DNA contamination that may cause overestimation of RNA concentration, potentially leading to reduced detection of low-expressing genes.
- Recommended RNA QC's 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. This procedure also increases the risk of RNA loss and has not been fully validated.
- 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 NonoString recommends 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 Breast Cancer 360™ panel includes a Breast Cancer 360™ Panel Standard containing a pool of synthetic DNA oligonucleotides that correspond to the target sequence of each of the 776 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. If multiple lots of Breast Cancer 360 CodeSet are used across one experiment, then at minimum the Panel Standard should be run once per lot.

> **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 Breast Cancer 360™ Panel Standard

1. The Breast Cancer 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 Breast Cancer 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 Breast Cancer 360™ Panel Standard in the hybridization reaction, following the nCounter XT Gene Expression Assay manual, MAN-10023.
6. The diluted Breast Cancer 360™ Panel Standard sample should not be re-frozen. If additional Panel Standards need to be run in the future, prepare a fresh dilution.

Breast Cancer 360™ Panel Assay

- Recommended RNA input is 100 ng for the Breast Cancer 360™ panel.
- If interested in PAM50 subtyping or additional BC 360 Data Analysis Services, then NanoString recommends increasing input to 250ng to ensure count data meet will meet our QC requirements.

  NOTE: NanoString recommends that the input of the assay not exceed 300 ng.
- Hybridization time ranges are from 15-24 hours, longer hybridization times, within the range of 15-24 hours can generate higher counts for low input samples.

  NOTE: If the recommended amount of RNA is not available, it may be possible to run a pilot study to test the robustness of the assay with lower RNA input amounts.

Data Processing and Analysis

nCounter® MAX or FLEX Settings:

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

  NOTE: FLEX must be run in Life Science setting.

nCounter® SPRINT Settings:

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

Signature Analysis

- For signature analysis, NanoString provides a BC 360 Data Analysis Service. For further information please contact a local representative or support using the email list below.
- Signatures in common between BC 360 and IO 360 were retrained to be more consistent in behavior between IO 360 and BC 360. This has resulted in some of these retrained signatures performing differently in newly generated reports versus the prior versions. To include the legacy version of these signatures in a new report, indicate so to the scientific lead when submitting a DAS request and they can be included as custom analysis.

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