

Protein-Only Hybridization Setup

Prepared samples are used as input for nCounter hybridization reactions containing NanoString Reporter and Capture probes. These overnight hybridization reactions enable specific hybridization of Reporter or 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 protein hybridizations.

Materials and Reagents

Table 1. Protein-Only Hybridization Reagents

Reagent	Description	Storage
Protein 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)

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 1,000xg 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.

Protein-only Hybridization Set Up

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 Protein TagSet 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 a hybridization master mix by adding the following reagents to the tube containing the protein TagSet.

Table 2. Protein TagSet Master Mix for one nCounter assay (12 reactions per nCounter assay)

Component	TagSet Master Mix (μL)	Per Reaction (μL)
Protein TagSet	In tube (28)	2
Hybridization Buffer	70	5
RNase-free Water	98	7
Total Volume	196	14

4. Flick or invert the tubes repeatedly to mix then briefly spin down the master mixes.
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. Dilute (if necessary) and denature prepared protein lysate (from [Protein Processing for Lysate Samples, MAN-10054](#)):

- a. For protein samples from FFPE, dilute the lysate 1:5 in Buffer T.

NOTE: This dilution is necessary to prevent saturation of the nCounter assay cartridge. The optimal dilution may vary depending on tissue type and size. Save the remaining undiluted sample at -80°C to re-run the assay if adjustments are to be made.

- b. Denature at 95°C for 5 min in a thermocycler with a heated lid at 100°C.
- c. Snap cool on ice, ice water, or quickly ramp the thermocycler to 4°C.
- d. If using nCounter SPRINT system, further dilute the denatured sample with an equal amount of nuclease-free water. A 1 μL aliquot of this sample is required per hybridization reaction. Additional dilution is not required with the MAX or FLEX analysis systems.
- e. Spin briefly, vortex, and spin briefly again to ensure a homogenous mixture.

7. Prepare hybridization reactions:
 - a. Add 14 μL of master mix to each well of a strip tube. Use a fresh tip for pipetting into each well.
 - b. Add 1 μL of **denatured diluted protein lysate** to each tube containing master mix.
 - c. Cap the strip tubes tightly and mix the by inverting the tubes several times and flicking to ensure complete mixing.
 - d. Spin briefly and immediately place the tubes in the pre-heated thermal cycler.
8. Incubate hybridization reactions for at least 16 hours. Maximum hybridization time should not exceed 48 hours.
9. 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.

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 the next section for 3D post-hybridization processing.

3D Post-Hybridization Processing

IMPORTANT: Use **only** Master Kit strip tubes on the Prep Station. 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 Protein-Only Hybridization strip tube reactions with the (sample-matched) RNA and/or SNV hybridization 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 \(MAN-10017\)](#).