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Nanostring SOP

Version 3.0

The purpose is to provide a protocol for determining gene expression from FFPE and FF samples using the nCounter platform from Nanostring

1. Equipment and Reagents

nCounter Master Kit
nCounter Code Sets
Nuclease Free Water
nCounter Prep plates
nCounter Cartridge
Thermal Cycler
Ice bucket
Pipettes
Nanostring Prep Station
Nanostring nCounter cartridge reader
Nanostring nSolver analysis software

2. Specimen

Formalin Embedded tissues.

Required Sample Input amount: 250 ng of RNA as ideal input. Specimens below this amount will be run at the maximum input possible, flagged as a deviation and analyzed taking into account this deviation from the ideal input.

3. Procedure

1.0 Hybridization of sample with Code Sets

1.1 Thaw out samples and code sets on ice

1.2 Cut the 12 tube and cap strip into two 6 tube sections using a razor or scissors (All consumables associated with the hybridization step can be found in the master kit stored at room temperature).

1.3 Set up the hybridization reaction

A. If using GX custom code sets:

- Add 130uL of hybridization buffer (provided in master kit) to the **REPORTER** code set tube. Invert tube several times to mix then gently centrifuge to collect volume at bottom of tube.
- Calculate input volume in which FFPE samples will have 250ng and FF samples will have 75ng total within the tube with a max volume of 5uL. Fill to 5uL with nuclease free water inside tube.
- To each tube containing 5uL of sample, pipette 20uL of the Reporter codeset/hybridization buffer mixture directly from the supplied reporter code set tube.
- Preheat a thermal cycler to 65°C using 30uL as volume
- Pipette 5uL of **CAPTURE** codeset directly from the Capture codeset tube into each tube of the 12 tube strip containing sample/Reporter/hybridization buffer mixture.
- Cap the 2, 6 tube strips (from the cut 12 tube strip), invert several time to mix and centrifuged briefly to collect sample at bottom of the tube.
- Place in preheated 65°C thermal cycler for 12-24 hours

B. If using XT gene expression code sets

- Add 70uL of hybridization buffer to the **REPORTER** code set tube. Invert several times to mix and gently centrifuge to collect volume at bottom of the tube.
- Calculate input volume in which FFPE samples will have 250ng and FF samples will have 75ng total within the tube with a max volume of 5uL. Fill to 5uL with nuclease free water inside tube.
- To each tube containing 5uL of sample, pipette 8uL of the Reporter codeset/hybridization buffer mixture directly from the supplied reporter code set tube.
- Preheat a thermal cycler to 65°C using 15uL as volume
- Pipette 2uL of **CAPTURE** codeset directly from the Capture codeset tube into each tube of the 12 tube strip containing sample/Reporter/hybridization buffer mixture.
- Cap the 2, 6 tube strips (from the cut 12 tube strip), invert several times to mix and centrifuged briefly to collect sample at bottom of the tube.
- Place in preheated 65°C thermal cycler

1.4 Run hybridization reaction at 65°C for a minimum of 12 hours and a maximum of 24 hours.

2.0 Setup and Running of the Prep Station

2.1 Setup

A. Take out 2 prep plates (supplied and stored at 4°C) and 1 cartridge (supplied and stored at -20°C) and allow to come to room temperature about 30 minutes.

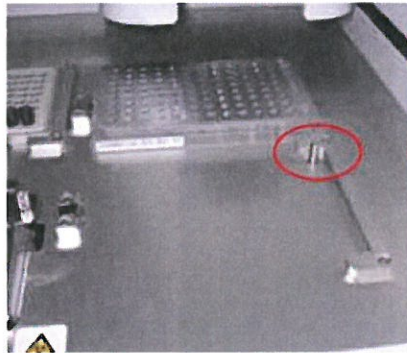
B. Centrifuge the prep plates in a table top centrifuge with plate bucket for 2 minutes at 2Kxg

- Check the bottom of the plates to see if there has been any bead loss due to shipping and handling.

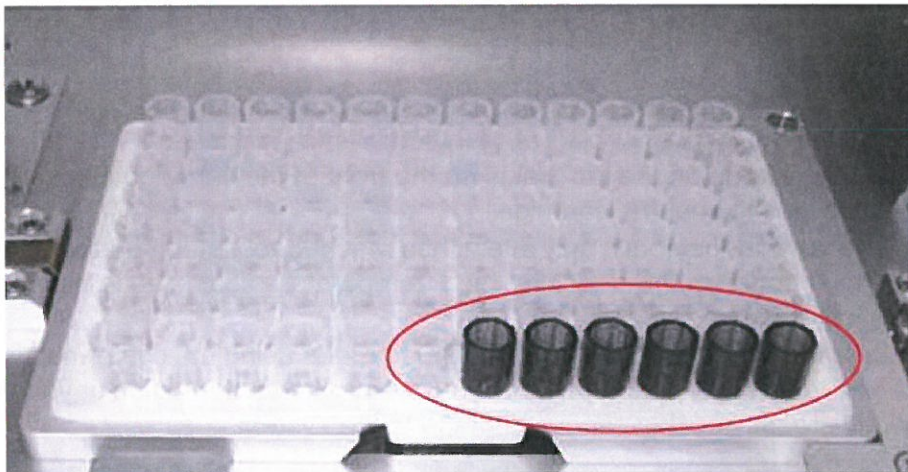
- If there is a substantial amount of bead loss in the wells you should not use the plate and should contact Nanostring for replacement

C. Dispose of all previous waste and set up new consumables of the prep station deck

- Empty the liquid waste receptacle by pulling on the tab on the tip waste receptacle toward you, thus releasing the mechanism and you can lift the liquid waste receptacle up out of its seat and can be disposed of in the proper waste stream.
- Empty the tips waste receptacle by pulling it out and dumping its contents into its appropriate waste stream.
- Place both waste receptacles back into their seat according to the same mechanism they were taken out.
- Place the two prep plates into their seats on the prep station deck by placing "A1" on the top left orientation so that the metal peg fits inside the space designated on the bottom of the plate and the plate will sit flat on the deck (See picture).



- Make sure that the metal clip on the left side of the seat snaps above the plate thus locking it into place.
- Place tip rack (provided in the master kit) into its cradle on the deck so that the darker 6 "foil puncher" tips are oriented in the bottom right of the cradle. (See picture)



- When placing the cradle back make sure that the clip on the left snaps above the cradle to lock it into place.
- Place the two tip sheaths in their respective seats.
- Lift up on the metal bracing lid that will hold the sample tubes in place
- Place 2 empty 12 tube strips in the top two receptacles

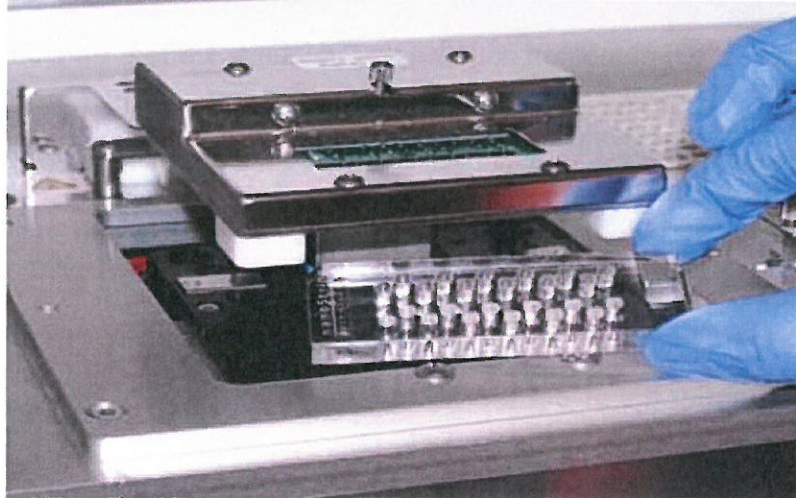
D. Loading hybridized samples

- Take the 2-6 tube strips containing the hybridization reaction off of the thermal cycler that has been running since the night before.
- Centrifuge the tube strips briefly to collect all sample to the bottom of the tube.
- Take sample tubes to the prep station, placing the samples in the third receptacle closest to the door of the station, making sure that the orientation of the strip is that the tubes contain samples 1-12 from left to right.
- Close the metal guard by pulling down the guard so that it sits firmly on top of the tube strips, having the openings of the guard correspond with the openings of the sample tubes.

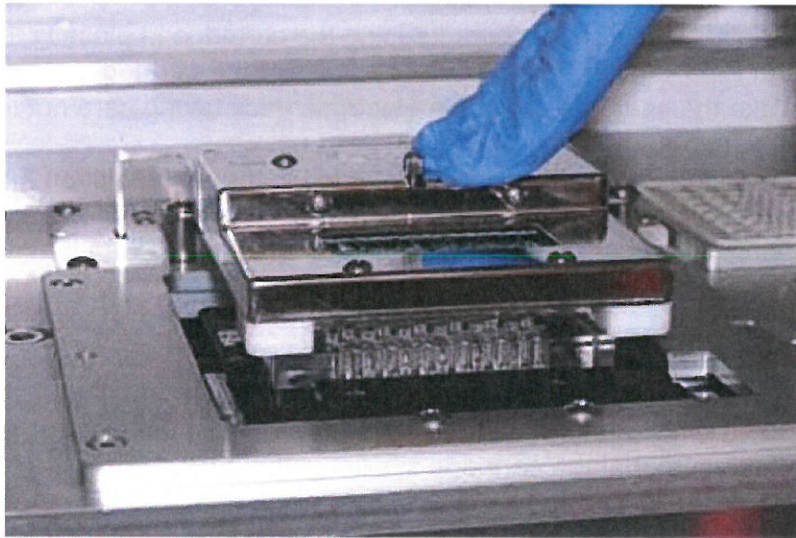


E. Loading cartridge array

- Gently pulling up on the tab that is on the front of the cartridge electrode deck will allow access to seat the cartridge.
- Open the cartridge pouch only after it has reached room temperature >20 minutes. (Opening pouch before reaching room temperature could cause moisture buildup on the cartridge)
- Place cartridge into its seat under the electrodes using the conformation shown in the picture. It is very important that you use proper conformation or you could bend the electrodes causing serious damage to the system.



- Once cartridge is in place and sits flat in its seat, gently push down on the top of the electrode deck with two fingers until mechanism engages and locks the cartridge into place. Note: If resistance is encountered check to make sure that the electrodes all fit properly into each of the 24 holes.



2.2 Performing a Prep Station run

A. Select a Sensitivity of Screening

- Select high sensitivity that will run for 3 hours
- Click next

B. Review set up of consumables and other pre run orders

- Continue to click next over the next several procedural windows, checking to make sure the setup is complete.

C. Sample selection

- Select the number of samples that you have loaded. Consumables must be run in samples of 6 or of 12.

D. Notification Option

- You may select an email go to your address to notify when the prep station run has completed.
- It is important that you seal the cartridge as quickly as possible to keep from evaporation and or quenching of the signal on the cartridge.

E. Process sample

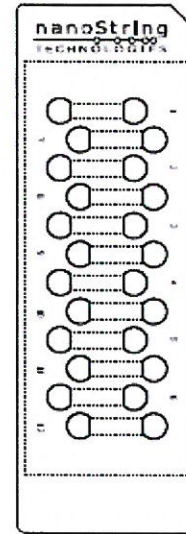
- Click the "Start" button on the "Start Processing" screen to begin the 3 hour run.

3.0 Setup and Running the nCounter Digital Analyzer

3.1 Set up of the nCounter Digital Analyzer

A. Prior to using a codeset for the first time it is necessary to upload its customized RLF file from the USB drive provided in the codeset box stored at -80°C

- From the main menu click on upload file
- Then click on upload RLF file
- Chose the RLF file to upload to the system and it will be saved Placing the cartridge in the Digital Analyzer
- Gently pull up on the lever keeping the cartridge in place in the electrode deck of the Prep Station. This will allow access to the cartridge
- Take the cartridge out of the electrode deck taking care not to bend the electrodes.
- Place the clear adhesive film provided in the master kit on the cartridge as to cover all the holes and will not run over the sides of the cartridge and will seal firmly to the cartridge with no wrinkles or bubbles. Film that runs over the side of the cartridge could cause the cartridge not to sit flat in the Analyzer and will distort data.
- Pull up on the tab closest to the door of the Analyzer to access the reading deck.
- Place the cartridge in the reading deck in a conformation that has the angled notch to the upper right hand corner as seen in the picture.
- Make sure that the cartridge sits flat in the deck.
- Close the lid to each reading deck making sure that the magnet on the tip of the lid engages with the magnet on the base of each deck.
- Proceed to running Digital Analyzer



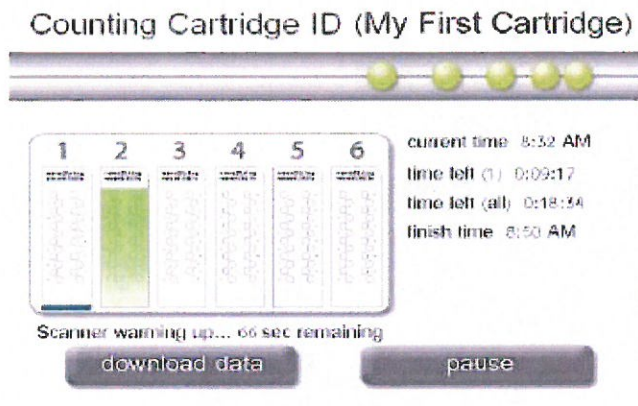
3.2 Running cartridges on the nCounter Digital Analyzer

A. Creating CDF file

- From the main menu select “start counting”
- On the next screen chose the analysis bay that you have placed your cartridge and click create CDF button
- Select all tubes that will have the same RLF file linked to it and click next
- Select the RLF file that the sample codeset should use in analysis (if you have more than one codeset on the same cartridge just repeat the last two steps labeling the tubes with alternate codesets and applying that codeset to those tubes.

B. Performing a run

- Once the CDF file is established press the next button
- Select the “high” resolution option for data analysis
- Select email alert option to have the unit send a confirmation when the run has completed and the zipped RCC data files will also be sent on the completion confirmation email
- Initiate imaging to begin the run, on high resolution the run should take 2.5 hours to complete. You can see the progression by the green bar filling up the cartridge in the display window.



C. Data Collection

- From the main menu select “start counting”
- Download the zipped RCC data file by either saving the attached file from the completion email, or by downloading onto a flash drive from the Digital Analyzer
- If you download from the Digital Analyzer you plug in your drive to the USB port on the right side of the screen on the machine. Select download files. Select the files you want to download, and then press the “download” button.
- Unzip the RCC files on a computer that contains the nSolver software and proceed with data analysis.

4.0 nCounter default QC protocol

Quality Control (QC) of data is performed according to nSolver™ Data Analysis Software Guidelines. Each NanoString experiment contains a set of positive controls comprised of a linear titration of in vitro transcribed synthetic RNA transcripts and corresponding probes and a set of negative controls consisting of probes with no sequence homology to human RNA sequences as well as a set of housekeeping genes. The normalization involves three steps: first a normalization with respect to the geometric mean of the positive control spike counts, then a normalization with respect to the geometric mean of a group of housekeeping reference genes, and finally a background correction, which consists of subtracting the mean + two standard deviation of the negative control counts.



4.1 Data Import

A. Uploading RCC files

- From the main interface, choose “import RCC Files”
- Select all files to be uploaded from the unzipped folder uploaded to your computer from the nCounter Digital Analyzer
- If necessary select “GX” as the type of assay performed
- Select QC measures that are default for “GX” assays

Import RCC Files

Run QC

1. Specify QC parameters for all data types to be imported.
2. Select appropriate data type in navigation bar at the left and set QC values.
3. QC will be performed automatically during the import process.

Execute QC on GX files

Flag lanes/samples where ANY of the following criteria are met:

- Imaging QC:** Flag lanes when percent FOV registration is less than
- Binding Density QC:** Flag lanes when binding density is outside of - range
- Positive Control Linearity QC:** Flag lanes when Positive Control R^2 value is less than
- Positive Control Limit of Detection QC:**
Flag lanes when .5fM positive control is less than or equal to standard deviations above the the negative controls.

Back Import Cancel

4.2 Starting a new Study

A. Click on experiment tab

B. Click on “New Study”

- Fill in any information that will help tract information in the new study

C. Click on “New Experiment”

- Fill in any information that will help tract information in the new experiment then click next
- Select the codesets on the left window pane that will be used in this experiment.
- Filter out any lanes that you do not want in to take into consideration.
- Proceed with default settings, unchecking the Ratios box on the top left of the ratios screen
- Complete the experiment

4.3 Viewing QC and Normalized flags and data

A. Viewing QC Flags

- Click arrow next to the experiment name
- Click on the “+” sign to expand the data choices
- Click on the “Raw Data” and scroll to the right to check for flags on QC measures
- Perform the same on the “Normalized Data” found just below the “Raw Data” selection

B. Viewing Data

- On “Raw Data” highlight the samples you would like to view data from and then click on the “Table” button.
- This will bring up a table that is oriented with the codeset genes on the left column proceeding downward and the samples on the top row proceeding to the right up to 12 rows.
- The numbers represented are raw counts of the codeset identifiers from the digital analyzer.
- The same can be done with the normalized data by repeating the last two steps under the “Normalized Data” selection.
- The numbers on the table created under normalized data are counts normalized underneath the default parameters set up during the “New Experiment” set up of data.

5. Data Analysis

The nCounter® system will count the number of molecules of each gene’s mRNA transcript in each sample. The quality of the data from the raw counts in each sample is assessed with the nSolver software version 4.0 using the quality control metrics described in section 5 below. Samples that meet all QC criteria will then undergo normalization and QC. Normalized data are used for more detailed analysis tailored to specific disease type and experimental goals.

5.1 Quality Control

Data quality is assessed by first checking the following QC metrics, samples will be removed from further analysis based on how they perform on each metric as described below.

A. Imaging QC

$$\frac{\text{FOV Counted (Fields of View successfully counted)}}{\text{FOV Count (Fields of View attempted)}} = \text{at least 75\%}$$

- a. If there is a flag on imaging QC (i.e. ratio < 75% +/- 3%) for one sample, then sample will removed from further analysis
- b. If there is an imaging QC flag (i.e. ratio < 75% +/- 3%) for all samples, then the cartridge should be rescan as this maybe a result of tilted cartridge during scan)

B. Binding Density QC

- a. The binding density should be 0.1 to 2.25.
 - i. If there is a binding density QC flag, further evaluation of the whole data set is required to determine the cause of the flag. (Note: binding density QC flag on its own does not result in critical data loss).

C. Positive Control Linearity QC

$$R^2 = \text{linear regression of } \frac{\text{Log}_2 \text{ Known [positive controls]}}{\text{Log}_2 \text{ Measured [positive controls]}} > 0.95$$

- a. If there is a positive control linearity QC flag (i.e. $R^2 < 0.95$), examine the positive and negative control values in the data;
 - i. if the sample was flagged because a single positive or negative control probe was much different than expected, sample will be included in further analysis
 - ii. If the sample was flagged because all the positive controls were extremely low (close to the negative control counts), then assay sensitivity is compromised and the sample should be excluded from further analysis.

D. Positive Control Limit of Detection QC

- a. The LOD metric is when POS_E (0.5fM concentration) is less than or equal to 2 standard deviations above the negative controls.
 - i. if the sample was flagged because a single positive or negative control probe was much different than expected, sample will be included in further analysis
 - ii. If the sample was flagged because all the positive controls were extremely low (close to the negative control counts), then assay sensitivity is compromised and the sample should be excluded from further analysis.

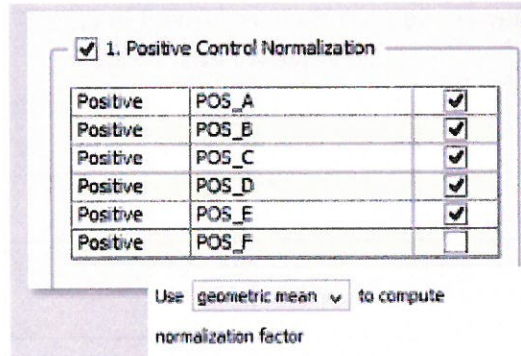
5.2 Normalization

Samples that pass all QC metrics will be included in sample normalization using both positive control normalization and Codeset content normalization.

A. Positive control normalization

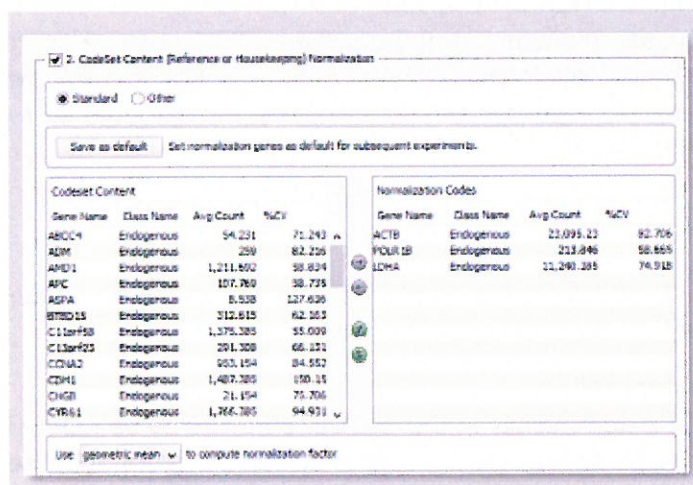
- a. This is done automatically in the nsolver software by selecting the positive control normalization box within the experiment wizard, then selecting the geometric mean to compute normalization. The process is as follows:
 - i. Calculate geometric mean of positive controls for each lane
 - ii. Calculate arithmetic mean of these geometric means for all sample lanes

- iii. Divide this arithmetic mean by the geometric mean of each lane to generate lane-specific normalization factor
- iv. Multiply the counts for every gene by its lane-specific normalization factor.



B. Codeset content/housekeeping gene normalization

- a. This is done automatically in the nsolver software by selecting codeset content normalization box within the experiment wizard, then select standard to compute normalization. The process is as follows:
 - i. Calculate geometric mean of all the housekeeping genes for each lane
 - ii. Calculate arithmetic mean of these geometric means for all sample lanes
 - iii. Divide this arithmetic mean by the geometric mean of each lane to generate lane-specific normalization factor
 - iv. Multiply the counts for every gene by its lane-specific normalization factor.



5.2 Statistical Analyses

Statistical analyses is performed by R version 3.3.0. Background is defined as the mean of the negative controls plus two standard deviations. R script is tailored to the specific disease type and experimental goals.

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[Signatures] and [dates]

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