

nCounter Gene Expression CodeSet RNA Hybridization Protocol User Manual

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Gene Expression CodeSet RNA Hybridization Protocol

This manual includes instructions for setting up hybridization reactions for gene expression assays that utilize NanoString's standard CodeSet chemistry for off-the-shelf XT panels as well as custom-designed XT assays.

For gene expression assays that utilize off-the-shelf Vantage RNA panels or custom-designed XT Elements assays, refer to the Vantage RNA Hybridization Protocol or XT Elements user manuals, respectively.

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Overview

NanoString's patented molecular barcodes provide a true digital detection technology capable of highly multiplexed analysis. CodeSet chemistry uses a Reporter CodeSet and a Capture ProbeSet to bind to the target. Excess probes are removed, and hybridized probes bind to the cartridge. The target-probe complexes are immobilized and aligned on the cartridge, and the barcodes are counted on nCounter[®] systems.

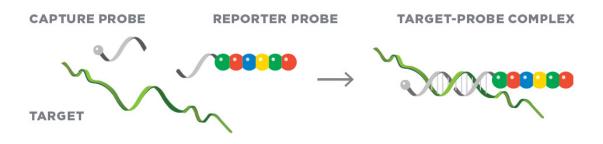


Figure 1. CodeSet chemistry: Capture and Reporter probes bind to the target.

Choose from NanoString's broad portfolio of expertly curated, ready-to-use gene expression panels. Each multiplex panel contains up to 770 genes and is customizable with additional unique targets. Simple workflow of less than 15 minutes hands-on time and streamlined data analysis in under 24 hours.

- Compatible with total RNA, FFPE, cell lysate, PBMC, plasma, serum, and more.
- No amplification, cDNA conversion or library prep with as little as 25 ng input material required.
- Primer pools available for use with multi-target enrichment for low input material or single-cell analysis.

All nCounter gene expression panels are sold in increments of 12 reactions. Master Kits (for MAX or FLEX systems) or SPRINT Reagents and Cartridges (for SPRINT) are also required and sold separately.



Materials and Reagents

Materials Supplied by NanoString

The materials listed in Table 1 are supplied by NanoString for CodeSet processing.

Table 1. Materials supplied by NanoString

Item	Description	Storage
Reporter CodeSet	Barcoded Reporter Probes	At or below -80°C
Capture ProbeSet	Capture Probes	At or below -80°C
Reporter Plus CodeSet (optional)	Barcoded Reporter Plus Probes	At or below -80°C
Capture Plus ProbeSet (optional)	Capture Plus Probes	At or below -80°C
Hybridization Buffer	Supplied with nCounter Master Kits and SPRINT Reagent Packs	RT (15–25°C)
Panel Standard (Optional)	Optional sample used for Calibration	At or below -20°C

Additional Materials Required

The additional materials listed in Table 2 are required to complete the hybridization setup.

Table 2. Additional materials required

ltem	Manufacturer	Part No.
Thermal Cycler	Various	Various
Microfuge or picofuge	Various	Various
NanoDrop or QuBit*	ThermoFisher	Various
Bioanalyzer 2100*	Agilent	G2939BA
12-tube PCR hybridization strip	Various	Various
Multi-channel pipettor	Various	Various
Pipettes for 0.5–10, 2–20, 20–200 μL*	Rainin	Various
96-well clear polystyrene round-bottom plates*	Corning	351177
Disposable gloves	Various	Various
RNeasy [®] Mini Kit* (optional)	QIAGEN	74104, 74106
Proteinase K Solution (20 mg/mL)**	ThermoFisher	AM2546

*Alternative products can be used if they offer similar function and reliability.

If using crude whole cell lysates as sample input, Proteinase K **must be added to the CodeSet Master Mix.

IMPORTANT: NanoString highly recommends verifying the integrity of total RNA samples via denaturing PAGE or Bioanalyzer before proceeding with hybridization. Recommended Total RNA sample: **50 ng to 300 ng** per hybridization assay. Recommended starting amount varies depending on your NanoString Instrument platform and your sample quality (see Table 3).



Sample Input Recommendations

Table 3 provides recommendations for unamplified total RNA required for the CodeSet hybridization.

Table 3. Nucleic Acid Sample In	nput Recommendations
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Analyte		MAX/FLEX	SPRINT
Linamplified Total DNA	Fresh Frozen	100 ng	50 ng
Unamplified Total RNA	FFPE	300 ng	150 ng

For additional information related to nucleic acid sample preparation based on sample type, see Preparing Nucleic Acid from FFPE Samples (MAN-10050) or Preparing RNA and Lysate from Fresh Frozen Samples (MAN-10051)

Important Probe Handling Instructions

- During setup, **do not vortex or pipette vigorously** to mix.
- Mixing should be done by **flicking or inverting** the tubes.
- If using a microfuge to spin down tubes, do not spin any faster than 1,000 x g for more than 30 seconds.
- **Do not "pulse"** to spin because that will cause the centrifuge to go to the maximum speed and may spin the probes out of solution.



Gene Expression CodeSet RNA Hybridization Protocol

GENERAL PROBE HANDLING WARNING: During setup of the reaction, do not vortex or pipette vigorously to mix as it may shear the 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 1,000 rpm for more than 30 seconds. Do not "pulse" to spin because that will cause the centrifuge to go to maximum speed and may spin the CodeSet out of solution.

IMPORTANT: Check the reagent labels before you begin to ensure the correct reagents are being utilized.

NOTE: A thermal cycler with a heated lid is required for this protocol. NanoString recommends a thermal cycler with a programmable heated lid. Models without programmable lids may reach a high temperature that causes tubes to melt or deform during extended or overnight hybridization times, and if used, should be set to ensure that the heated lid does not exceed 110°C.

1. **Pre-heat** the thermal cycler to **65°C** with a heated lid at **70°C**.

NOTE: If using **cell lysates**, see MAN-10051, Preparing RNA and Lysates from Fresh Frozen Samples.

NOTE: If using **Panel Plus** or **CodeSet Plus**, refer to the CodeSet RNA Hybridization Protocol with Panel Plus or CodeSet Plus.

2. **Remove** Reporter CodeSet and Capture ProbeSet tubes from the freezer and thaw at room temperature. Invert or flick the tube several times to mix well and briefly spin down reagents.

IMPORTANT: 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 at room temperature before using.

3. **Create a hybridization Master Mix** by adding the following reagents to the tube containing the Reporter CodeSet. Do not remove the Reporter CodeSet from the tube, add components directly into the CodeSet tube. **Do not add the Capture ProbeSet to the master mix**.

Component	Master Mix (µL)	Per Reaction (µL)
Reporter CodeSet	In tube (42)	3
Hybridization Buffer	70	5
Total Volume	112	8

Table 4. CodeSet Master Mix for one nCounter assay (12 reactions + 2 reactions of dead volume)*

***IMPORTANT:** If using **crude whole cell lysates** as sample input, add Proteinase K to the CodeSet Master Mix at a final concentration of 200 μ g/mL (for 20 mg/ml Proteinase K solution in the final hybridization volume of 15 μ l, add **2.1 \muL** into the 14-reaction hybridization Master Mix).

4. Flick or invert the tube repeatedly to mix then briefly spin down the Master Mix.

- 5. Label a 12-tube PCR hybridization strip. If necessary, ensure the strip will fit in a microfuge or picofuge by cutting both the strip tube and its lid in half prior to setting up the reactions, taking care not to crack the tubes.
- 6. **Prepare hybridization reactions** using a fresh tip for pipetting into each well:
 - a. Add 8 µL of Master Mix to each well of a strip tube.
 - b. Add 5 µL of sample (or diluted Panel Standard) to each tube containing Master Mix. If you are using a Panel Standard, see the section Panel Standard Usage for details.
 - c. Mix the Capture ProbeSet tube by inverting or flicking, and briefly spin down the contents.
 - d. Add 2 μL of Capture ProbeSet to each tube.
 - e. **Cap the strip tubes tightly and mix** by inverting the tubes several times and flicking to ensure complete mixing.
 - f. Spin briefly and immediately place the tubes in a pre-heated 65°C thermal cycler.
- 7. Incubate hybridization reactions for at least 16 hours. Maximum hybridization time should not exceed 48 hours.
- 8. **Incubate at 4°C** once desired hybridization time is reached and process the following day. Do not leave the reactions at 4°C for more than 24 hours or increased background may result.

NOTE: Counts continue to accumulate with time at 65°C, with total counts typically increasing 5% per hour between **16 and 24 hours**. Although a 16-hour incubation is adequate for most purposes, a longer incubation increases sensitivity by increasing counts without significantly increasing background.

 Once the hybridization reactions have been removed from the thermal cycler, proceed immediately to an nCounter Prep Station or SPRINT as described in the nCounter Analysis System User Manual (MAN-C0035) or nCounter SPRINT User Manual (MAN-10017).



Quick Reference GX CodeSet RNA Hybridization Setup

Please read through the full protocol before beginning. This illustrated workflow is intended for quick reference at the bench.

1) Prepare for hybridization

□ Preheat thermocycler to **65° C** with a heated lid at **72°C**.

Create & aliquot Master Mix

Add 70 µL of Hybridization Buffer

create Master Mix.

to the Reporter CodeSet tube to

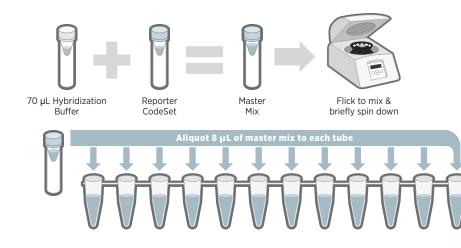
□ Flick to mix, then briefly spin down

□ Aliquot 8 µL of Master Mix into each tube of a labeled 12-tube strip.

□ Thaw codeset & samples.



Preheat to 65°C with a heated lid at 72°C





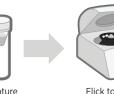
contents.

□ Add **5 µl of sample** to each tube.

Note: If using less than 5 μ L of sample, add RNAse-free water to each tube to bring the volume to 13 μ L.

4) Add Capture ProbeSet

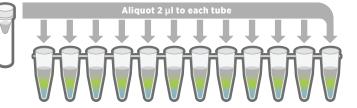
- □ Flick-mix Capture ProbeSet and spin down briefly.
- □ Add **2 µL of Capture ProbeSet** to each tube.
- □ Cap tightly, **flick-mix**, and **spin down briefly**.



Capture ProbeSet

5 µL of sample (each)

> Flick to mix & briefly spin down





Flick to mix & briefly spin down



Immediately place tubes in the thermocycler at 65° C for
 16-24 hours. Maximum hybridization time should not exceed 48 hours.





65°C for 16-24 hours (48 hours maximum)

OPTION A: Use nCounter[®] MAX/FLEX



6a) Load nCounter MAX/FLEX

See MAN-CO035, nCounter Analysis System User Manual for details.

OPTION B: Use nCounter [®] SPRINT
(b) Load nCounter SPRINT
See MAN-10017, nCounter SPRINT Profiler User Manual for details.

Panel Standard Usage

Some panels are available with a Panel Standard. The Panel Standard contains a pool of synthetic DNA oligonucleotides that correspond to the target sequence of each of the probe sets in the panel. This enables normalization of the data to control for potential user, instrument, and lot-to-lot variation, which is important when comparing data from studies run at different times. Additionally, panel standard is required for some analytical methods to allow proper normalization and calculation of gene signatures or other advanced metrics.

NanoString recommends that at a minimum, Panel Standard be run at least once per purchased lot of panel. A typical study may include the panel standard more frequently, e.g., one lane of panel standard for every two to three cartridges. The most rigorous studies will include one lane of Panel Standard per cartridge to enable the most controlled data analysis.

IMPORTANT: 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 Panel Standard

- The Panel Standard is supplied at a concentrated stock solution in 4.5 μL and stored at less than -20°C.
 Remove one tube of 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 Panel Standard in the hybridization reaction, the same as you would add sample.
- 6. The diluted Panel Standard sample **should not be re-frozen**. If additional Panel Standard need to be run in the future, prepare a fresh dilution from a new vial.



Gene Expression CodeSet RNA Hybridization Protocol with Panel Plus or CodeSet Plus

GENERAL PROBE HANDLING WARNING: During setup of the reaction, do not vortex or pipette vigorously to mix as it may shear the 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 1,000 rpm for more than 30 seconds. Do not "pulse" to spin because that will cause the centrifuge to go to maximum speed and may spin the CodeSet out of solution.

IMPORTANT: Check the reagent labels before you begin to ensure the correct reagents are being utilized.

IMPORTANT: For CodeSet Plus scans, a combined RLF **must** be acquired in order to properly count both the probes in the base panel as well as the add-in genes in the Plus portion of the panel. See Preparing the RLF for more information.

NOTE: A thermal cycler with a heated lid is required for this protocol. NanoString recommends a thermal cycler with a programmable heated lid. Models without programmable lids may reach a high temperature that causes tubes to melt or deform during extended or overnight hybridization times, and if used, should be set to ensure that the heated lid does not exceed 110°C.

1. **Pre-heat** the thermal cycler to **65°C** with a heated lid at **70°C**.

NOTE: If you are using **cell lysates**, see MAN-10051, Preparing RNA and Lysates from Fresh Frozen Samples.

NOTE: If you are not using **Panel Plus** or **CodeSet Plus**, use the CodeSet Hybridization Setup.

2. **Remove** Reporter CodeSet, Capture ProbeSet, Reporter Plus and Capture Plus tubes from the freezer and thaw at room temperature. Invert or flick the tube several times to mix well and briefly spin down reagents.

IMPORTANT: 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 at room temperature before using.

3. **Create a hybridization master mix** by adding the following reagents to the tube containing the Reporter CodeSet (Table 5). Do not remove the Reporter CodeSet from the tube, add components directly into the CodeSet tube. **Do not add the Capture ProbeSet or Capture Plus to the master mix**.



Component	Master Mix (µL)	Per Reaction (µL)
Reporter CodeSet	In tube (42)	3
Reporter Plus	28	2
Hybridization Buffer	70	5
Total Volume	140	10

 Table 5. CodeSet + Panel/CodeSet Plus Master Mix for one nCounter assay (12 reactions + 2 reactions of dead volume)*

***IMPORTANT:** If using **crude whole cell lysates** as sample input, add Proteinase K to the CodeSet Master Mix at a final concentration of 200 μ g/mL (for 20 mg/ml Proteinase K solution in the final hybridization volume of 18 μ l, add **2.5 \muL** into the 14-reaction hybridization Master Mix).

- 4. Flick or invert the tube repeatedly to mix then briefly spin down the Master Mix.
- 5. **Label** a 12-tube PCR hybridization strip. If necessary, ensure the strip will fit in a microfuge or picofuge by cutting both the strip tube and its lid in half prior to setting up the reactions, taking care not to crack the tubes.
- 6. Prepare hybridization reactions using a fresh tip for pipetting into each well:
 - a. Add 10 µL of Master Mix to each well of a strip tube.
 - b. Add 5 μL of sample (or diluted Panel Standard) to each tube containing Master Mix. If you are using a Panel Standard, see Panel Standard Usage for details.
 - c. **Mix the Capture ProbeSet and Capture Plus tubes** by inverting or flicking, and briefly spin down the contents.
 - d. **Create a Master Capture** by adding **14** μ L of the Capture Plus directly into the Core Capture ProbeSet tube containing **28** μ L. Mix by inverting or flicking, and briefly spin down the contents.
 - e. Add 3 µL of Master Capture to each tube.

NOTE: Final hyb volume for Core CodeSet + Panel/CodeSet Plus will be 18 μL.

- f. **Cap the strip tubes tightly and mix** by inverting the tubes several times and flicking to ensure complete mixing.
- g. Spin briefly and immediately place the tubes in a pre-heated 65°C thermal cycler.
- 7. Incubate hybridization reactions for at least 16 hours. Maximum hybridization time should not exceed 48 hours.
- 8. Incubate at 4°C once desired hyb time is reached and process the following day. Do not leave the reactions at 4°C for more than 24 hours or increased background may result.

NOTE: Counts continue to accumulate with time at 65°C, with total counts typically increasing 5% per hour between **16 and 24 hours**. Although a 16-hour incubation is adequate for most purposes, a longer incubation increases sensitivity by increasing counts without significantly increasing background.



 Once the hybridization reactions have been removed from the thermal cycler, proceed immediately to an nCounter Prep Station or SPRINT as described in the nCounter Analysis System User Manual (MAN-C0035) or nCounter SPRINT User Manual (MAN-10017).

Preparing the RLF

All nCounter Plus reagents are accompanied by an add-in library file (ALF), which specifies the association between each Plus reagent and its target. Information from the ALF must be merged with the reporter library file (RLF) from the original CodeSet prior to scanning on the Digital Analyzer. Failure to merge an ALF with the original RLF will result in no count information being collected for targets of Plus reagents. Counts that are not scanned are not recoverable on MAX/FLEX instruments.

To obtain a merged RLF file, email NanoString at bioinformatics@nanostring.com. Include both the ALF for your Plus product and the RLF for the CodeSet that you want to spike into the Plus product. A new merged RLF will be generated and emailed to the requestor's address that contains all probe information for both the Plus product and the original CodeSet.

IMPORTANT: Failure to use the merged RLF during the subsequent scan will result in incomplete barcode scanning and the loss of data from a subset of the included probes.



Quick Reference

GX CodeSet RNA CodeSet/Panel Plus Hybridization Protocol

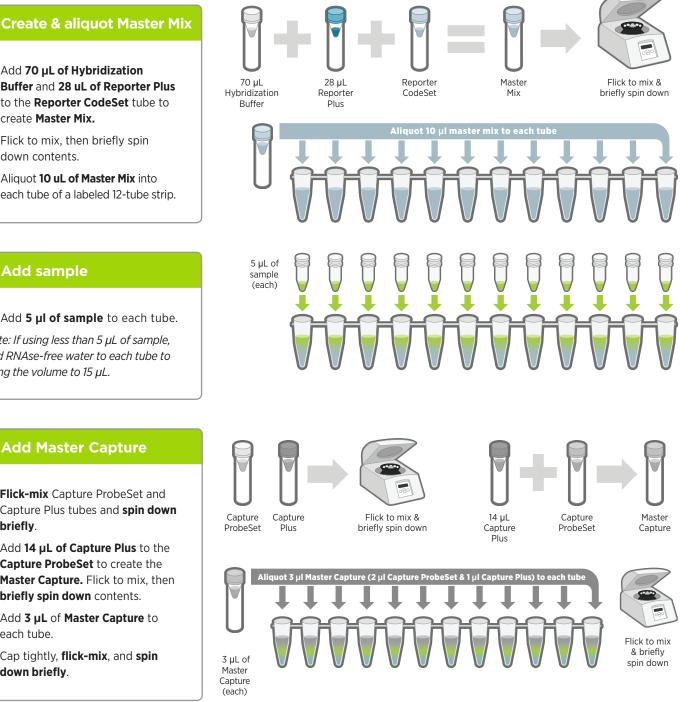
Please read through the full protocol before beginning. This illustrated workflow is intended for guick reference at the bench.

Prepare for hybridization

- Preheat thermocycler to 65° C with a heated lid at 72°C.
- **Thaw samples** and Codeset and CodeSet Plus tubes.



Preheat to 65°C with a heated lid at 72°C



- Add 70 µL of Hybridization Buffer and 28 uL of Reporter Plus to the Reporter CodeSet tube to create Master Mix.
- □ Flick to mix, then briefly spin down contents.
- □ Aliquot 10 uL of Master Mix into each tube of a labeled 12-tube strip.

Add sample

Add **5 µl of sample** to each tube.

Note: If using less than 5 µL of sample, add RNAse-free water to each tube to bring the volume to 15 µL.

Add Master Capture

- □ Flick-mix Capture ProbeSet and Capture Plus tubes and **spin down** briefly.
- Add 14 μL of Capture Plus to the Capture ProbeSet to create the Master Capture. Flick to mix, then briefly spin down contents.
- □ Add **3 µL** of **Master Capture** to each tube.
- □ Cap tightly, **flick-mix**, and **spin** down briefly.



Immediately place tubes in the thermocycler at 65° C for
 16-24 hours. Maximum hybridization time should not exceed 48 hours.





65°C for 16-24 hours (48 hours maximum)

OPTION A: Use nCounter[®] MAX/FLEX



6a) Load nCounter MAX/FLEX

See MAN-CO035, nCounter Analysis System User Manual for details.

OPTION B: Use nCounter [®] SPRINT
6b Load nCounter SPRINT
See MAN-10017, nCounter SPRINT Profiler User Manual for details.

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