CITOGEN

User Guide:

Pre-test Recommendations for
NanoString nCounter Assays.

nCounter Service

To ensure an appropriate experimental design and subsequent acceptance by the laboratory for execution, it is necessary to be aware of the following information.

The following outline provides a simplified overview of the GeoMx assay process.

1. Preparatory Considerations

The laboratory must receive RNA meeting the following acceptance criteria for the assay.

QUALITATIVE EVALUATION:
NanoDrop must be used to determine the 260/280 and 260/230 ratios. Recommended ratios for RNA are:

- 260/280: ~2.0
- 260/230: 2.0

QUANTITATIVE EVALUATION:
Qubit must be used for quantification.

Sample Type	Minimum Volume	Concentration Ranges	Recommended Concentration
FFPE Tissue RNA	15 µL	20 - 60 ng/µL	60 ng/µL
Blood, Plasma, Cells, Tissue RNA, etc. (CodeSets panels <400 genes)	15 µL	20 - 40 ng/µL	40 ng/µL
Blood, Plasma, Cells, Tissue RNA, etc. (CodeSet panels >400 genes)	15 µL	20 - 40 ng/µL	40 ng/µL

DV200

For FFPE tissue extractions, it is necessary to assess the **DV200** quality metric, which represents the percentage of RNA fragments over 200 nucleotides and shows high correlation with sample performance. **NanoString recommends DV200 to be at least 50% of the sample** for optimal hybridization performance. The appropriate quantity can be estimated using the following equation:

100/% sample >200 nt) x 100 ng

The percentage of samples above 200 nt can be estimated using BioAnalyzer/TapeStation or similar. This calculation is a tool to help increase the suitable sample quantity, although not a complete predictor of success (especially for cases with less than 25% of fragments above 200 nt and extremely low concentration (<20 ng/µL)).

For samples that do not reach the indicated concentration, attempts can be made to concentrate them using columns (such as Amicon Ultra YM-3, 3000 kDa MWCO by Millipore).

Due to the wide variety of cases, a DV200 value lower than 50% can be studied and evaluated to proceed with the study. Contact the Specialist to evaluate your case.

1.1. Required Quantity for the Service

The laboratory must receive RNA samples quantified by Nanodrop, including their concentration and the A260/A280 and A260/A230 ratios. The minimum volume to send to the laboratory is 15 µl. This surplus facilitates our quality control through quantification by Qubit and DV200. Although Nanodrop quantification is useful for analyzing ratios, we always quantify the samples due to possible discrepancies with Qubit results. Any excess samples will be returned upon completion of the study. If you have limited samples and cannot send this volume, please contact the laboratory to discuss your particular case (laboratorio@cagt.es).

Sample Type	Minimum Volume (µL)	Minimum concentration (ng/µL)	Minimum TOTAL Amount (ng)
FFPE Tissue RNA	15	60	900
Total ARN (CodeSets panels<400 genes)	15	30	450
Total ARN (CodeSets panels>400 genes)	15	20	300

Some panels allow for less total RNA input (from 10-50ng) and have an additional "Low RNA Input kit" available for amplification before hybridization and nCounter reading. Contact us for this particular case.

Always store RNA at -80°C until use/shipping.

CITOGEN performs viability checks on received samples to confirm that they meet the requirements for the assay. If any of these checks indicate that the samples are not compliant to continue the assay, you will be notified to confirm the next steps.

1.2. Considerations According to the Type of Starting Sample

Recommendations regarding the starting sample for nCounter assays have been developed using purified total RNA from a variety of tissues, of which mRNA typically constitutes between 5% and 10% (5-10 ng in a total RNA sample of 100 ng).

Blood Samples

Blood samples can be analyzed using purified total RNA, unpurified blood lysates, or specific blood fractions such as PBMCs isolated from whole blood. NanoString recommends using commercially available kits to collect and purify the starting blood RNA, although kits for other biological fluids such as sputum or urine can also be used. For unpurified RNAs, NanoString recommends collecting blood lysate samples in specialized PAXgene® tubes, although EDTA or Tempus tubes can also be used.

FFPE or Paraffinized Samples

For FFPE or paraffinized samples, sections of 5 µm can be used, although optimal performance is generally achieved with sections of 10-20 µm thickness due to the higher percentage of intact cells in larger sections. NanoString recommends taking serial sections for histological or pathological evaluation before and after cutting the sections to be used for nucleic acid extraction. A wide variety of extraction methods can be used to isolate nucleic acids from paraffinized samples. Regardless of the extraction method employed, it is important to quantify and check the quality of the extracted RNA material before hybridization. The minimum surface area of starting material for 5 µm thick sections to obtain a concentration of 10 ng/µL and 50 ng of RNA is 48 mm2, although an increase in the number of tissue sections improves the likelihood of obtaining the recommended RNA concentrations for these assays. See Table 1 for minimum recommendations on the number of sections and sizes.

Table 1. Minimum Number of Cuts According to Tissue Area and Thickness

Tumor Surface Area (mm^2)	Minimum Number of Cuts (assuming 5µm** of tissue)
2-4	12
5-7	8
8-15	6
16-23	3
24-27	2
>48	1

Table 1 **Samples with tumor surface <50% (by total surface) must be macrodissected to remove non-tumor tissue. Similarly, non-tumor or normal lymphoid structures containing non-contiguous lymphoid anatomical structures should be removed by macrodissection before RNA extraction.

Other Samples

Contact the Specialist to evaluate your case.

1.3. Other considerations

NanoString does not recommend a specific RNA extraction kit but recommends always eluting in a volume of elution <30 µL, as well as re-passing the eluate through the column, as the final concentration may increase.

NanoString recommends digestion with Proteinase K for 2-3 hours. Certain fatty tissues (breast, skin, etc.) may require longer digestion, up to 24 additional hours, if the tissue withstands it.

Previously extracted but untreated RNA can be used; however, contaminants from the preservation method may decrease assay efficiency. DNAase treatment step is not necessary but highly recommended to remove genomic DNA from samples, which can cause an overestimation of the starting RNA amount and lead to reduced detection of genes with lower expression.

2. Laboratory Confirmation

Once all the information about the samples and the experimental design of the study has been collected, the laboratory will coordinate the sample collection. They must always be accompanied by the nCounter Sample Shipment Form.

3. Assay Execution

Received samples undergo the RNA quality controls. For FFPE samples, the DV200 value is analyzed using a fragment analyzer. If viability controls are correct and meet the requirements, the nCounter study is carried out.

4. QC and Data Delivery

Once the assay is completed, the laboratory will perform a quality control of the obtained data, and they are sent in RCC (Reporter Code Count) format for further analysis in nSolver. nSolver is an integrated analysis platform for storage, quality control/normalization, and data analysis of nCounter data. It is free to access and can be easily downloaded through this link (https://nanostring.com/products/ncounter-analysis-system/ncounter-analysis-solutions/). It generates customized exports, basic statistical results, and publication-quality figures quickly and easily, without requiring bioinformatics expertise.

5. Disposition of Samples Post-Analysis

Once the analysis is completed, if there is excess sample, please indicate your preference regarding:

- Return of samples to the same address.
- Return of samples to another address.
- Destruction of samples.

We will contact you to coordinate the shipping details. If you have any questions or need more information, please do not hesitate to contact us at laboratorio@cagt.es.

6. Notes on User Responsability

This user guide has been provided to assist you with the use of the nCounter platform. By reaching the end of this guide, it is assumed that the user has read it in its entirety. The laboratory advises that achieving optimal results is directly related to the understanding and proper application of the instructions provided in this guide. Any deviation from the instructions or procedures may adversely affect the results. Therefore, the laboratory is not responsible for suboptimal results or problems arising from not having read and properly followed this user guide. It is strongly recommended to follow all detailed instructions to ensure correct usage and obtain the best possible results. Thank you for your understanding and cooperation.