nCounter®

Hyb & CNV Assay

Setting Up an nCounter Hybridization Reaction

1. **Pre-heat thermocycler to 65°C with the lid set to 70°C.** It is important that the lid temperature adjust with the temperature on the heat block and not be set to 95°C.

2. **Prepare mastermix for 12 samples.**
   - Thaw Reporter CodeSet and Capture ProbeSet on ice.
     If a precipitate is present in the Reporter CodeSet, heat the tube at 75°C for 10 min and cool at room temperature beore using.
   - Pipette 130µL of hybridization buffer into Reporter CodeSet aliquot.
   - Mix by flicking or inverting tubes and spin down in picofuge.
     Do not vortex or centrifuge in high speed minifuge.

3. **Aliquot 20µL of mastermix into strip tubes.**
   - If necessary, carefully cut the strip in half to fit into the centrifuge. Pay close attention to the sample numbers once the strip is halved and handle cut strip gently.

4. **Add sample to reaction.**

Gene Expression	Add 5µL (100ng) of total RNA or the equivalent of 10,000 lysed cells.
miRNA Analysis	Denature 5µL of prepared miRNA sample at 85°C for 5-mins, snap cool on ice for 2-mins, add to hybridization reaction.
CNV Analysis	Denature 5µL (200–300ng) of fragmented DNA at 95°C for 5-mins, snap cool on ice for 2-mins, add to hybridization reaction.

5. **Add 5µL of Capture ProbeSet into each tube.** Use a fresh tip for each tube.

6. **Cap tubes and immediately transfer to 65°C thermocycler.**
   Limiting reaction time at room temp will minimize background counts.

7. **Incubate at 65°C for 12–30 hours.**
   Samples should remain at 65°C until **Step 4** of the Prep Station Quick Start Guide.

CNV Sample Preparation for 12 Samples

- **Fragment your genomic DNA samples.**
  It is important to use reagents supplied with the kit as well as RNAse free water, tips, and gloves during all steps of this protocol.

  Combine the following to a 10µL final volume:
  - 200–600ng (600ng recommended) of genomic DNA in 7µL of RNAse-free water, Tris pH 8.0, or a similar buffer
  - 1µL of 10x AluI Fragmentation Buffer
  - 1µL of 10x CNV DNA Prep Control
  - 1µL of 5U/µL AluI Fragmentation Enzyme

  Incubate digest at 37°C for at least 2 hours in a heat block or thermocycler with a heated lid. If you have 300ng or less of DNA, it is advisable to set up a 5µL reaction by reducing the volume of water or buffer added to the DNA so that all the DNA can be added to the reaction.

For more comprehensive information, sign in to our customer resources site (**www.nanostring.com/sign/**) and go to **Support > Customer Resources*** to view the manuals and other technical product literature. For technical support, please e-mail **support@nanostring.com** or in the U.S./Canada, call **1 888 358 6266**.

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miRNA Sample Prep

MicroRNA Sample Preparation for 12 Samples

1 Program thermocycler protocols.

Annealing Protocol

Temperature	Time
94°C	1 min
65°C	2 min
45°C	10 min
48°C	hold
Total Time	**13 min**

Ligation Protocol

Temperature	Time
48°C	3 min
47°C	3 min
46°C	3 min
45°C	5 min
65°C	10 min
4°C	hold
Total Time	**24 min**

Purification Protocol

Temperature	Time
37°C	1 hr
70°C	10 min
4°C	hold
Total Time	**1 hr 10 min**

2 Prepare total RNA samples.

Using RNAse-free water, normalize total RNA samples to 33ng/µL in a total of 3µL to provide 100ng input (there is no need to enrich for small RNAs). Samples must be free of chaotropic salts and organic solvents.

3 Prepare controls.

Add 1µL of miRNA Assay Controls to 499µL of RNAse-free water in a sterile microfuge tube. Vortex and briefly spin down. Store on ice.

4 Anneal samples.

- Combine 13µL of Annealing Buffer, 26µL of nCounter miRNA Tag Reagent, and 6.5µL of the miRNA Assay Controls dilution prepared in **Step 3** to create an annealing mastermix. Mix well by pipetting.
- Dispense 3.5µL of the annealing mastermix into provided 12 x 0.2mL strip tubes.
- Add 3µL of total RNA sample (100ng) into each tube with mastermix. Cap tube, flick to mix and spin down.
- Place strip in thermocycler and initiate **Annealing Protocol**.

5 Ligate samples.

- Combine 19.5µL of PEG and 13µL of Ligation Buffer in a microfuge tube and mix well by pipetting to prepare a ligation mastermix. PEG should be pipetted very slowly to ensure an accurate measurement.
- When the thermocycler has reached 48°C, remove tubes, add 2.5µL of the ligation mastermix to each tube in the strip. Cap tubes, flick to mix and spin down. Do not turn off thermocycler.
- Incubate tubes at 48°C in the thermocycler for 5-mins.
- While tubes remain in thermocycler, carefully uncap strips, add 1µL of ligase to each tube. Check the tip at each pipetting step to ensure all ligase was dispensed. There is no need to mix. To keep track of ligase addition, it can be helpful to line up 12 tips in front of the thermocycler discarding each tip after use.
- Immediately recap tubes in thermocycler, initiate thermocycler **Ligation Protocol**.

6 Clean up ligation.

- Remove tubes from thermocycler, carefully uncap strips, add 1µL of Ligation Clean-Up Enzyme to each reaction. Flick to mix and spin down.
- Place tubes in thermocycler and initiate Purification Protocol.
- Add 40µL of RNAse-free water. Samples may be stored at -20°C for up to several weeks before hybridization.

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