SNV Hybridization Setup

Amplified genomic DNA samples are used as input into nCounter hybridization reactions containing NanoString Reporter and SNV Capture probes. These overnight hybridization reactions enable specific hybridization of reporter and capture probes to their targets. 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 SNV Chemistry.

Materials and Reagents

Table 1. Hybridization Reagents

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 (M, S, and T)	Target-specific oligonucleotide probes	At or below -80°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.

Hybridization Setup

IMPORTANT: Check the reagent labels before you begin to ensure the correct SNV panel 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 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 working stocks of all the Probe Pools by adding 22 µL of nuclease-free water or TE buffer directly to each tube. 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.

4. Denature the amplified DNA samples (from DNA Amplification for SNV Analysis, MAN-10052) at 95°C for 10 minutes. After heat denaturation, immediately place the DNA on ice to rapidly cool and to minimize any reannealing that may otherwise occur. Leave the amplified DNA on ice for at least 5 minutes.

   IMPORTANT: Heat denaturation followed by ramping down to 4°C within the thermal cycler does NOT cool the DNA rapidly enough to minimize reannealing.

   NOTE: If using nCounter SPRINT system, dilute the denatured sample with an equal amount of nuclease-free water (10 µL). A 5 µL aliquot of this sample is required per hybridization reaction. Dilution is not required with the MAX or FLEX analysis systems.

5. Create a hybridization Master Mix by adding the following reagents to the tube containing the appropriate TagSet.

   Table 2. SNV Hybridization Master Mix

Component	SNV Master Mix (µL)	Per Reaction (µL)
SNV TagSet	In tube (28)	2
Hybridization Buffer	70	5
Diluted SNV Assay Probe T Pool	7	0.5
Diluted SNV Assay Probe S Pool	7	0.5
Diluted SNV Assay Probe M Pool	28	2
Total Volume	**140**	**10**

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

7. Label a 12-tube PCR strip for the hybridization. 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.

8. Prepare hybridization reactions:
   a. Add 10 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 **denatured** sample to each tube containing Master Mix.

      IMPORTANT: Amplified DNA must be denatured prior to addition to the hybridization reaction.

   c. Cap the strip tubes tightly and mix by inverting the tubes several times and flicking to ensure complete mixing.
   d. Spin briefly and immediately place the tubes in a pre-heated 65°C thermal cycler.
9. Incubate hybridization reactions for at least 16 hours. Maximum hybridization time should not exceed 20 hours.
10. 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 20 hours. Although a 16-hour incubation is adequate for most purposes, a longer incubation increases sensitivity by increasing counts without significantly increasing non-specific background.

11. 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 DNA SNV Hybridization strip tube reactions with the (sample-matched) RNA 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)