# TagSet Hybridization Setup

## Overview

Prepared samples are used as input into nCounter hybridization reactions containing NanoString Reporter and Capture probes. These overnight hybridization reactions enable specific hybridization of Reporter and Capture probes to their target. After hybridization, reactions are purified and imaged on nCounter® systems.

This manual provides instructions for producing hybridization Master Mix and setting up nCounter hybridizations using TagSet chemistry.

## Contents

# Materials and Reagents

## Materials Supplied by NanoString

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

**Table 1.** Materials supplied by NanoString

| Reagent | Description | Storage |
|---|---|---|
| TagSet | Barcoded Reporter and Capture Probes | At or below -80°C |
| Hybridization Buffer | Supplied with nCounter Master Kits and SPRINT Reagent Packs | RT (15–25°C) |
| Probe Pools (A and B) | Probe A hybridizes to a specific Reporter Tag<br>Probe B hybridizes to a universal Capture Tag | At or below -80°C |

## Additional Materials Required

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

**Table 2.** Additional materials required to run the TagSet hybridization

| Item | Manufacturer | Part # |
|---|---|---|
| Thermal Cycler | Various | Various |
| Microfuge or picofuge | Various | Various |
| NanoDrop ND-2000* | NanoDrop Technologies | N/A |
| Bioanalyzer 2100* | Agilent | G2940CA |
| Disposable gloves | Various | Various |
| 12-tube PCR hybridization strip | Various | Various |
| Multi-channel pipettor | Various | Various |
| Pipettes for 0.5–10 µL* | Rainin® | L-10XLS+ |
| Pipettes for 2–20 µL* | Rainin® | L-20XLS+ |
| Pipettes for 20–200 µL* | Rainin® | L-200XLS+ |
| 96-well clear polystyrene round-bottom plates* | Corning® | 351177 |
| RNeasy® Kit* (optional) | QIAGEN® | 74104, 74106 |
| Proteinase K Solution (20 mg/mL)** | Invitrogen™ | 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 TagSet 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 type and your sample quality (see Table 3).

## Sample Input Recommendation

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

**Table 3.** Nucleic Acid Sample Input Recommendation

| Analyte | | MAX/FLEX | SPRINT |
|---|---|---|---|
| Unamplified Total RNA | Fresh Frozen | 100 ng (~20 ng/µL) | 50 ng (~10 ng/µL) |
| | FFPE | 300 ng (~60 ng/µL) | 150 ng (~30 ng/µL) |

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.

## XT TagSet Hybridization Setup

**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 **67°C** with a heated lid at **72°C**.

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

   **NOTE:** If using **XT TagSet Extensions**, use the XT TagSet Extensions Hybridization Setup.

2. **Remove** TagSet and Probe Pool tubes from the freezer and thaw at room temperature. Invert or flick the tubes several times to mix well and briefly spin down reagents.

3. **Create 30X Working Stocks of all the Master Stock Probe Pools** by following Table 4. (If you have not created the Master Probe Stocks, see Creating Master Stocks before continuing to Working Stocks). The amount of Master Probe Stock and diluent used is dependent on the number of reactions being run. See Table 4 for quantities. For example, for 12 reactions, use 4 µL of Master Stock and 29 µL of TE-Tween for a final volume of 33 µL**. Invert or flick the tubes several times to mix well** and **briefly spin down** reagents. Diluted probe pools should not be stored for long-term use.

   **Table 4**. Diluting Master Stocks to generate Working Pools

| Number of Reactions | Aliquot from Master Probe Stock (µL) | TE-Tween (µL) | Final Volume (µL) |
|---|---|---|---|
| 12 | 4 | 29 | 33 |
| 24 | 4 | 29 | 33 |
| 36 | 5 | 37 | 42 |
| 48 | 7 | 51 | 58 |
| 60 | 8 | 59 | 67 |
| 72 | 10 | 73 | 83 |
| 84 | 11 | 81 | 92 |
| 96 | 13 | 95 | 108 |
| 144 | 19 | 139 | 158 |
| 192 | 26 | 191 | 217 |

4. **Create a hybridization Master Mix** by adding the following reagents to the tube containing the TagSet (Table 5). **Do not remove the TagSet from the tube**, add components directly into the TagSet.

   **Table 5**. TagSet Master Mix for one nCounter assay (12 reactions + 2 reactions of dead volume)*

| Component | TagSet Master Mix (µL) | Per Reaction (µL) |
|---|---|---|
| TagSet | In tube (28) | 2 |
| Hybridization Buffer | 70 | 5 |
| 30x Probe A Pool | 7 | 0.5 |
| 30x Probe B Pool | 7 | 0.5 |
| **Total Volume** | **112** | **8** |

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

5. **Flick or invert the tube repeatedly** to mix then briefly spin down the Master Mix.
6. **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.
7. **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 up to 7 µL** of RNA sample to each tube
   c. **If necessary, add RNAse-free water** to each tube to bring the volume of each reaction to **15 µL**.
   d. **Cap the strip tubes tightly and mix** by inverting the tubes several times and flicking to ensure complete mixing.
   e. **Spin briefly and immediately place** the tubes in a **pre-heated 67°C** thermal cycler.
8. **Incubate hybridization reactions** for at least **16 hours**. Maximum hybridization time should not exceed **30 hours**.
9. **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 67°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.

10. 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).

    If using this protocol in conjunction with other 3D-compatible products, see 3D Post-Hybridization Processing.

## Quick Reference

# TagSet Hybridization Setup

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

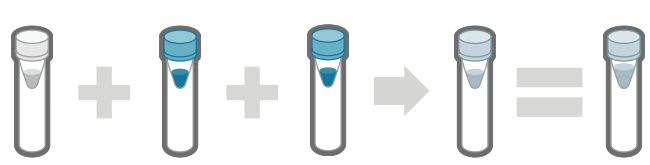
## 1 Prepare for hybridization

- ☐ Preheat thermocycler to **67° C**.
- ☐ **Thaw TagSet tubes and samples** at room temperature

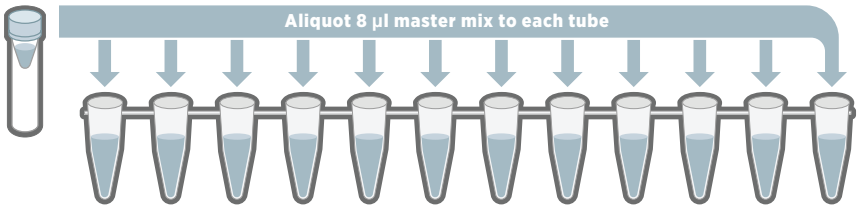
Preheat to 67°C

## 2 Create & aliquot Master Mix

- ☐ Add the following to the TagSet tube to create the Master Mix:
  - ☐ **70 µL** of Hybridization Buffer
  - ☐ **7 µL** of 30X Working Probe Pool A
  - ☐ **7 µL** of 30X Working Probe Pool B
- ☐ **Flick to mix**, then briefly spin down contents
- ☐ Aliquot **8 µL** of Master Mix into each tube of a labeled 12-tube strip



70 µL Hybridization Buffer | 7 µL Working Probe Pool A | 7 µL Working Probe Pool B | TagSet | Master Mix | Flick to mix & briefly spin down

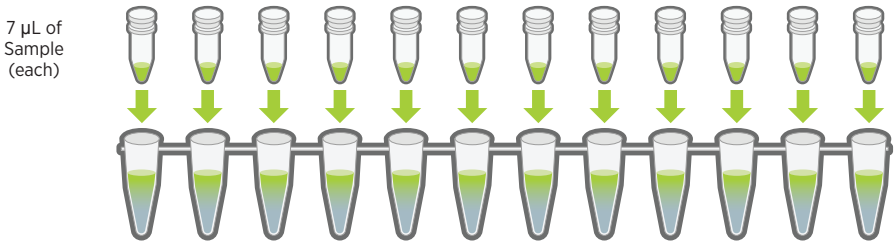



## 3 Add sample

- ☐ Add up to **7 µL** of the sample to each tube

*Note: If using less than 7 µL of sample, add RNAse-free water to each tube to bring the volume to 15 µL*

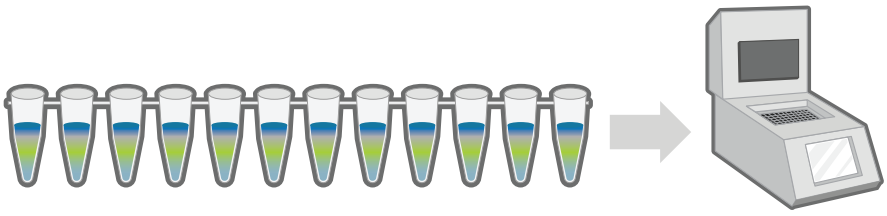
- ☐ **Cap the strip tubes tightly**, then invert and flick several times
- ☐ **Spin down briefly**




Flick to mix & briefly spin down

## 5 Hybridize

☐ **Immediately** place tubes in the thermocycler at **67° C** for **16-24 hours.** Maximum hybridization time should not exceed 30 hours.



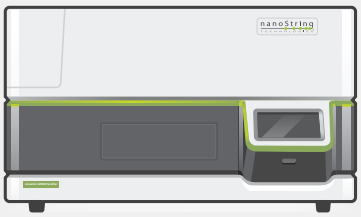
67°C for 16 hours
(30 hours maximum)

## **OPTION A:** Use nCounter® MAX/FLEX

### 6a Load nCounter MAX/FLEX

See **MAN-C0035, 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.

## 3D Post-Hybridization Processing

**IMPORTANT:** **Only** strip tubes provided with the Master Kit can be used on the Prep Station. Specific strip tubes are not required for use with the Sprint. If you need additional strip tubes, contact NanoString.

1. After the parallel hybridization reactions are completed, **remove the strip tubes** from the thermal cycler and **spin briefly**.
2. Using a multichannel pipette, **pool the full volume** (**15 µL**) of the TagSet (RNA) Hybridization strip tube reactions with the (sample-matched) Protein strip tube reactions. Pooled sample will be processed and analyzed in a single nCounter cartridge lane. **Proceed immediately to the next step**.
3. **Run** the mixed hybridization reactions on the nCounter platform:
   - **MAX/FLEX**: Run the full volume of the mixed hybridization reactions immediately on an nCounter Prep Station as described in the nCounter Analysis System User Manual (MAN-C0035) **For 3D applications, it is recommended to run the Prep Station in "Standard" mode.**
   - **SPRINT:** Run **30 µL** of the mixed hybridization reactions immediately on an nCounter SPRINT Profiler as described in the nCounter SPRINT User Manual (MAN-10017)

## Creating Master Stocks

**NOTE:** Some oligo suppliers will provide pooled oligos which can be used in place of creating your own Master Stocks. If utilizing this service, specify a pool of Probe As at a final concentration of **5 nM** per oligo and a separate pool of Probe Bs at a final concentration of **25 nM** per oligo. Pooled probes provided by oligo suppliers at the recommended concentrations must still be diluted to create Working Pools (see next section) before addition to the hybridization reaction.

**IMPORTANT:** Always create separate Master Stocks for Probe A, Probe B, and Protector Probes. Do not create a combined Master Stock containing Probe A, Probe B, and Protector Probes in the same tube; elevated background and lowered reaction sensitivity may result.

1. **Prepare the following concentrations** for resuspending individual oligonucleotides:
   - Probe A oligonucleotides at a **1 µM** concentration
   - Probe B oligonucleotides at a **5 µM** concentration
   - Protector probes at a **2 µM** concentration.

   Oligonucleotides should be resuspended in TE (10 mM Tris pH 8, 1 mM EDTA) or a similar buffer and stored frozen under conditions recommended by the supplier. To prepare Master Probe Stocks, begin by removing the appropriate Probe A and Probe B oligonucleotides from storage and thawing them on ice.
2. **Create** Probe A Master Stock
   a. **Pipet 5 µL** of each Probe A (starting concentration **1 µM**) into a 1.7 mL microfuge tube.
   b. **Add TE** to a final combined volume of **1 mL**.
   c. The **final concentration** of each Probe A in the Probe A Master Stock will be **5 nM**.
   d. **Store in aliquots** at -20°C or -80°C as recommended by the supplier.
3. **Create** Probe B Master Stock
   a. **Pipet 5 µL** of each Probe B (starting concentration **5 µM**) into a 1.7 mL microfuge tube.
   b. **Add TE** to a final combined volume of **1 mL**.
   c. The **final concentration** of each Probe B in the Probe B Master Stock will be **25 nM**.
   d. **Store in aliquots** at -20°C or -80°C as recommended by the supplier.
4. **Create** Protector Probe Master Stock (optional; for fusion gene reactions only)
   a. **Pipet 5 µL** of each Protector Probe (starting concentration **2 µM**) into a 1.7 mL microfuge tube.
   b. **Add TE** to a final combined volume of **1 mL**.
   c. The **final concentration** of each Protector Probe in the Protector Probe Master Stock will be **10 nM**.

d. **Store in aliquots** at -20°C or -80°C as recommended by the supplier.

**NOTE:** The probes in the Master Stocks must be appropriate for the targets being queried. If reporter tags are reassigned to new targets, new Master Stocks containing the specific set of appropriate probes must be created. **Do not add additional probes** to existing Master Stocks. If using an Extension TagSet as well as a Core TagSet, create separate Master Stocks for the Extension Probes.

**IMPORTANT:** Minimize freeze-thaw cycles by storing Master Stocks in appropriate aliquots at -20°C or -80°C. **Thaw each aliquot only once** and then place at 4°C for use in creating multiple Working Pools. A suitable aliquot size for the workflow can be calculated from the information in Table 4 based on your expected reaction throughput. Follow the supplier's guidance on stability of the oligonucleotide stocks at 4°C.

## nCounter Extension Products

The nCounter Extension products and reagents are unique universal Reporter and Capture Probes that increase the flexibility of the nCounter platform. Probes for up to 36 additional targets are available in 12, 24, and 36 extra genes (24 for Vantage RNA TagSet). These are combined with an existing TagSet prior to overnight hybridization. Because the original TagSet contains the proper controls, the Extension reagents do not have them.

- Extension products are not compatible with each other, Plex2, or ChIP-String assays.
- Purchase of additional Master Kit reagents is not required.

### nCounter Vantage Protein Products

Protein Plus reagents are compatible with XT formulations only. The Protein Plus reagent provided in nCounter Vantage Protein Assays enables combined analysis of proteins and RNA in a single reaction. Two separate vials containing the Antibody Mix and Protein Plus TagSet are included. Separate hybridization protocols are provided for combining the Protein Plus reagent with XT CodeSet assays or XT TagSet assays.

### Preparing the RLF

When creating nCounter Extension TagSet probes with the bioinformatics team, you will be provided with an RLF that merges the Core TagSet with your Extension TagSet. Failure to use a merged RLF will result in no count information being collected for targets of the Extension reagents.

If you have questions, email NanoString at bioinformatics@nanostring.com.

### Data Analysis Guidelines

Extension reagents do not include built-in ERCC standards and should only be used in conjunction with TagSets already containing these controls. Extension probes are designed to minimize off-target effects and cross-hybridization through bioinformatic screens that look for secondary structure, high local GC content, runs of polynucleotides, and homology to areas of the transcriptome for the organism of interest. To empirically assess interactions between TagSet and Extension reagents, NanoString recommends comparing the same samples using the TagSet alone vs. the TagSet and Extension reagents together. No interactions are expected.

After sample imaging, data may be imported into the nSolver Analysis Software for downstream analysis. To download the latest version, go to http://www.nanostring.com/products/nSolver. See the nSolver Analysis Software User Manual for additional information on using the nSolver application.

## XT TagSet Extensions Hybridization Setup

**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 **67°C** with a heated lid at **72°C**.

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

   **NOTE:** If **not using XT TagSet Extensions**, use the XT TagSet Hybridization Setup.

2. **Remove** TagSet and Probe Pool tubes from the freezer and thaw at room temperature. Invert or flick the tubes several times to mix well and briefly spin down reagents.

3. **Create 30X Working Stocks of all the Master Stock Probe Pools** by following Table 6. (If you have not created the Master Probe Stocks, see Creating Master Stocks before continuing to Working Stocks). The amount of Master Probe Stock and diluent used is dependent on the number of reactions being run. See Table 6 for quantities. For example, for 12 reactions, use 4 µL of Master Stock and 29 µL of TE-Tween for a final volume of 33 µL**. Invert or flick the tubes several times to mix well** and **briefly spin down** reagents. Diluted probe pools should not be stored for long-term use.

**Table 6**. Diluting Master Stocks to generate Working Pools

| Number of Reactions | Aliquot from Master Probe Stock (µL) | TE-Tween (µL) | Final Volume (µL) |
|---|---|---|---|
| 12 | 4 | 29 | 33 |
| 24 | 4 | 29 | 33 |
| 36 | 5 | 37 | 42 |
| 48 | 7 | 51 | 58 |
| 60 | 8 | 59 | 67 |
| 72 | 10 | 73 | 83 |
| 84 | 11 | 81 | 92 |
| 96 | 13 | 95 | 108 |
| 144 | 19 | 139 | 158 |
| 192 | 26 | 191 | 217 |

4. **Create a hybridization Master Mix** by adding the following reagents to the tube containing the TagSet (Table 7). Do not remove the TagSet from the tube; add components directly into the TagSet.

**Table 7**. TagSet + Extension Master Mix for one nCounter assay (12 reactions + 2 reactions of dead volume)*

| Component | TagSet Master Mix (µL) | Per Reaction (µL) |
|---|---|---|
| TagSet | In tube (28) | 2 |
| Hybridization Buffer | 70 | 5 |
| Extension TagSet | 28 | 2 |
| 30x Probe A Pool | 7 | 0.5 |
| 30x Probe B Pool | 7 | 0.5 |
| 30x Extension Probe A Pool | 7 | 0.5 |
| 30x Extension Probe B Pool | 7 | 0.5 |
| **Total Volume** | **154** | **11** |

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

5. **Flick or invert the tube repeatedly** to mix then briefly spin down the Master Mix.
6. **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.
7. **Prepare hybridization reactions** using a fresh tip for pipetting into each well:
   a. **Add 11 µL** of Master Mix to each well of a strip tube.
   b. **Add up to 4 µL** of RNA sample to each tube
   c. **If necessary, add RNAse-free water** to each tube to bring the volume of each reaction to **15 µL**.
   d. **Cap the strip tubes tightly and mix** by inverting the tubes several times and flicking to ensure complete mixing.
   e. **Spin briefly** and immediately place the tubes in a **pre-heated 67°C** thermal cycler.
8. **Incubate hybridization reactions** for at least **16 hours**. Maximum hybridization time should not exceed **30 hours**.
9. **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 67°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.

10. 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).

    If using this protocol in conjunction with other 3D-compatible products, see 3D Post-Hybridization Processing.

## Quick Reference

# TagSet Extension Hybridization Setup

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

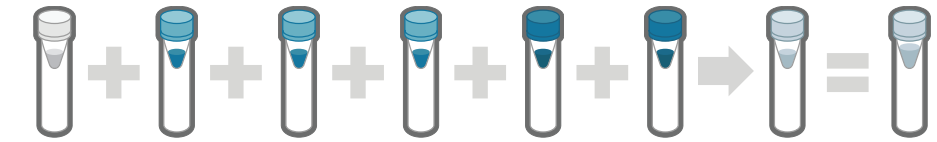
## 1 Prepare for hybridization

- ☐ Preheat thermocycler to **67° C**.
- ☐ **Thaw** TagSet tubes, Extension TagSet tubes, and samples at **room temperature (RT)**

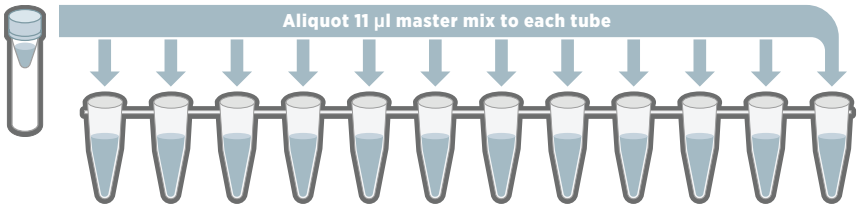
Preheat to 67°C

## 2 Create & aliquot Master Mix

- ☐ Add the following to the TagSet tube to create Master Mix:
  - ☐ **70 µL** of Hybridization Buffer
  - ☐ **28 µL** of Extension TagSet
  - ☐ **7 µL** of 30X Probe A Pool
  - ☐ **7 µL** of 30X Probe B Pool
  - ☐ **7 µL** of 30X Extension Probe A Pool
  - ☐ **7 µL** of 30X Extension Probe B Pool
- ☐ Flick to mix, then briefly spin down contents.
- ☐ Aliquot **11 µL** of Master Mix into each tube of a labeled 12-tube strip.




Flick to mix & briefly spin down

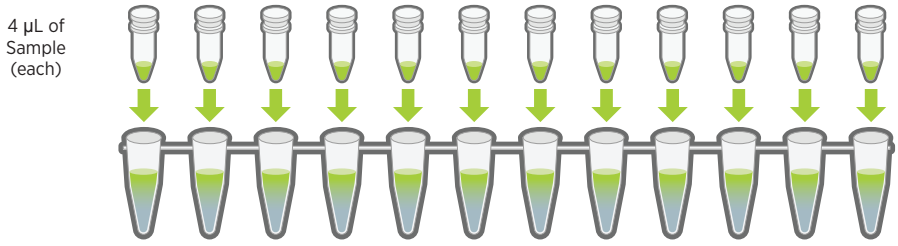



## 3 Add sample

- ☐ Add up to **4 µL** of the sample to each tube.

*Note: If using less than 4 µL of sample, add RNAse-free water to each tube to bring the volume to 15 µL*

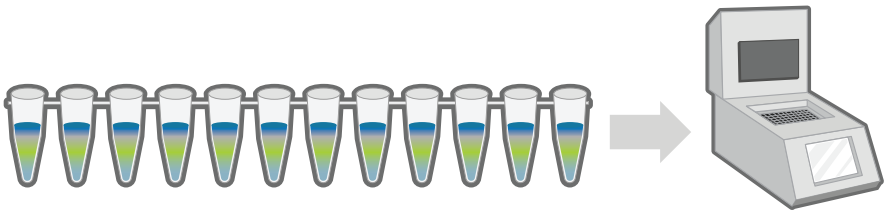
- ☐ **Cap the strip tubes tightly**, then invert and flick several times
- ☐ **Spin down briefly**




Flick to mix & briefly spin down

## 5 Hybridize

☐ **Immediately** place tubes in the thermocycler at **67° C** for **16-24 hours.** Maximum hybridization time should not exceed 30 hours.



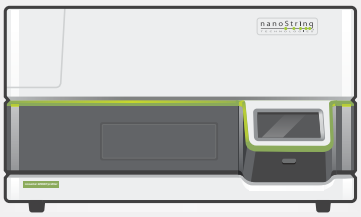
67°C for 16 hours
(30 hours maximum)

## **OPTION A:** Use nCounter® MAX/FLEX

### 6a Load nCounter MAX/FLEX

See **MAN-C0035, 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.