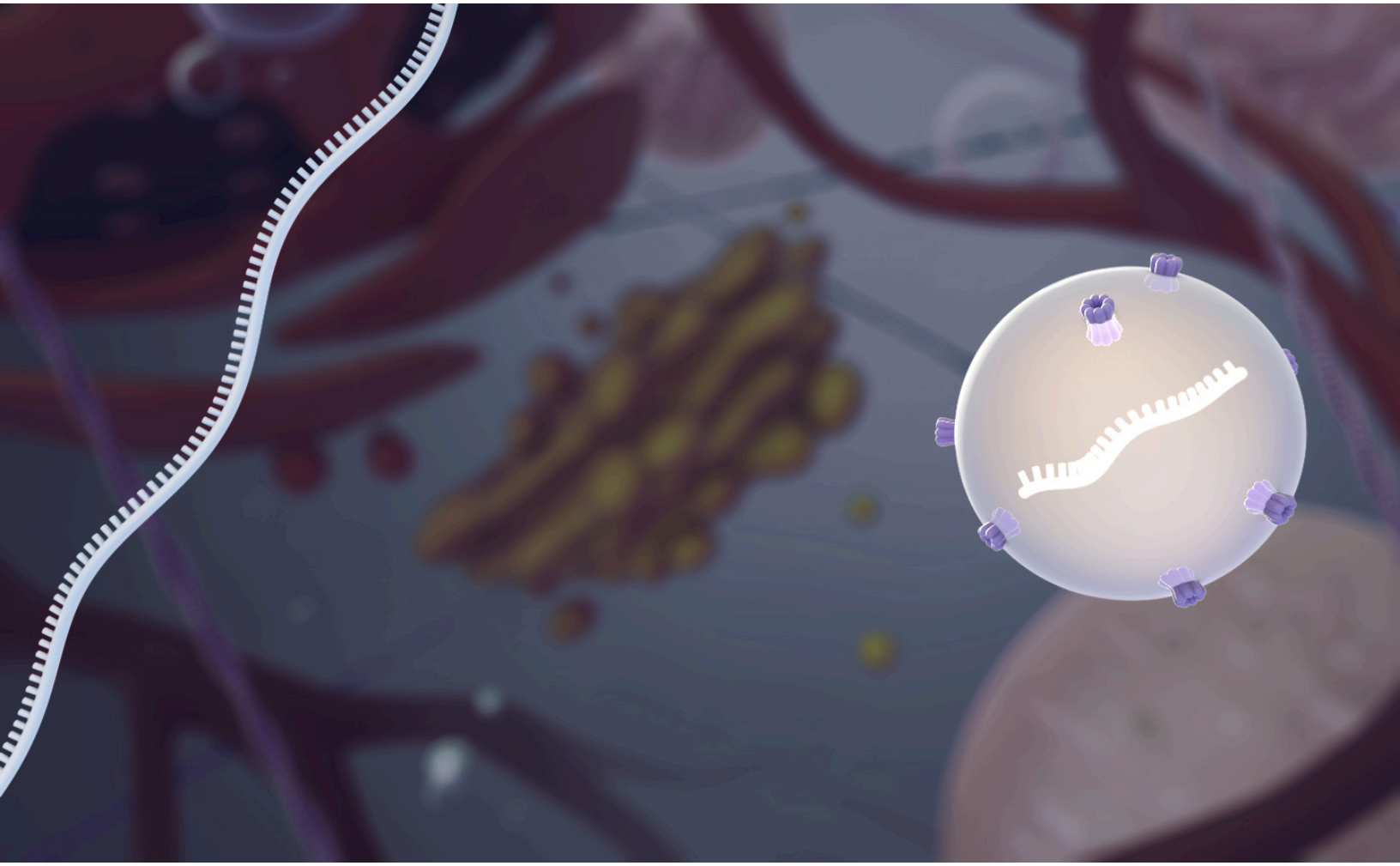
nanoString™



nCounter® miRGE™ Assay

User Manual

Contents

Introduction

The nCounter miRGE (miRNA and Gene Expression) Assay is designed to provide a single-tube, ultra-sensitive, reproducible, and highly multiplexed method for detecting subsets of both mRNAs and miRNAs in total RNA across all biological levels of expression. After processing with an nCounter miRNA Sample Preparation Kit, messenger RNA and tagged miRNAs are detected in total RNA without the use of reverse transcription or amplification using molecular barcodes called nCounter Reporter Probes. The assay can be run on total RNA isolated from any source, including Formalin-Fixed Paraffin Embedded (FFPE) samples.

This manual describes in detail the methods for both the miRNA Sample Preparation and the miRNA CodeSet Hybridization. For instructions on post-hybridization processing and data analysis please see the nCounter® Analysis System User Manual (MAN-C0035), nCounter® SPRINT Profiler User Manual (MAN-10017), and the Gene Expression Data Analysis Guidelines (MAN-C0011).

Product Workflow

Table 1. Workflow for the nCounter miRGE Assay

	Process	Hands-on Time
Day 1: Manual Processing	miRGE Sample Preparation Protocol	30 minutes
	miRGE CodeSet Hybridization Setup Protocol	5 minutes
Day 2: Automated Processing	**Process**	**Hands-on Time**
	Set up either the Prep Station run or SPRINT run	30 minutes
	Move Cartridge to Digital Analyzer for Data Collection (MAX/FLEX only)	5 minutes

Overview of miRNA Sample Preparation

The nCounter miRNA Sample Preparation Kit, provided with the miRGE Assay, includes reagents for ligating unique oligonucleotide tags onto miRNAs of interest, allowing these short RNAs to be detected with great specificity and sensitivity. The miRNA tag ligation protocol is compatible with total RNA, preserving mRNA and allowing it to be detected in a downstream hybridization.

> **NOTE**: It is important to note that the while the steps involved for sample preparation in this manual refer to miRNA, mRNA is also present in the sample and will be preserved for detection downstream. Throughout the rest of this section this manual will only refer to the miRNA in the sample preparation steps.

Sample preparation involves a multiplexed annealing of the specific tags to their target miRNA, a ligation reaction, and an enzymatic purification to remove the unligated tags. Sequence specificity between each miRNA and its appropriate tag is ensured by careful, stepwise control of annealing and ligation temperatures. Control miRNA included with the nCounter miRGE Assay allows the user to monitor the ligation efficiency and specificity through each step of the reaction.

The total hands-on time for the sample preparation reaction is approximately 30 minutes, with an elapsed time of approximately 2 hours.

Overview of CodeSet Hybridization and Downstream Processing

NanoString's technology is based on the direct molecular barcoding and digital detection of target molecules using a color-coded probe pair. The probe pair consists of a Reporter Probe, which carries the signal on its 5' end, and a Capture Probe, which carries a biotin moiety on its 3' end. The complexity of the color codes, comprised of four colors in six positions, allows a large diversity of targets present in the same sample to be individually resolved and identified during data collection. During the overnight hybridization reaction, probe pairs are present in large excess to target nucleic acids in order to ensure that each target finds a probe pair.

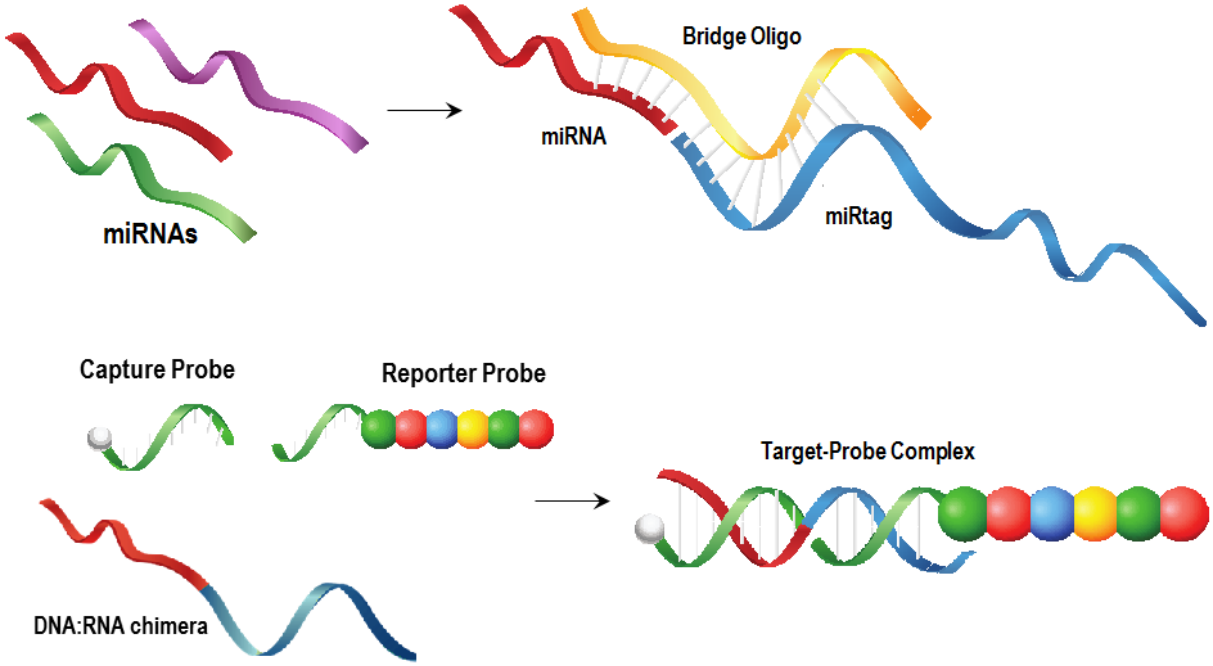


Figure 1. miRNA-specific preparation to utilize the general nCounter technology downstream

The nCounter miRGE Assay can be run on either nCounter Analysis system. The MAX/FLEX is comprised of two instruments, the Prep Station used for post- hybridization processing, and the Digital Analyzer used for data collection. The SPRINT Profiler is a single instrument that handles both the post- hybridization processing and the data collection together.

After hybridization, excess probes are washed away using a two-step magnetic bead-based purification. Magnetic beads derivatized with short nucleic acid sequences that are complementary to the Capture Probe and the Reporter Probes are used sequentially. First, the hybridization mixture containing target/probe complexes is allowed to bind to magnetic beads complementary to sequences on the Capture Probe. Wash steps are performed to remove excess Reporter Probes and non-target cellular transcripts. After washing, the Capture Probes and target/probe complexes are eluted off the beads and are hybridized to magnetic beads complementary to sequences on the Reporter Probe. An additional

wash is performed to remove excess Capture Probes. Finally, the purified target/probe complexes are eluted off the beads and immobilized on the cartridge for data collection.

Digital images are processed and the barcode counts are tabulated in a comma separated value (CSV) format. Data can be analyzed using the nSolver™ software or other analysis programs.

Materials and Equipment

Table 2. Materials and Reagents provided in the miRGE Assay Kit

Item	Reagents	Storage
nCounter miRGE Assay Kit, custom for **Human**, **Mouse**, or **Rat** *Catalog #:* *- MIR-P1CC-48 (48 Assays)* *- MIR-P1CC-96 (96 Assays)* *- MIR-P1CC-192 (192 Assays)* *- MIR-P1CC-384 (384 Assays)* *- MIR-P1CC-576 (576 Assays)*	Custom miRGE Reporter CodeSet Custom miRGE Capture ProbeSet	-80°C -80°C
nCounter miRNA Sample Prep Kit *(part of the miRGE Assay kit)*	Annealing Buffer miRNA Tag Reagent, Species Specific PEG Ligation Buffer Ligase Ligation Clean-up Enzyme miRNA Assay Controls	-20°C -20°C -20°C -20°C -20°C -20°C -80°C

Table 3. Additional materials required (not provided).

Material	Recommended Supplier	Catalog number
nCounter Master Kit *(for MAX/FLEX users)*	NanoString	NAA-AKIT-012
nCounter SPRINT Reagent Pack *(for SPRINT users)*	NanoString	SPRINT-REAG-KIT
nCounter SPRINT Cartridge *(for SPRINT users)*	NanoString	SPRINT-CAR-1.0
Total RNA Extraction Kit†	QIAGEN miRNeasy (or equivalent)	Various
DEPC-treated (or RNAse-free) water	Various	Various
Pipettes for 0.5–10 µL*	Rainin (or equivalent)	Various
Pipettes for 2.0–20 µL*	Rainin (or equivalent)	Various
Pipettes for 20–200 µL*	Rainin (or equivalent)	Various
Manual multi-channel pipette for 200 µL*	Rainin	L12-200XLS+
12-strip standard tubes*	Bioexpress	T-3034-1
Disposable gloves	Various	Various

† Extraction kit must preserve the small RNA fraction.

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

Table 4. Required equipment for use of the miRGE Assay.

Instruments
NanoString nCounter SPRINT, MAX, or FLEX analysis system
Spectrophotometer (NanoDrop Technologies or equivalent)
Picofuge with strip tube adaptor (Stratagene or equivalent)
Calibrated thermal cycler with a heated lid*
DNA Engine thermal cycler or hybridization oven** (MJ Research/BioRad PTC 200 or equivalent)
Fluorometer***

** See Thermal Cycler Guidelines below.*

*** Hybridization oven can be used for the miRNA Hybridization Protocol only. A thermal cycler with a heated lid is required for the miRNA Sample Preparation Protocol.*

**** NanoString recommends using a Qubit™ Fluorometer for RNA and DNA quantification.*

Thermal Cycler Guidelines

Please note that a thermal cycler with a heated lid is required for this protocol. NanoString recommends using a model with a programmable heated lid. Models without programmable lids may reach a very high temperature that causes tubes to melt or deform. However, programmable lids may offer different levels of control.

- Ideally, 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.
- Some heated lids cannot be assigned a floating temperature. In this situation, program the heated lid to be 5°C greater than the maximum temperature during the protocol. The heated lid should not exceed 110°C.

Thermal Cycler Protocols

Table 5. Annealing, Ligation, and Purification protocols

Protocol	Temp	Time
Annealing Protocol	94°C	1 min
	65°C	2 min
	45°C	10 min
	48°C	Hold
	Total Time	**13 minutes**
Ligation Protocol	48°C	3 min
	47°C	3 min
	46°C	3 min
	45°C	5 min
	65°C	10 min
	4°C	Hold
	Total Time	**24 minutes**
Purification Protocol	37°C	1 hour
	70°C	10 min
	4°C	Hold
	Total Time	**1 hour 10 minutes**

Sample Guidelines/Recommendations

The nCounter miRGE Assay requires purified total RNA as input material. NanoString Technologies recommends the use of approximately 100 ng of total RNA, as this quantity of input material generates robust signal for most tissue and cell isolates. Total RNA purified from any cell or tissue type may be used in the assay, including Formalin-Fixed, Paraffin-Embedded (FFPE) material, though highly fragmented samples may require additional input (see below). Unpurified lysates may not be used with the nCounter miRGE Assay, as the denaturants in the homogenization buffer will inhibit the miRNA sample preparation reaction.

The quality of the purified RNA is critically important, as residual contaminants left over from lysis and RNA extraction can impact assay performance by inhibiting the enzymatic ligation and purification steps. Typical lysis or extraction contaminants that can inhibit the assay include:

- Guanidinium Isothiocyanate (lysis buffer)
- Guanidinium HCl (initial wash buffer)
- Phenol (organic extraction)
- Ethanol (secondary wash buffer)

Purified RNA quality can be evaluated via a spectrophotometer by measuring absorbance at 230 nm (A230), 260 nm (A260) and 280 nm (A280). The A260/A280 ratio can help identify contamination with proteins, whereas the A260/A230 ratio can help identify contamination with organic compounds, such as phenol and guanidinium salts. NanoString recommends a 260/280 ratio of 1.9 or greater and a 260/230 ratio of 1.8 or greater for optimal results.

RNA integrity may be evaluated using a fragment analysis system to measure nucleic acid fragmentation. NanoString recommends that at least 50% of the sample be greater than 300 nucleotides (nt) in length for optimal performance. RNA samples that exhibit greater levels of fragmentation may still be used, but input levels may need to be increased (see below).

Appropriate input may be estimated with the following equation: (100/percent of sample >300 nt) x 100 ng. The percent of samples greater than 300 nt can be estimated by having the BioAnalyzer or Tape Station calculate the percent of the sample between 50-300nt and subtracting that quantity from 100%. This calculation is a tool to help estimate ideal input, but not a complete predictor of success; it is less predictive in samples with less than 25% of fragments greater than 300 nt and samples with extremely low concentration (<10 ng/µL).

Understanding and Troubleshooting RNA Sample Contamination

Significant absorbance at 280 nm can indicate contamination with protein. Such contamination may lead to an overestimation of the RNA concentration, resulting in a lower-than-anticipated signal in the assay.

Significant absorbance at 230 nm is indicative of contamination with phenol or guanidinium; a "pure" RNA sample should have a A260/A230 ratio above 2.0. Extra washes with a secondary wash buffer or ethanol can help to minimize carry-through. It is also important that residual secondary wash buffer be removed prior to elution/resuspension.

IMPORTANT:

- At very low RNA concentrations (under ~10 ng/µL), the A260/A230 ratio may be unreliable as an indicator of contamination, due to limited nucleic acid absorbance at 260 nm. NanoString recommends preparing samples with a concentration of > 33 ng/µL, allowing 100 ng of total RNA to be added to the sample preparation reaction in the available 3 µL volume.
- Some RNA extraction protocols suggest that better yield can be achieved by re-eluting the column with the initial eluate. NanoString does not recommend this, as the extra elution can generate significant carry through of guanidinium and organic contamination. If re-elution must be performed, it should be preceded by at least 2 additional column washes with the secondary ethanol-based wash buffer (for a total of 4 secondary washes).
- Ethanol is not evident spectrophotometrically, but can be eliminated by a one-minute post-wash centrifugation in a clean collection tube, as is suggested in most kit protocols. Additionally, air-drying the filter for five minutes can help if ethanol contamination persists.

miRGE Sample Preparation Protocol

nCounter miRGE assays require *purified* total RNA as input material. See the Sample Guidelines/Recommendations for additional information on sample input considerations.

All experiments should be designed in sets of twelve samples. The protocol below is for one set of 12 samples. All reagents are supplied in 12-reaction aliquots.

1. Prepare a 1:5000 dilution of the miRNA Assay Controls. To do this, we recommend performing a serial dilution. First, make a 1:500 dilution by adding 499 µL DEPC H_20 to 1 µL of the miRNA Assay Controls in a sterile microcentrifuge tube. Mix the 1:500 dilution by vortexing and briefly spin down. Add 10 µL of the 1:500 dilution to 90 µL DEPC H_20 in a second sterile microcentrifuge tube to generate the final 1:5000 dilution. Mix the final dilution by vortexing and briefly spin down. Store on ice.
2. Prepare an annealing master mix by combining 13 µL of Annealing Buffer, 26 µL of nCounter miRNA Tag Reagent and 6.5 µL of the 1:5000 miRNA Assay Controls dilution prepared in Step 1. Mix well by pipetting up and down.
3. Aliquot 3.5 µL of the annealing master mix into each tube of a 12 x 0.2 mL strip tube.
4. Add 3 µL of RNA sample to each tube. Cap tubes and flick tubes gently to mix. Spin down.
5. Place strip in thermal cycler and initiate Annealing Protocol (Table 5).
6. Combine 19.5 µL PEG and 13 µL Ligation Buffer to prepare a ligation master mix.

   CAUTION: PEG is viscous and should be pipetted slowly to ensure accurate transfer of volume into the mix. Mix well by pipetting up and down.
7. Following completion of the Annealing Protocol, when the thermal cycler has reached 48°C, add 2.5 µL of the ligation master mix to each tube. (Do not turn off the thermal cycler; you will need the block to be at 48°C in Step 8 and Step 9). Flick tubes gently to mix and spin down.
8. Return tubes to 48°C thermal cycler, close lid, and incubate at 48°C for 5 min.

   CAUTION: For Step 9, **do not remove tubes from the thermal cycler**. Maintaining the temperature of the tubes at 48°C is critical for optimal assay performance. Tubes should never be removed from heat block during this step.
9. Open thermal cycler, carefully remove caps from tubes, leaving strip in place in the heat block, and add 1.0 µL of Ligase directly to each tube while incubating at 48°C. Check the pipette tip to make certain all of the Ligase was added to the reaction. There is no need to mix.

   NOTE: To keep track of Ligase addition to sequential samples, it can be helpful to line up 12 tips in front of thermal cycler, discarding each tip after use.
10. Immediately after addition of Ligase to the final tube, recap tubes (leaving tubes in heat block), close thermal cycler, and initiate Ligation Protocol (Table 5).
11. After completion of Ligation Protocol, add 1 µL Ligation Clean-Up Enzyme to each reaction. The tubes can be removed from the heat block for this step. Flick tubes gently to mix. Spin down.
12. Return tubes to thermal cycler and initiate Purification Protocol (Table 5).
13. After completion of Purification Protocol, proceed immediately with miRGE CodeSet Hybridization Setup Protocol. Be sure to denature your sample before proceeding with CodeSet Hybridization (Step 5 of the miRGE CodeSet Hybridization Protocol).

miRGE CodeSet Hybridization Setup Protocol

Setting Up nCounter Hybridization Assays

> **CAUTION**: During the setup of your assay, do not vortex or pipet reactions vigorously to mix as it may shear the Reporter Probes. Mixing should be done by flicking or inverting the tubes. Do not spin tubes any faster than 1,000 rpm for more than 30 seconds; do not "pulse" microfuge to spin as that will cause the centrifuge to go to maximum speed and you may spin your CodeSet out of solution.

The final hybridization reaction will contain the following components: 10 µL Reporter CodeSet, 10 µL hybridization buffer, the entire miRNA Sample Preparation reaction volume (~10 µL), and 5 µL Capture ProbeSet. The order of addition of components is important; please follow the protocol exactly.

1. Remove aliquots of both the Reporter CodeSet and Capture ProbeSet reagent from the freezer and thaw on ice. Invert the aliquots several times to mix well and briefly spin down reagent at <1000 rpm (see the above **CAUTION**).
2. Create a master mix containing 130 µL of the Reporter CodeSet and 130 µL of hybridization buffer by adding the hybridization buffer to the tube containing the Reporter CodeSet. **Do not add the Capture ProbeSet to the Master Mix.** Invert to mix and spin down master mix.

Table 6.Hybridization Master Mix and Reaction Contents

Component	Hyb Master Mix (µL)	Per Reaction (µL)
miRGE Reporter CodeSet	In tube (130)	10
Hybridization Buffer	130	10
Sample from the miRNA Sample Preparation Protocol	-	~10
miRGE Capture ProbeSet	-	5
Total Volume	**260**	**~35**

3. Label a provided 12-tube strip and cut it in half so it will fit in a picofuge.
4. Add 20 µL of master mix to each of the 12 tubes.

> **CAUTION**: It is advisable to use a fresh tip for each pipetting step to pipet the correct volume. The CodeSet has components that can start to wick up into the tip and not dispense the correct amount if you use the same tip to dispense master mix into all of the hybridization tubes.

5. Denature samples from the miRNA sample prep protocol at 85°C for 5 minutes and quick-cool on ice. Add the entire reaction volume (~10 µL) from the miRNA Sample Preparation Protocol for each sample to each tube.

6. Pre-heat thermal cycler to 65°C. Program the thermal cycler using 35 µL volume, calculated temperature, heated lid and "forever" time setting. 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 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.

7. Add 5 µL of Capture ProbeSet to each tube immediately before placing at 65°C. Cap tubes and mix the reagents by inverting the strip tubes several times and flicking with your finger to ensure complete mixing. Briefly spin down at <1000 rpm and immediately place the strip tube in the 65°C thermal cycler. Minimizing the time between the addition of the Capture ProbeSet and the placement of the reaction at 65°C will increase the sensitivity of your assay.

8. Incubate hybridization assays for at least 12 hours. Hybridizations should be left at 65°C until ready for processing. Maximum hybridization time should not exceed 30 hours.

9. Once removed from the thermal cycler, proceed immediately to post-hybridization processing with the nCounter Analysis System User Manual (MAN-C0035) or the nCounter SPRINT Profiler User Manual (MAN-10017). **Do not store hybridizations at 4°C**.

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