



EMORY
UNIVERSITY

**Emory Integrated
Genomics Core**

EIGC.009_nCounter XT Assay_Nanostring

Standard Operating Procedure Staff Review Page:

I have read and understand the procedure listed above.

Employee name	Date SOP review complete

Standard Operating Procedure Approval Page:

Date Implemented: February 3, 2020

Annual Review and Approval

Michael Zwick, PhD
Laboratory Director

Note: *No laboratory personnel should use this protocol without proper training by the laboratory supervisor or other designated person.*

Safety: All employees are required to follow universal safety protocols when working with potentially infectious biohazardous materials. Safety procedures are outlined in the Emory University Bloodborne Pathogen Exposure Control Plan (see Employee Safety Notebook).

Introduction to nCounter Assays with XT Formulation

Nanostring's nCounter technology is designed to provide a sensitive, reproducible, and highly multiplexed method for detecting various small molecules across all levels of biological expression. Nanostring's flagship gene expression assays utilize dual-probe system to provide a method for specifically and sensitively detecting mRNAs with molecular barcodes without the use of enzymes for reverse transcription or amplification.

nCounter Assay Overview

Nanostring nCounter technology is based on digital detection and direct molecular barcoding of individual target molecules through the use of a unique probe pair for each target of interest. The probe pair consists of a color-coded Reporter probe, which carries the visible signal on its 5' end, and a Capture probe, which carries a biotin moiety on 3' end. Each Reporter probe has six positions, and each position can be one of four colors. A large diversity of probes can be mixed together with the sample in a single well, with each Reporter Probe interacting with a specific target. These target-probe complexes are then individually resolved and identified during data collection.

Probes are placed into a reaction in massive excess to target nucleic acid molecules to ensure that each target finds a probe pair. After hybridization, excess probes are washed away using a two-step magnetic bead-based purification on a nCounter instrument. These magnetic beads are derivatized with short nucleic acid that are complementary to the Capture probe sequences or the Reporter probes and are used sequentially. First, the hybridization mixture is allowed to bind to the magnetic beads complementary to the capture probes. Wash steps are performed to remove excess Reporter probes and non-target cellular transcripts. The target-probe complexes and unbound Capture probes are then eluted off the beads and allowed to bind to a second set of beads complementary to the Reporter probes. Wash steps are performed to remove excess Capture Probes. Finally, the purified target-probe complexes are eluted off the beads and are immobilized and aligned for data collection.

Data collection is performed using epifluorescence microscopy and CCD capture technology on an nCounter instrument to yield hundreds of thousands of target molecule counts. Digital images are processed within the nCounter instrument, and the Reporter Probe counts are tabulated in a comma separated value (CSV) format for convenient data analysis with Nanostring's free nSolver Analysis Software.

nCounter CodeSet Chemistry

Nanostring's nCounter CodeSet chemistry utilizes target-specific Reporter and Capture Probes, collectively referred to as a CodeSet, that directly hybridize to a target of interest. Each probe pair consists of a color-coded Reporter and a Capture Probe to which a target-specific sequence has been covalently attached.

*For nCounter miRNA Expression Assay please, use nCounter miRNA Expression Assay User Manual

Workflow

1. **Set up sample hybridization (pre-pcr room)**
2. **Hybridization (post-pcr)**
3. **Create CDF file**
4. **Initiate run on Prep Station (post-pcr)**
5. **Seal cartridge and transfer from Prep Station to Digital Analyzer (post-pcr)**
6. **Download data and analyze it in nSolver 4.0 (post-pcr)**
7. **Post RLF, RCC, and nSolver export files to client data folder.**

nCounter Assays with XT Formulation

nCounter Analysis Systems require the use of consumables to perform post-hybridization processing and image scanning. The reagents required are defined by the instrumentation used.

MAX/FLEX Consumables

The nCounter Master Kit provides all the consumables and reagents you need to perform any NanoString assay on the nCounter FLEX Analysis Systems. With each Master Kit order, you receive the nCounter Cartridges, Prep Plates, and Prep Pack, which includes racked tips and foil piercers, 12-tube strips, strip tube caps, tube sheaths, cartridge well seals and hybridization buffer. Master Kits are available in packs of 12, 48 or 192 assays.

- Twelve samples can be processed in a single nCounter Cartridge (except for Breast Cancer and Pancreatic Cancer O-360 panels, that must have 1 control)
- All reagents and consumables assembled in one kit
- No prep or dilutions required
- Arrive ready to run

nCounter Master Kit components are shipped in separate boxes under different shipping conditions. Ensure prep plates are stored in the orientation marked on the side of the box.

COMPONENT	DESCRIPTION
<p>nCounter Cartridge</p>	<p>Consumable for both nCounter MAX Analysis System and nCounter FLEX Analysis System</p> <ul style="list-style-type: none"> • 12 samples processed per cartridge
<p>nCounter Prep Plates</p>	<p>The Prep Plates are foil sealed 96-well plates that are used by the Prep Station and contain:</p> <ul style="list-style-type: none"> • wash buffers • magnetic beads • immobilization and imaging reagents
<p>nCounter Prep Pack</p>	<p>nCounter Prep Pack contents:</p> <ul style="list-style-type: none"> • Racked tips and foil piercers • 12-tube strips • Tip sheaths • 12-strip tube caps • Cartridge well seals • Hybridization buffer <p>*If hybridization buffer has been stored in cold conditions and precipitate is observed, warm tubes at 37°C until salts have dissolved.</p>
STORAGE CONDITIONS	
<p>Cartridge</p>	<p>Store for up to 1 year at -20°C.</p>
<p>nCounter Prep Plate</p>	<p>Stable for up to 1 year at 4°C.</p>
<p>Hybridization Buffer & Prep Pack</p>	<p>Store for up to 2 years at room temperature. Remaining Prep Pack materials, store at room temperature. No expiration date.</p>

Sample Input Recommendations and QC

Use a Nanodrop or M200 Infinite Pro Tecan (plate reader) to measure RNA sample concentration and purity. Nanostring recommendations are a 260/280 ratio of 1.7 to 2.3 and a 260/230 ratio of 1.8 to 2.3.

After measuring the concentration of RNA, evaluate RNA quality by using an Agilent Bioanalyzer to measure nucleic acid fragmentation. If previously measured concentration is 30 ng/µl or below, use the Agilent Pico kit. If above, use the Agilent Nano kit.

Nanostring recommends that at least 20% of the area under the trace be greater than 200 nucleotides in length for optimal performance. (DV200 has to be greater than 20%).

Nanostring recommends RNA concentration to be higher than 20 ng/µl.

Recommended sample input mass for nCounter XT assays are:

- Total RNA (CodeSets < 400 genes) - 100 ng
- Total RNA (CodeSets > 400 genes) - 50 ng

Information on number of genes in the panel can be found on the Nanostring website.

If DV200 is lower than 50% and greater than 20%, smear-adjusted input must be implemented in order to determine how much sample should be used.

$$\begin{aligned} &(\text{Recommended input mass}/\text{DV200\%}) * 100 = \text{Smear-adjusted input} \\ &(\text{Smear-adjusted input}/\text{Sample concentration}) = \text{Sample input} \end{aligned}$$

If DV200 is below 20%, input is a fixed 250 ng.

If DV200 is higher than 50%, input is a fixed 50 ng or 100 ng depending on number of CodeSet genes.

If the input of the sample is below 0.5 µl, use a 1:10 dilution to avoid pipetting errors.

Sample ID	External ID	ng/µl	A260/280	260/230	RIN	DV200 (%)	50ng/ scaling	Dilution	Input	H2O to 5 µl

Note: Both factors (concentration and %DV200) come into play when considering the quality of a sample. If there is an option between the samples with lower concentration and higher %DV200 and higher concentration and lower %DV200, Nanostring recommends choosing samples with higher %DV200. However, if the concentration is very low (<10 ng/ul) then it may not really matter how intact the RNA is.

Hybridization Protocol for nCounter XT CodeSet Panels

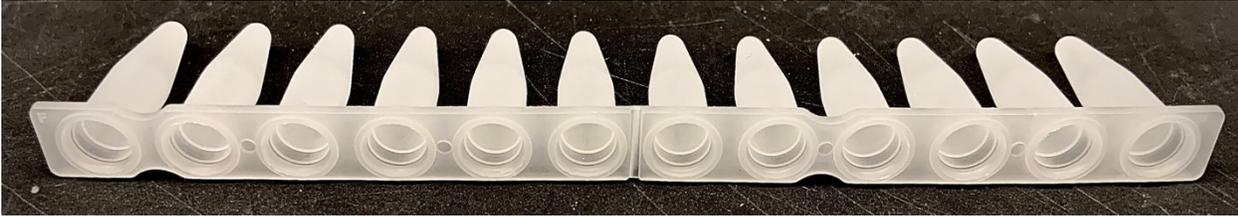
Set up a hybridization reaction at room temperature for each sample with the following components (do not combine all reagents into a master mix – see detailed protocol):

1. Up to 5 μ l of sample RNA
2. 5 μ l of hybridization buffer
3. 3 μ l of Reporter CodeSet
4. 2 μ l of Capture ProbeSet

If using total RNA:

1. Remove aliquots of both Reporter CodeSet and Capture ProbeSet reagents from the freezer and thaw at room temperature protected from light.
2. Invert several times to mix well and spin down reagents. During setup do not vortex or pipette vigorously to mix any reagents, as it may shear the Reporter CodeSet. Mixing should be done by flicking or inverting the tubes. If using a microfuge to spin down tubes, do not spin any faster than 1000 RCF for more than 30 seconds. Do not “pulse” to spin because this will cause the centrifuge to go to maximum speed and may spin the CodeSet out of solution.
3. After it has thawed, inspect the tube of Reporter CodeSet to make sure no colored precipitate is present. If you see a colored precipitate, heat the entire tube to 75°C for 10 minutes and cool to room temperature before using.
4. Create a master mix by adding 70 μ l of hybridization buffer to the tube containing the Reporter CodeSet. Do not remove Reporter CodeSet from original tube. Invert repeatedly to mix and spin down master mix. Do not add Capture ProbeSet to the master mix. Add 8 μ l of master mix into each strip tube.

Twelve-well notched strip tubes are provided by Nanostring and have notches after the first and eighth tube for orientation purposes. There are three regions – first with one tube, second with seven tubes, and third with four tubes.



Orientation of the strip should always be that the first tube before the first notch holds sample number one. This is important because of the orientation of the strip on the Flex instrument.

- 5. Add water to the sample to bring the volume up to a maximum of 5 μ L, if necessary. Add the sample into each tube following instructions about orientation.**
- 6. Invert the Capture ProbeSet to mix and spin down the contents in micro centrifuge, briefly, by placing the tubes onto micro centrifuge with two tubes overlap on each end. Do not close the lid to spin. Do not spin faster than 1000 RCF for more than 30 seconds . Add 2 μ l of Capture ProbeSet immediately before placing at 65°C. Cap tubes and mix reagents by inverting the tubes several times and flicking with a finger to ensure complete mixing. Briefly spin down and immediately place the tubes in a pre-heated 65°C thermal cycler. Minimizing the time between addition of the Capture ProbeSet and incubation at 65°C will increase assay sensitivity.**
- 7. Incubate reactions for 16-18 hours, and ramp the reactions down to 4°C, using program “GEX-ST “in the Sprint folder of the BioRad quad thermal cycler. Do not leave the reactions at 4°C for more than 24 hours or increased background may result.**

Prepare Consumables on the Prep Station

- 1. Remove one blank sample cartridge from -20°C storage. Allow it to warm to room temperature (about half an hour), then remove it from the foil package.**
- 2. Remove two prep plates from 4°C storage. Allow the plates to warm to room temperature (about half an hour). Remove and discard the clear lids, then centrifuge at 2000 g for 2 minutes. Confirm the presence of magnetic beads in only last row of the plate.**

Load Consumables on the Prep Station (Run in High Sensitivity mode)

1. Place the prep plates on the deck with green labels forward.
2. Load the tips with the black, piercer tips oriented in the bottom right corner.
3. Place the tip sheaths in the slots located in front of the tip rack.
4. Remove the foil from the cartridge.
5. Raise the electrode fixture and place the cartridge under it. The notch in the cartridge should be in the back left, and the barcode sticker should be on the right. A properly aligned cartridge will be tilted with the right side higher than the left; the right side should be able to bounce up and down with light pressure from your index finger.
6. Lower the electrode fixture to insert the electrodes into the cartridge wells.
7. Place empty Nanostring-provided strip tubes into the heater block.
8. Uncap the hybridized sample strip tube and place it into the metal tube holder, aligning the notches of the strip tubes with the metal pegs.
9. Close the heater lid.
10. Close the Flex door firmly and touch “Start Processing” to initiate the run.

Validate Deck Layout on the Prep Station

The Prep Station will automatically check to confirm that consumables are placed correctly on the deck. Wait approximately ten minutes for the system check to complete. If successful, the instrument will begin piercing the foil on the prep plates and start the run. Run lasts for 3 hours and 10 minutes.

Cover Cartridge after Run on the Prep Station

Upon completion of the run, use the clear adhesive film supplied in the Prep Pack to seal the holes on the top of the sample cartridge. Do not leave processed cartridges uncovered, as evaporation of the liquids in the well will result in data loss. Cartridges should be scanned immediately but can be stored in the dark at 4°C for up to one week.

Upload RLF to the Digital Analyzer

Retrieve the green Nanostring USB, which can be found inside the -80°C CodeSet box, and allow it to come to room temperature. (Mark the USB with Project and Order number). Every CodeSet from Nanostring is shipped with an RLF (Reporter Library File). The RLF contains barcode definitions for the group of probes which comprise the CodeSet.

To upload the RLF, insert the USB into the port off the right side of the Digital Analyzer touch screen and follow the menu commands from the Main Menu. **Upload Files > Upload RLF.***

***Nanostring recommends uploading the RLF from each shipment of the CodeSet to ensure that the most up to date version is used.**

Upload CDF to the Digital Analyzer

On the same, green Nanostring USB will be a CDF file template. This file is used to set up the run on the Digital Analyzer.

First, edit the extension of the file from “CDF” to “CSV” to allow it to be opened and edited in Excel. Input the cartridge ID (which can be found on the foil package), the sample names, date, FOV value of 555, and associated RLF. The RLF name should be copied directly from the RLF filename on the green USB to avoid typos.

Save the file and change the extension back to “CDF”. To upload, insert the USB into the Digital Analyzer and follow the menu commands from the Main Menu. **Upload Files > Upload CDF.**

Begin Analyzer Run

1. Click “Start Counting” on the Main Menu and select a stage position on the touch screen.
2. Insert the cartridge in the corresponding stage position while holding it lengthwise. The cartridge will only fit in the proper orientation with the notched corner on the right.
3. Close the magnetic clip and push down to ensure that the cartridge is flat.
4. Under Cartridge Definition Mode, select “Load Existing” and select the previously created CDF file.
5. Touch “Done” to return to the Select Stage Position Screen. Click “Done” a second time if no additional scans will be programmed. Repeat above steps if additional cartridges will be scanned.
6. Touch “Start” to begin scanning first cartridge.

Data Analysis

1. Insert a green, Nanostring USB drive into the Digital Analyzer USB port.
2. Once the scan has completed, touch the “Download Data” button.
3. Select the corresponding Cartridge ID(s), and touch “Download Data”.
4. Data will be in zip files within the RCC folder.
5. Transfer the zip files to the client order folder on the PPMS drive and unzip them.
6. Open nSolver 4.0.
7. Import RLF file, if necessary.
8. Import unzipped RCC files using default QC settings.

9. **Select all files for the order and export the data table using the “custom text” option. Leave all options defaulted.**
10. **Open the nSolver data table file in Excel and check the positive and negative controls. The negative controls should follow a dilution pattern that should be similar for all samples.**
11. **Zip the nSolver data table file, the raw RCC files, and the RLF file and post to the client data folder on the PPMS drive.**
12. **Upon completion store the USB in the boxes (Nanostring USB's), that are located in the drawer in 7310 marked Nanostring. USB's are marked based on panel, and each panel has its own compartment in the boxes. Boxes are clearly labeled on the lid with exact position of the compartments.**