nCounter®
miRNA Expression Assay
User Manual

FOR RESEARCH USE ONLY. Not for use in diagnostic procedures.

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nanoString®

About this Manual

This manual describes the methods for miRNA Sample Preparation and miRNA CodeSet Hybridization. For instructions on post-hybridization processing and data analysis, please see the instrument-specific user manuals (nCounter Pro Analysis System User Manual (MAN-10147), nCounter Analysis System User Manual for MAX/FLEX systems (MAN-C0035), nCounter SPRINT Profiler User Manual (MAN-10017)) and the Gene Expression Data Analysis Guidelines (MAN-C0011).

Changes in this Revision (MAN-C0009-08)

- Updates to text for clarity and accuracy.
- Updates to list of required materials.
- Increased volume of ligation master mix (step 7 on page 8) to ensure sufficient volume of viscous solution.
- Adjusted recommended hybridization time to 16-24 hours to align with best practices.

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Introduction

NanoString® patented molecular barcodes provide a true digital detection technology capable of highly multiplexed analysis. The nCounter® miRNA Expression Assay is designed to provide an ultra-sensitive and reproducible method to detect miRNAs without the use of reverse transcription or amplification. The assay is run on total RNA isolated from any source, including formalin-fixed paraffin embedded (FFPE) samples, and allows for detection across all biological levels of expression.

This assay involves sample processing where unique oligonucleotide tags are annealed and subsequently ligated with miRNAs of interest via a target specific bridge oligo (Figure 1, top). Sequence specificity between each miRNA and its appropriate tag is ensured by careful, stepwise control of annealing and ligation temperatures. This sample processing allows the short miRNAs to be detected with great specificity and sensitivity using NanoString Codeset chemistry. CodeSet chemistry consists of a Reporter CodeSet and a Capture ProbeSet that hybridize to the specific targets of interest (Figure 1, bottom). Excess probes are removed, and target-probe complexes are immobilized and aligned on the cartridge. Their barcodes are then counted on an nCounter system.

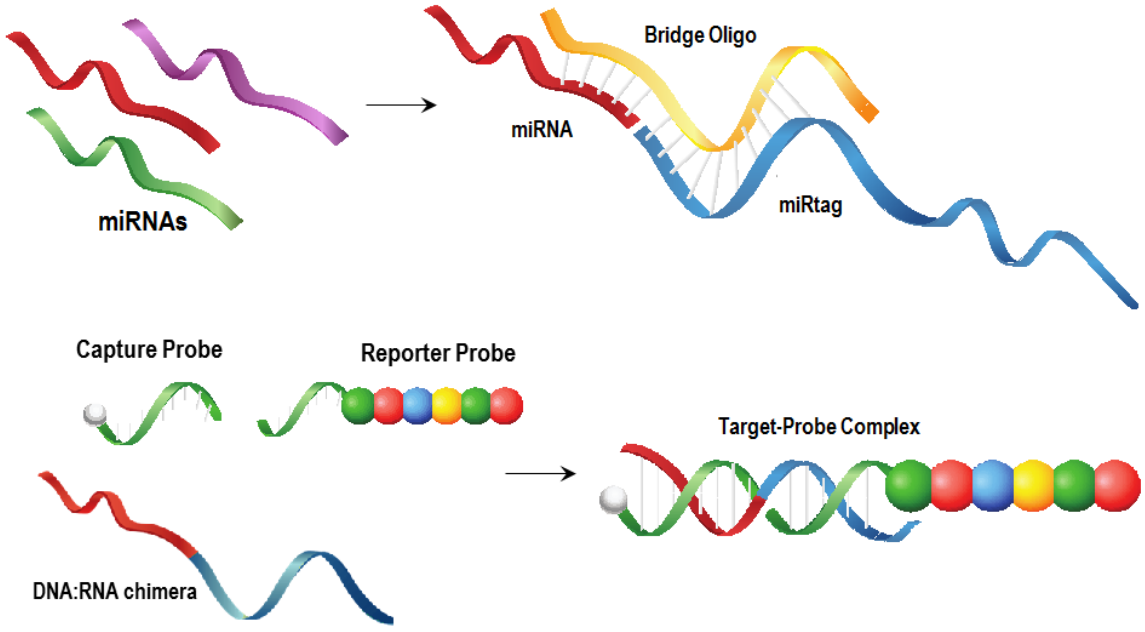

Figure 1. miRNA-specific preparation to utilize nCounter technology downstream.

The tabulated barcodes from the nCounter system can be analyzed using NanoString's nSolver™ Analysis Software or other analysis programs, such as the ROSALIND™ cloud platform.

nCounter miRNA gene expression panels are sold in increments of 12 reactions. Master Kits (for MAX, FLEX, or Pro Analysis Systems) or SPRINT Reagents and Cartridges (for SPRINT Profilers) are also required and sold separately (see Table 2).

Workflow

nCounter technology allows a simple workflow of less than 45 minutes hands-on time and streamlined data analysis in under 24 hours to profile miRNAs of interest.

Table 1. Workflow for the nCounter miRNA Assay.

	Process	Hands-on Time
Day 1 (2 hr total)	miRNA Sample Preparation Protocol	30 minutes
	miRNA CodeSet Hybridization Setup Protocol	5 minutes
Day 2 (up to 6 hr total)	**Process**	**Hands-on Time**
	Set up either the Prep Station run or SPRINT run	5 minutes
	Move Cartridge to Digital Analyzer for Data Collection (Pro/MAX/FLEX only)	5 minutes

Materials and Equipment

Materials Supplied by NanoString

Table 2. NanoString-provided materials required to run the nCounter miRNA Assay.

Item	Reagents	Storage
miRNA CodeSet: - ***Human v3:*** *Catalog # CSO-MIR3-12* - ***Mouse v1.5:*** *Catalog # CSO-MMIR15-12* - ***Rat v1.5:*** *Catalog # CSO-RMIR15-12*	miRNA Reporter CodeSet miRNA Capture ProbeSet	-80°C -80°C
nCounter miRNA Sample Prep Kit: - ***Human:*** *Catalog # Hu-MIRTAG-12* - ***Mouse:*** *Catalog # Mu-MIRTAG-12* - ***Rat:*** *Catalog # Rn-MIRTAG-12*	Annealing Buffer miRNA Tag Reagent PEG Ligation Buffer Ligase Ligation Clean-up Enzyme miRNA Assay Controls	-20°C -20°C -20°C -20°C -20°C -20°C -80°C
nCounter Master Kit (*for MAX/FLEX/Pro*) - *Catalog # NAA-AKIT-012*	nCounter Sample Cartridge Prep Plate Prep Pack, including Hybridization Buffer	-20°C 4°C 15–25°C
nCounter SPRINT Reagent Pack (*for SPRINT*) - *Catalog # SPRINT-REAG-KIT*	nCounter SPRINT Reagent C nCounter SPRINT Reagent A, B, and Hybridization Buffer	4°C 15–25°C
nCounter SPRINT Cartridge (*for SPRINT*) - *Catalog # SPRINT-CAR-1.0*	nCounter SPRINT Cartridge	-20°C

Additional Materials Required

Table 3. Additional materials required (not provided by NanoString).

Material	Supplier
Pipettes for 0.5–10 µL, 2–20 µL, 20–200 µL, and 200-1000 µL	Various
Multi-channel pipette for 20 µL (optional)	
RNase-free pipette tips with aerosol barriers	
1.5-mL microcentrifuge tubes	
0.2-mL strip tubes and caps, nuclease-free (SPRINT users only; these are provided in Master Kits for MAX/FLEX/Pro users)	
Disposable gloves	
Molecular biology-grade nuclease-free water	

Equipment

Table 4. Required equipment to run the nCounter miRNA Assay.

Equipment	Supplier
Spectrophotometer or fluorometer	NanoDrop Technologies® spectrophotometer, or ThermoFisher® Qubit™ fluorometer
Picofuge or mini-centrifuge with strip tube adaptor	Stratagene® or equivalent
Thermal cycler with a programmable heated lid (see Thermal Cycler Guidelines, below)	Various
NanoString nCounter Pro, MAX, or FLEX Analysis System or SPRINT Profiler	NanoString Technologies, Inc

Thermal Cycler Guidelines

Please note that a thermal cycler with a heated lid is required for this protocol. NanoString recommends a model with a *programmable* heated lid, to avoid high temperatures that cause tubes to melt or deform.

- 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.
- Otherwise, program the heated lid to be 5°C greater than the maximum temperature reached in the protocol. The heated lid should not exceed 110°C.

Sample Guidelines and Recommendations

The nCounter miRNA Expression Assay requires purified total RNA as input material. NanoString recommends approximately 100 ng of total RNA to generate robust signal for most tissue and cell isolates. Total RNA purified from any cell or tissue type may be used, including formalin-fixed, paraffin-embedded (FFPE) material. A variety of kits are available to extract RNA from FFPE (such as Qiagen® miRNeasy®, Catalog # 217504). **Ensure that the selected kit preserves the small RNAs.**

For plasma, serum, or other cell-free sample-derived RNA, please see the Tech Note for nCounter miRNA Expression Analysis in Plasma and Serum Samples (MK1432) for important sample preparation considerations. Unpurified lysates **may not** be used with the nCounter miRNA Expression assay, as the denaturants in the homogenization buffer will inhibit the sample preparation reaction.

The quality of the purified RNA is critically important for the nCounter miRNA assay. Residual contaminants left over from lysis and RNA extraction can impact assay performance by inhibiting the enzymatic ligation and purification steps. Typical lysis or extraction contaminants that can inhibit the assay include:

- Guanidinium Isothiocyanate (lysis buffer)
- Guanidinium HCl (initial wash buffer)
- Phenol (organic extraction)
- Ethanol (secondary wash buffer)

Purified RNA quality can be evaluated via a spectrophotometer by measuring absorbance at 230 nm (A_{230}), 260 nm (A_{260}) and 280 nm (A_{280}). Significant absorbance at 230 nm, or a low A_{260}/A_{230} ratio, can indicate contamination with organic compounds such as phenol or guanidinium. Extra washes with a secondary wash buffer or ethanol can help to minimize carry-through. Be sure to remove residual secondary wash buffer prior to elution/resuspension. Significant absorbance at 280 nm, or a low A_{260}/A_{280} ratio, can indicate contamination with protein. Such contamination may lead to an overestimation of the RNA concentration, resulting in a lower-than-anticipated signal in the assay.

NanoString recommends a 260/280 ratio of 1.9 or greater and a 260/230 ratio of 1.8 or greater for optimal results. **Please note that for plasma, serum, or other cell-free sample-derived RNA, the mRNA content will be too low to obtain accurate absorbance measurements.**

IMPORTANT:

- At very low RNA concentrations (under ~10 ng/µL), the A_{260}/A_{230} ratio may be unreliable as an indicator of contamination, due to limited nucleic acid absorbance at 260 nm. For tissue and cell derived RNA, NanoString recommends preparing samples with a concentration of > 33 ng/µL, allowing 100 ng of total RNA to be added to the sample preparation reaction in the available 3 µL volume.
- Some RNA extraction protocols suggest that better yield can be achieved by re-eluting the column with the initial eluate. NanoString does not recommend this, as the extra elution can generate significant carry-through of guanidinium and organic contamination. If re-elution must be performed, it should be preceded by at least 2 additional column washes with the secondary ethanol-based wash buffer (for a total of 4 secondary washes).
- Ethanol is not evident spectrophotometrically. Excess ethanol can be eliminated by a one-minute post-wash centrifugation in a clean collection tube, as is suggested in most kit protocols, and/or by air-drying the filter for 5 minutes.

miRNA Sample Preparation Protocol

nCounter miRNA assays require *purified* total RNA as input material. See the Sample Guidelines and Recommendations on page 6 for additional information on sample input considerations.

All experiments should be designed in sets of 12 samples. NanoString reagents are supplied in 12-reaction aliquots. The protocol below is for one set of 12 samples.

1. Program a thermal cycler according to the protocols in Table 5.

Table 5. Annealing, Ligation, and Purification protocols.

Protocol	Temp	Time
Annealing Protocol	94°C	1 min
	65°C	2 min
	45°C	10 min
	48°C	Hold
	Total Time 13 minutes	
Ligation Protocol	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 minutes	
Purification Protocol	37°C	1 hour
	70°C	10 min
	4°C	Hold
	Total Time 1 hour 10 minutes	

2. Prepare a 1:500 dilution of the miRNA Assay Controls: combine 499 µL of nuclease-free water with 1 µL of the miRNA Assay Controls in a sterile microcentrifuge tube. Mix by vortexing and briefly spin down. Store on ice.

   NOTE: Assay control RNA included in the nCounter miRNA Sample Preparation Kit allows the user to monitor the ligation efficiency and specificity through each step of the reaction.

3. Prepare an annealing master mix: combine 13 µL of Annealing Buffer, 26 µL of nCounter miRNA Tag Reagent and 6.5 µL of the 1:500 miRNA Assay Controls dilution prepared in Step 2. Mix well by pipetting up and down.

4. Aliquot 3.5 µL of the annealing master mix into each tube of a 0.2-mL strip tube.

5. Add 3 µL of RNA sample (recommended concentration ≥ 33 ng/µL) to each tube. Cap tubes and flick gently to mix. Spin down.

6. Place strip in thermal cycler and initiate the Annealing Protocol (Table 5).

7. Combine 22.5 µL PEG and 15 µL Ligation Buffer to prepare a 15X ligation master mix. Mix well by pipetting up and down.

   NOTE: PEG is viscous and should be pipetted slowly to ensure accurate transfer of volume into the mix.

8. Following completion of the Annealing Protocol, when the thermal cycler has reached 48°C, add 2.5 µL of the ligation master mix to each tube. (**Do not** turn off the thermal cycler; the block must be at 48°C in Steps 9 and 10). Flick tubes gently to mix and spin down.

9. Return tubes to 48°C thermal cycler, close lid, and incubate at 48°C for 5 min.

   IMPORTANT: For Step 10, **do not remove tubes from the thermal cycler**. Maintaining the temperature of the tubes at 48°C is critical for optimal assay performance.

10. Open thermal cycler, carefully remove caps from tubes (leaving strip in place in the heat block) and add 1 µL of Ligase directly to the bottom of each tube while tubes remain at 48°C. Check the pipette tip to be sure all the Ligase was added to the reaction. There is no need to mix.

    NOTE: To keep track of Ligase addition to sequential samples, it can be helpful to line up 12 tips in front of thermal cycler, discarding each tip after use.

11. Immediately after addition of Ligase to the final tube, recap tubes (leaving tubes in heat block), close thermal cycler, and initiate the Ligation Protocol (Table 5).

12. After completion of the Ligation Protocol, add 1 µL Ligation Clean-Up Enzyme to each reaction. The tubes can be removed from the heat block for this step. Flick tubes gently to mix, then spin down.

13. Return tubes to thermal cycler and initiate the Purification Protocol (Table 5).

14. After completion of the Purification Protocol, add 40 µL nuclease-free water to each sample. Mix well and spin down.

    NOTE: At this point, purified sample preparation reactions may be stored at –20°C for several weeks.

15. Proceed with the miRNA CodeSet Hybridization Setup Protocol (page 9).

    NOTE: Be sure to denature your samples as part of the CodeSet Hybridization Protocol (Step 6.b. of the following protocol).

miRNA CodeSet Hybridization Setup Protocol

Reporter CodeSet and Capture ProbeSet Handling Instructions:

- During setup, do not vortex or pipette vigorously to mix. Instead, gently flick or invert the tubes.
- To spin down contents of tubes, a picofuge or mini-centrifuge is recommended. If using a centrifuge, spin at <3000x*g* for <10 seconds. Do not "pulse" spin as it will cause the centrifuge to go to maximum speed and may spin the probes out of solution.

The order of addition of components is important; **follow the protocol exactly.**

1. **Pre-heat** a thermal cycler to 65°C with a heated lid at 70°C; set the time interval to "infinite". **Do not** set the thermal cycler to ramp down to 4°C at the end of the incubation time.

   NOTE: A thermal cycler with a heated lid is required for this protocol. NanoString recommends a thermal cycler with a *programmable* heated lid. See Thermal Cycler Guidelines on page 5.

2. **Remove** Reporter CodeSet and Capture ProbeSet reagents from the -80°C freezer and thaw on ice, shielded from light. Once thawed, invert several times to mix well and briefly 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 to room temperature before using.

3. Create a **hybridization master mix** by adding 130 µL Hybridization Buffer to the tube containing 130 µL Reporter CodeSet (Table 6). **Do not add the Capture ProbeSet to the master mix.**

Table 6. Hybridization master mix for one nCounter assay (12 reactions + 2 reactions of dead volume).

Component	Hybridization Master Mix (µL)	Per Reaction (µL)
miRNA Reporter CodeSet	130 (in tube)	10
Hybridization Buffer	130	10
Total Volume of Master Mix	**260**	**20**

4. **Flick or invert the hybridization master mix tube repeatedly** to mix, then briefly spin down.

5. **Label a strip tube.** If necessary, cut strip in half to fit in a picofuge with strip tube adaptor, and label both halves. For MAX/FLEX/Pro users, use the strip tubes provided with the nCounter Master Kits, ensuring that the notch is positioned between tubes 1-2 and 8-9.

6. Prepare the hybridization reactions (Table 7) using a new pipette tip at every step:

   a. **Add 20 µL of hybridization master mix** to each tube of the prepared strip tube.

b. **Denature samples** from the miRNA Sample Preparation Protocol at 85°C for 5 minutes, then quick-cool on ice.

c. **Add 5 µL of each sample** from the miRNA Sample Preparation Protocol to their respective tubes of the strip tube.

d. A**dd 5 µL of Capture ProbeSet** to each tube.

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

e. **Cap the strip tube tightly and mix** by inverting or flicking to ensure complete mixing.

f. **Spin briefly and immediately place the strip tube in a preheated 65°C thermal cycler**.

Table 7. Hybridization reaction contents.

Component	Per Reaction (µL)
Hybridization Master Mix	20
Sample from the miRNA Sample Preparation Protocol	5
miRNA Capture ProbeSet	5
Total Reaction Volume	**30**

7. Incubate hybridization reactions for 16-30 hours. Hybridizations should be left at 65°C until ready for processing, although maximum hybridization time should not exceed 30 hours.

   NOTE: NanoString recommends 20-22 hours of hybridization time for this assay.

8. Once removed from the thermal cycler, proceed immediately to post-hybridization processing with the nCounter Pro Analysis System User Manual (MAN-10147), nCounter Analysis System User Manual for MAX/FLEX systems (MAN-C0035), or nCounter SPRINT Profiler User Manual (MAN-10017). **Do not store hybridizations at 4°C.**

Technical Support

For technical support, please contact support@nanostring.com.

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