# CodeSet Hybridization Setup

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 of 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 CodeSet chemistry.

## Materials and Reagents

**Table 1.** CodeSet Hybridization Reagents

| Reagent | Description | Storage |
|---|---|---|
| Reporter CodeSet | Barcoded Reporter Probes | At or below -80°C |
| Capture ProbeSet | Capture Probes | At or below -80°C |
| Hybridization Buffer | Supplied with nCounter Master Kits and SPRINT Reagent Packs | RT (15–25°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.

## Nucleic Acid Sample Input Recommendations

**Table 2.** Nucleic Acid Sample Input Recommendations

| 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 Nucleic Acid from Fresh Frozen Samples (MAN-10051).

## Hybridization Setup

**IMPORTANT:** Check the reagent labels before you begin to ensure the correct reagents are being utilized.

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

   **NOTE:** A thermal cycler with a heated lid is required for this protocol. NanoString recommends a thermal cycler with a programmable heated lid. 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.

2. Remove Reporter CodeSet and Capture ProbeSet tubes from the freezer and thaw at room temperature. Invert or flick the tube several times to mix well and briefly spin down reagents.

3. Create a hybridization master mix by adding the following reagents to the tube containing the Reporter CodeSet.

   **Table 3.** CodeSet Master Mix for one nCounter assay (12 reactions)

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

4. Flick or invert the tube repeatedly to mix then briefly spin down the Master Mix.

5. 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.

6. Prepare hybridization reactions:

   a. Add 8 µL of Master Mix to each well of a strip tube. Use a fresh tip for pipetting into each well.

   b. Add 5 µL of sample to each tube containing Master Mix (see Table 2).

   c. Mix the Capture ProbeSet tube by inverting or flicking, and briefly spin down the contents.

   d. Add 2 µL of Capture ProbeSet to each tube.

   e. Cap the strip tubes tightly and mix by inverting the tubes several times and flicking to ensure complete mixing.

   f. Spin briefly and immediately place the tubes in a pre-heated 65°C thermal cycler.

7. Incubate hybridization reactions for at least 16 hours. Maximum hybridization time should not exceed 48 hours.

8. 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:** Selecting a fixed hybridization time followed by a ramp down to 4°C ensures equivalent hybridization time for all assays being directly compared in the same series of experiments. Counts continue to accumulate with time at 65°C, with total counts typically increasing 5% per hour between 16 and 24 hours. Although a 16-hour incubation is adequate for most purposes, a longer incubation increases sensitivity by increasing counts without significantly increasing background.

9. 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 the next section for 3D post-hybridization processing.

## 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 CodeSet (RNA) Hybridization strip tube reactions with the (sample-matched) SNV and 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)