nCounter®
Gene Expression Panels and Custom CodeSet
User Manual

MAN-10056-06 | August 2022

nanoString®

About this Manual

This manual is intended for use with NanoString® Technologies Gene Expression Panels and Custom CodeSets, which utilize standard CodeSet chemistry.

For assays that use TagSet chemistry, such as Vantage Fusions or nCounter® Elements assays, please refer to those products' respective user manuals in the NanoString University Document Library.

For instructions on post-hybridization processing and data analysis, please see the instrument-specific user manuals (nCounter Pro Analysis System User Manual (MAN-10147), nCounter Analysis System User Manual for MAX/FLEX systems (MAN-C0035), nCounter SPRINT Profiler User Manual (MAN-10017)) and the Gene Expression Data Analysis Guidelines (MAN-C0011).

Changes in this Revision (MAN-10056-06)

- Updates to text for clarity and accuracy.
- Updates to list of required materials.
- Updates to Quick Reference Guide.
- Panel Standard information moved to Appendix I.

Table of Contents

Introduction

NanoString® patented molecular barcodes provide a true digital detection technology capable of highly multiplexed analysis. CodeSet chemistry consists of a Reporter CodeSet and a Capture ProbeSet that hybridize specific targets of interest (Figure 1). Excess probes are removed, and hybridized target-probe complexes are immobilized and aligned on a cartridge. This is followed by imaging and counting of the barcodes on an nCounter® system.

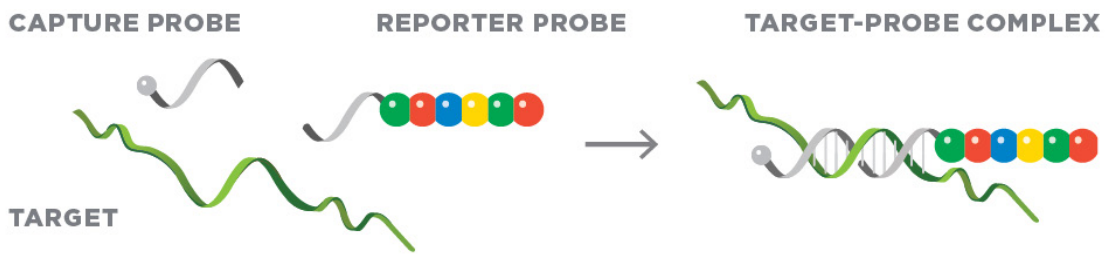


Figure 1. CodeSet chemistry: Capture and Reporter probes bind to the target.

NanoString nCounter Assays

Choose from NanoString's broad portfolio of expertly curated, ready-to-use gene expression panels. Each multiplex panel contains up to 770 genes and can be customized by adding up to 55 additional unique targets. nCounter technology follows a simple workflow with less than 15 minutes of hands-on time and streamlined data analysis for results 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 or 5000 cells input material required.
- Primer pools available for use with multi-target enrichment for low input material.

All nCounter gene expression panels are sold in increments of 12 reactions. Master Kits (for MAX, FLEX, or Pro Analysis Systems) or SPRINT Reagents and Cartridges (for SPRINT Profilers) are also required and sold separately (see Table 1).

Materials and Equipment

Materials Supplied by NanoString

Table 1. NanoString-provided materials required for CodeSet hybridization.

Item	Reagents	Storage
GX Panel or CodeSet - *Catalog # varies*	Reporter CodeSet Capture ProbeSet	-80°C -80°C
Panel Plus / CodeSet Plus (optional) - *Catalog # varies*	Reporter Plus CodeSet Capture Plus ProbeSet	-80°C -80°C
Panel Standard (optional) - *Catalog # varies*	Optional sample used for Calibration	-20°C
nCounter Master Kit (*for Pro or MAX/FLEX*) - *Catalog # NAA-AKIT-012*	nCounter Sample Cartridge Prep Plate Prep Pack, including Hybridization Buffer	-20°C 4°C 15–25°C
nCounter SPRINT Reagent Pack (*for SPRINT*) - *Catalog # SPRINT-REAG-KIT*	nCounter SPRINT Reagent C nCounter SPRINT Reagent A, B, and Hybridization Buffer	4°C 15–25°C
nCounter SPRINT Cartridge (*for SPRINT*) - *Catalog # SPRINT-CAR-1.0*	nCounter SPRINT Cartridge	-20°C

Additional Materials Required

Table 2. Additional materials required (not provided by NanoString).

Item	Supplier, Catalog #
Pipettes for 0.5–10, 2–20, 20–200 µL	Various
Multi-channel pipette for 20 µL (optional)	
RNase-free pipette tips with aerosol barriers	
0.2-mL strip tubes and caps, nuclease-free (SPRINT users only; these are provided in Master Kits for MAX/FLEX/Pro users)	
Disposable gloves	
Molecular biology-grade nuclease-free water	
Proteinase K Solution (20 mg/mL)*	ThermoFisher®, AM2546

If using crude whole cell lysates as sample input, Proteinase K **must be added to the CodeSet Master Mix (see Table 4 and Table 5).*

Equipment

Table 3. Required equipment to process an nCounter CodeSet.

Equipment	Supplier, Catalog #
Thermal cycler with a programmable heated lid (see Thermal Cycle Guidelines, below)	Various
Picofuge or mini-centrifuge with strip tube adaptor	Various
NanoDrop™ spectrophotometer or equivalent	ThermoFisher, ND-2000
Bioanalyzer™ 2100	Agilent®, G2939BA
NanoString nCounter® Pro, MAX, or FLEX Analysis System or SPRINT Profiler	NanoString Technologies, Inc

Thermal Cycler Guidelines

Please note that a thermal cycler with a heated lid is required for this protocol. NanoString recommends a model with a programmable heated lid, to avoid high temperatures that cause tubes to melt or deform.

- NanoString recommends a thermal cycler with a heated lid that can adjust throughout the protocol. The heated lid should be set to 5°C greater than the current incubation temperature at any moment.
- Otherwise, program the heated lid to be 5°C greater than the maximum temperature reached in the protocol. The heated lid should not exceed 110°C.

Sample Input Recommendations

Please refer to Preparing Nucleic Acid from FFPE Samples (MAN-10050) or Preparing RNA and Lysate from Fresh Frozen Samples (MAN-10051) for guidance to extract RNA and determine its concentration and purity.

IMPORTANT: NanoString strongly recommends verifying the integrity of total RNA samples via denaturing PAGE or Bioanalyzer before proceeding with hybridization.

The recommended input amount of total RNA for CodeSet hybridization depends on the sample type and the nCounter platform (see Table 3).

Table 3. Nucleic acid sample input recommendations.

Sample Type		Pro or MAX/FLEX	SPRINT
Unamplified Total RNA	From Fresh Frozen	100 ng	50 ng
	From FFPE	300 ng	150 ng

Panel Standard

If using a Panel Standard with your nCounter assay, please refer to Appendix I. Use of the Panel Standard on page 17 for instructions.

Gene Expression Panel / CodeSet Hybridization Protocol

Reporter CodeSet and Capture ProbeSet Handling Instructions:

- During setup, do not vortex or pipette vigorously to mix. Instead, gently flick or invert the tubes.
- To spin down contents of tubes, a picofuge or mini-centrifuge is recommended. If using a centrifuge, spin at <3000x*g* for <10 seconds. Do not "pulse" spin as it will cause the centrifuge to go to maximum speed and may spin the probes out of solution.

IMPORTANT: Check the reagent labels before you begin to ensure use of the correct reagents. If using **Panel Plus** or **CodeSet Plus**, refer to Gene Expression Panel with Panel Plus / CodeSet with CodeSet Plus Hybridization Protocol on page 13.

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

   NOTE: A thermal cycler with a heated lid is required for this protocol. NanoString recommends a thermal cycler with a *programmable* heated lid. See Thermal Cycler Guidelines on page 6.

2. **Remove** Reporter CodeSet and Capture ProbeSet tubes from the -80°C freezer and thaw at room temperature, shielded from light. Once thawed, invert or flick the tube several times to mix well, then briefly spin down.

   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 to room temperature before using.

3. **Create a hybridization master mix** by adding the hybridization buffer to the tube containing the Reporter CodeSet (Table 4). Do not remove the Reporter CodeSet from the tube. **Do not add the Capture ProbeSet to the master mix**.

   Table 4. Hybridization master mix for one nCounter assay (12 reactions + 2 reactions of dead volume).

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

   *IMPORTANT: If using **crude whole cell lysates** as sample input, add Proteinase K to the hybridization master mix at a final concentration of 200 µg/mL (for 20 mg/ml Proteinase K solution in the final hybridization volume of 15 µl, add **2.1 µL** into the 14-reaction hybridization master mix).

4. **Flick or invert the tube repeatedly** to mix, then briefly spin down.

5. **Label a strip tube**. If necessary, cut strip in half to fit in a picofuge with strip tube adaptor, and label both halves. For MAX/FLEX/Pro users, use the strip tubes provided with the nCounter Master Kits, ensuring that the notch is positioned between tubes 1-2 and 8-9.

6. **Prepare hybridization reactions** using a new pipette tip at every step:
   a. **Add 8 µL** of hybridization master mix to each tube of the prepared strip tube.
   b. **Add 5 µL** of sample (or diluted Panel Standard - see Appendix I. Use of the Panel Standard on page 17 for details) to each tube containing hybridization master mix. If adding less than 5 µL, add nuclease-free water to bring the reaction volume to 13 µL.
   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 the pre-heated **65°C** thermal cycler.
7. **Incubate hybridization reactions** for at least **16 hours**. Maximum hybridization time should not exceed **48 hours**. It is recommended to keep a consistent hybridization time across experiments.

   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.
8. **(Optional) Incubate at 4°C** on the thermal cycler following desired hybridization time before proceeding to processing on the nCounter system. Do not leave the reactions at 4°C for more than 24 hours or increased background may result.
9. Once the hybridization reactions have been removed from the thermal cycler, **proceed immediately to loading on an nCounter Prep Station or SPRINT Profiler**. Please refer to instrument-specific user manuals (nCounter Pro Analysis System User Manual (MAN-10147), nCounter Analysis System User Manual for MAX/FLEX Systems (MAN-C0035), nCounter SPRINT Profiler User Manual (MAN-10017)).

Quick Reference Guide

GX CodeSet RNA Hybridization Setup

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

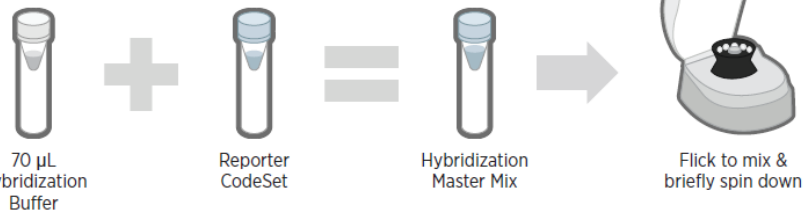
1 Prepare for hybridization

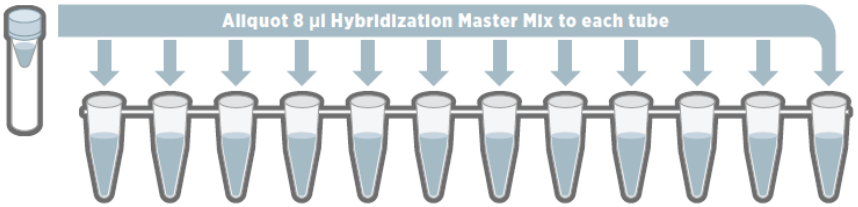
- ☐ Preheat thermocycler to **65° C** with a heated lid at **70° C**.
- ☐ **Thaw** Codeset and samples.

Preheat to 65° C with a heated lid at 70° C

2 Create & aliquot Hybridization Master Mix

- ☐ Add **70 µL of Hybridization Buffer to the Reporter CodeSet tube** to create **Hybridization Master Mix**.
- ☐ Flick to mix, then briefly spin down contents.
- ☐ Aliquot **8 µL of Hybridization Master Mix** into each tube of a labeled 12-tube strip.

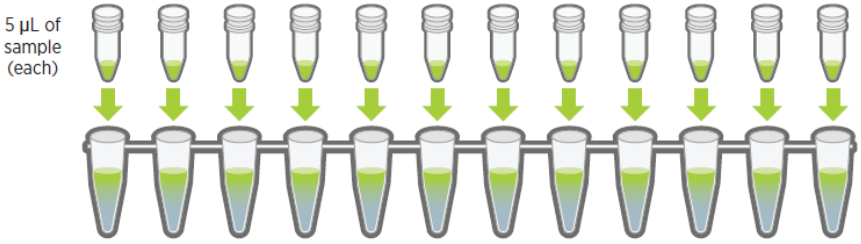




3 Add sample

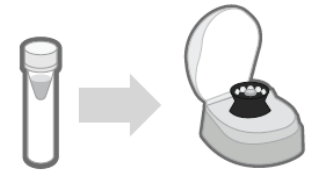
- ☐ Add **5 µl of sample** to each tube.

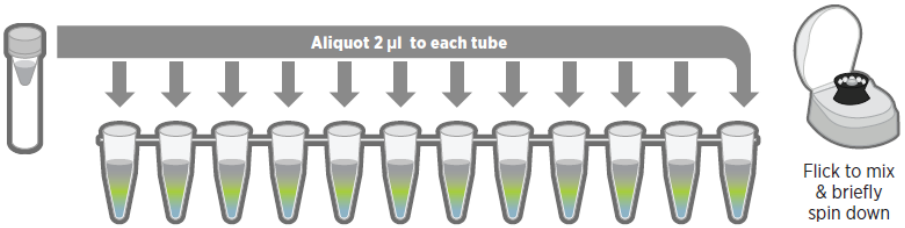
Note: If using less than 5 µL of sample, add RNAse-free water to each tube to bring the volume to 13 µL



4 Add Capture ProbeSet

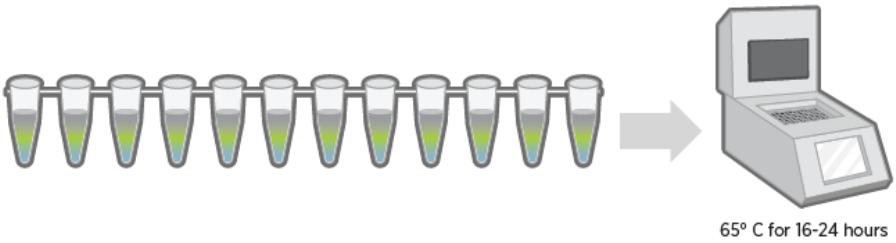
- ☐ **Flick-mix** Capture ProbeSet and **spin down briefly**.
- ☐ Add **2 µL of Capture ProbeSet** to each tube.
- ☐ Cap tightly, **flick-mix**, and **spin down briefly**.





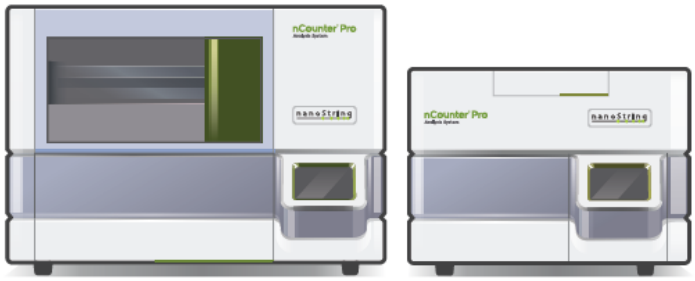
5 Hybridize

☐ **Incubate** hybridization reactions at **65° C for 16-24 hours.** Hybridizations should be left at 65° C until ready for processing.



65° C for 16-24 hours

OPTION A: Use nCounter® Pro or MAX/FLEX



6a Load nCounter Pro or MAX/FLEX

See **MAN-C0035, nCounter Analysis System User Manual** or **MAN-10147, nCounter Pro 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.

nCounter Panel Plus Products

All off-the-shelf nCounter Gene Expression panels are customizable by adding 6 to 55 user-defined probes. These additional probes are referred to as a Panel Plus product. Similarly, customized CodeSets can be supplemented with CodeSet Plus products.

Preparing a Merged Reporter Library File (RLF)

All nCounter Plus products are accompanied by an add-in library file (ALF) that specifies the association between each Plus probe pair and its target. Information from the ALF must be merged with the reporter library file (RLF) from the CodeSet the Plus product is being added to, prior to scanning the cartridge or the barcodes on the nCounter Digital Analyzer or SPRINT Profiler. Failure to merge an ALF with an nCounter CodeSet RLF will result in no count information being collected for targets of Plus products.

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 into which you will spike the Plus product. A new merged RLF will be generated and emailed to the requestor that contains all probe information for both the Plus product and the original CodeSet.

IMPORTANT: When using a Plus product, you MUST use a merged RLF to ensure counting of Plus targets. Ensure that you have the merged RLF file before beginning the hybridization protocol.

Gene Expression Panel with Panel Plus / CodeSet with CodeSet Plus Hybridization Protocol

Reporter CodeSet and Capture ProbeSet Handling Instructions:

- During setup, do not vortex or pipette vigorously to mix. Instead, gently flick or invert the tubes.
- To spin down contents of tubes, a picofuge or mini-centrifuge is recommended. If using a centrifuge, spin at <3000x*g* for <10 seconds. Do not "pulse" spin as it will cause the centrifuge to go to maximum speed and may spin the probes out of solution.

IMPORTANT: Check the reagent labels before you begin to ensure use of the correct reagents. If you are NOT using **Panel Plus or CodeSet Plus**, refer to the Gene Expression Panel / CodeSet Hybridization Protocol on page 8.

IMPORTANT: When using a Panel Plus product, you MUST use a merged RLF to ensure counting of Panel Plus targets. Ensure that you have the merged RLF file before beginning the hybridization protocol. See Preparing a Merged Reporter Library File (RLF) on page 12 for more information.

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

   NOTE: A thermal cycler with a heated lid is required for this protocol. NanoString recommends a thermal cycler with a *programmable* heated lid. See Thermal Cycler Guidelines on page 6.

2. **Remove** Reporter CodeSet, Capture ProbeSet, Reporter Plus and Capture Plus tubes from the -80°C freezer and thaw at room temperature, shielded from light. Once thawed, invert or flick the tube several times to mix well, then 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 hybridization master mix**.

Table 5. Hybridization master mix for Panel + Panel Plus or CodeSet + CodeSet Plus (12 reactions + 2 reactions of dead volume)

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

*IMPORTANT: If using **crude whole cell lysates** as sample input, add Proteinase K to the hybridization master mix at a final concentration of 200 µg/mL (for 20 mg/ml Proteinase K solution in the final hybridization volume of 18 µl, add **2.5 µL** into the 14-reaction hybridization master mix).

4. **Flick or invert the hybridization master mix tube repeatedly** to mix, **then briefly spin down**.
5. **Label** a strip tube. If necessary, cut strip in half to fit in a picofuge with strip tube adaptor, and label both halves. For MAX/FLEX/Pro users, use the strip tubes provided with the nCounter Master Kits, ensuring that the notch is positioned between tubes 1-2 and 8-9.
6. **Prepare hybridization reactions** using a fresh tip for pipetting into each well:
   a. **Add 10 µL** of hybridization master mix to each tube of the prepared strip tube.
   b. **Add 5 µL** of sample (or diluted Panel Standard - see Appendix I. Use of the Panel Standard on page 17 for details) to each tube containing hybridization master mix. If adding less than 5 µL, add nuclease-free water to bring the reaction volume to 15 µL.
   c. **Mix the Capture ProbeSet and Capture Plus tubes** by inverting or flicking, and briefly spin down the contents.
   d. **Create a Capture Master Mix** by adding **14 µL** of the Capture Plus directly into the Core Capture ProbeSet tube containing **28 µL**. Mix by inverting or flicking, and briefly spin down the contents.
   e. **Add 3 µL** of Capture Master Mix to each tube.

   NOTE: Final hybridization volume for Core CodeSet + Panel Plus or CodeSet Plus is **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 the pre-heated **65°C** thermal cycler.
7. **Incubate hybridization reactions** for at least **16 hours**. Maximum hybridization time should not exceed **48 hours**. It is recommended to keep a consistent hybridization time across experiments.

   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.
8. **(Optional) Incubate at 4°C** on the thermal cycler following desired hybridization time before proceeding to processing on the nCounter system. Do not leave the reactions at 4°C for more than 24 hours or increased background may result.
9. Once the hybridization reactions have been removed from the thermal cycler, **proceed immediately to loading on an nCounter Prep Station or SPRINT Profiler**. Please refer to instrument-specific user manuals (nCounter Pro Analysis System User Manual (MAN-10147), nCounter Analysis System User Manual for MAX/FLEX systems (MAN-C0035), nCounter SPRINT Profiler User Manual (MAN-10017)).

Quick Reference Guide

Panel Plus/CodeSet Plus RNA Hybridization Setup

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

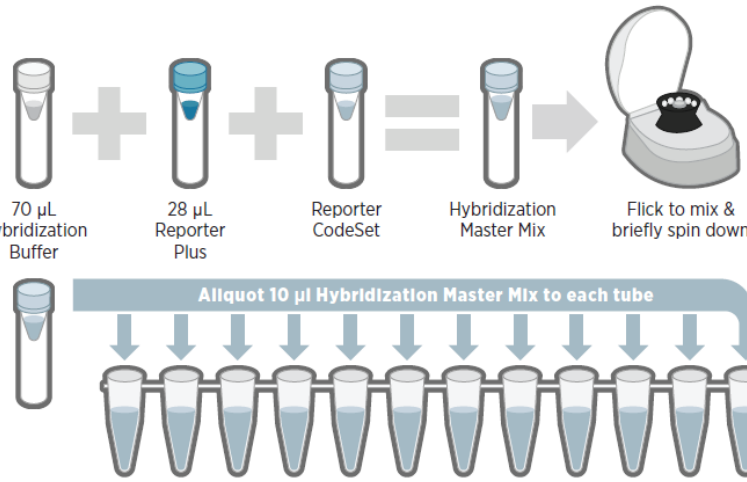
1 Prepare for hybridization

- ☐ Preheat thermocycler to **65° C** with a heated lid at **70°C**.
- ☐ **Thaw samples** and Codeset and CodeSet Plus tubes.

Preheat to 65°C with a heated lid at 70°C

2 Create & aliquot Hybridization Master Mix

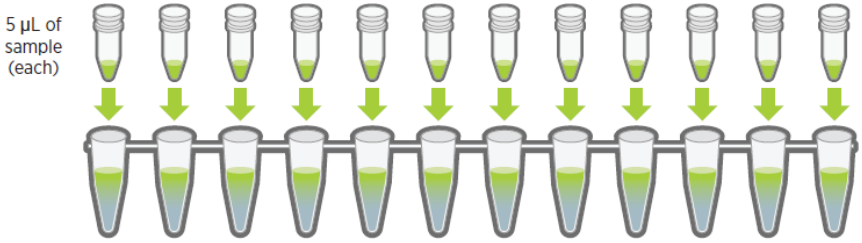
- ☐ Add **70 µL of Hybridization Buffer** and **28 µL of Reporter Plus** to the **Reporter CodeSet** tube to create **Hybridization Master Mix.**
- ☐ Flick to mix, then briefly spin down contents.
- ☐ Aliquot **10 µL of Hybridization Master Mix** into each tube of a labeled 12-tube strip.



3 Add sample

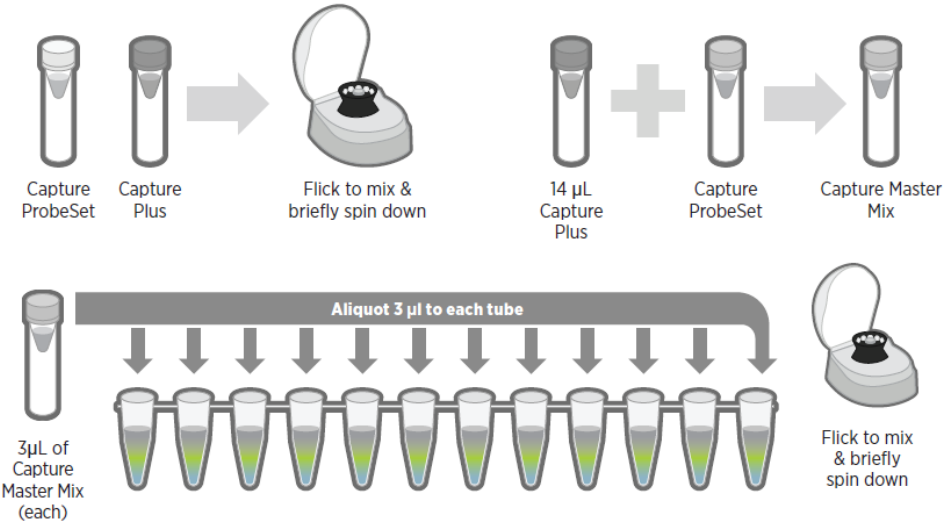
- ☐ Add **5 µl of sample** to each tube.

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



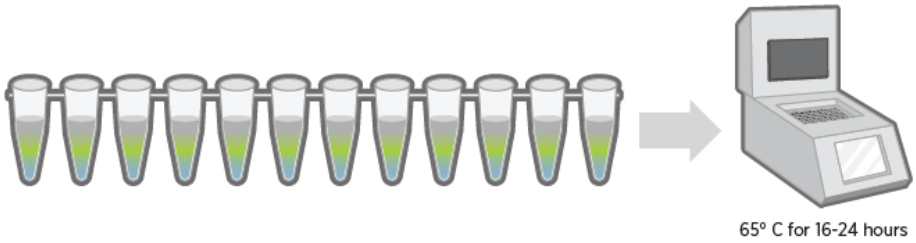
4 Add Capture Master Mix

- ☐ **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 **Capture Master Mix.** Flick to mix, then **briefly spin down** contents.
- ☐ Add **3 µL** of **Capture Master Mix** to each tube.
- ☐ Cap tightly, **flick-mix**, and **spin down briefly**.

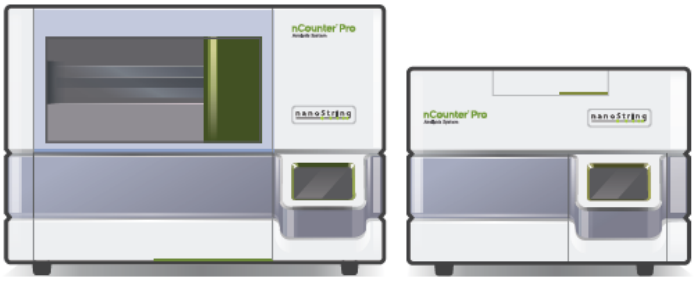


5 Hybridize

☐ **Incubate** hybridization reactions at **65° C for 16-24 hours.** Hybridizations should be left at 65° C until ready for processing.



OPTION A: Use nCounter® Pro or MAX/FLEX



6a Load nCounter Pro or MAX/FLEX

See **MAN-C0035, nCounter Analysis System User Manual** or **MAN-10147, nCounter Pro 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.

Appendix I. Use of the Panel Standard

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 additional 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 sites or over an extended period of time. Additionally, panel standard is required in some analytical methods for 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.

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.

1. The Panel Standard is supplied as a concentrated stock solution in 4.5 µL and stored at -20°C or below. **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 (at **step 6b** on page 10 or 14).
6. The diluted Panel Standard sample **should not be re-frozen**. If additional Panel Standard must be run in the future, prepare a fresh dilution from a new vial.

Technical Support

For technical support, please contact support@nanostring.com.

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Trademarks

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