

nCounter™ Gene Expression Assay Manual

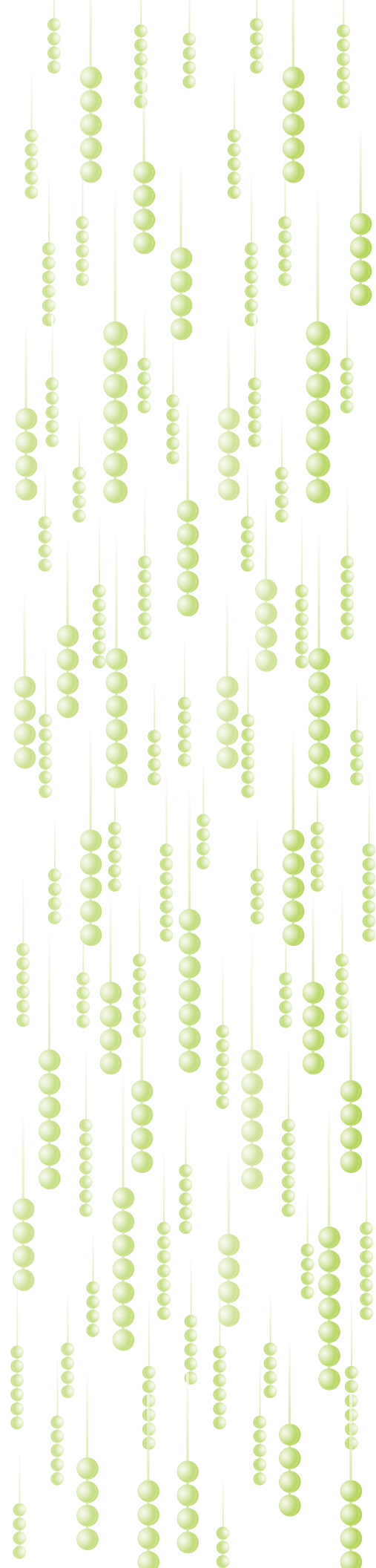
Total RNA and Cell Lysate Protocols



www.nanostring.com

206.378.6266
888.358.6266

v.20090807



For research use only. Not for use in diagnostic procedures.

Limited License

Subject to the terms and conditions of the nCounter™ Analysis System contained in the product quotation, NanoString grants you a limited, non-exclusive, non-transferable, non-sublicensable, research use only license to use the proprietary nCounter Analysis System only in accordance with the manual and other written instructions provided by NanoString. Except as expressly set forth in the terms and conditions, no right or license, whether express, implied or statutory, is granted by NanoString under any intellectual property right owned by, or licensed to, NanoString by virtue of the supply of the proprietary nCounter Analysis System. Without limiting the foregoing, no right or license, whether express, implied or statutory, is granted by NanoString, to use the nCounter Analysis System with any third party product not supplied or licensed to you by NanoString, or recommended for use by NanoString in a manual or other written instruction provided by NanoString.

Trademarks

NanoString Technologies®, NanoString™, nCounter™ and Molecules That Count™ are registered trademarks or trademarks of NanoString Technologies, Inc., in the United States and/or other countries. All other trademarks and/or service marks not owned by NanoString that appear in this manual are the property of their respective owners.

Copyright

© 2008-2009 NanoString Technologies, Inc. All rights reserved.

Contents

Chapter 1: Introduction

NanoString Technology	1
Principles and Procedures	1
The nCounter™ Expression Assay Overview	2
Materials Required	3
Contact Information	4

Chapter 2: nCounter™ Gene Expression Protocols

Setting Up Twelve nCounter™ Assays	5
Setting Up a Single nCounter™ Assay	7

Introduction

The nCounter™ Gene Expression Assay is designed to provide an ultra sensitive, reproducible and highly multiplexed method for detecting gene expression across all levels of biological expression. This assay provides a method for detecting mRNAs with molecular barcodes called nCounter Reporter Probes without the use of reverse transcription or amplification.

This manual describes in detail the methods for setting up the nCounter hybridization assay. Please see the *nCounter™ Prep Station User Manual*, *nCounter™ Digital Analyzer User Manual*, and *nCounter™ Data Analysis Guidelines* for instructions on post-hybridization processing and data analysis.

This manual covers the nCounter Gene Expression Assay protocol and provides instruction for both the Total RNA Standard Protocol and the Cell Lysate Protocol. The Total RNA Standard Protocol can also be used with total RNA isolated from blood and formalin-fixed paraffin embedded (FFPE) samples.

NanoString Technology

Principles and Procedures

NanoString technology is based on digital detection and direct molecular barcoding of target molecules through the use of a color coded probe pair. The probe pair consists of a Reporter Probe, which carries the signal on its 5' end, and a Capture Probe which carries a biotin on the 3' end. The color codes carry six positions and each position can be one of four colors, thus allowing for a large diversity of tags that can be mixed together in a single well for direct hybridization to target and yet still be individually resolved and identified during data collection.

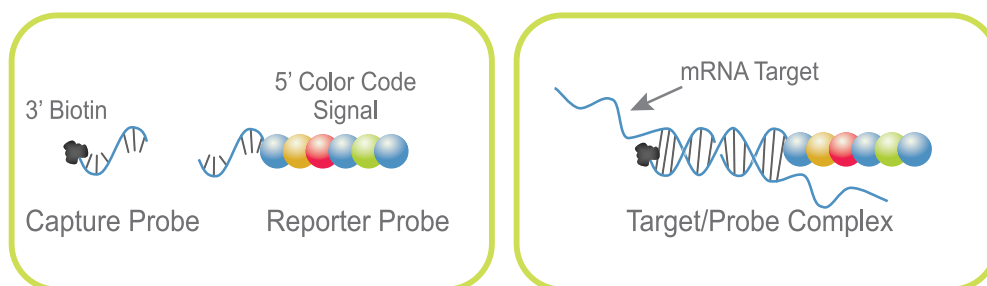


Figure 1.1 Capture and Reporter Probes (left) and, Probe pair bound to an mRNA (right)

Probe pairs are placed into a reaction in massive excess to target mRNA to ensure that each target finds a probe pair. After hybridization, excess probes are washed away using a two step magnetic bead-based purification on the nCounter™ Prep Station.

Magnetic beads derivatized with short nucleic acid sequences that are complementary to the Capture Probe and the Reporter Probes are used sequentially. First, the hybridization mixture is allowed to bind to the magnetic beads by the Capture Probe. Wash steps are performed and excess Reporter Probes and non-target cellular transcripts are removed during wash steps. After washing, the Capture Probes and Target/Probe complexes are eluted off of the beads and are hybridized to magnetic beads complementary to the Reporter Probe. Wash steps are performed and excess Capture Probes are washed away. Finally, the purified Target/Probe complexes are eluted off and are immobilized in the cartridge for data collection.

Data Collection is carried out in the nCounter™ Digital Analyzer. Digital images are processed and the barcode counts are tabulated in a comma separated value (CSV) format.

The nCounter™ Expression Assay Overview

The nCounter Expression Assay is run on the nCounter System. The system is comprised of two instruments, the nCounter Prep Station used for post-hybridization processing, and the Digital Analyzer used for data collection.

Figure 1.2 illustrates the typical workflow for the nCounter Expression Assay.

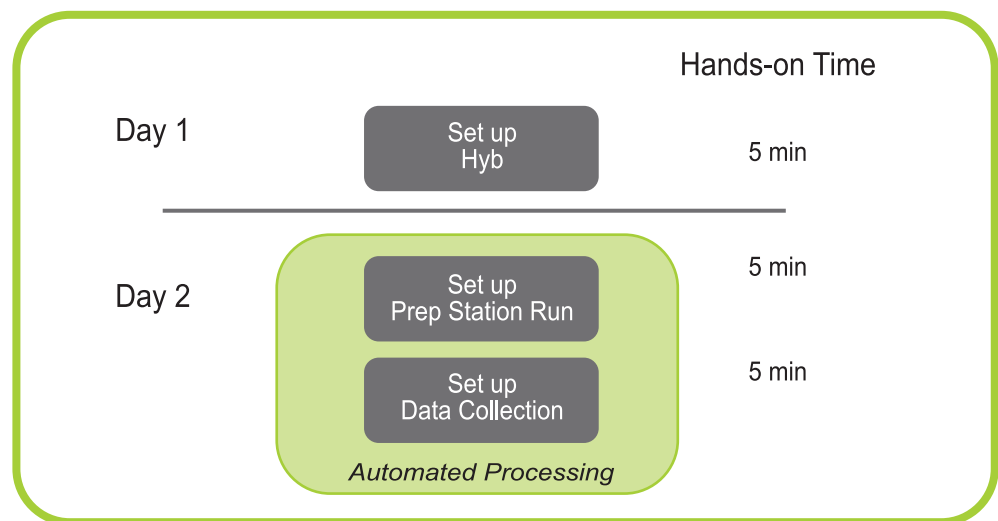


Figure 1.2 Suggested workflow for the nCounter™ Expression Assay

Materials Required

The following tables list the recommended materials and instrumentation required to run the nCounter Gene Expression Assay.

Table 1.1 Materials Required for Total RNA Standard Protocol

Material	Manufacturer	Part Number
nCounter GX CodeSet	NanoString Technologies	GXA-PICS-xxx
nCounter Master Kit	NanoString Technologies	NAA-AKIT-xxx
QIAGEN RNeasy® Kit	QIAGEN	74104 or 74106
Disposable gloves	various	
Total RNA - 100 ng per hybridization assay*		

*If total RNA has been isolated by some other method, please contact NanoString customer support (customersupport@nanosttring.com). We highly recommend verifying the integrity of your total RNA samples via denaturing PAGE or Bioanalyzer (Agilent Technologies) before proceeding with hybridization.

Table 1.2 Materials Required for Cell Lysate Protocol

Material	Manufacturer	Part Number
nCounter GX CodeSet	NanoString Technologies	GXA-PICS-xxx
nCounter Master Kit	NanoString Technologies	NAA-AKIT-xxx
RLT Buffer	QIAGEN*	79216
Disposable gloves	various	
Cell lysate 2,500-10,000 cells		

*QIAGEN buffers and a QIAGEN cell lysate procedures (with modifications outlined in Chapter 2) have been tested internally at NanoString Technologies. If using a cell lysis procedure other than QIAGEN'S, please contact NanoString customer support (customersupport@nanosttring.com) for additional information.

Table 1.3 Instruments Required for Total RNA Standard Protocol and Cell Lysate Protocol

Instrument	Manufacturer	Part Number
NanoDrop ND-1000*	NanoDrop Technologies	N/A
Bioanalyzer 2100*	Agilent	G2940CA
Pipette for 0.5-10 µL*	Rainin	L-10
Pipette for 2-20 µL*	Rainin	L-20
Pipette for 20-200 µL*	Rainin	L-200
Tube-Strip Picofuge*	Stratagene	400540
DNA Engine Thermocycler or hybridization oven	MJ Research/BioRad	PTC-200G†
		PTC-1148†
		PTC-0220G†
		PTC-0221G†
		PTC-0240G†
nCounter Prep Station	NanoString Technologies	NCT-PREP-120
nCounter Digital Analyzer	NanoString Technologies	NCT-DIGA-120
Memory Stick	NanoString Technologies	N/A

*nCounter system performance data was generated on model PCT-200 DNA engines. Other instruments may produce non-standard results when used with the nCounter assay.

† Any one of these instruments will meet the requirements of the nCounter Assay.

Contact Information

NanoString Technologies

530 Fairview Ave N, Suite 2000

Seattle, WA 98109

Phone: 1-888-358-NANO (1-888-358-6266)

Fax: 1-206-378-6288

E-mail: support@nanostring.com

nCounter™ Gene Expression Protocols

This chapter outlines the nCounter Gene Expression Assay protocol and provides instruction for both the Total RNA Standard Protocol and the Cell Lysate Protocol. Instruction for setting up 12 nCounter Assays or a single nCounter Assay are provided.

Setting Up Twelve nCounter™ Assays



GENERAL PROBE HANDLING WARNING: During setup of your assay, do not vortex or pipet vigorously to mix as it may shear the Reporter Probes. Mixing should be done by flicking or inverting the tubes. Also, if you use a microfuge to spin down tubes, do not spin any faster than 1,000 rpm for more than 30 seconds and do not “pulse” it to spin because that will cause the centrifuge to go to maximum speed and you may spin your CodeSet out of solution.

The final hybridization reaction will contain the following components: 10 μL Reporter CodeSet, 10 μL hybridization buffer, a total volume of 5 μL of sample RNA (100 ng), and 5 μL Capture ProbeSet. The order of addition of components is important, please follow the protocol exactly.

1. If following the Total RNA Standard Protocol go to [Step 3](#).
2. If following the Cell Lysate Protocol: Lyse Cells according to Qiagen recommendations (see Qiagen RNeasy Mini Handbook, supplied with product numbers 74104 and 74106) with the following modifications:
 - a. Cells should be lysed at concentration between 2,500 and 10,000 cells/ μL of RLT buffer. The nCounter cell lysate hybridization procedure has been optimized for \sim 10,000 mammalian cells/reaction or the equivalent of approximately 100 ng of total RNA.
 - b. Cell lysates should be aliquoted and stored at -80°C . Avoid freeze/thaw cycles.
3. Remove aliquots of both Reporter CodeSet and Capture ProbeSet reagent from the freezer and thaw on ice. Invert several times to mix well and spin down reagent.
4. Create a master mix containing 130 μL of the Reporter CodeSet and 130 μL of hybridization buffer. RNase-free water may also be added to this mix if the volume of the individual RNA samples is less than 5 μL and is constant. (Add enough water for 13 assays to allow one assay’s worth of dead volume.)
Do not add the Capture ProbeSet to the master mix. Invert to mix and spin down master mix.
5. Label a provided 12 tube strip and cut it in half so it will fit in a picofuge.

6. Add 20 μL of mastermix to each of the 12 tubes (if you added water to the master mix, adjust volumes). It is advisable to use a fresh tip for each pipetting step to accurately pipet the correct volume. The CodeSet has components that can start to wick up into the tip and not dispense the correct amount if you use the same tip to dispense master mix into all of the hybridization tubes.
7. Add sample according to your protocol type as follows:
 - a. If following the Total RNA Standard Protocol: Add total RNA sample (maximum volume 5 μL) for a total of 100 ng to each tube. Go to [Step 8](#).
 - b. If following the Cell Lysate Protocol: Add cell lysate sample (maximum volume 4 μL) for a total of approximately 10,000 cells per hybridization assay. Using less than 10,000 cells/reaction will result in fewer counts/gene.
 - c. If using attenuation mix(es), add 1 μL of each mix. Note: this reagent can also be added to the master mix if all reactions are to be attenuated.
8. If necessary, add RNase-free water to each tube to bring the volume of each assay to 25 μL .
9. Pre-heat thermocycler to 65°C. Program the thermocycler using 30 μL volume, calculated temperature, heated lid and “forever” time setting. **Do not set the thermocycler to ramp down to 4°C at the end of the run.**

If a thermocycler is not available, a 65°C hybridization oven may be used. (The use of a thermocycler is recommended if possible. Due to less stringent temperature control, assay results may be more variable in a hybridization oven.) To use a hybridization oven, place a large beaker full of water in the oven to ensure a humid environment. **Do not place the samples in the water beaker;** evaporative loss causes the water temperature to be below the air temperature in the oven. Place the samples in a dry rack in the middle of the oven shelf, or tape the strip tubes to a rotator in the center of the oven. Make sure that the strip tubes and/or rack do not touch the sides or bottom of the oven. Failure to follow these instructions may result in uneven hybridization temperatures which can compromise the results.
10. Add 5 μL of Capture ProbeSet to each tube immediately before placing at 65°C. Cap tubes and mix the reagents by inverting the strip tubes several times and flicking with your finger to ensure complete mixing. Briefly spin down and immediately place the strip tube in the 65°C thermocycler. Minimizing the time between the addition of the Capture ProbeSet and the placement of the reaction at 65°C will increase the sensitivity of your assay.
11. Incubate hybridization assays for at least 12 hours. Hybridizations should be left at 65°C until ready for processing. Maximum hybridization time should not exceed 30 hours.
12. Once removed from the thermocycler, proceed immediately to post-hybridization processing with the nCounter Prep Station. Do not store hybridizations at 4°C.

Setting Up a Single nCounter™ Assay

During setup of your assay, do not vortex or pipet vigorously to mix as it may shear the Reporter Probes. Mixing should be done by flicking or inverting the tubes. Also, if you use a microfuge to spin down tubes, do not spin any faster than 1,000 rpm for more than 30 seconds and do not “pulse” it to spin because that will cause the centrifuge to go to maximum speed and you may spin your CodeSet out of solution.

The final hybridization reaction will contain the following components: 10 μ L Reporter CodeSet, 10 μ L hybridization buffer, a total volume of 5 μ L of sample RNA (100 ng), and 5 μ L Capture ProbeSet. **If you are making a master mix of any components, DO NOT include the Capture ProbeSet.** It is important that the Capture ProbeSet be added individually to each assay, immediately before the reaction is transferred to 65°C.

1. If following the Total RNA Standard Protocol go to [Step 3](#).
2. If following the Cell Lysate Protocol: Lyse Cells according to Qiagen recommendations (see Qiagen RNeasy Mini Handbook, supplied with product numbers 74104 and 74106) with the following modifications:
 - a. Cells should be lysed at concentration between 2,500 and 10,000 cells/ μ L of RLT buffer. The nCounter cell lysate hybridization procedure has been optimized for ~10,000 mammalian cells/reaction or the equivalent of approximately 100 ng of total RNA.
 - b. Cell lysates should be aliquoted and stored at –80°C. Avoid freeze/thaw cycles.
3. Remove aliquots of both Reporter CodeSet and Capture ProbeSet reagent from the freezer and thaw on ice. Invert several times to mix and spin down reagent.
4. Label a provided 12 tube strip and cut it in half so it will fit in a picofuge.
5. Add 10 μ L of Reporter CodeSet reagent to each tube. Store remaining Reporter CodeSet at –80°C.
6. Add 10 μ L of hybridization buffer to each tube.
7. Add sample according to your protocol type as follows:
 - a. If following the Total RNA Standard Protocol: Add total RNA sample (maximum volume 5 μ L) for a total of 100 ng to each tube. Go to [Step 8](#).
 - b. If following the Cell Lysate Protocol: Add cell lysate sample (maximum volume 4 μ L) for a total of approximately 10,000 cells per hybridization assay. Using less than 10,000 cells/reaction will result in fewer counts/gene.
 - c. If using attenuation mix(es), add 1 μ L of each mix.
8. Add RNase-free water to bring the final volume of each hybridization assay to 25 μ L.
9. Pre-heat thermocycler to 65°C. Program the thermocycler using 30 μ L volume, calculated temperature, heated lid and “forever” time setting. **Do not set the thermocycler to ramp down to 4°C at the end of the run.** Alternatively, a 65°C hybridization oven may be used when a large beaker full of water is placed in the oven to ensure a humid environment.

- 10.** Add 5 μ L of Capture ProbeSet reagent to each tube. Cap tubes and mix the reagents by inverting the strip tubes several times and flicking with your finger to ensure complete mixing. Briefly spin down and immediately place the strip tube in the 65°C thermocycler. Minimizing the time between the addition of the Capture ProbeSet and the placement of the reaction at 65°C will increase the sensitivity of your assay.
- 11.** Store remaining Capture ProbeSet at –80°C.
- 12.** Incubate hybridization assays for at least 12 hours. Hybridizations should be left at 65°C until ready for processing. Maximum hybridization time should not exceed 30 hours.
- 13.** Once removed from the thermocycler, proceed immediately to post-hybridization processing with the nCounter Prep-station. Do not store hybridizations at 4°C.