nCounter Low RNA Input Kit

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Introduction

The nCounter Low RNA Input Kit enables the generation of high-quality gene expression profiling of up to 800 gene targets from as little as 1 ng of sample. The kit is optimized for use with RNA from formalin-fixed paraffin-embedded (FFPE) tissue as well as crude cell lysates. Additionally, the kit can be utilized in the study of low-expressing genes. The streamlined, user-friendly workflow and reliable results enable gene expression studies of small samples or low-expressing genes to be completed quickly and efficiently.

The kit produces enough target for detection in an nCounter hybridization assay via a two-step process: Input RNA is converted to cDNA, which is then amplified using target-specific primers. After enrichment, samples can be directly analyzed with nCounter hybridization reagents that assay target sequences internal to the corresponding low-input primers. No clean up or dilution of the enriched sample is required prior to analysis, simplifying the workflow and maximizing sensitivity.

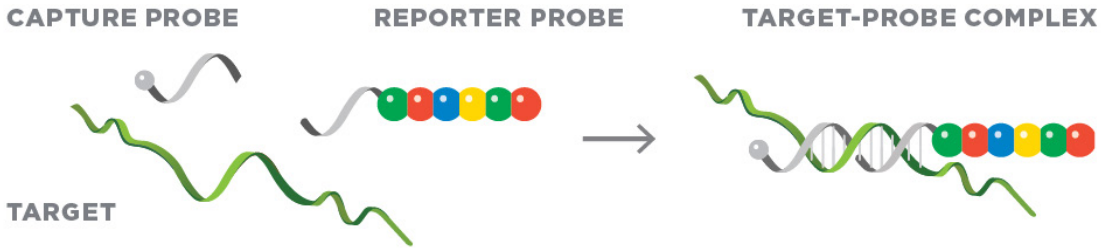


Figure 1. CodeSet chemistry: Capture and Reporter probes bind to the target.

Primers

Primer pools are available for many NanoString Gene Expression panels. Please visit the Low RNA Input Kit page for a list of primer pools. Low-input primers are provided at a final concentration of 500 nM per oligo in TE Buffer (pH 7.5).

Upon request, NanoString will provide low-input primer designs flanking the target sequences for custom CodeSets. As part of the CodeSet design process, NanoString can also provide information to design alternate primers if desired. For additional information or questions about primer design, please contact support@nanostring.com.

Materials and Equipment

Table 1. Materials provided with the nCounter Low RNA Input Reagent Kit

Kit	Reagents	Description	Storage
nCounter Low RNA Input Reagent Kit (48 rxns) Catalog #: LOW-RNA-48, See specific Primer Pool	10X RT Enzyme Mix	Contains reverse transcriptase	-20°C
	10X RT Primer Mix	Contains primers for RT	-20°C
	5X dT Amp Master Mix	Contains amplification polymerase and buffer	-20°C

Table 2. Additional materials required (not provided)

Item	Manufacturer	Catalog Number
Primers	NanoString**	Various
Pipettes for 10–1,000 µL*	Various	Various
Manual multi-channel pipette for 200 µL*	Rainin	L12-200XLS+
12-strip standard tubes*	BioExpress	T-3034-1

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

***Contact support@nanostring.com on how to order low-input primers for custom design.*

Table 3. Equipment required for use with the nCounter Low RNA Input Reagent Kit

Equipment
NanoString nCounter SPRINT, nCounter MAX, or nCounter FLEX Analysis System
Calibrated thermal cycler with heated lid
Microfuge or picofuge
Pipettes (p1000, p100, p20, p10)

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.

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 1,000x*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.

Sample Type Considerations

The nCounter Low RNA Input Reagent Kit is compatible with RNA obtained from a variety of sources, including formalin-fixed, paraffin-embedded (FFPE) samples. To ensure optimal performance with nCounter hybridization reagents in downstream assays, NanoString recommends utilizing the per reaction input amounts shown in Table 4.

Table 4. Sample Input Recommendations

Sample Type	Minimum Recommended Input Amount	
	Mass	Concentration
Purified Fresh/Frozen RNA	500 pg	0.125 ng/µL
Purified FFPE RNA	10 ng	2.5 ng/µL
Cell Lysate	1 ng (or ~100 cells)	200 cells/µL

FFPE

Successful amplification requires RNA fragments of sufficient length. As FFPE-derived RNA is often degraded, NanoString recommends evaluating RNA quality via an Agilent Bioanalyzer (or similar system) prior to amplification. Best results will typically be achieved with RNA samples for which at least 30% of the RNA fragments are 200 nucleotides in length or greater.

Cell Lysates

For applications involving small numbers of initial cells, such as flow-sorting, NanoString recommends sorting directly into a chemical- or detergent-based buffer (such as iScript or Cells-to-Ct) to maximize the concentration of cells in the lysate (up to ~2,000 cells/µL).

- Using a chemical- or detergent-based buffer at concentrations > 2,000 cells/µL is not recommended as this may result in incomplete cell lysis.
- It is important to remove and wash growth medium from cells as it may inhibit lysis and result in reduced assay performance.
- High concentrations of lysis buffer can inhibit the reverse transcription and subsequent amplification steps. **It is important that lysis buffer make up no more than 10% (or 0.5 µL) of the 5 µL RT reaction volume.**

The nCounter Low RNA Input Reagent Kit is incompatible with lysates prepared with chaotropic buffers, e.g., buffers containing guanidine salts, such as RLT.

Sample Prep for Total RNA

cDNA Conversion

IMPORTANT: Set up reactions *and* keep all components **on ice** during processing unless otherwise specified. Up to 4 µL of sample may be used per reaction. If sample dilution is required, dilution in RNase-free H_2O is recommended.

1. **Remove 10X RT Primer Mix** from the freezer and **thaw** at room temperature. Invert several times to mix well and spin down reagent.
2. Add up to **4 µL** of diluted sample to each well of a strip tube (see Table 4).
3. Make a **RT master mix** by combining the components as shown below in an RNase-free 0.5 mL tube. Gently **flick to mix** and **spin down** (at less than 1,000x*g*).

Table 5. RT Master Mix

Reagent	Per sample	12 samples	24 samples	36 samples	48 samples
10X RT Enzyme Mix	0.5 µL	8 µL	15 µL	22 µL	30 µL
10X RT Primer Mix	0.5 µL	8 µL	15 µL	22 µL	30 µL

4. Add 1 µL RT master mix to each sample (discard the remaining master mix volume).
5. Cap tubes; gently flick to mix and spin down.
6. Place strip tubes in a **thermal cycler** with a heated lid (which should be set to 5°C greater than the current incubation temperature at any moment) and run the following protocol:

Table 6. cDNA Conversion Thermocycler Settings

Step	Temperature	Time
Primer anneal	25˚C	10 min
First strand cDNA synthesis	42˚C	60 min
Enzyme inactivation	85˚C	5 min
Hold	4˚C	Forever

7. Proceed to multiplexed target enrichment. Keep reactions on ice or at 4˚C if proceeding directly. For long term storage, cDNA should be stored at -80˚C.

NOTE: Up to 1 µL of the RT reaction may be reserved for troubleshooting purposes.

Multiplexed Target Enrichment

IMPORTANT: Set up reactions *and* keep all components **on ice** during processing unless otherwise specified. Save Master Kit strip tubes for the hybridization step for use on the nCounter platform.

1. **Remove Low-input Primers** from the freezer and **thaw** at room temperature. Invert several times to mix well and spin down reagent.
2. Make an **amplification master mix** by combining the components as shown below in an RNase-free 0.5 mL tube. Gently **flick to mix** and **spin down**.

Table 7. Amplification Master Mix

Reagent	Per sample	12 samples	24 samples	36 samples	48 samples
5X dT Amp Master Mix	1.5 µL	21 µL	42 µL	63 µL	84 µL
Low-input Primers 500 nM per primer	1 µL	14 µL	28 µL	42 µL	56 µL

3. Add **2.5 µL Amplification Master Mix** directly to each converted cDNA sample (previously, thaw cDNA on ice if it has been stored at -80ºC).
4. Since strip tube caps may be warped in the thermal cycler, place **new** caps on tubes. Gently **flick to mix** and **spin down**.
5. **Select** the recommended number of **amplification cycles** based on the sample type.

Table 8. Amplification Cycle Recommendations*

Total RNA	Minimum recommended # of Cycles
Purified fresh/frozen RNA	8
Purified FFPE RNA	10
Cell Lysate	8

***** *Optimal cycle number may be sample specific*

6. Place strip tubes in a **thermal cycler** with a heated lid (set to 5°C greater than the current incubation temperature) and run the following protocol:

Table 9. PCR Thermocycler Settings

Step		Temperature	Time
Initial Denaturation		95˚C	10 minutes
# of cycles (see Table 8)	Denature	95˚C	15 seconds
	Anneal	60˚C	4 minutes
Hold		4˚C	Forever

IMPORTANT: After multiplexed target enrichment, **incubate** the prepared samples for **2 minutes at 95˚C** and then **snap cool on ice for at least two minutes** prior to proceeding to the hybridization reaction setup. The **entire volume of the MTE** reaction should be carried over **into the hybridization reaction** setup for maximum sensitivity. For long term storage, amplified product should be stored at -80˚C.

NOTE: Successful amplification of purified RNA samples results in production of dsDNA which may be quantified on a Qubit or similar instrument capable of distinguishing dsDNA from ssDNA and RNA. Presence of genomic DNA in a sample will interfere with this type of quantification.

Low RNA Input Hybridization Protocol

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

1. **Pre-heat** the thermal cycler to **65°C** with a heated lid at **70°C**. Program the thermal cycler using **18 µL** volume, calculated temperature, and heated lid.
2. **Remove Reporter CodeSet and Capture ProbeSet tubes** from the freezer and **thaw** at room temperature. Invert several times to mix well and spin down reagent.

   IMPORTANT: After it has thawed, inspect the Reporter CodeSet tube 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 70 µL of hybridization buffer to the Reporter probes tube. Do not remove the Reporter probes from the tube. **Do not add the Capture ProbeSet to the master mix.**

   Table 10. Hybridization Master Mix for one LI assay.

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

4. Gently **flick to mix** and **spin down**.
5. **Label** the provided **12-tube hybridization strip**. Use **only** the strip tube(s) provided with the Master Kit. Ensure that they fit in your microfuge or picofuge and cut the strip in half if necessary.
6. **Add 8 µL of master mix** to each of the 12 tubes. (If water was added to the master mix, increase this volume as necessary). Use a fresh tip for each pipetting step to accurately measure the correct volume.
7. Add up to 8 µL of the prepared MTE reaction to each tube.
8. **If necessary**, add RNase-free water to bring the volume of each assay to **16 µL**.
9. **Invert the Capture ProbeSet tube** to mix and spin down the contents.
10. **Add 2 µL of Capture ProbeSet** to each tube. Use a fresh tip for each pipetting step to accurately measure the correct volume. Cap tubes and mix the reagents by inverting the tubes several times and flicking with a finger to ensure complete mixing. **Briefly spin down** and **immediately** place the tubes in the pre-heated 65°C thermal cycler.

    NOTE: Minimizing the time between addition of the Capture ProbeSet and incubation at 65°C will increase assay sensitivity.

11. **Incubate reactions for at least 16 hours**. Maximum hybridization time should not exceed 48 hours. Ramp reactions down to 4°C and process the following day. Do not leave the reactions at 4°C for more than 24 hours or increased background may result.

    NOTE: The purpose of selecting a fixed hybridization time followed by a ramp down to 4°C is to ensure equivalent hybridization times of all assays being directly compared in the same series of experiments. Counts continue to accumulate with time, 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 will increase sensitivity by increasing counts without significantly increasing background.

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

Quick Start Guide

Low RNA Input Hybridization Protocol

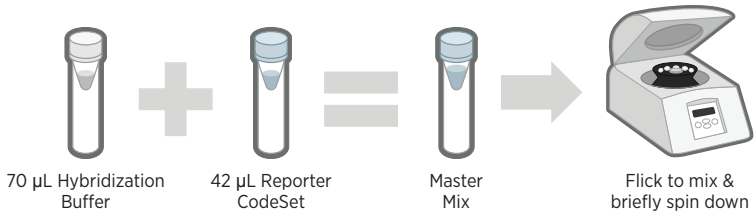
1 Prepare for hybridization

- ☐ Preheat thermocycler to **65° C**.
- ☐ **Thaw** Reporter CodeSet, Capture ProbeSet & samples.

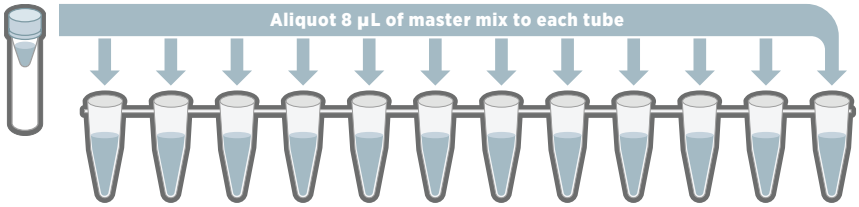
Preheat to 65°C

2 Create & aliquot Master Mix

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



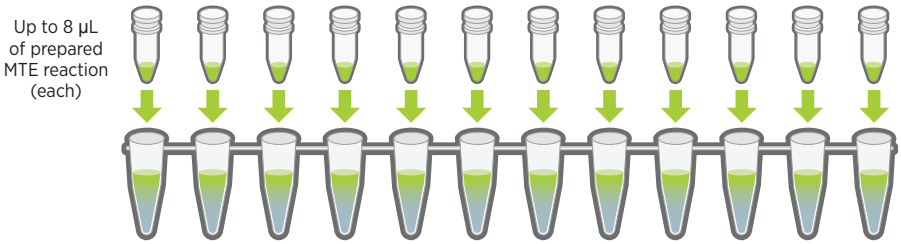
70 µL Hybridization Buffer | 42 µL Reporter CodeSet | Master Mix | Flick to mix & briefly spin down



3 Add sample

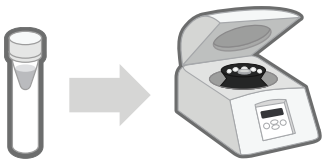
- ☐ Add **up to 8 µL of prepared MTE reaction** to each tube.

Note: If necessary, add RNase-free water to bring the volume of each assay to 16 µL.

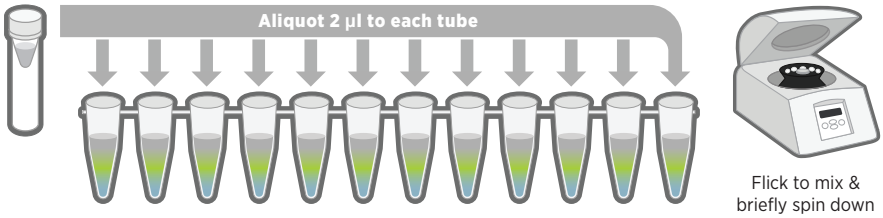


4 Add Capture ProbeSet

- ☐ **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**.



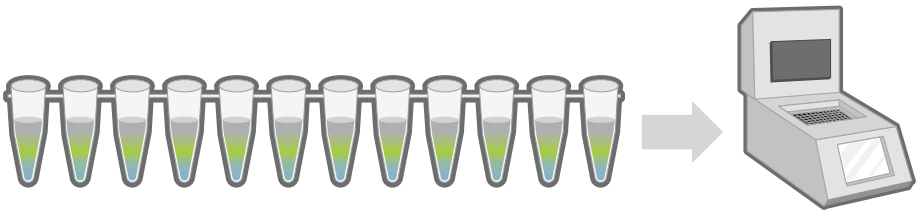
Capture ProbeSet | Flick to mix & briefly spin down



Flick to mix & briefly spin down

5 Hybridize

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



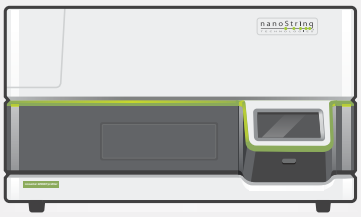
65°C for 16-24 hours
(48 hours maximum)

OPTION A: Use nCounter® MAX/FLEX

6a Load nCounter MAX/FLEX

See **MAN-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.

Using Plus Reagents with Low RNA Input Assays

Sample Prep for Total RNA

cDNA Conversion

IMPORTANT: Set up reactions *and* keep all components **on ice** during processing unless otherwise specified. Up to 4 µL of sample may be used per reaction. If sample dilution is required, dilution in RNase-free H_2O is recommended.

1. **Remove 10X RT Primer Mix** from the freezer and **thaw** at room temperature. Invert several times to mix well and spin down reagent.
2. Add up to **4 µL** of diluted sample to each well of a strip tube (see Table 4).
3. Make a **RT master mix** by combining the components as shown below in an RNase -free 0.5 mL tube. Gently **flick to mix** and **spin down**.

Table 11. RT Master Mix

Reagent	Per sample	12 samples	24 samples	36 samples	48 samples
10X RT Enzyme Mix	0.5 µL	8 µL	15 µL	22 µL	30 µL
10X RT Primer Mix	0.5 µL	8 µL	15 µL	22 µL	30 µL

4. Add **1 µL RT master mix** to each sample (discard the remaining master mix volume).
5. **Cap tubes** and gently **flick to mix**. Briefly **spin down** at less than 1,000x*g*.
6. Place strip tubes in a **thermal cycler** with a heated lid (which should be set to 5°C greater than the current incubation temperature at any moment) and run the following protocol:

Table 12. cDNA Conversion Thermocycler Settings

Step	Temperature	Time
Primer anneal	25°C	10 min
First strand cDNA synthesis	42°C	60 min
Enzyme inactivation	85°C	5 min
Hold	4°C	Forever

7. Proceed to **multiplexed target enrichment**. Keep reactions on ice or at 4°C if proceeding directly. For long term storage, cDNA should be stored at -80°C.

NOTE: Up to 1 µL of the RT reaction may be reserved for troubleshooting purposes.

Multiplexed Target Enrichment for Panel Plus

IMPORTANT: Set up reactions *and* keep all components **on ice** during processing unless otherwise specified.

1. **Remove all Low-input Primers** from the freezer and **thaw** at room temperature. Invert several times to mix well and spin down reagent.
2. Make an **amplification master mix** by combining the components as shown below in an RNase-free 0.5 mL tube. Gently **flick to mix** and **spin down**.

Table 13. Amplification Master Mix

Reagent	Per sample	12 samples	24 samples	36 samples	48 samples
5X dT Amp Master Mix	1.5 µL	21 µL	42 µL	63 µL	84 µL
Low-input Primers 500 nM per primer	1 µL	14 µL	28 µL	42 µL	56 µL
Low-input Panel Plus Primers 500 nM per primer	1 µL	14 µL	28 µL	42 µL	56 µL

3. Add **3.5 µL Amplification Master Mix** directly to each converted cDNA sample (previously, thaw cDNA on ice if it has been stored at -80°C).
4. Since strip tube caps may be warped in the thermal cycler, place **new** caps on tubes. Gently **flick to mix** and **spin down**.
5. **Select** the recommended number of **amplification cycles** based on the sample type.

Table 14. Amplification Cycle Recommendations*

Total RNA	Minimum recommended # of cycles
Purified fresh/frozen RNA	8
Purified FFPE RNA	10
Cell Lysate	8

** Optimal cycle number may be sample specific*

6. Place strip tubes in a **thermal cycler** with a heated lid (set to 5°C greater than the current incubation temperature) and run the following protocol:

Table 15. PCR Thermocycler Settings

Step		Temperature	Time
Initial Denaturation		95°C	10 minutes
# of cycles (see Table 8)	Denature	95°C	15 seconds
	Anneal	60°C	4 minutes
Hold		4°C	Forever

IMPORTANT: **After** multiplexed target enrichment, **incubate** the prepared samples for **2 minutes at 95°C and then snap cool on ice for at least two minutes** prior to proceeding to the hybridization reaction setup. The **entire volume of the MTE** reaction should be carried over **into the hybridization reaction** setup for maximum sensitivity. For long-term

storage, amplified product should be stored at -80°C.

Low RNA Input Hybridization Protocol for Panel Plus

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

1. **Pre-heat** the thermal cycler to **65°C** with a heated lid at **70°C**. Program the thermal cycler using **21 µL** volume, calculated temperature, and heated lid.
2. **Remove Reporter CodeSet, Capture ProbeSet, and Plus tubes** from the freezer to thaw at room temperature. Invert several times to mix well and 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 Reporter CodeSet tube. Do not remove the Reporter CodeSet from this tube**. Do not add the Capture ProbeSet or Capture Plus reagent to the master mix.**

   Table 17. Hybridization Master Mix with Plus reagents for one LI assay.

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

4. Gently **flick to mix** and **spin down**.
5. **Label** the provided **12-tube hybridization strip**. Use **only** the strip tube(s) provided with the Master Kit. Ensure that they fit in your microfuge or picofuge and cut the strip in half if necessary.
6. Add **10 µL** of the **Reporter/Hybridization Buffer master mix** to each of the tubes. Use a fresh tip for each pipetting step to accurately pipette the correct volume.
7. Add up to **8 µL** of **prepared MTE reaction** to each tube.
8. If necessary, add RNase-free water to bring the volume of each assay to **18 µL**.
9. Add **14 µL** of **Capture-Plus reagent to** the thawed aliquot of **Capture ProbeSet**. Gently **flick to mix** and **spin down**.
10. Add **3 µL** of **Capture ProbeSet and Plus reagent mix** to each tube (**21 µL total volume) immediately** before **placing at 65°C**. Use a fresh tip for each pipetting step to accurately pipette the correct volume. Cap tubes and mix the reagents by inverting the tubes several times and flicking with a finger to ensure complete mixing. Briefly spin down and place the tubes in the pre-set 65°C thermal cycler.

11. **Incubate** reactions **for at least 16 hours**. Maximum hybridization time should not exceed 48 hours. Ramp reactions down to 4°C and process the following day. Do not leave the reactions at 4°C for more than 24 hours or increased background may result.

    NOTE: The purpose of selecting a fixed hybridization time followed by a ramp down to 4°C is to ensure equivalent hybridization times of all assays being directly compared in the same series of experiments. Counts continue to accumulate with time, 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 will increase sensitivity by increasing counts without significantly increasing background.

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

Quick Reference

Low RNA Input Plus Hybridization Protocol

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

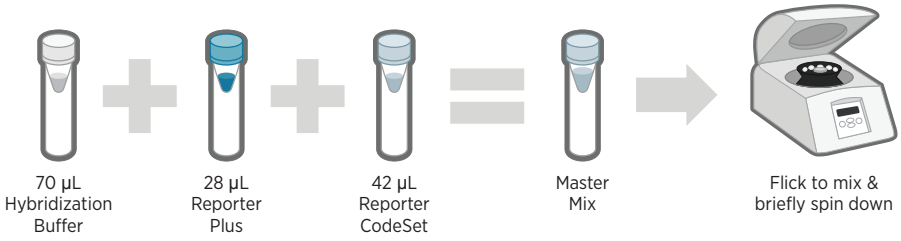
1 Prepare for hybridization

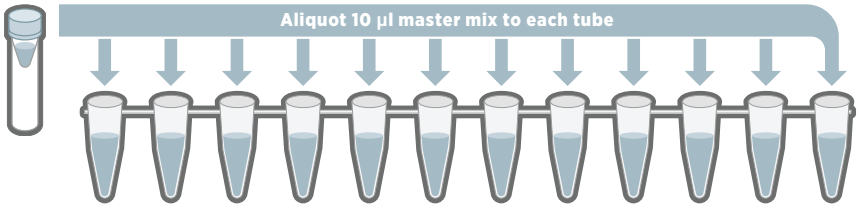
- ☐ Preheat thermocycler to **65° C**.
- ☐ **Thaw samples** and Codeset and CodeSet Plus tubes

Preheat to 65°C

2 Create & aliquot Master Mix

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

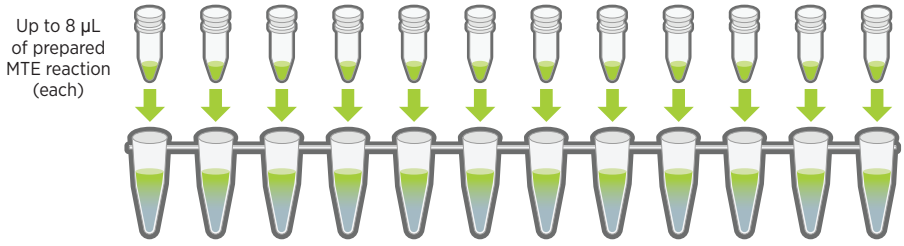




3 Add sample

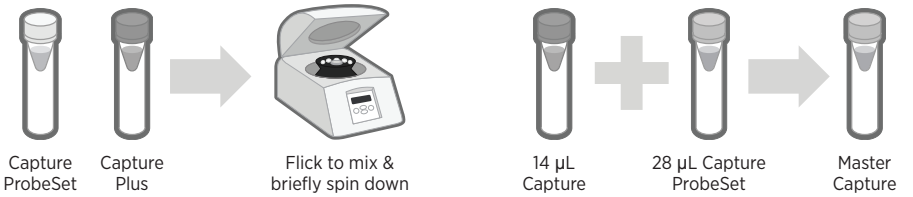
- ☐ Add **up to 8 µL of prepared MTE reaction** to each tube.

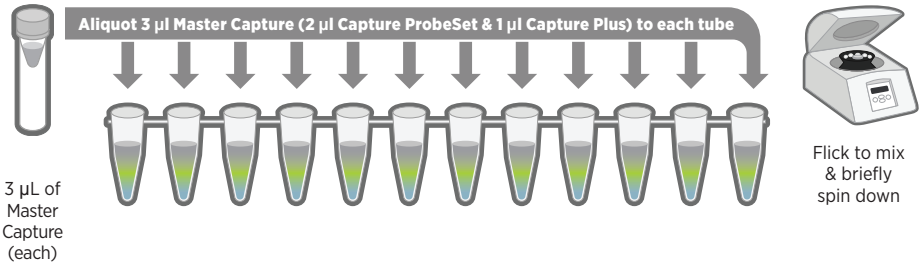
*Note: **If necessary**, add RNase-free water to bring the volume of each assay to **18 µL**.*



4 Add Master Capture

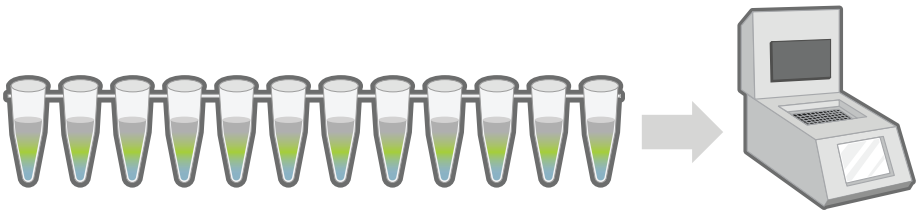
- ☐ **Flick-mix** Capture ProbeSet and Capture Plus tubes and **spin down briefly**.
- ☐ Add **14 µL of Capture Plus** to the **Capture ProbeSet** to create the **Master Capture.** Flick to mix, then **briefly spin down** contents.
- ☐ Add **3 µL** of **Master Capture** to each tube.
- ☐ Cap tightly,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





5 Hybridize

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



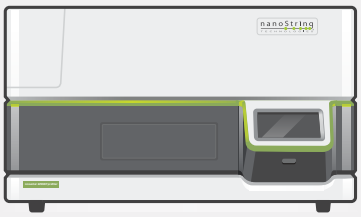
65°C for 16-24 hours
(48 hours maximum)

OPTION A: Use nCounter® MAX/FLEX

6a Load nCounter MAX/FLEX

See **MAN-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.

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