nCounter®
RUO PAM50 CodeSet
User Manual

MAN-10125-03 | August 2022

nanoString®

Changes in this Revision

- Updates to text for clarity and accuracy.
- Updates to list of required materials.
- Added RNA Extraction and RNA Input Recommendations (page 5).
- Updates to Quick Reference Guide.

Table of Contents

Introduction

PAM50 is a 50-gene signature that is used to classify breast cancer into four molecular intrinsic subtypes: Luminal A, Luminal B, Basal-like, and HER2-enriched. The clinical and prognostic characteristics of these subtypes have been extensively studied. The companion PAM50 signature report calculates subtypes by comparing expression for PAM50 genes and centroids of the gene sets for each of the four subtypes, resulting in a set of correlation values for each sample.

These signatures are for research use only (RUO) and not for clinical or diagnostic use.

The prepared RNA samples are used as input for nCounter® hybridization reactions containing NanoString Reporter and Capture probes. These overnight hybridization reactions enable specific hybridization of Reporter and Capture probes to their target (**Figure 1**). After hybridization, reactions are purified and imaged on an nCounter system.

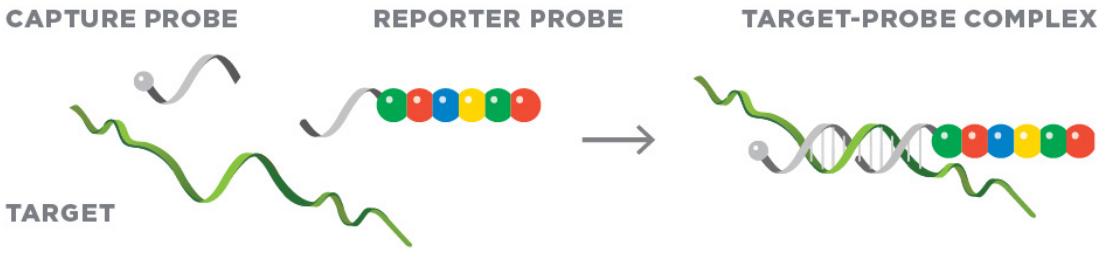


Figure 1. CodeSet chemistry: Capture and Reporter probes bind to the target.

All nCounter gene expression panels are sold in increments of 12 reactions. Additional materials to process samples on an nCounter Analysis System or SPRINT Profiler are required and sold separately (see Table 1).

Workflow

1. Prepare FFPE RNA samples and assess quantity and quality (refer to Preparing RNA from FFPE Samples (MAN-10050)).
2. Set up RNA hybridization to the RUO PAM50 CodeSet (0.5 hours).
3. Hybridize overnight in thermal cycler with a heated lid (15–24 hours).
4. Purify and bind hybridized RNA targets on an nCounter Analysis System (Pro or MAX/FLEX) or SPRINT Profiler.

Materials and Equipment

Materials Supplied by NanoString

Table 1. NanoString-provided materials required to run nCounter RUO PAM50 Assay.

Item	Reagents	Storage
RUO PAM50 CodeSet - *Catalog # RUO-PAM50-12*	RUO PAM50 Reporter CodeSet RUO PAM50 Capture ProbeSet	-80°C -80°C
RUO PAM50 Reference Sample (optional) - *Catalog # NALG-REF-PAM50*	RUO PAM50 Reference Sample	-80°C
RUO PAM50 Panel Plus (optional) - *Catalog # varies*	RUO PAM50 Reporter Plus CodeSet tube RUO PAM50 Capture Plus CodeSet tube	-80°C -80°C
nCounter Master Kit (*for MAX/FLEX/Pro*) - *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
Multi-channel pipette for 200 µL	Various
Single-channel pipettes for 2 µL, 20 µL, 200 µL, 1000 µL	
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	

Equipment

Table 3. Required equipment to run the nCounter RUO PAM50 Assay.

Equipment	Supplier
Thermal cycler with a programmable heated lid	Various
Standard benchtop centrifuge with a fixed-angle rotor that fits 1.5 mL tubes	
Picofuge or mini-centrifuge with 1.5 mL-tube rotor and strip tube rotor	
NanoString nCounter® Pro, MAX, or FLEX Analysis System or SPRINT Profiler	NanoString

Sample Preparation Recommendations

RNA Extraction

See Preparing RNA from FFPE Samples (MAN-10050) for guidance to extract RNA and determine its concentration and purity.

Isolated RNA is expected to meet the following specifications:

- RNA concentration: ≥ 12.5 ng/µL
- RNA purity: A260/A280 ratio between 1.7 and 2.3

RNA Input to RUO PAM50 Assay

The recommended RNA input for the assay is 250 ng. The acceptable RNA input range for hybridization is 125–500 ng.

- Calculate the volume (in microliters) of RNA sample to add to the hybridization reaction by dividing the desired sample input (e.g., 250 ng) by the measured concentration.
- If the calculated concentration of the sample is between 12.5 ng/µL and 25 ng/µL, add the maximum volume of 10 µL.
- For samples that require less than 10 µL, calculate the volume of water required to generate a 10 µL final sample volume.

RUO PAM50 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 RUO PAM50 Panel Plus, refer to RUO PAM50 Panel Plus Hybridization Protocol on page 11.

1. **Pre-heat** a thermal cycler to **65°C** with a heated lid at **70°C**; set the calculated reaction volume to 30 µL and the time interval to "infinite". **Do not** set the thermal cycler to ramp down to 4°C at the end of the run.

   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, if used, should be set to ensure that the heated lid does not exceed 110°C.

2. **Remove** Reporter CodeSet and Capture ProbeSet from the -80° freezer and thaw at room temperature, shielded from light. Once thawed, invert or gently flick the tubes several times to mix well and briefly spin down reagents. Store reagents on ice if not proceeding immediately.

   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 Hybridization Buffer to the tube containing the Reporter CodeSet at room temperature. Do not remove the Reporter CodeSet from the tube; add the Hybridization Buffer directly into the CodeSet tube. **Do not add the Capture ProbeSet to the master mix**.

   Table 4. RUO PAM50 Master Mix for one nCounter assay (12 reactions + 1 reaction dead volume).

Component	CodeSet Master Mix (µL)	Per Reaction (µL)
Reporter CodeSet	65 (in tube)	5
Hybridization Buffer	130	10
Total Volume	**195**	**15**

4. **Gently 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 new pipette tip at every step:
   a. **Add 15 µL of hybridization master mix** to each well of the prepared strip tube.
   b. **Add up to 10 µL of sample** to each tube containing hybridization master mix. If using less than 10 µL of sample, add nuclease-free water to each tube to bring the volume to 25 µL.
   c. **Mix the Capture ProbeSet tube** by inverting or flicking, and briefly spin down the contents.
   d. **Add 5 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 at 65°C for 15–21** hours. Hybridizations should be left at 65°C until ready for processing.
8. Once the hybridization reactions have been removed from the thermal cycler, **proceed immediately to loading 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)).

RUO PAM50 Hybridization Protocol

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

1 Prepare for hybridization

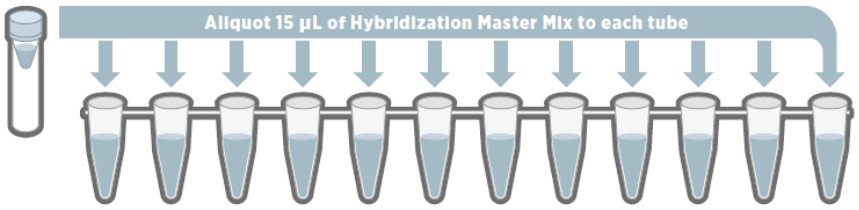
- ☐ Preheat thermocycler to **65° C** with heated lid at **70° C**.
- ☐ **Thaw** CodeSet & samples.

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

2 Create & aliquot Hybridization Master Mix

- ☐ Add **130 µL of Hybridization Buffer to the Reporter CodeSet tube** to create Hybridization Master Mix.
- ☐ Flick to mix, then briefly spin down contents.
- ☐ Aliquot **15 µL of Hybridization Master Mix** into each tube of a labeled 12-tube strip.

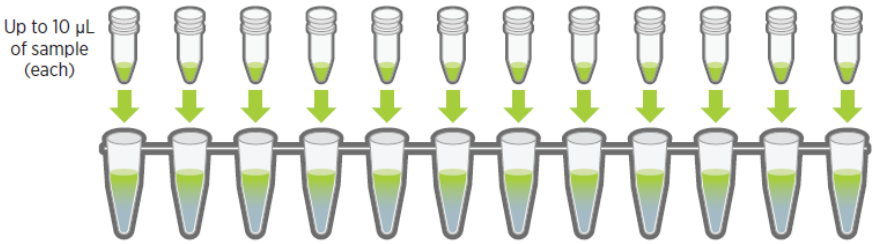
130 µL Hybridization Buffer | 65 µL Reporter CodeSet | 195 µL Hybridization Master Mix | Flick to mix & briefly spin down



3 Add sample

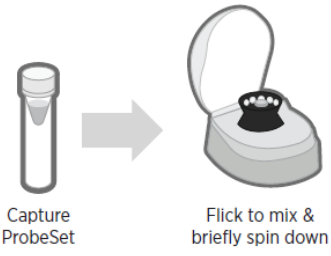
- ☐ Add **up to 10 µl of sample** to each tube.

Note: *If using less than 10 µL of sample, add RNAse-free water to each tube to bring the volume to 25 µL.*

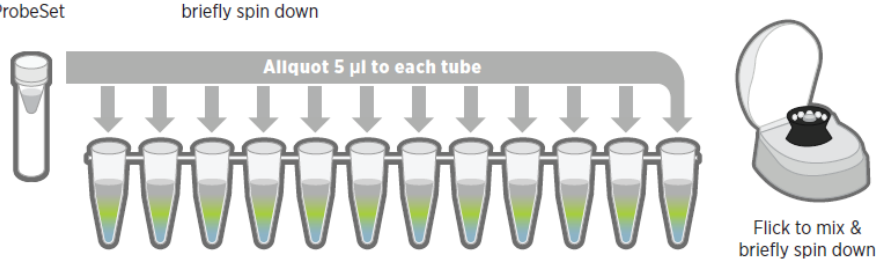


4 Add Capture ProbeSet

- ☐ **Flick-mix** Capture ProbeSet and **spin down briefly**.
- ☐ Add **5 µL of Capture ProbeSet** to each tube.
- ☐ Cap tightly, **flick-mix**, and **spin down briefly**.

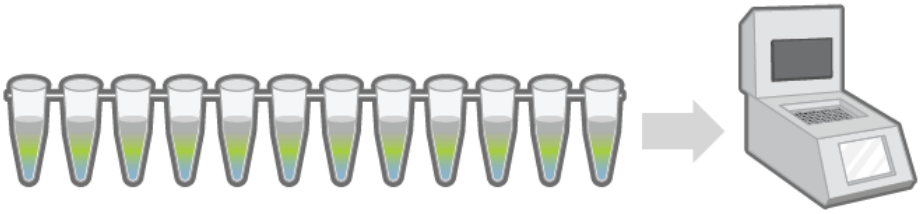


Capture ProbeSet | Flick to mix & briefly spin down

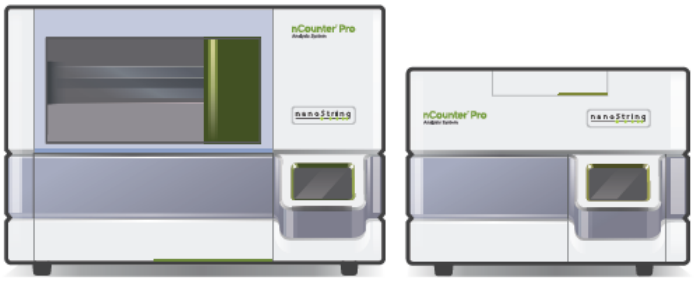


5 Hybridize

☐ **Incubate** hybridization reactions at **65° C for 15-21 hours.** Hybridizations should be left at 65° C until ready for processing.



OPTION A: 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: 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.

RUO PAM50 Panel 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, refer to the RUO PAM50 Hybridization Protocol on page 6.

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 the Merged Reporter Library File (RLF) on page 10 for more information.

1. **Pre-heat** a thermal cycler to **65°C** with a heated lid at **70°C**, set the calculated reaction volume to 30 µL, and the time interval to "infinite". **Do not** set the thermal cycler to ramp down to 4°C at the end of the run.

   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, if used, should be set to ensure that the heated lid does not exceed 110°C.

2. **Remove** Reporter CodeSet, Capture ProbeSet, Reporter Plus, and Capture Plus tubes from the -80° 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. Store reagents on ice if not proceeding immediately.

   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 Reporter Plus CodeSet and Hybridization Buffer to the tube containing the Reporter CodeSet at room temperature. Do not remove the Reporter CodeSet from the tube — add components directly into the CodeSet tube. **Do not add the Capture ProbeSet to the hybridization master mix**.

Table 5. RUO PAM50 Hybridization Master Mix for one nCounter assay (12 reactions + 1 reaction dead volume).

Component	Hybridization Master Mix (µL)	Per Reaction (µL)
Reporter CodeSet	65 (in tube)	5
Reporter Plus CodeSet	26	2
Hybridization Buffer	130	10
Total Volume	**221**	**17**

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 new pipette tip at every step:
   a. **Add 17 µL of hybridization master mix** to each well of the prepared strip tube.
   b. **Add up to 10 µL of sample** to each tube containing hybridization master mix. If using less than 10 µL of sample, add nuclease-free water to each tube to bring the volume to 27 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. Mix by inverting or gently flicking, and briefly spin down the contents.
   e. **Add 6 µL** of Capture Master Mix to each tube.

      NOTE: Final hybridization volume for **RUO PAM50 CodeSet + Panel Plus** is **33 µL**.

   f. **Cap the strip tubes tightly and mix** by inverting the tubes several times and gently 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 at 65°C for 15–24 hours**. Hybridizations should be left at **65°C** until ready for processing.
8. Once the hybridization reactions have been removed from the thermal cycler, **proceed immediately to 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

RUO PAM50 Panel Plus Hybridization Protocol

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

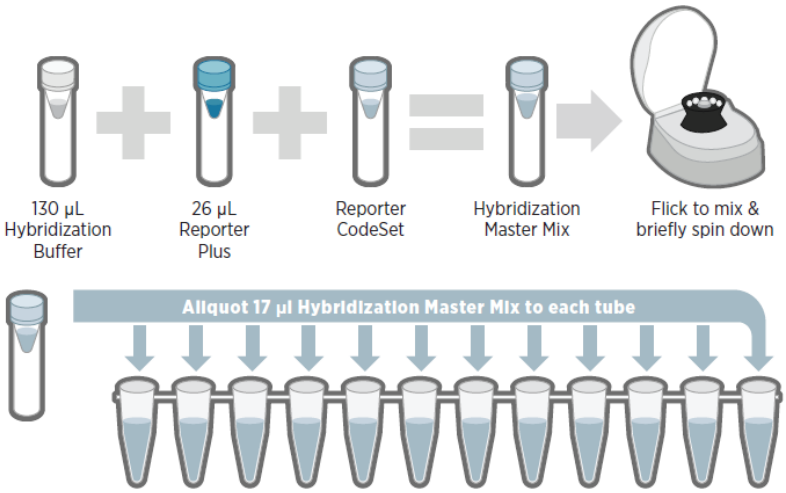
1 Prepare for hybridization

- ☐ Preheat thermocycler to **65° C** with 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

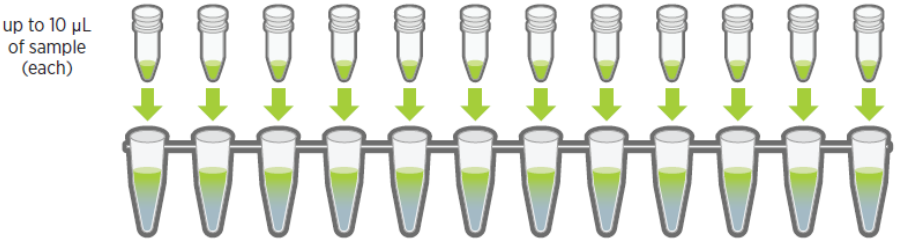
- ☐ Add **130 µL of Hybridization Buffer** and **26 µL of Reporter Plus** to the **Reporter CodeSet** tube to create **Hybridization Master Mix.**
- ☐ Flick to mix, then briefly spin down contents.
- ☐ Aliquot **17 µL of Hybridization Master Mix** into each tube of a labeled 12-tube strip.



3 Add sample

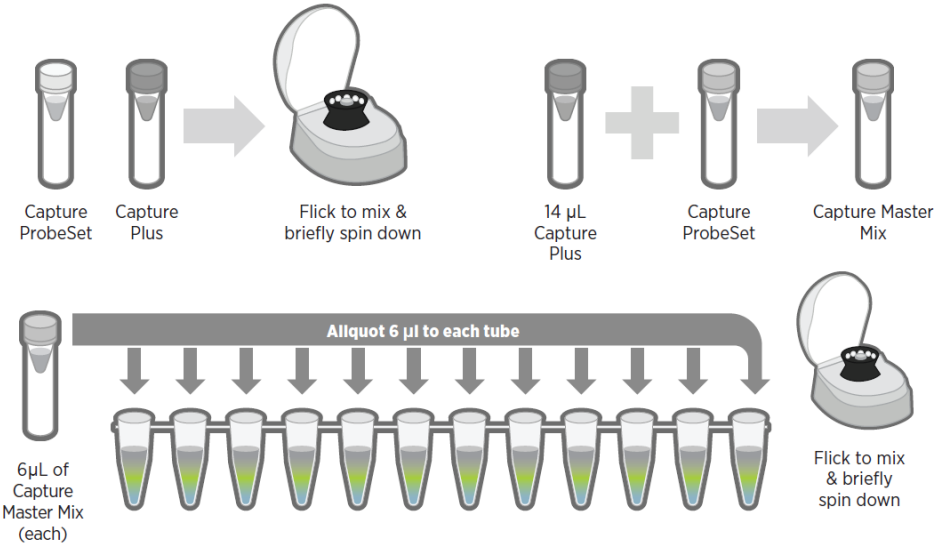
- ☐ Add **up to 10 µl of sample** to each tube.

Note: *If using less than 10 µL of sample, add RNAse-free water to each tube to bring the volume to 25 µL.*



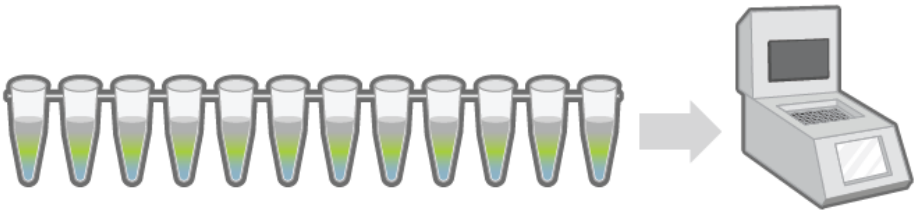
4 Add Capture Master Mix

- ☐ **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 **6 µL** of **Capture Master Mix** to each tube.
- ☐ Cap tightly, **flick-mix**, and **spin down briefly**.



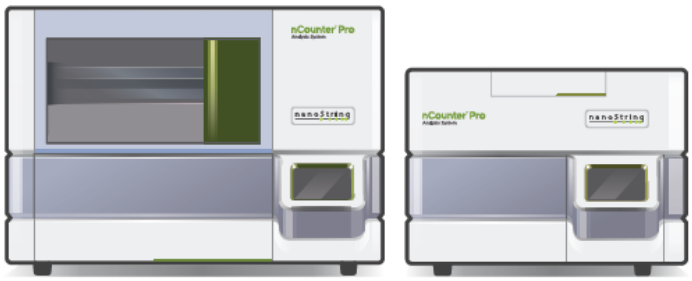
5 Hybridize

☐ **Incubate** hybridization reactions at **65° C for 15-24 hours.** Hybridizations should be left at 65° C until ready for processing.



65° C for 15-24 hours

OPTION A: nCounter® Pro or MAX/FLEX



OPTION B: nCounter® SPRINT

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.

6b Load nCounter SPRINT

See **MAN-10017, nCounter SPRINT Profiler User Manual** for details.

Technical Support

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

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NanoString Technologies, Inc.
530 Fairview Avenue North
Seattle, Washington 98109
T (888) 358-6266
F (206) 378-6288
info@nanostring.com

Sales Contacts
United States: us.sales@nanostring.com
EMEA: europe.sales@nanostring.com
Asia Pacific & Japan: apac.sales@nanostring.com
Other Regions: info@nanostring.com