nanoString TECHNOLOGIES®

nCounter® XT Assay
User Manual

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1 Introduction to nCounter Assays with XT Formulation

NanoString's nCounter technology is designed to provide a sensitive, reproducible, and highly-multiplexed method for detecting various small molecules across all levels of biological expression. Our flagship gene expression assays utilize a dual-probe system to provide a method for specifically and sensitively detecting mRNAs with molecular barcodes without the use of enzymes for reverse transcription or amplification. Other assay types can detect miRNA, DNA, or protein with minor variations in protocol and reagents.

A. Selecting Your Protocol

This manual describes the procedures for setting up all nCounter XT hybridization assays. nCounter XT is a reformulated version of the fundamental assay chemistries (CodeSet and TagSet) used by standard nCounter reagents. All differences apply to the CodeSet or TagSet only; nCounter Master Kits can be used with either formulation. Additional information is provided in the *Tech Note: Validation and Impact of nCounter XT Formulation*.

Gene expression assays, Plex^2 assays, DNA assays, and assays for single cells or low input material in this manual are for use with nCounter XT CodeSets only, which are supplied in a solid green box (**FIGURE 1.1**).

If you are using Legacy nCounter CodeSets for these assays, do not use this manual. Also, do not use this manual for miRNA expression assays or miRGE™ assays. Instead, use one of the protocols listed below, which contain the original instructions for Legacy nCounter reagents.

- *nCounter Gene Expression Assay User Manual*
- *nCounter DNA Assay User Manual*
- *nCounter Single Cell Gene Expression Protocols*
- *nCounter miRNA Expression Assay User Manual*
- *nCounter miRGE Assay Manual*

FIGURE 1.1 Examples of the new nCounter XT CodeSet packaging, including a green box and updated tube labels.

B. nCounter Assay Overview

NanoString's nCounter technology is based on digital detection and direct molecular barcoding of individual target molecules through the use of a unique probe pair for each target of interest. The probe pair consists of a color-coded Reporter probe, which carries the visible signal on its 5' end, and a Capture probe, which carries a biotin moiety on the 3' end. Each Reporter probe has six positions, and each position can be one of four colors. A large diversity of probes can be mixed together with the sample in a single well, with each Reporter probe interacting with a specific target. These target-probe complexes are then individually resolved and identified during data collection.

Probes are placed into a reaction in massive excess to target nucleic acid molecules to ensure that each target finds a probe pair. After hybridization (typically an overnight process), excess probes are washed away using a two-step magnetic bead-based purification on an nCounter instrument. These magnetic beads are derivatized with short nucleic acid sequences that are complementary to the Capture probes or the Reporter probes and are used sequentially. First, the hybridization mixture is allowed to bind to the magnetic beads complementary to the Capture probes. Wash steps are performed to remove excess Reporter probes and non-target cellular transcripts. The target-probe complexes and unbound Capture probes are then eluted off the beads and allowed to bind to a second set of beads complementary to the Reporter probes. Wash steps are performed to remove excess Capture probes. Finally, the purified target-probe complexes are eluted off the beads and are immobilized and aligned for data collection.

Data collection is performed using epifluorescence microscopy and CCD capture technology on an nCounter instrument to yield hundreds of thousands of target molecule counts. Digital images are processed within the nCounter instrument, and the Reporter Probe counts are tabulated in a comma separated value (CSV) format for convenient data analysis with NanoString's free nSolver™ Analysis Software or the application of your choice.

nCounter technology makes lab work and sample analysis a simpler process by limiting the variables in experiments. The end result is very precise and accurate measurements, enabling you to gather data on your targets of interest rapidly and with minimal intervention.

After completing sample hybridization, see the appropriate User Manual for your nCounter instrument as well as the nCounter Data Analysis Guidelines or the nSolver Analysis Software User Manual for instructions on post-hybridization sample processing and data analysis.

C. nCounter CodeSet Chemistry

NanoString's nCounter CodeSet chemistry utilizes target-specific Reporter and Capture Probes, collectively referred to as a CodeSet, that directly hybridize to a target of interest (**FIGURE 1.2**). Each probe pair consists of a color-coded Reporter and a Capture Probe to which a target-specific sequence has been covalently attached. During an overnight hybridization, the specific Reporter and Capture Probes hybridize directly to the single-stranded RNA or DNA target molecule in solution. nCounter CodeSet chemistry-based assays are described in Chapters 2, 3, 5, 6, and 7 of this manual.

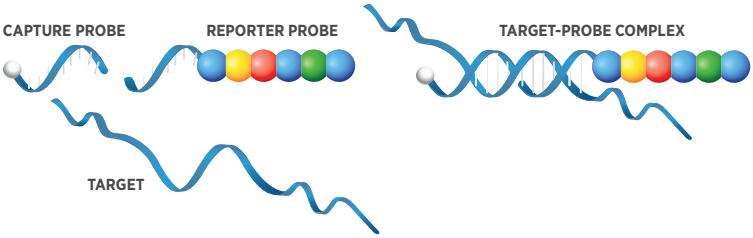

FIGURE 1.2 Capture and Reporter Probes (left) hybridize with a single-stranded target to form a double-stranded probe-target complex (right).

D. nCounter TagSet Chemistry

NanoString's nCounter TagSet chemistry consists of target-specific oligonucleotide probe pairs, fluorescently-labeled specific Reporter Tags, and a biotinylated universal Capture Tag, collectively called a TagSet (**FIGURE 1.3**). Like nCounter CodeSet Reporter Probes, nCounter TagSet Reporter Tags each have a unique pattern of six spots of color, creating fluorescent barcodes that can be individually resolved and counted during data collection.

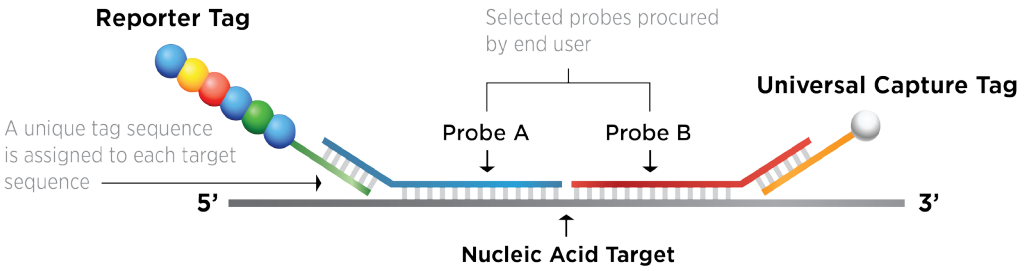

FIGURE 1.3 Oligonucleotide probes hybridize with Reporter and Capture Tags and the target nucleic acid to create a Tag Complex.

Unlike nCounter CodeSet probes, the specific Reporter Tags and universal Capture Tag hybridize to a pair of target-specific oligonucleotide probes, which in turn hybridize directly to the single-stranded RNA or DNA target. Probe A hybridizes to a specific Reporter Tag and the 5' region of the target nucleic acid sequence. Probe B hybridizes to the universal Capture Tag and the 3' region of the target nucleic acid sequence. Each complete structure—containing the target RNA or DNA, two oligonucleotide probes, and the Reporter and Capture Tags—is referred to as a Tag Complex. nCounter TagSet chemistry-based assays are described in Chapters 4 and 8 of this manual.

E. nCounter Instruments

All nCounter assays can be run on the nCounter instruments, which perform post-hybridization processing and data collection with minimal hands-on time (**TABLE 1.1** and **TABLE 1.2**). Follow instructions on the touchscreen that will guide you step-by-step through performing runs.

TABLE 1.1 Suggested workflow for using the nCounter Analysis System.

Day 1	**Manual Processing**	**Hands-on Time**
	Set up sample hybridization	5 minutes*
Day 2	**Automated Processing**	**Hands-on Time**
	Enter run information and initiate run on instrument	5 minutes
	Transfer cartridge from Prep Station to Digital Analyzer	5 minutes

TABLE 1.2 Suggested workflow for using the nCounter *SPRINT™* Profiler.

Day 1	**Manual Processing**	**Hands-on Time**
	Set up sample hybridization	5 minutes*
Day 2	**Automated Processing**	**Hands-on Time**
	Enter run information (using Web Application; optional)	5 minutes
	Initiate run on instrument	5 minutes

** Some assays may require additional time for sample preparation.*

F. Sample Type Considerations

Sample input recommendations for nCounter assays were developed using purified total RNA from a variety of tissues, of which mRNA typically composes 5–10% (~5–10 ng in a sample of 100 ng total RNA). Use a NanoDrop™ or other spectrophotometer to measure RNA sample quality. NanoString recommends an A260/A280 ratio of 1.7–2.3 and an A260/A230 ratio of 1.8–2.3.

Many other sample types provide high-quality results with minor adjustments to sample volume or concentration as outlined below. Please consult with your Field Applications Scientist or contact NanoString Support at **support@nanostring.com** if you have any questions about how to ensure the best results from your experiment.

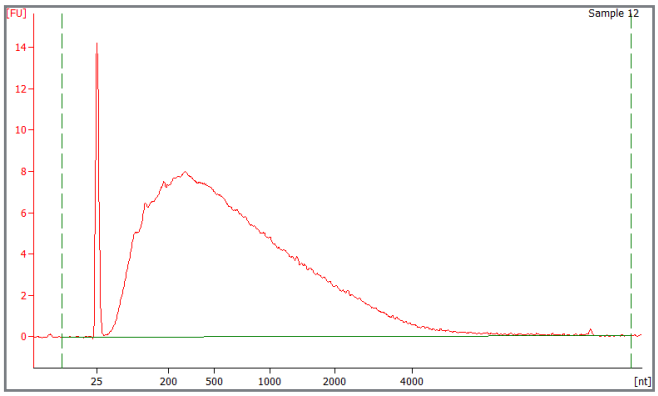

FIGURE 1.5 Ideal fragmentation profile for nucleic acids. This fragmentation profile was obtained using an Agilent 2100 Bioanalyzer and an Agilent RNA 6000 Nano Kit to assay 300 ng of total RNA purified with a commercial FFPE extraction kit. The majority of the sample is greater than 300 nucleotides.

Formalin-fixed, paraffin-embedded (FFPE)-derived samples have been shown to provide high-quality results due to NanoString's enzyme-free chemistry*, and mRNA degradation does not typically affect data quality since probes recognize a relatively short 100-base target region. NanoString recommends increasing the sample input up to 300 ng in some cases to provide better results. Carefully evaluate RNA quality using an Agilent Bioanalyzer® to measure nucleic acid fragmentation. NanoString recommends that at least 50% of the area under the trace be greater than 300 nucleotides in length for optimal performance (**FIGURE 1.5**).

Blood samples can be assayed using purified total RNA, unpurified blood lysates, or specific blood fractions such as PBMCs isolated from whole blood. NanoString recommends the use of a commercially available kit to collect and purify RNA from blood; kits may also be used for other biological fluids such as sputum or urine. For unpurified RNA, NanoString recommends collecting blood lysate samples in specialized PAXgene® tubes. Additional information on the attentuation of highly expressed genes in some sample types is provided in the *Tech Note: Strategies for Successful Gene Expression Assays*.

**Single-cell assays require amplification prior to sample hybridization.*

NOTE: Recommendations for preparing DNA samples are provided in *Chapter 6: nCounter XT DNA Assays*. For questions on additional sample types outside the scope of this section, contact NanoString Support at **support@nanostring.com**.

G. Sample Input Recommendations

The nCounter Analysis System and nCounter *SPRINT* Profiler utilize different methods for sample processing and digital imaging, although the underlying nCounter chemistry is unchanged. NanoString recommends using 50% less sample for assays performed on the nCounter *SPRINT* Profiler compared to the nCounter Analysis System to avoid saturation of the imaging surface, which can reduce data quality.

Use **TABLE 1.3** to determine the recommended sample input for most assays included in this manual. (These recommendations do not apply to the RNA:Protein assay, which is optimized for cell number. Instead, see the sample preparation instructions in Chapter 5.) With the exception of single cell assays, these recommendations apply to sample mass only; sample volume does not vary between systems.

TABLE 1.3 Recommended sample input mass for nCounter XT assays.

Sample Type	nCounter Analysis System (*MAX™/FLEX™*)	nCounter *SPRINT™* Profiler
FFPE-derived RNA	300 ng	150 ng
Total RNA (CodeSets < 400 genes)	100 ng	50 ng
Total RNA (CodeSets > 400 genes)	50 ng	25 ng
Fragmented DNA	300 ng	150 ng
ChIP DNA (unamplified)	10 ng	5 ng
ChIP DNA (whole genome amplification)	100 ng	50 ng
Single Cell or Low Input Material	up to 8 µL of amplified sample	up to 5 µL of amplified sample

Using Whole Cell Lysates in nCounter XT Gene Expression Assays

NanoString recommends a minimum of 5,000 to 10,000 cell equivalents per nCounter XT hybridization reaction for most applications. The required number of cells for any given application will ultimately be dependent on the abundance of the mRNA targets of interest in the sample to be assayed. Furthermore, the maximum sample input when using cell lysates depends on the type of lysis buffer used.

Detergent-based lysis buffers that do not contain chaotropic salts are fully compatible with nCounter reagents; as much as 5 µl may be added to each nCounter XT hybridization reaction. Other lysis buffers that contain chaotropic salts may alter nucleic acid hybridization thermodynamics and are compatible with nCounter XT reagents with some modifications to protocol. These include Buffer RLT and other buffers with a high concentration of guanidine isothiocyanate. NanoString recommends using no more than 1.5 µL of these lysis buffers per nCounter XT hybridization reaction. For this reason, NanoString recommends the use of Buffer RLT for applications in which cells can be pelleted in order to achieve a minimum cell concentration of 3,500–6,500 cells per µL. See **TABLE 1.4** for additional details.

TABLE 1.4 Sample input recommendations for using cell lysates with nCounter XT assays.

Initial Number of Cells	Recommended Lysis Buffer	nCounter Analysis System (*MAX*™/*FLEX*™)	nCounter *SPRINT*™ Profiler	Sample Volume (either system)
50,000 cells or more	RLT or other buffer with a high concentration of guanidine isothiocyanate	~6,500 cells/µL	~3,500 cells/µL	Up to 1.5 µL
50,000 cells or less	iScript™ RT-qPCR Sample Preparation Reagent or other detergent/chemical lysis buffer	~2,000 cells/µL	~1,000 cells/µL	Up to 5 µL

To prepare cell lysates, NanoString recommends following guidance provided by the lysis buffer supplier. **TABLE 1.5** contains a list of suggested lysis buffers and associated catalog numbers.

TABLE 1.5 Suggested lysis buffers.

Lysis Buffer	Supplier	Catalog Number
iScript RT-qPCR Sample Preparation Reagent	BioRad®	170-8899
Cells-to-CT™	Life Technologies®	4391851C
Buffer RLT	QIAGEN®	79216

Preparing Cell Lysates with Non-chaotropic Buffers

For applications involving small numbers of initial cells, such as flow-sorting, NanoString recommends sorting directly into a chemical- or detergent-based buffer (such as Cells-to-Ct) in order to maximize the concentration of cells in the lysate (up to ~2,000 cells/µL). NanoString does not recommend using a chemical- or detergent-based buffer at concentrations > 2,000 cells/µL because this may result in incomplete cell lysis. Additionally, it is important to remove growth medium from cells as it may inhibit lysis and result in reduced assay performance.

Preparing Cell Lysates with Chaotropic Buffers

To prepare cell lysates with Buffer RLT (QIAGEN), or if using other buffers containing guanidine isothiocyanate, NanoString recommends following the guidance provided in the QIAGEN RNeasy® protocol (see pp 16–27 of RNeasy Mini Handbook v.06/2012 for important notes). For most mammalian cell lines grown in tissue culture, the basic steps are:

1. Harvest an appropriate number of cells, and pellet by centrifugation for 5 minutes at 300 RCF in a microcentrifuge tube. Carefully remove all supernatant by aspiration. Failure to remove all supernatant may dilute lysis buffer and result in incomplete cell lysis.
2. Disrupt cells by adding QIAGEN Buffer RLT. Addition of β-mercaptoethanol to RLT is optional but may improve RNase inactivation in cell lines expressing high levels of RNase. Use 10 µL β-mercaptoethanol per 1 mL RLT. NanoString does not recommend lysis of highly concentrated material (i.e., > 20,000 cells/µL) as this may result in incomplete lysis and reduced assay performance.
3. Homogenize cells by vortexing for 1 minute. Centrifuge briefly to recover all material to bottom of tube. (It is not necessary to centrifuge cellular debris and remove the supernatant. Hybridization can be performed using the complete lysate.)
4. Proceed immediately to hybridization (using no more than 1.5 µL in each hybridization reaction) or freeze lysate at -80°C.

H. Plus Products

The nCounter Plus products and reagents are unique universal Reporter and Capture Probes that increase the flexibility of the nCounter platform. Probes for up to 30 additional targets are combined with an existing CodeSet prior to overnight hybridization. Because the original CodeSet contains the proper controls, the Plus reagents do not have them.

- Plus products are not compatible with each other, Plex2, or ChIP-String assays.
- Purchase of additional Master Kit reagents is not required.

Panel-Plus and CodeSet-Plus

Panel-Plus and CodeSet-Plus products are compatible with both Legacy and XT formulations.

IMPORTANT: CodeSet/Panel-Plus reagents are compatible with both Codeset (see *Chapter 2, nCounter XT CodeSet Gene Expression Assays*) and TagSet (see *Chapter 4, nCounter Vantage RNA Assays*) chemistry assays. Please use the protocol appropriate for the assay chemistry with which you are using your CodeSet/Panel-Plus reagents.

nCounter Vantage Protein Products

Protein Plus reagents are compatible with XT formulations only. The Protein Plus reagent provided in nCounter Vantage Protein Assays enables combined analysis of proteins and RNA in a single reaction. Two separate vials containing the Antibody Mix and Protein Plus TagSet are included. Separate hybridization protocols are provided for combining the Protein Plus reagent with XT CodeSet assays or XT TagSet assays (see *Chapter 5, nCounter Vantage RNA:Protein Assays,* for detailed protocols).

Preparing the RLF

All nCounter Plus reagents are accompanied by an add-in library file (ALF), which specifies the association between each Plus reagent and its target. Information from the ALF must be merged with the reporter library file (RLF) from the original CodeSet prior to scanning on the Digital Analyzer. Failure to merge an ALF with the original RLF will result in no count information being collected for targets of Plus reagents.

To generate a merged RLF file, email NanoString at bioinformatics@nanostring.com. Include both the ALF for your Plus product and the RLF for the CodeSet into which you wish to spike the Plus product. A new RLF will be generated and emailed to the requestor's address that contains all probe information for both the Plus product and the original CodeSet.

Data Analysis Guidelines

Plus reagents do not include built-in ERCC standards and should only be used in conjunction with CodeSets already containing these controls. Plus probes are designed to minimize off-target effects and cross-hybridization through bioinformatic screens that look for secondary structure, high local GC content, runs of polynucleotides, and homology to areas of the transcriptome for the organism of interest. To empirically assess interactions between CodeSet and Plus reagents, NanoString recommends comparing the same samples using the CodeSet alone vs. the CodeSet and Plus reagents together. No interactions are expected. For Protein Plus, NanoString also recommends running a no template control for the standalone CodeSet and combination CodeSet and Protein Plus assay to confirm no change in background.

After sample imaging, data may be imported into the nSolver Analysis Software for downstream analysis. To download the latest version, go to http://www.nanostring.com/products/nSolver. See the nSolver Analysis Software Manual for additional information on using the nSolver application.

When running a study using different production lots of the Reporter CodeSet contained in a given Panel Kit, it is good practice to run a common calibrator (reference) sample with every lot of the Reporter CodeSet. This enables any differences in individual probe binding efficiencies to be normalized, thereby allowing the counts between the two lots to be directly compared. For details, contact your local Field Applications Scientist or e-mail support@nanostring.com.

The lot number of the Reporter CodeSet is found on the Reporter CodeSet tube and begins with the letters "RC".

I. Recommended Materials

TABLE 1.6 lists materials and instrumentation that are recommended or required to run nCounter XT Assays. Additional materials are recommended for RNA purification in **TABLE 1.7**. Information for cell lysates is provided in *Section F: Sample Input Recommendations*.

TABLE 1.6 Materials recommended for all nCounter assays.

Material	Manufacturer	Part Number(s)
Disposable gloves	Various	Various
NanoDrop ND-2000*	NanoDrop Technologies®	N/A
Bioanalyzer® 2100*	Agilent®	G2940CA
Pipette for 0.5–10 µL*	Rainin®	L-10XLS+
Pipette for 2–20 µL*	Rainin	L-20XLS+
Pipette for 20–200 µL*	Rainin	L-200XLS+
Microcentrifuge or picofuge	Various	Various
Thermal cycler†	Various	Various

nCounter performance data were generated using a Bio-Rad® DNA Engine®. Other instruments can be used but should have a programmable heated lid. Contact NanoString Support at **support@nanostring.com with questions about the compatibility of products not listed here.*

TABLE 1.7 Additional materials recommended for gene expression assays using total RNA (standard protocol).

Material	Manufacturer	Part Number(s)
RNeasy® Kit (or an equivalent kit from another manufacturer)*	QIAGEN®	74104 74106
Total RNA sample: 25 ng to 100 ng per hybridization assay*		

**NanoString highly recommends verifying the integrity of total RNA samples via denaturing PAGE or Bioanalyzer before proceeding with hybridization.*

IMPORTANT: All assays require PCR tubes to perform the sample hybridization reaction. Ensure that these tubes meet the guidelines provided by the thermal cycler manufacturer. Strip tubes may be helpful, but individual tubes may also be used.

While any thermal cycler-compatible tube will work for hybridization, those tubes will NOT work for the PrepStation. Any hybridizations done in non-NanoString-supplied strip tubes MUST be transferred to the strip tubes supplied in the Master Kit.

Thermal Cycler Guidelines

Thermal cyclers are produced by a wide variety of manufacturers and possess a wide variety of features. NanoString recommends using a model with a programmable heated lid. Models without programmable lids may reach a very high temperature that causes tubes to melt or deform. However, programmable lids may offer different levels of control.

- Ideally, 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.
- Some heated lids cannot be assigned a floating temperature. In this situation, program the heated lid to be 5°C greater than the maximum temperature during the protocol. The heated lid should not exceed 110°C.

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2 nCounter XT CodeSet Gene Expression Assays

This chapter explains the procedures for setting up hybridizations for all mRNA gene expression assays with nCounter XT CodeSets. These include protocols for using total RNA or cell lysates with custom CodeSets and pre-defined panels.

Do not use these instructions with Legacy nCounter CodeSets. Instead, refer to the *nCounter Gene Expression Assay User Manual*.

A. Hybridization Protocol for nCounter XT CodeSet Gene Expression Assays Including Panels

IMPORTANT: Are these CodeSet or TagSet assays and/or panels? Check before you begin. If you are using nCounter XT **TagSet** Assays and/or Panels, refer to *Chapter 4, nCounter XT TagSet Gene Expression Assays*.

GENERAL PROBE HANDLING WARNING: During setup of the assay, do not vortex or pipette vigorously to mix as it may shear the Reporter CodeSet. Mixing should be done by flicking or inverting the tubes. If using a microfuge to spin down tubes, do not spin any faster than 1,000 RCF for more than 30 seconds. Do not "pulse" to spin because that will cause the centrifuge to go to maximum speed and may spin the CodeSet out of solution.

IMPORTANT: Pre-heat the thermal cycler to 65°C. Program the thermal cycler using 15 µL volume, calculated temperature, and heated lid.

Set up a hybridization reaction at room temperature for each sample with the following components: 3 µL of Reporter CodeSet, 5 µL of hybridization buffer, up to 5 µL (25–100 ng) of sample RNA, and 2 µL of Capture ProbeSet. See **TABLE 1.3** in Chapter 1 for the sample input recommendations specific to each instrument.

1. If using total RNA, go to Step 3.
2. If using cell lysates, see **TABLE 1.4** in Chapter 1 and the accompanying instructions for preparing cell lysates.
3. Remove aliquots of both Reporter CodeSet and Capture ProbeSet reagent from the freezer and thaw at room temperature. Invert several times to mix well and spin down reagent.

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 at room temperature before using.

4. Create a master mix by adding 70 µL of hybridization buffer to the tube containing the Reporter CodeSet. Do not remove the Reporter CodeSet from this tube. 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 14 assays to allow two assays' worth of dead volume.) **Do not add the Capture ProbeSet to the master mix.** Invert repeatedly to mix and spin down master mix.
5. Label the hybridization tubes. If using strip tubes, ensure they fit in a microfuge or picofuge and cut the strip in half if necessary.
6. Add 8 µL of master mix to each of the 12 tubes. (If water was added to the master mix, increase this volume as necessary). Use a fresh tip for each pipetting step to accurately measure the correct volume.
7. Add up to 5 µl of sample to each tube.

NOTE: If working with lysates, a reduced input volume may be recommended. For details, please see *Chapter 1, Section G, Using Whole Cell Lysates in nCounter XT Gene Expression Assays*.)

8. If using attentuation mixes (optional; see the Tech Note ***Strategies for Successful Gene Expression Assays***), add 1 µL of each attentuation mix to each tube. Alternatively, add 14 µL to the master mix in Step 4 if all reactions are to be attenuated.
9. If necessary, add RNase-free water to bring the volume of each assay to 13 µL.
10. Invert the Capture ProbeSet tube to mix and spin down the contents. Add 2 µL of Capture ProbeSet to each tube immediately before placing at 65°C. Cap tubes and mix the reagents by inverting the tubes several times and flicking with a finger to ensure complete mixing. Briefly spin down and immediately place the tubes in the pre-heated 65°C thermal cycler.

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

11. Incubate reactions for at least 16 hours. Maximum hybridization time should not exceed 48 hours. 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: The purpose of selecting a fixed hybridization time followed by a ramp down to 4°C is to ensure equivalent hybridization times of all assays being directly compared in the same series of experiments. Counts continue to accumulate with time, 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 will increase sensitivity by increasing counts without significantly increasing background.

B. Workflow for nCounter XT Gene Expression Assays

1. Create Master Mix

Add 70 µL to Reporter CodeSet

Hybridization Buffer + **Reporter CodeSet** = **Master Mix**

Invert to mix and spin down.

Thaw at room temperature, then invert to mix and spin down.

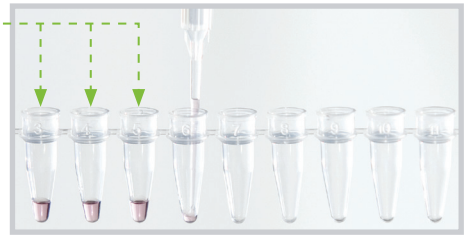
Invert to mix and spin down.

2. Set up Hybridization Reactions

To each tube, add:

- 8 µL Master Mix
- 1.5-5 µL Sample
- RNase-free Water *(if needed)*

13 µL total volume per tube

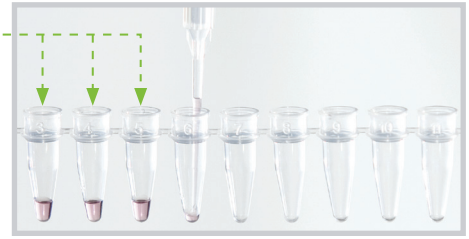


3. Complete Hybridization Reactions

To each tube, add:

- 2 µL Capture ProbeSet

15 µL final volume after addition of Capture ProbeSet



1. Cap tubes.
2. Mix by inverting tubes.
3. Briefly spin down.
4. Incubate at 65°C.

C. Using CodeSet-Plus/Panel-Plus Reagents with nCounter XT Gene Expression Assays

GENERAL PROBE HANDLING WARNING: During setup of the assay, do not vortex or pipette vigorously to mix as it may shear the Reporter CodeSet. Mixing should be done by flicking or inverting the tubes. If using a microfuge to spin down tubes, do not spin any faster than 1,000 RCF for more than 30 seconds. Do not "pulse" to spin because that will cause the centrifuge to go to maximum speed and may spin the CodeSet out of solution.

IMPORTANT: Pre-heat the thermal cycler to 65°C. Program the thermal cycler using 18 µL volume, calculated temperature, and heated lid.

Set up a hybridization reaction at room temperature for each sample with the following components: 3 µL of Reporter CodeSet, 2 µL of Reporter Plus, 5 µL of hybridization buffer, up to 5 µL (25–100 ng) of sample RNA, 2 µL of Capture ProbeSet, and 1 µL of Capture Plus. See TABLE 1.3 in Chapter 1 for the sample input recommendations specific to each instrument.

1. If using total RNA, go to Step 3.
2. If using cell lysates, see TABLE 1.4 in Chapter 1 and the accompanying instructions for preparing cell lysates.
3. Remove aliquots of Reporter CodeSet, Capture ProbeSet, and Plus reagents from the freezer to thaw at room temperature. Invert several times to mix well and spin down reagents.
4. Add 28 µL of Reporter Plus reagent to the thawed Reporter CodeSet. Invert several times to mix well, and spin down reagents.
5. Create a master mix by adding 70 µL of hybridization buffer to the tube containing the Reporter CodeSet and Reporter Plus reagents. Do not remove the Reporter CodeSet from this tube. 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 14 assays to allow two assay's worth of dead volume.) **Do not add the Capture ProbeSet or Capture Plus reagent to the master mix.** Invert repeatedly to mix and spin down master mix.
6. Label the hybridization tubes. If using strip tubes, ensure they fit in a microfuge or picofuge and cut the strip in half if necessary.
7. Add 10 µL of master mix to each of the tubes. (If water was added to this mix, increase this volume as necessary.) Use a fresh tip for each pipetting step to accurately pipette the correct volume.
8. Add up to 5 µl of sample to each tube.

NOTE: If working with lysates, a reduced input volume may be recommended. For details, please see *Chapter 1, Section G, Using Whole Cell Lysates in nCounter XT Gene Expression Assays*.)

9. If using attentuation mixes (optional; see the Tech Note ***Strategies for Successful Gene Expression Assays***), add 1 µL of each attentuation mix to each tube. Alternatively, add 14 µL to the master mix in Step 4 if all reactions are to be attenuated.
10. If necessary, add RNase-free water to bring the volume of each assay to 15 µL.
11. Add 14 µL of Capture Plus reagent to the thawed aliquot of Capture ProbeSet. Invert several times to mix well, and spin down reagents.
12. Add 3 µL of Capture ProbeSet and Capture Plus reagent mix to each tube immediately before placing at 65°C. Cap tubes and mix the reagents by inverting the tubes several times and flicking with a finger to ensure complete mixing. Briefly spin down and place

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

13. Incubate reactions for at least 16 hours. Maximum hybridization time should not exceed 48 hours. 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: The purpose of selecting a fixed hybridization time followed by a ramp down to 4°C is to ensure equivalent hybridization times of all assays being directly compared in the same series of experiments. Counts continue to accumulate with time, 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 will increase sensitivity by increasing counts without significantly increasing background.

3 nCounter XT Plex² Expression Assays

This chapter explains the procedures for setting up hybridizations for all Plex² expression assays with nCounter XT CodeSets. These include protocols for using total RNA or cell lysates with modifications for setting up 24 or 48 Plex² assays using a single nCounter cartridge.

Do not use these instructions with Legacy nCounter CodeSets. Instead, refer to the *nCounter Gene Expression Assay User Manual*.

A. Hybridization Protocol for 24 Plex² Assays (2PLEX)

GENERAL PROBE HANDLING WARNING: During setup of the assay, do not vortex or pipette vigorously to mix as it may shear the Reporter CodeSet. Mixing should be done by flicking or inverting the tubes. If using a microfuge to spin down tubes, do not spin any faster than 1,000 RCF for more than 30 seconds. Do not "pulse" to spin because that will cause the centrifuge to go to maximum speed and may spin the CodeSet out of solution.

IMPORTANT: Instructions for setting up sample hybridization reactions are for a single sub-CodeSet and 12 assays. They must be repeated for each sub-CodeSet—two times for 2PLEX assays. After hybridization, the reactions will be combined in a single set of 12 tubes before sample processing and data collection. Ensure that any given sub-CodeSet will be used only one time in each lane of the nCounter Cartridge.

IMPORTANT: To facilitate downstream analysis, the same sample ("calibration sample") must be assayed using both sub-CodeSets in the same lane at least once per study (**FIGURE 3.1**). This means that the calibration sample must be hybridized one time with each sub-CodeSet. For more information, see the ***nCounter Expression Data Analysis Guidelines***.

FIGURE 3.1 Hybridization setup for 24 Plex² Assays. CS signifies a sub-CodeSet (CS1 or CS2). A single calibration sample (red tube) is assayed with each sub-CodeSet once per study. All other tubes contain unique samples.

IMPORTANT: Pre-heat the thermal cycler to 65°C. Program the thermal cycler using 15 µL volume, calculated temperature, and heated lid.

Set up a hybridization reaction at room temperature for each sample with the following components: 3 µL of Reporter CodeSet, 5 µL of hybridization buffer, up to 5 µL of sample RNA (25-100 ng), and 2 µL of Capture ProbeSet. See **TABLE 1.3** in Chapter 1 for the sample input recommendations specific to each instrument.

1. If following the Total RNA Protocol, go to Step 3.
2. If following the Cell Lysate Protocol see **TABLE 1.4** in Chapter 1 and the accompany instructions for preparing cell lysates.
3. Remove aliquots of both Reporter CodeSet and Capture ProbeSet reagent from the freezer and thaw at room temperature. Invert several times to mix well and spin down reagent.

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 at room temperature before using.

4. Each sub-CodeSet will require a separate master mix. Create a master mix by adding 70 µL of hybridization buffer to the tube of Reporter CodeSet. Do not remove the Reporter Probes from this tube. 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 14 assays to allow one assay's worth of dead volume.) **Do not add the Capture ProbeSet to the master mix.** Invert repeatedly to mix and spin down master mix.
5. Label the hybridization tubes. If using strip tubes, ensure they fit in a microfuge or picofuge and cut the strip in half if necessary.
6. Add 8 µL of master mix to each of the 12 tubes for each sub-CodeSet (if you added water to the master mix, increase this volume as necessary). NanoString recommends using a fresh tip for each pipetting step to accurately pipette the correct volume. The CodeSet has components that can wick up into the tip. You may not dispense the correct amount if you use the same tip.
7. Add up to 5 µl of sample to each tube.

NOTE: If working with lysates, a reduced input volume may be recommended. For details, please see *Chapter 1, Section G, Using Whole Cell Lysates in nCounter XT Gene Expression Assays*.)

8. If using attentuation mixes (optional; see the Tech Note ***Strategies for Successful Gene Expression Assays***), add 1 µL of each attentuation mix to each tube. Alternatively, add 14 µL to the master mix in Step 4 if all reactions are to be attenuated.
9. If necessary, add RNase-free water to bring the volume of each assay to 13 µL.
10. Invert the Capture ProbeSet tube to mix and spin down the contents. Add 2 µL of Capture ProbeSet to each tube immediately before placing at 65°C. Cap tubes and mix the reagents by inverting the tubes several times and flicking with a finger to ensure complete mixing. Briefly spin down and immediately place the tubes in the pre-heated 65°C thermal cycler.

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

11. Incubate reactions for at least 16 hours. Maximum hybridization time should not exceed 48 hours. 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: The purpose of selecting a fixed hybridization time followed by a ramp down to 4°C is to ensure equivalent hybridization times of all assays being directly compared in the same series of experiments. Counts continue to accumulate with time, 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 will increase sensitivity by increasing counts without significantly increasing background.

12. Remove tubes from the thermal cycler, and combine the full hybridization volumes from both sub-CodeSets into a single set of tubes. The example in **FIGURE 3.2** uses two 12-tube strips and involves pipetting the contents of Strip 2 into Strip 1. Be sure to maintain tube orientation so that the calibration samples are combined in Tube 1 (red tube). The resulting final volume will be 30 µL per well.
13. Recap the tubes. Flick them with a finger to mix and briefly spin down contents. Proceed immediately to post-hybridization processing as described in your instrument manual. Do not store hybridizations at 4°C.

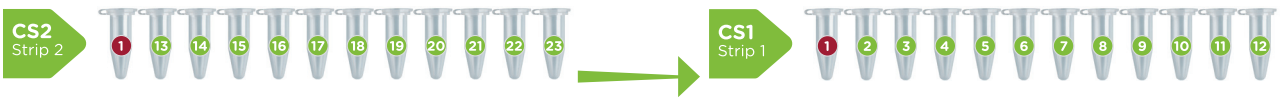


FIGURE 3.2 Post-hybridization mixing step. After hybridization, add the full hybridization reaction volume from each tube in sub-CodeSet 2 into the corresponding tube from sub-CodeSet 1, maintaining tube orientation. The resulting final volume will be 30 µL per well.

B. Hybridization Protocol for 48 Plex² Assays (4PLEX)

GENERAL PROBE HANDLING WARNING: During setup of the assay, do not vortex or pipette vigorously to mix as it may shear the Reporter CodeSet. Mixing should be done by flicking or inverting the tubes. If using a microfuge to spin down tubes, do not spin any faster than 1,000 RCF for more than 30 seconds. Do not "pulse" to spin because that will cause the centrifuge to go to maximum speed and may spin the CodeSet out of solution.

IMPORTANT: Instructions for setting up sample hybridization reactions are for a single sub-CodeSet and 12 assays. They must be repeated for each sub-CodeSet—four times for 4PLEX assays. After hybridization, the reactions will be combined in a single set of 12 tubes before sample processing and data collection. Ensure that any given sub-CodeSet will be used only one time in each lane of the nCounter Cartridge. If you are using the *SPRINT* Profiler, please contact NanoString Support (support@nanostring.com).

IMPORTANT: To facilitate downstream analysis, the same sample ("calibration sample") must be assayed using all four sub-CodeSets in the same lane at least once per study (**FIGURE 3.3**). This means that the calibration sample must be hybridized one time with each sub-CodeSet. For more information on the calibration sample, see the ***nCounter Expression Data Analysis Guidelines.***

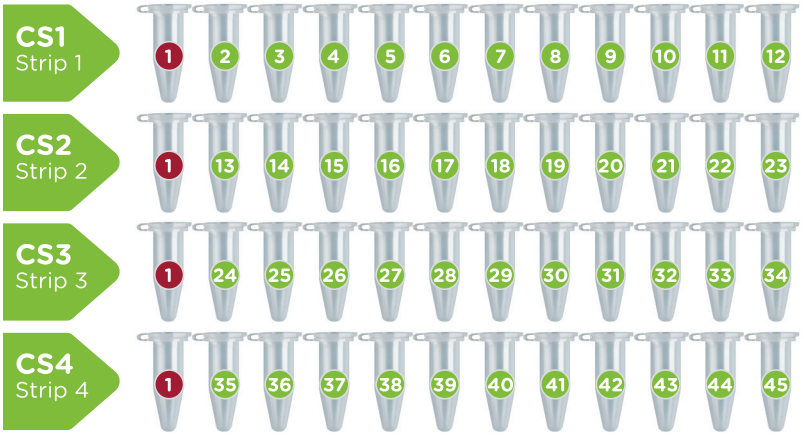


FIGURE 3.3 Hybridization setup for 48 Plex² Assays. CS signifies a sub-CodeSet (CS1, CS2, CS3, or CS4). A single calibration sample (red tube) is assayed with each sub-CodeSet once per study. All other tubes contain unique samples.

IMPORTANT: Pre-heat the thermal cycler to 65°C. Program the thermal cycler using 15 µL volume, calculated temperature, and heated lid.

Set up a hybridization reaction at room temperature for each sample with the following components: 3 µL of Reporter CodeSet, 5 µL of hybridization buffer, up to 5 µL of sample RNA (37.5-150 ng), and 2 µL of Capture ProbeSet. See **TABLE 1.3** in Chapter 1 for the sample input recommendations specific to each instrument.

NOTE: 4PLEX assays require 50% more sample input than standard recommendations due to the dilution that occurs when the hybridization reactions for each sub-CodeSet are combined in Step 12.

1. If following the Total RNA Protocol, go to Step 3.
2. If following the Cell Lysate Protocol see **TABLE 1.4** in Chapter 1 and the accompany instructions for preparing cell lysates.
3. Remove aliquots of both Reporter CodeSet and Capture ProbeSet reagent from the freezer and thaw at room temperature. Invert several times to mix well and spin down reagent.

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 at room temperature before using.

4. Each sub-CodeSet will require a separate master mix. Create a master mix by adding 70 µL of hybridization buffer to the tube of Reporter CodeSet. Do not remove the Reporter CodeSet from this tube. 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 repeatedly to mix and spin down master mix.
5. Label the hybridization tubes. If using strip tubes, ensure they fit in a microfuge or picofuge and cut the strip in half if necessary.
6. Add 8 µL of master mix to each of the 12 tubes for each sub-CodeSet (if you added water to the master mix, adjust volumes). It is advisable to use a fresh tip for each pipetting step to accurately pipette 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.
7. Add up to 5 µl of sample to each tube.

NOTE: If working with lysates, a reduced input volume may be recommended. For details, please see *Chapter 1, Section G, Using Whole Cell Lysates in nCounter XT Gene Expression Assays*.)

8. If using attentuation mixes (optional; see the Tech Note ***Strategies for Successful Gene Expression Assays***), add 1 µL of each attentuation mix to each tube. Alternatively, add 14 µL to the master mix in Step 4 if all reactions are to be attenuated.
9. If necessary, add RNase-free water to bring the volume of each assay to 13 µL.
10. Invert the Capture ProbeSet tube to mix and spin down the contents. Add 2 µL of Capture ProbeSet to each tube immediately before placing at 65°C. Cap tubes and mix the reagents by inverting the tubes several times and flicking with a finger to ensure complete mixing. Briefly spin down and immediately place the tubes in the pre-heated 65°C thermal cycler.

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

11. Incubate reactions for at least 16 hours. Maximum hybridization time should not exceed 48 hours. 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: The purpose of selecting a fixed hybridization time followed by a ramp down to 4°C is to ensure equivalent hybridization times of all assays being directly compared in the same series of experiments. Counts continue to accumulate with time, 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 will increase sensitivity by increasing counts without significantly increasing background.

12. Remove tubes from the thermal cycler and combine the full hybridization volumes from all four sub-CodeSets into a single set of tubes. The example in **FIGURE 3.4** uses four 12-tube strips and involves pipetting the contents of Strips 2, 3, and 4 into Strip 1. Be sure to maintain tube orientation so that the calibration samples are combined in Tube 1 (red tube). The final volume will be 60 µL per well.
13. Recap the tubes. Flick them with a finger to mix and briefly spin down contents. Proceed immediately to post-hybridization processing as described in your instrument manual. Do not store hybridizations at 4°C.

FIGURE 3.4 Post-hybridization mixing step. After hybridization, add the full hybridization reaction volume from each tube in sub-CodeSets 2, 3, and 4 into the corresponding tube from sub-CodeSet 1, maintaining tube orientation. The resulting final volume will be 60 µL per well.

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4 nCounter Vantage RNA Assays

This chapter explains the procedures for setting up hybridizations for all TagSet chemistry-based gene expression panels. These include protocols for using total RNA or cell lysates with pre-defined Vantage panels.

A. Using Extension TagSets and Designing Probes with nDesign Gateway

Prior to Extension assay setup, oligonucleotide probes should be ordered separately for each target RNA or DNA. The oligonucleotide probes should contain both tag- and target-specific sequences that effectively link each target to a specific Reporter Tag and to the universal Capture Tag. Because Reporter and Capture Tags do not directly hybridize with the targets of interest, generating new oligonucleotide probes enables a common TagSet to be used to label and detect many different RNA or DNA targets.

NanoString provides an online tool, **nDesign Gateway**, which enables users to design probes for gene expression assays. Users may select specific probe sequences for human, mouse, and rat genes of interest and then establish associations between targets and Reporter Tags. Design data created using nDesign Gateway can be downloaded and submitted to a third-party oligonucleotide manufacturer.

Please see the instructions provided on the nDesign Gateway home page for additional information on the use of this tool. Alternatively, contact NanoString Support (support@nanostring.com) to discuss other options and tools available to facilitate CodeSet design.

TagSet chemistry panels are provided with all components required to run an assay, including Probe A and Probe B oligonucleotide probes. To add additional targets to an ongoing experiment, use an Extension TagSet, which is available for 12 or 24 targets and does not include controls. The unique Reporter Tags in the Extension TagSets do not coincide with any of those found in the core TagSets.

B. Hybridization Protocol for nCounter Vantage RNA Assays (TagSet Chemistry)

Do not use these instructions with Legacy nCounter CodeSets. Instead, refer to the *nCounter Gene Expression Assay User Manual*.

IMPORTANT: Are these CodeSet or TagSet assays and/or panels? Check before you begin. If you are using nCounter XT **CodeSet** Assays and/or Panels, refer to *Chapter 2, nCounter XT CodeSet Gene Expression Assays*.

IMPORTANT: Do not vortex or pipette vigorously as this may shear the Reporter Tags. Mix only by flicking or inverting tubes. A picofuge is preferable when spinning down solutions due to its low speed. If using a microfuge, do not "pulse" the machine as it may reach maximum speed and spin the TagSet out of solution. Spin at less than 1,000 RCF for no more than 30 seconds.

Each final hybridization reaction will have a volume of 15 µL and contain the following components: 5 µL of hybridization buffer, 2 µL of TagSet, 0.5 µL of 30X Working Probe A Pool (0.6 nM each Probe A), 0.5 µL of 30X Working Probe B Pool (3 nM each Probe B), and up to 7 µL of sample RNA. To set up 12 assays, master mix is created for 14 reactions to allow for dead volume in pipetting (see **FIGURE 4.1**)

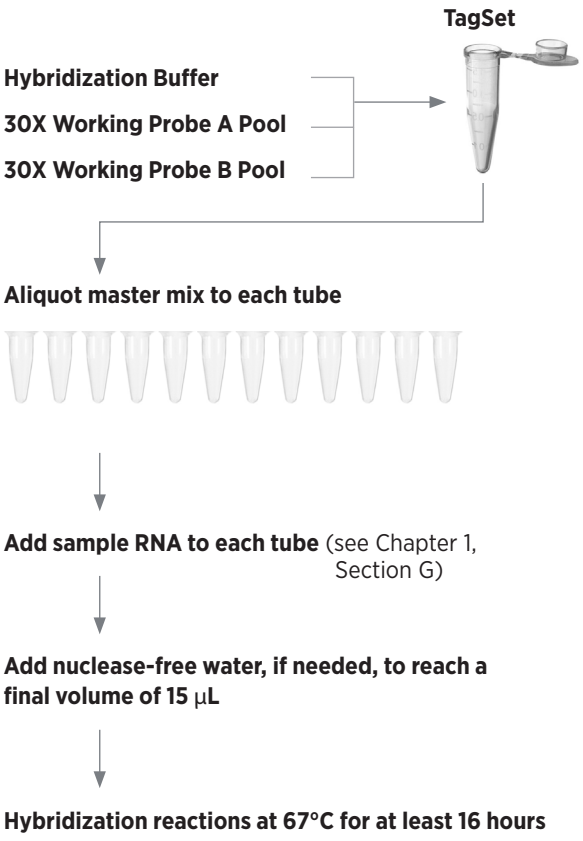

FIGURE 4.1 Creating master mix and preparing RNA hybridization reactions.

Core TagSet Only

	Each Reaction	14 Reactions
Hybridization Buffer	5 µL	70 µL
TagSet	2 µL	28 µL
30X Working Probe A Pool	0.5 µL	7 µL
30X Working Probe B Pool	0.5 µL	7 µL
Sample*	Up to 7 µL	
Total Volume	15 µL	

Core TagSet + Extension TagSet

	Each Reaction	14 Reactions
Hybridization Buffer	5 µL	70 µL
TagSet	2 µL	28 µL
Extension TagSet	2 µL	28 µL
30X Working Probe A Pool	0.5 µL	7 µL
30X Working Probe B Pool	0.5 µL	7 µL
30X Extension Probe A Pool	0.5 µL	7 µL
30X Extension Probe B Pool	0.5 µL	7 µL
Sample*	Up to 4 µL	
Total Volume	15 µL	

**NanoString recommends preparing samples with a high concentration to facilitate reducing the sample volume if an extension TagSet is later necessary.*

1. Remove an aliquot of nCounter XT TagSet from the freezer and thaw it on ice. Invert several times to mix well, and briefly spin down the reagent at less than 1,000 RCF.
2. Create a 30X Probe A Pool working dilution by adding 22 µL of TE to the 3 µL Probe A Pool stock aliquot provided.
3. Create a 30X Probe B Pool working dilution by adding 22 µL of TE to the 3 µL Probe B Pool stock aliquot provided.
4. Each core TagSet tube contains 28 µL of reagent. Create a master mix by adding reagents directly into the TagSet tube. First add 70 µL of hybridization buffer, followed by 7 µL of the 30X Working Probe A Pool. Mix well by flicking the tube and briefly spin down at less than 1,000 RCF before adding 7 µL of the 30X Working Probe B Pool. Mix and spin again.
   a. If using an Extension TagSet, also add 28 µL of the Extension TagSet reagent and 7 µL of both 30X Extension Probe A Pool and 30X Extension Probe B Pool to the master mix (154 µL total).
   b. If all samples have the same volume, additional nuclease-free water may be added to the master mix now instead of to individual tubes during Step 8. Increase the aliquot in Step 6 as necessary.
5. Label a 12-tube strip.
6. Add 8 µL (or 11 µL if using an Extension TagSet and Step 4a was followed) of master mix to each of the 12 tubes using a fresh pipette tip for each well.
7. Add sample to each of the 12 tubes (maximum volume of 7 µL for core TagSet only, or 4 µL for core TagSet plus Extension TagSet) to each tube.

NOTE: If working with lysates, a reduced input volume may be recommended. For details, please see *Chapter 1, Section G, Using Whole Cell Lysates in nCounter XT Gene Expression Assays*.)

IMPORTANT: If using cell lysates, adding more than 4 µL of sample will have an adverse impact on hybridization efficiency due to the denaturing effect of the lysis buffer.

8. If necessary, add nuclease-free water to each tube to bring the volume of each reaction to 15 µL.
9. Program the thermal cycler to use a 15 µL volume, calculated temperature, and heated lid. Set at 67°C for the duration of hybridization. To minimize the potential for evaporation, the thermal cycler lid should be set at 5°C above the block temperature. Cap tubes and mix the reagents by inverting the strip tubes several times and flicking with a finger to ensure complete mixing. Briefly spin down the hybridization reactions at less than 1,000 RCF and immediately place the strip tubes in the thermal cycler.
10. Incubate reactions for at least 16 hours. Maximum hybridization time should not exceed 48 hours. 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: The purpose of selecting a fixed hybridization time followed by a ramp down to 4°C is to ensure equivalent hybridization times of all assays being directly compared in the same series of experiments. Counts continue to accumulate with time, 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 will increase sensitivity by increasing counts without significantly increasing background.

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5 nCounter Vantage RNA:Protein Assays

This chapter contains instructions for nCounter Vantage RNA:Protein and Protein assays, which combine gene expression and protein analysis in a single assay. The following protocol is designed for use with either the nCounter Vantage RNA:Protein Immune Cell Profiling Panel or the nCounter Vantage RNA:Protein Immune Cell Signaling Panel. Branched workflows allow for sample collection, RNA and protein sample preparation, and hybridization (FIGURE 5.1).

If you are using the Immune Cell Profiling Panel to detect extracellular proteins, follow the instructions labeled with the symbol .

If you are using the Immune Cell Signaling Panel to detect intracellular proteins, follow the instructions labeled with the symbol .

NOTE: RNA sample preparation and the hybridization reaction protocols are the same for both assays.

A. Overview

nCounter technology can be used to detect a variety of nucleic acids, including mRNA, miRNA, and DNA. However, other molecules can also be detected using intermediate proxies. NanoString has developed a method for protein analysis using antibodies specific to proteins of interest that have been barcoded with unique synthetic DNA oligonucleotides. Each DNA oligonucleotide is then recognized by a unique Reporter probe that contains a fluorescent barcode. Reporter probes are imaged and counted by the nCounter Analysis System to provide a direct, digital readout of protein expression. This allows for an integrated RNA:Protein workflow.

The procedures described in this chapter are compatible with intact cell suspensions from cell lines, PBMCs, and other primary human cells. FFPE and fresh frozen tissue are not compatible with the procedures described in this chapter. Following sample preparation, the RNA and protein components are combined in a single hybridization reaction. Contact NanoString Support (**support@nanostring.com**) to receive additional assistance with this assay.

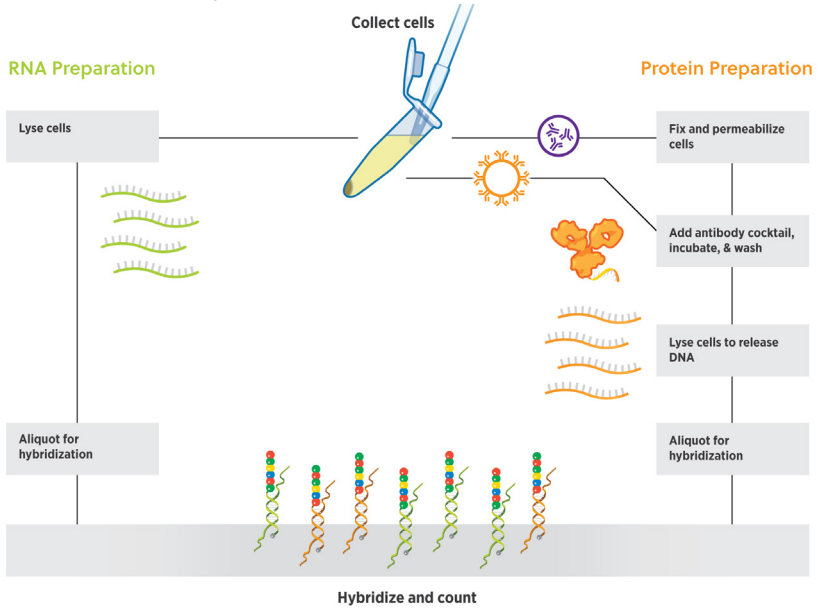


FIGURE 5.1 Illustration of the nCounter Vantage RNA:Protein Assay workflow. Cells are collected and divided into two fractions, which are separately prepared for analysis of RNA or protein expression. The protein sample preparation uses DNA-linked antibodies to recognize proteins of interest. Samples are then denatured to release the DNA oligos. The two analyte preparations are combined for a single nCounter Vantage assay.

Materials and Reagents

Some materials are not provided by NanoString and must be purchased separately (**TABLE 5.2**). These are in addition to the materials recommended for all nCounter assays, which are listed in Chapter 1.

TABLE 5.1 NanoString-provided reagents for Vantage RNA:Protein and Protein Assays. nCounter Vantage Protein (D) can be used as a stand-alone Protein assay, whereas nCounter Vantage Protein (R) must be used in a combined hybridization reaction with a NanoString RNA assay.

Kit	Catalog Number	Reagents
nCounter Vantage RNA:Protein	VRPC-HIPS-12 VRPC-HISS-12	Reporter CodeSet Capture ProbeSet Protein Plus Antibody Mix
nCounter Vantage Protein (D)	VPODC-HIPS-12 VPODC-HISS-12	Protein TagSet Antibody Mix
nCounter Vantage Protein (R)	VPRXC-HIPS-12 VPRXC-HISS-12	Protein Plus Antibody Mix

TABLE 5.2 Additional materials required for use with RNA:Protein and Protein Immune Cell Profiling Panels.

Material	Manufacturer	Part Number(s)
Pipettes for 10–1,000 µL*	Various	Various
Refrigerated centrifuge with swinging bucket rotor for 96-well plates	Various	Various
96-well clear polystyrene **round-bottom** plates (Do not use other 96-well plates for this assay)	Corning®	351177
Salmon sperm DNA (10 mg/mL)*	Sigma-Aldrich®	D7656
Dextran sulfate sodium salt (200 kDA)*	Sigma-Aldrich®	67578-5G
Buffer RLT	QIAGEN®	79216
1X phosphate buffered saline (PBS; pH 7.4)*	Life Technologies®	10010-023
TruStain FcX™†	BioLegend®	422301 or 422302
Cell Staining Buffer	BioLegend®	420201

†BioLegend TruStain FcX is only required for samples that contain human Fc receptor (e.g., PBMCs).

**Alternative products can be used if they offer similar function and reliability.*

TABLE 5.3 Additional materials required for use with RNA:Protein and Protein Immune Cell Signaling Panels.

Material	Manufacturer	Part Number(s)
Pipettes for 10–1,000 µL	Various	Various
Refrigerated centrifuge with swinging bucket rotor for 96-well plates	Various	Various
96-well clear polystyrene **round-bottom** plates (Do not use other 96-well plates for this assay)	Corning®	351177
Salmon sperm DNA (10 mg/mL)	Sigma-Aldrich®	D7656
Dextran sulfate sodium salt (200kDa)*	Sigma-Aldrich®	67578-5G
Buffer RLT	QIAGEN®	79216
1X phosphate buffered saline (PBS; pH 7.4)*	Life Technologies®	10010-023
TruStain FcX™†	BioLegend®	422301 or 422302
Fix buffer concentrate	eBioscience®	00-5123-43
Fix Diluent	eBioscience®	00-5223-56
Permeabilization /Wash buffer 10X	eBioscience®	00-8333
RNase/DNase Free H_2O*	ThermoFisher®	4387937
Brefeldin A solution 1000X#	Biolegend®	420601

†BioLegend TruStain FcX is only required for samples that contain human Fc receptor (e.g., PBMCs).

#Brefeldin A solution is only recommended for fresh cell suspensions. Incubation of cryopreserved cells with Brefeldin A may lead to a significant loss in cell viability.

**Alternative products can be used if they offer similar function and reliability.*

TABLE 5.4 Additional materials suggested for use with RNA:Protein and Protein Immune Cell Profiling Panels and RNA:Protein and Protein Immune Cell Signaling Panels.

Material	Manufacturer	Part Number(s)
Manual multi-channel pipette for 200 µL*	Rainin®	L12-200XLS+
12-strip standard tubes*	Bioexpress®	T-3034-1
15 mL conical tubes*	FisherBrand®	S50712
Hemocytometer*	Various	Various
Trypan Blue*	Various	Various

**Alternative products can be used if they offer similar function and reliability.*

B. Sample Collection

NOTE: Sections D and E contain overnight blocking steps. Before beginning your assay, please read these sections.

NOTE: NanoString has found that PBMC samples or other primary human cells have less total RNA per cell than typical cell lines and are more difficult to pellet. Therefore, the required cell input for primary cells (500K total cells, with 300K allocated to RNA sample preparation) is greater than that required for cell lines (150K cells total, with 50K allocated to RNA sample preparation).

NOTE: Automated cell counters can inflate primary cell numbers. To ensure accurate cell input, it is recommended that you manually count your cells to determine a scaling factor for your automated cell counter model.

IMPORTANT: Use the recommended centrifugation settings to avoid damage to cells. Never exceed 400 RCF. All centrifugations should be performed at 4°C. Ensure that your centrifuge is properly calibrated to ensure that it does not dip below freezing during all centrifugation steps.

1. Cool phosphate-buffered saline (PBS) on ice.
2. Use a hemocytometer or other cell-counting device to determine the concentration of total viable cells in each sample.
3. Collect a minimum of 150,000 cells (or 500,000 cells from primary cell samples) in a 1.7 mL microcentrifuge tube for each sample. Very dilute samples may require a larger conical tube.
4. Centrifuge cells at 400 RCF for 5 minutes at 4°C and discard the supernatant by pipetting, being careful not to disturb the pellet.
5. Re-suspend cells by adding 400 µL of cold 1X PBS and pipette gently at least 5 times. (final concentration of 1,250 cells per µL for primary cells or 375 cells per µL for cell lines). If performing a Protein-only assay, adjust re-suspension volume accordingly.

See **Section C** for information on RNA preparation.

See **Section D** for information on Protein preparation with the Immune Cell Profiling Panel.

See **Section E** for information on Protein preparation with the Immune Cell Signaling Panel.

Note that different assays require different rows in the round-bottom 96-well plate. Refer to **FIGURE 5.2** before beginning your assay.

FIGURE 5.2 Illustration of the plate-based workflow. After cells are collected, they are divided into two fractions and moved to Row A of a 96-well plate for processing of 12 samples. Immune Cell Profiling protein sample preparation requires only Row A of a 96-well for 12 samples. Immune Cell Signaling protein sample preparation requires three rows of a 96-well plate for processing of 12 samples and requires movement of samples between each row, as described in the protocol. RNA Preparation requires only Row A of a 96-well plate for 12 samples.

C. RNA Preparation

NOTE: If using the Immune Cell Profiling assay, NanoString recommends pre-blocking the RNA plate with the block/wash buffer described in *Section D1*. A tighter, more uniform pellet will result. Make sure to discard the block/wash buffer before proceeding to Step 1. DO NOT pre-block the RNA plate if performing the Immune Cell Signaling, as the buffers described in *Section E1* are not compatible with this function.

1. Transfer an aliquot equivalent to 50,000 cells from each sample prepared in *Section B: Sample Collection* into Row A of a **round-bottom** 96-well RNA plate. (Increase the aliquot to 300,000 cells if using primary cell samples.) Set this plate on ice until ready to move to Step 2.
2. Centrifuge the plate of RNA samples at 400 RCF for 5 minutes at 4°C. Discard the supernatant by inverting and carefully flicking the plate followed by blotting gently on a paper towel only once.
3. Lyse the RNA samples.
   a. If using cell lines, re-suspend in 10 µL of 1X PBS and pipette gently, and then add 4 µL of Buffer RLT. Thoroughly mix samples by pipetting, and proceed to Step 4.
   b. If using primary cells, add 15 µL of Buffer RLT directly to each primary cell pellet. Do not add PBS. Thoroughly mix samples by pipetting, and proceed to Step 4.
4. If using the nCounter Analysis System (*MAX/FLEX*), proceed to Step 5 without diluting samples.
   a. If performing this assay using the nCounter *SPRINT* Profiler, perform a 1:2 dilution of the prepared RNA samples by combining equal volumes of RNA sample and RNase-free water in a new row on the sample plate or in separate tubes.
5. Transfer the RNA samples to a 12-strip tube, cap, and keep on ice until ready to perform *Section F: Vantage RNA:Protein Hybridization Assay*. If not using immediately, samples can be stored at -80°C.

NOTE: Lysed cells may form a viscous solution. Take care when pipetting to ensure accurate volumes are used.

D. Immune Cell Profiling Protein Preparation

Advanced Preparation

1. Prepare 25 mL of block/wash buffer (approximately 20 mL is required to assay 12 samples).
   a. Add 250 mg of dextran sulfate to 24.75 mL of Cell Staining Buffer. It may require 10 minutes on a rotator/rocker to dissolve.
   b. Denature salmon sperm DNA by heating for 10 minutes at 95°C. Keep on ice when finished.
   c. Add 250 µL of denatured salmon sperm DNA to the mixture of dextran sulfate and buffer. Store block/wash buffer at 4°C for up to 1 week until ready for use, and keep on ice during sample collection and preparation.
2. Pre-block the protein sample plates by adding 300 µL of block/wash buffer to each of the 12 wells in Row A. Block the plate on a flat surface for at least 1 hour at room temperature or overnight at 4°C.

Protein Sample Preparation

3. Remove the block/wash buffer from the pre-blocked plate.
4. Transfer an aliquot equivalent to 100,000 cells prepared in *Section B: Sample Collection* from each sample into Row A. (Increase the aliquot to 200,000 cells if using primary cell samples.) Set this plate on ice.
5. Centrifuge at 400 RCF for 5 minutes at 4°C. Discard the supernatant by inverting and flicking the plate only once and blotting on a fresh paper towel.
   a. Do not remove any additional supernatant with a pipette. A small volume (20–25 µL) of remaining liquid is acceptable.

6. For primary cells and cell lines that contain proteins that bind to human Fc receptor (e.g., CD16, CD64, and/or CD32), an additional step is required to block Fc receptor-mediated antibody binding. If this step does not apply, proceed to Step 7.
    a. Prepare the Fc receptor blocking solution for 13 samples by diluting 65 µL of BioLegend TruStain FcX in 585 µL of block/wash buffer (The extra sample is dead volume to ensure accurate pipetting.)
    b. Add 50 µL of Fc receptor blocking solution to each well, directly into the pellet, and mix gently.
    c. Incubate samples for 10 minutes at room temperature. Do not centrifuge the samples.
    d. Add 150 µL block/wash buffer to each sample, mix by pipetting gently, and proceed to Step 8.
7. Re-suspend cells in 200 µl block/wash buffer.
8. Incubate cells for 30 minutes at 4°C.
9. Add 10 µL of antibody mix (Ab Mix) to each sample. Use a new tip for each sample. Mix by pipetting gently.
10. Incubate cells for 1 hour at 4°C on a flat surface.
11. Centrifuge samples at 400 RCF for 5 minutes at 4°C. Discard the supernatant.
12. Perform four washes by repeating Steps 12a–c.
    a. Re-suspend the cells in 200 µL of block/wash buffer and mix by pipetting gently.
    b. Centrifuge samples at 400 RCF for 5 minutes at 4°C. Discard the supernatant.
    c. If bubbles are present after discarding supernatant, centrifuge the plate at 400 RCF for 1 minute before adding block/wash buffer. Ensure complete removal of the block/wash buffer on your final wash before lysis.

    ☐☐☐☐ - Check off each box as you perform each wash.

NOTE: Maintaining sufficient cell quantities through four washes is critical for optimal assay performance. If the cell pellet is not visible after the third wash, reserve a 10 µL aliquot of the re-suspended cells before the final centrifugation. Cells in this aliquot can be counted to determine yield. Loss of more than 70% during sample preparation may reduce assay performance.

13. Lyse the washed cells by adding 20 µL of Buffer RLT to the pellet, and thoroughly mix by pipetting.
14. Dilute the cell lysates.
    a. If starting with an aliquot of 100K cells for typical cell lines, transfer 4 µL of sample to a new tube in a 12-tube strip. If starting with an aliquot of 200K cells for primary cell samples, transfer only 2 µL of sample to a new tube.
    b. Dilute each sample to a final volume of 80 µL by adding 76 µL of PBS (typical cell lines) or 78 µL of PBS (primary cell samples).
    c. If performing this assay using the nCounter Analysis System (*MAX/FLEX*), then proceed to Step 15. If performing this assay using the nCounter *SPRINT* Profiler, add an additional 80 µL of PBS to dilute the samples further.
15. Denature the diluted cell lysates for 15 minutes in a thermal cycler at 95°C with a heated lid at 100°C, and then immediately ramp down to 4°C or quickly cool on ice for 2 minutes.
16. Keep the protein samples on ice until ready to perform *Section F, RNA:Protein Hybridization Assay*. If not using immediately, it is recommended to store diluted and undiluted samples at -80°C.
    - If using CodeSet assays, follow subsection *XT CodeSet Vantage RNA:Protein Hybridization*.
    - If using TagSet Vantage assays, follow subsection *XT TagSet Vantage RNA:Protein Hybridization*.

E. Immune Cell Signaling Protein Preparation

Advanced Preparation

1. Prepare 25 mL of permeabilization/wash buffer (~20 mL is required to assay 12 samples).
   a. Add 2.5 mL of 10X Permeabilization/Wash Buffer to 22.5 mL of DEPC H_2O.
   b. Add 250 mg of dextran sulfate to 24.75 mL of permeabilization/wash buffer. It may require 10 minutes on a rotator/rocker to dissolve.
   c. Denature salmon sperm DNA by heating for 10 minutes at 95°C. Keep on ice when finished.
   d. Add 250 µL of denatured salmon sperm DNA to the mixture of dextran sulfate and buffer. Store permeabilization/wash buffer at 4°C for up to 1 week until ready for use.
2. Prepare 1X Fix buffer by adding 700 µl Fix buffer concentrate to 2.1 mL of Fix Diluent. Note this buffer must be prepared fresh on the day of sample collection.
3. **Optional:** Add 1000X Brefeldin A to a tissue culture flask containing cells at 1 µl per mL of culture media. Incubate at 37° for 4 hours. Please note that extended treatments with Brefeldin A may be toxic to the cells and reduce viability and thus cell yield when performing this assay. Depending on cell type and experimental question, length of Brefeldin A treatment may need to be optimized. Do not treat cryopreserved cells with Brefeldin A as it may lead to a significant loss in cell viability.

Protein Sample Preparation

4. Transfer an aliquot equivalent to 100,000 cells from each sample prepared in *Section B: Sample Collection* into Row A of a new round-bottom 96-well plate. (Increase the aliquot to 200,000 cells if using primary cell samples.) Set this plate on ice.
5. Centrifuge the plate of protein samples at 400 RCF for 5 minutes at 4°C. Discard the supernatant by inverting and flicking the plate only once and blotting on a fresh paper towel.
   a. Do not remove any additional supernatant with a pipette. A small volume (20–25 µL) of remaining liquid is acceptable.
6. Fix cells by adding 200 µl 1X fix buffer from *Advance Preparation* to each well in **Row A** and re-suspend cells by pipetting.
7. Pre-block the sample plate by adding 300 µl permeabilization/wash buffer from Advance Preparation above to **Row B** of the sample plate (**FIGURE 5.2**).
8. Incubate cells in **Row A** and pre-block **Row B** for 30 minutes at room temperature.
9. Centrifuge samples at 400 RCF for 5 minutes. Discard the supernatant.

NOTE: After fixing cells, centrifugation steps can be performed at room temperature.

10. Resuspend cells in **Row A** with 200 µl 1X permeabilization/wash buffer and transfer cells into the pre-blocked **Row B** (**FIGURE 5.2**).
11. Centrifuge samples at 400 RCF for 5 minutes. Discard the supernatant.
12. For primary cells and cell lines that contain proteins that bind to human Fc receptor (e.g., CD16, CD64, and/or CD32), an additional step is required to block Fc receptor-mediated antibody binding. If this step does not apply, proceed to Step 13.
    a. Prepare the Fc receptor blocking solution for 13 samples by diluting 65 µL of BioLegend TruStain FcX in 585 µL of permeabilization/wash buffer (The extra sample is dead volume to ensure accurate pipetting.)
    b. Add 50 µL of Fc receptor blocking solution to each well, directly into the pellet, and mix gently.
    c. Incubate samples for 10 minutes at room temperature. Do not centrifuge the samples.
    d. Add 150 µL permeabilization/wash buffer to each sample, mix by pipetting gently, and proceed to Step 14.
13. Re-suspend cells in 200 µl permeabilization/wash buffer.
14. Incubate cells for 30 minutes at room temperature.
15. Add 10 µL of antibody mix (Ab Mix) to each sample. Use a new tip for each sample. Mix by pipetting gently.
16. Incubate cells for 1 hour at room temperature on a flat surface.
17. Centrifuge samples at 400 RCF for 5 minutes. Discard the supernatant from Row A and B.

18. Perform four washes as described below:
    a. Re-suspend the cells in 200 µL of permeabilization/wash buffer and mix by pipetting gently.
    b. Centrifuge samples at 400 RCF for 5 minutes. Discard the supernatant.
    c. If bubbles are present after discarding supernatant, centrifuge the plate at 400 RCF for 1 minute before adding permeabilization/wash buffer.
    d. Repeat Steps 18a–c an additional two times.
    e. On the fourth wash, add 200 µL of permeabilization/wash buffer and mix by pipetting gently. Transfer resuspended cells to **Row C** (**FIGURE 5.2**).
    f. Centrifuge samples at 400 RCF for 5 minutes. Discard the supernatant.

    ☐☐☐☐ -Check off each box as you perform each wash

NOTE: Maintaining sufficient cell quantities through four washes is critical for optimal assay performance. If the cell pellet is not visible after the third wash, reserve a 10 µL aliquot of the re-suspended cells before the final centrifugation. Cells in this aliquot can be counted to determine yield. Loss of more than 70% during sample preparation may reduce assay performance.

19. Lyse the washed cells by adding 20 µL of Buffer RLT to the pellet, and thoroughly mix by pipetting.

NOTE: Lysed cells may form a viscous solution. Take care when pipetting to ensure accurate volumes are used.

20. Transfer the lysate to strip tube and incubate at 95°C for 5 minutes to ensure complete cell lysis.
21. Dilute the cell lysates.
    a. If starting with an aliquot of 100K cells for typical cell lines, transfer 4 µL of sample to a new tube in a 12-tube strip. If starting with an aliquot of 200K cells for primary cell samples, transfer only 2 µL of sample to a new tube.
    b. Dilute each sample to a final volume of 80 µL by adding 76 µL of PBS (typical cell lines) or 78 µL of PBS (primary cell samples).
    c. If performing this assay using the nCounter Analysis System (*MAX/FLEX*), then proceed to Step 22. If performing this assay using the nCounter *SPRINT* Profiler, add an additional 80 µL of PBS to dilute the samples further.
22. Denature the diluted cell lysates for 15 minutes in a thermal cycler at 95°C with a heated lid at 100°C, and then immediately ramp down to 4°C or quickly cool on ice for 2 minutes.
23. Keep the protein samples on ice until ready to perform *Section F, RNA:Protein Hybridization Assay*. If not using immediately, it is recommended to store diluted and undiluted samples at -80°C.
    - If using CodeSet assays, follow subsection *XT CodeSet Vantage RNA:Protein Hybridization*.
    - If using TagSet Vantage assays, follow subsection *XT TagSet Vantage RNA:Protein Hybridization*.

F. Hybridization Protocol for nCounter Vantage RNA:Protein Assays

GENERAL PROBE HANDLING WARNING: During setup of the assay, do not vortex or pipette vigorously to mix as it may shear the Reporter Probes. Mixing should be done by flicking or inverting the tubes. If using a microfuge to spin down tubes, do not spin any faster than 1,000 RCF for more than 30 seconds. Do not "pulse" to spin because that will cause the centrifuge to go to maximum speed and may spin the CodeSet out of solution.

IMPORTANT: Are these CodeSet or TagSet assays? Check the reagent labels before you begin and refer to the appropriate protocol below.

IMPORTANT: If you are using nCounter Vantage Protein with a Custom CodeSet, NanoString suggests running a no-template control to ensure an accurate assessment of background signal.

XT CodeSet Vantage RNA:Protein Hybridization

1. Remove aliquots of Reporter CodeSet, Protein Plus Reagent, and Capture ProbeSet from the freezer and thaw at room temperature. Invert several times to mix well and spin down reagents.

IMPORTANT: After they have thawed, inspect the tubes of Reporter CodeSet and Protein Plus Reagent 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 at room temperature before using.

2. Create a master mix by adding 70 µL of hybridization buffer and 28 µL of Protein Plus Reagent to the tube containing the Reporter CodeSet. Do not remove the Reporter CodeSet from this tube. **Do not add the Capture ProbeSet to the master mix**. Invert repeatedly to mix and spin down master mix.
3. Label the hybridization tubes.
4. Add 10 µL of master mix to each of the 12 tubes. Use a fresh tip for each pipetting step to accurately measure the correct volume.

IMPORTANT: If not already done in Section D, Step 15 or Section E, Step 20, denature protein samples for 15 minutes in a thermal cycler at 95°C with a heated lid at 100°C, and then immediately ramp down to 4°C or quickly cool on ice for 2 minutes. Denaturation is critical for optimal assay performance.

5. Add the prepared RNA sample from Section C, Step 5 to each tube. Add 3 µL if using typical cell lines or 1 µL if using primary cell samples (e.g., tumor samples or PBMCs).
6. Add 2 µL of the prepared protein sample from Section D, Step 16 or Section E, Step 23 to each tube.
7. Invert the Capture ProbeSet to mix and spin down the contents. Add 2 µL of Capture ProbeSet to each tube immediately before placing at 65°C. Cap tubes and mix the reagents by inverting the tubes several times and flicking with a finger to ensure complete mixing. Briefly spin down and immediately place the tubes in the pre-heated 65°C thermal cycler.

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

8. Incubate reactions for at least 16 hours. Maximum hybridization time should not exceed 48 hours. Do not leave the reactions at 4°C for more than 24 hours or increased background may result.

NOTE: The purpose of selecting a fixed hybridization time followed by a ramp down to 4°C is to ensure equivalent hybridization times of all assays being directly compared in the same series of experiments. Counts continue to accumulate with time, 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 will increase sensitivity by increasing counts without significantly increasing background.

XT TagSet Vantage RNA:Protein Hybridization

IMPORTANT: Pre-heat the thermal cycler to 67°C with a heated lid at 72°C.

1. Remove an aliquot of nCounter XT TagSet, Probe A pool, Probe B pool, and Protein Plus from the freezer and thaw on ice. Invert several times to mix well and spin down reagents.

IMPORTANT: After they have thawed, inspect the tubes of Protein Plus Reagent 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 at room temperature before using.

2. Create a 30X Probe A Pool working dilution by adding 22 µL of TE to the 3 µL aliquot of Probe A provided.
3. Create a 30X Probe B Pool working dilution by adding 22 µL of TE to the 3 µL aliquot of Probe B provided.
4. Create a master mix by adding (a) 70 µL of hybridization buffer, (b) 28 µL of Protein Plus Reagent, (c) 7 µL diluted Probe A, and (d) 7 µL diluted Probe B to the tube containing TagSet. Do not remove TagSet from this tube. Invert repeatedly to mix and spin down master mix.

IMPORTANT: If not already done in Section D, Step 15 or Section E, Step 20, denature protein samples for 15 minutes in a thermal cycler at 95°C with a heated lid at 100°C, and then immediately ramp down to 4°C or quickly cool on ice for 2 minutes. Denaturation is critical for optimal assay performance.

5. Label the hybridization tubes.
6. Add 10 µL of master mix to each of the 12 tubes. Use a fresh tip for each pipetting step to accurately measure the correct volume.
7. Add the prepared RNA sample from Section C, Step 5 to each tube. Add 3 µL if using typical cell lines or 1 µL if using primary cell samples (e.g., tumor samples or PBMCs).
8. Add 2 µL of the prepared protein sample from Section D, Step 16 or Section E, Step 23 to each tube.
9. Cap tubes and mix the reagents by inverting the tubes several times and flicking with a finger to ensure complete mixing. Briefly spin down and immediately place the tubes in the pre-heated 67°C thermal cycler.
10. Incubate reactions for at least 16 hours. Maximum hybridization time should not exceed 48 hours. 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: The purpose of selecting a fixed hybridization time followed by a ramp down to 4°C is to ensure equivalent hybridization times of all assays being directly compared in the same series of experiments. Counts continue to accumulate with time, 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 will increase sensitivity by increasing counts without significantly increasing background.

Vantage Protein-only Hybridization Assay

GENERAL PROBE HANDLING WARNING: During setup of the assay, do not vortex or pipette vigorously to mix as it may shear the Protein TagSet. Mixing should be done by flicking or inverting the tubes. If using a microfuge to spin down tubes, do not spin any faster than 1,000 RCF for more than 30 seconds. Do not "pulse" to spin because that will cause the centrifuge to go to maximum speed and may spin the TagSet out of solution.

IMPORTANT: Pre-heat the thermal cycler to 65°C with a heated lid at 70°C.

1. Remove an aliquot of Protein TagSet from the freezer and thaw at room temperature. Invert several times to mix well and spin down reagents.

IMPORTANT: Inspect the thawed Protein TagSet 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 at room temperature before using.

2. Create a master mix by adding 70 µL of hybridization buffer and 84 µL of RNase-free water to the tube containing the Protein TagSet. Do not remove the Protein TagSet from this tube. Invert repeatedly to mix and spin down master mix.
3. Label the hybridization tubes. If using strip tubes, ensure they fit in a microfuge or picofuge and cut the strip in half if necessary.
4. Add 13 µL of master mix to each of the 12 tubes. Use a fresh tip for each pipetting step to accurately measure the correct volume.
5. Add 2 µL of the prepared protein sample to each tube from Section D, Step 16 or Section E, Step 23.
6. Cap tubes and mix the reagents by inverting the tubes several times and flicking with a finger to ensure complete mixing. Briefly spin down and immediately place the tubes in the pre-heated 65°C thermal cycler.
7. Incubate reactions for at least 16 hours. Maximum hybridization time should not exceed 48 hours. 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.

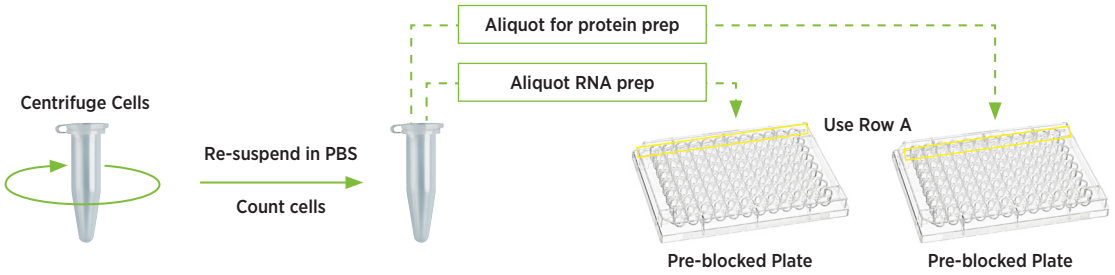
NOTE: The purpose of selecting a fixed hybridization time followed by a ramp down to 4°C is to ensure equivalent hybridization times of all assays being directly compared in the same series of experiments. Counts continue to accumulate with time, with total counts typically increasing 5% per hour between 16 and 24 hours. Although a 16-hour incubation is adequate for most purposes, a longer incubation will increase sensitivity by increasing counts without significantly increasing background.

G. Workflow for nCounter Vantage RNA:Protein Assays

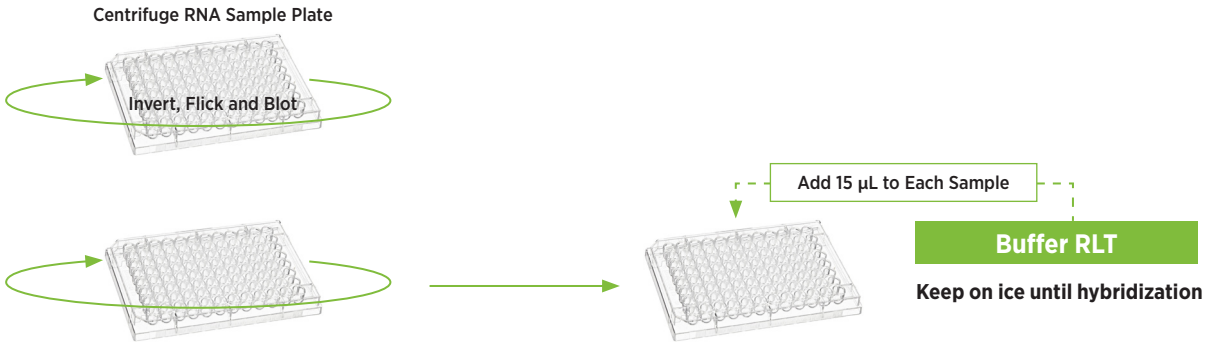
1. Perform Advanced Preparation

2. Collect Samples

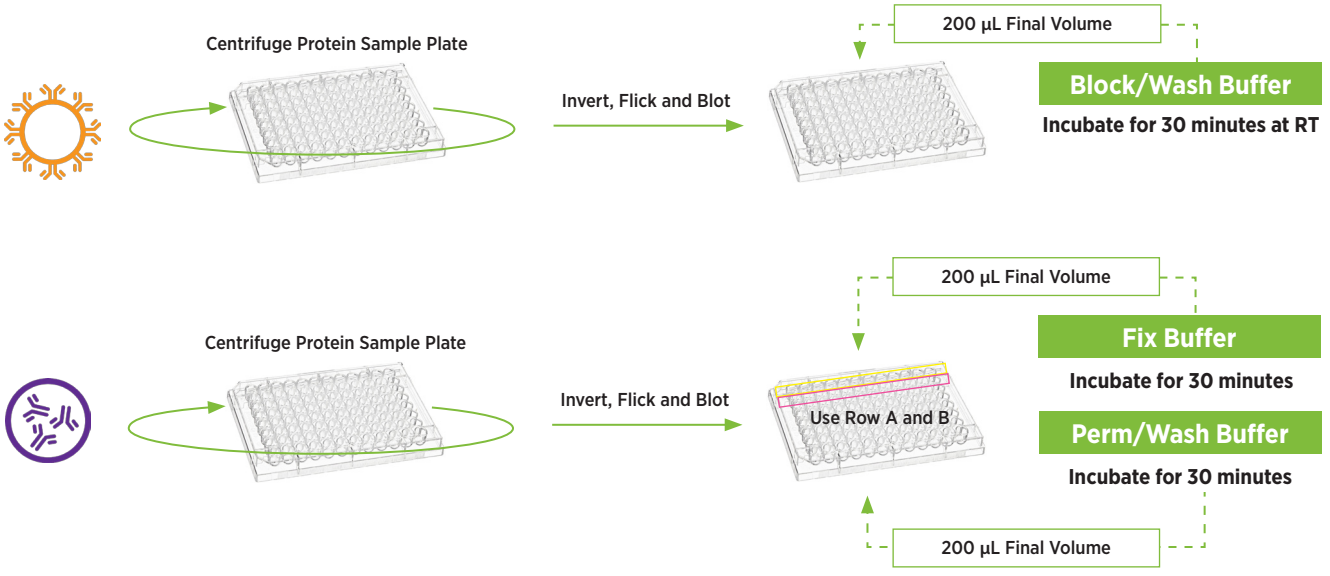
Collect 150,000 cells (or 500,000 cells from primary cell samples) from each sample in a 1.7 mL microcentrifuge tube.



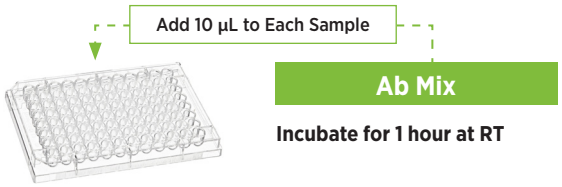
3. Prepare RNA Samples



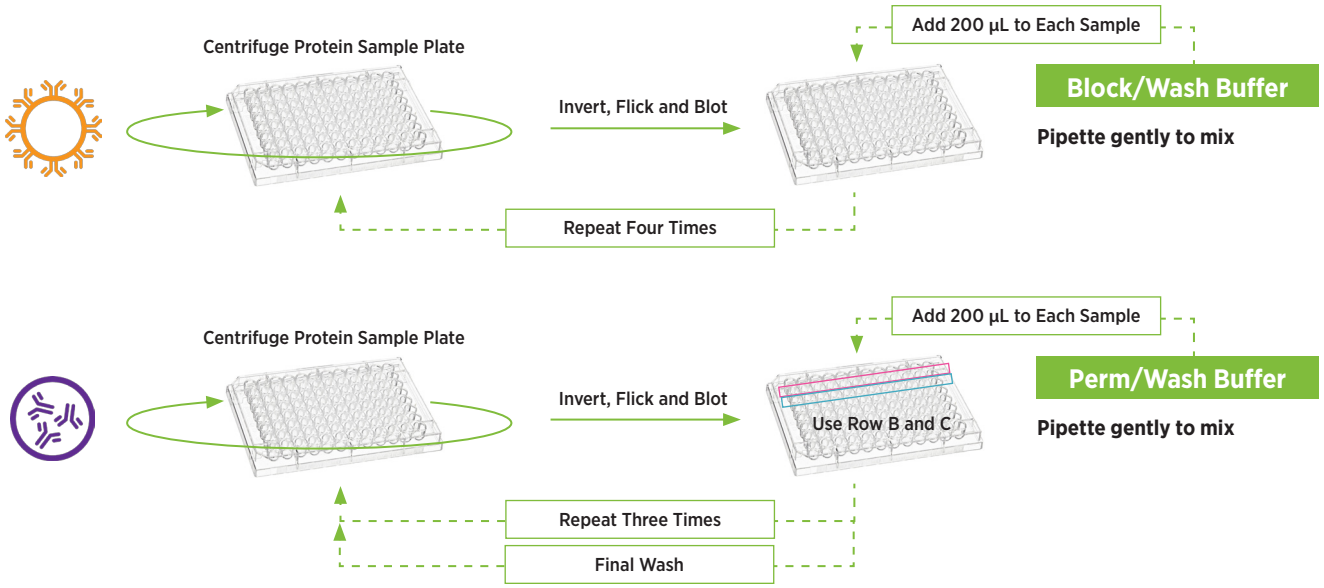
4. Prepare Protein Samples



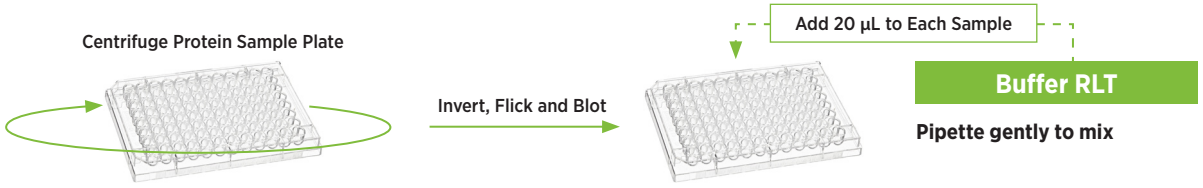
5. Add Antibody Mix to Protein Samples



6. Wash Protein Samples



7. Lyse Protein Samples

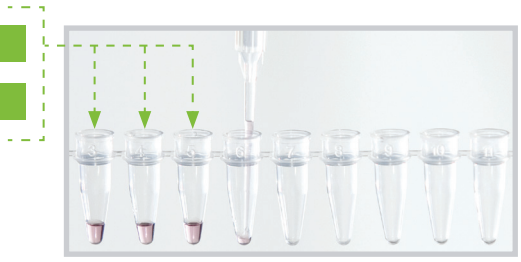


8. Dilute and Denature Protein Samples

1. Dilute the cell lysates by transferring an aliquot of each sample to a 12-tube strip. See the assay protocol for guidance specific to your sample characteristics and instrument.

To each tube, add:

- Protein Sample
- PBS



2. Denature diluted protein samples for 15 minutes at 95°C.
3. Ramp down to 4°C or quickly cool on ice for 2 minutes.

9. Create Master Mix (CodeSet Chemistry Only)

Add 70 µL to Reporter CodeSet

Hybridization Buffer +

Invert to mix and spin down.

Add 28 µL to Reporter CodeSet

Protein Plus Reagent + **Reporter CodeSet** = **Master Mix**

Thaw at room temperature, then invert to mix and spin down.

Thaw at room temperature, then invert to mix and spin down.

Invert to mix and spin down.

Create Master Mix (TagSet Chemistry Only)

Add 70 µL to TagSet

Hybridization Buffer +

Invert to mix and spin down.

Add 7 µL to TagSet

Diluted Probe A +

Thaw on ice, then invert to mix and spin down. Dilute with 22 µL TE

Add 7 µL to TagSet

Diluted Probe B +

Thaw on ice, then invert to mix and spin down. Dilute with 22 µL TE

Add 28 µL to TagSet

Protein Plus Reagent + **TagSet** = **Master Mix**

Thaw on ice, then invert to mix and spin down.

Thaw on ice, then invert to mix and spin down.

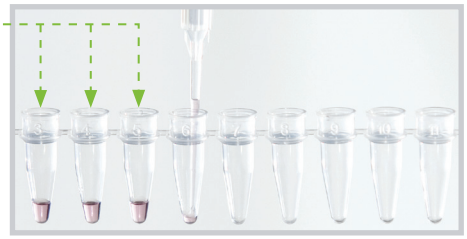
Invert to mix and spin down.

10. Set up Hybridization Reactions

To each tube, add:

- Master Mix
- RNA Sample
- Protein Sample
- RNase-free Water *(if needed)*

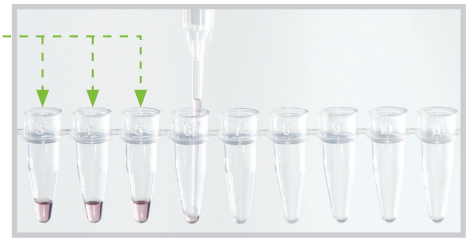
See assay protocol for sample input instructions



11. Complete Hybridization Reactions

To each tube, add:

Capture ProbeSet



1. Cap tubes.
2. Mix by inverting tubes.
3. Briefly spin down.
4. Incubate at 65°C for CodeSet Vantage and 67°C for TagSet Vantage.

6 nCounter XT DNA Assays

This chapter explains the procedures for setting up hybridizations for all DNA assays with nCounter XT CodeSets. These include the Custom CNV Assay, CNV panel products (Karyotype and Cancer CN), and ChIP-String assays.

Do not use these instructions with Legacy nCounter CodeSets. Instead, refer to the *nCounter DNA Assay User Manual*.

A. Overview

The nCounter XT DNA Assays are based on NanoString's core technology for measuring abundance of nucleic acids via digital detection of color-coded barcodes. However, DNA has several characteristics such as increased stability and a double-helix configuration that require additional sample preparation.

See the ***nSolver Analysis Software User Manual*** and product-specific ***nCounter Data Analysis Guidelines*** for instructions on data analysis.

B. General Considerations for DNA Assays

The nCounter DNA Assays include the Copy Number Assays, ChIP-String Assays, or assays for DNA isolated from other experimental conditions (e.g., MeDIP, NGS libraries, bacterial or viral gDNA, etc.). Much like NanoString's RNA-based assays, the nCounter DNA assays are compatible with a wide variety of DNA sample types. However, additional handling steps are generally required for processing DNA on an nCounter instrument. Below are some important considerations before using an nCounter assay with DNA samples. If you are considering using samples that are not addressed in this manual, contact NanoString Support at **support@nanostring.com**.

Fragmentation

Purified genomic DNA is typically longer in length (> 20 kb) than the average RNA (~1.5 kb) and too long for efficient counting. In addition, DNA is stable under the hybridization conditions used. Thus, regardless of sample type the DNA must be fragmented prior to analysis on nCounter. In the case of immunoprecipitated material (ChIP-String Assays), the DNA has typically been fragmented during the IP process and additional fragmentation is not required.

A DNA prep kit is supplied with all nCounter CNV assays and contains the reagents necessary for fragmentation of genomic DNA via the restriction endonuclease AluI. Digestion of human genomic DNA with Alu1 results in an average fragment size of 500 base pairs. Optimal target fragments for hybridization (~100–500 base pairs) are selected in probe design process.

Other fragmentation methods may be compatible with nCounter DNA assays. For the most accurate results when using FFPE samples, NanoString recommends fragmentation via Covaris AFA technology, with an optimal target size between 200–300 bases. It may also be possible to use chemical, standard sonication, or mechanical fragmentation methods if fragment length is controlled adequately. For best results, all samples (including reference samples) should have similar fragmentation profiles. Contact NanoString Support at **support@nanostring.com** to review guidelines for each approach. The nCounter DNA Assays include the Copy Number Assays, ChIP-String Assays, or assays for DNA isolated from other experimental conditions (e.g., MeDIP, NGS libraries, bacterial or viral gDNA, etc.). Much like NanoString's RNA-based assays, the nCounter DNA assays are compatible with a wide variety of DNA sample types. However, additional handling steps are generally required for processing DNA on an nCounter instrument. Below are some important considerations before using an nCounter assay with DNA samples. If you are considering using samples that are not addressed in this manual, contact NanoString Support at **support@nanostring.com**.

Denaturation

DNA is typically double-stranded in its biological conformation whereas RNA is single-stranded, and nCounter probes are designed to hybridize with a single-stranded target. Therefore, DNA must be denatured prior to hybridization and data collection.

IMPORTANT: The sample denaturation temperature must be 95°C. Incomplete denaturation may result in decreased counting efficiency.

DNA Sample Input Guidelines

NanoString recommends using 150–300 ng of input genomic DNA for all nCounter DNA assays. This amount is three times greater than the mass of RNA input required for the nCounter gene expression assays. (See **TABLE 1.3** in Chapter 1 for the sample input recommendations specific to each instrument.) A larger sample mass is required because most non-repetitive genomic DNA sequences have, on average, only two copies per cell in normal diploid samples. In contrast, the amount of a given mRNA sequence can range from one to many thousands of copies per cell depending on its level of expression.

The low copy number in individual cells provides some flexibility to optimize assay results by adjusting the sample amount. If the sample amount is not limiting, increasing input DNA amount to 300–600 ng may provide better resolution for single copy changes and may increase accuracy for highly degraded FFPE samples (see the *Tech Note: Analyzing FFPE Specimens with the nCounter CNV Assay* for more details). For DNA that has been enriched (via immunoprecipitation, NGS exome capture, target enrichment, etc.), the input requirement is typically lower than for standard genomic DNA. For these sample types, the recommended starting input ranges from 2.5–50 ng depending on the level of enrichment and the nCounter instrument model.

The maximum sample input volume for hybridization with DNA is 10 µL. Thus, sample concentration must be high enough to provide adequate sample mass (as discussed above).

DNA Sample Quality Control

DNA used in the nCounter assay MUST be free of contaminating RNA for accurate copy number analysis. DNA used in the assay should be free of contaminating RNA, which can negatively impact the quality of the data in two ways:

- Although probes are designed to be non-exonic, non-coding strand sequences, contaminating RNA may result in reduced counting efficiency.
- RNA may also result in over estimation of DNA concentration when measured by UV absorbance, leading to lower DNA input amounts than recommended and lower counts.

For pure genomic DNA preparations, NanoString recommends that the A260/A280 ratio should be between 1.7 and 1.9, and that the A260/A230 ratio should be between 1.3 and 2.0. Fluorescence-based assays (e.g., QuBit® from Life Technologies®) that use dyes specific for DNA may provide the most accurate concentration measurements if RNA contamination is suspected.

DNA extracted from FFPE is typically degraded due to the fixation and storage process. The nCounter FFPE protocol and analysis guidelines have been optimized to produce accurate results and recommend that the average size of DNA extracted from FFPE tissues be greater than 1 kb (prior to fragmentation). See the section on *DNA Fragmentation Quality Control* for an example of size variability of FFPE samples.

Materials Recommended for FFPE Sample Processing and Quality Control

TABLE 6.1 Additional instruments recommended for DNA assays using FFPE samples

Instrument	Manufacturer
Bioanalyzer 2100	Agilent Technologies®
Covaris AFA Instrument (various models)	Covaris, Inc.®
NanoDrop ND-2000	NanoDrop Technologies®

C. Types of DNA Assays

NanoString's nCounter DNA assays can be divided into two categories: Copy Number Assays and ChIP-String Assays.

nCounter Copy Number Assays

nCounter Copy Number assays enable precise quantification of copy number and include the following products:

- The nCounter Custom CNV Assay measures copy number variation for up to 800 loci in a single reaction. Probes are designed to target customer-defined regions in the genome of interest.
- The nCounter Karyotype Assay is a fixed-content panel designed to measure invariant regions (i.e., non-CNV) covering all human chromosomes and intended for detection of large chromosomal abnormalities (e.g., aneuploidy).
- The nCounter Cancer CN Assay is a fixed-content panel designed to measure 87 genes commonly amplified or deleted in cancer cells. To increase performance when analyzing FFPE samples of varying quality, three probes have been designed for each gene.

All nCounter Copy Number Assays utilize the same protocol (described below) and require DNA fragmentation prior to sample hybridization to ensure accurate analysis.

nCounter ChIP-String Assays

Chromatin immunoprecipitation (ChIP) is a technique used to study protein-DNA interactions. This method is widely used to determine which specific proteins or histone modifications are associated with certain genomic regions or features—such as promoters, enhancers, and insulators—or whether a gene is in an active, repressed, or poised state.

ChIP generally involves cross-linking of proteins to DNA, fragmentation of the cross-linked DNA via sonication, and immunoprecipitation with an antibody to a specific protein or histone modification. The antibody-protein-DNA complexes are then purified, the proteins are removed, and the DNA is isolated. The result of a successful ChIP preparation is a relatively pure and highly enriched DNA fragment population. The enriched DNA fragments can then be interrogated using the nCounter ChIP-String assay via the protocol below. NanoString does not offer reagents or support for performing the initial ChIP protocol. Users are expected to identify and optimize ChIP protocols based on their specific reagents and research needs.

Once samples are prepared via a user-defined ChIP method, the nCounter ChIP-String Assay allows the user to study up to 800 candidate genomic regions that correspond to the binding sites of the protein or locations of histone modifications of interest. The sensitivity of this assay allows for direct detection of enriched ChIP'd DNA and thus requires as little as 10 ng of DNA input material. The ChIP process generally produces fragmented DNA of appropriate length. However, chromatin with average length over 500 base pairs should be further fragmented to a range of 250–500 base pairs.

D. DNA Fragmentation Guidelines

All nCounter DNA assays require that the genomic DNA be fragmented prior to hybridization. Starting material for ChIP-String is typically fragmented during the immunoprecipitation process and may not require additional fragmentation (however, read the section on *Special Considerations for ChIP-String Assays*).

Two methods of fragmentation are acceptable: AluI restriction enzyme digestion or Covaris AFA-based fragmentation. When using intact (i.e., non-degraded) genomic DNA from cell lines, blood, or fresh or frozen tissue, NanoString recommends using AluI-based fragmentation. For degraded genomic DNA, either from FFPE samples or other sources, NanoString recommends the Covaris-based fragmentation method, although AluI can also be used. If samples were previously fragmented by other methods or do not meet the optimal fragmentation profiles described below, please contact NanoString Support (**support@nanostring.com**) or a Field Application Scientist for guidance.

AluI Restriction Digest Fragmentation

IMPORTANT: If samples were fragmented as part of experimental set-up (e.g., ChIP or NGS library prep) and have an average length under 500 base pairs, this step may be omitted. However, it is critical that all samples have similar fragmentation profiles for accurate copy number results (see the section on *DNA Fragmentation Quality Control*). Contact NanoString Support at **support@nanostring.com** for additional information.

IMPORTANT: If setting up a master mix, add reagents for 13 reactions to account for dead volume. When using a master mix, a minimum digest volume of 10 µL (300 ng DNA) is recommended. If your DNA concentration is less than 29 ng/µL, NanoString recommends ethanol precipitation with a carrier such as linear acrylamide (see the *Covaris AFA Fragmentation* protocol for an example). Vacuum drying is not recommended as this method concentrates salts and other components that may inhibit the restriction digest.

1. Set up the restriction digest in a 0.2–0.5 mL PCR tube*. Recommended DNA input is 300 ng in 7 µL of sample (minimum of 200 ng). The minimum concentration of genomic DNA should be 29 ng/µL prior to its addition to the restriction digest.

   The non-sample components can be set up in a master mix for multiple samples if necessary (see Note above). Use the following volumes for each component for a total volume of 10 µL per digest:

   - 1 µL 10X AluI Fragmentation Buffer (supplied with kit)
   - 1 µL 10X CNV DNA Prep Control† (supplied with kit)
   - 1 µL AluI fragmentation enzyme (5 U/µL, supplied with kit)
   - 7 µL containing 300 ng DNA in RNase-free water*, Tris pH 8.0, or similar

2. Mix and spin briefly to bring contents to the bottom of each tube.
3. Incubate the AluI restriction digest at 37°C for 1–2 hours in a heat block or a thermal cycler with the heated lid turned on.

   Optional: You may wish to check the quality of the digest on 1% agarose gel or Bioanalyzer DNA chip. See the section on *DNA Fragmentation Quality Control* for an example of a typical size distribution for human genomic DNA digested with AluI.

4. When the digest is complete, denature samples at 95°C for 5 minutes. Immediately cool samples on ice for 2 minutes. Keep on ice until ready to set up the hybridization reaction.
5. Proceed to the hybridization protocol. Any remainder of the digested DNA sample can be stored at -20°C for future use. Remember to denature the sample (Step 4) prior to use.

**If desired, the DNA Prep Kit reagent can be separated into 14 µL aliquots for storage (14 µL is enough to prepare one master mix for 12 restriction digests).*

†RNA is a component of the Reporter CodeSet. To reduce the risk of RNase contamination during set-up, use RNase-free water, tips, and gloves.

Covaris AFA Fragmentation

1. Begin with approximately three 10 µm slices of FFPE tissue*.
2. Isolate DNA from FFPE tissue using an accepted protocol (an example of a commonly used kit is the QIAamp DNA FFPE Tissue Kit from QIAGEN). Ensure that the genomic DNA is free of contaminating RNA.
3. Confirm DNA yield by checking concentration with a NanoDrop™ instrument or a fluorescent-based dye detection method.
4. Dilute between 500 ng and 1 µg of DNA in 130 µL of 10 mM Tris.
5. Fragment the diluted DNA with a Covaris AFA instrument. Use the settings defined by the manufacturer to produce 200 bp fragments (actual settings may vary depending on the instrument model).
6. After fragmentation is complete, assay 1 µL of sample (approximately 4 ng) on an Agilent® 2100 Bioanalyzer using a High Sensitivity DNA Kit to confirm the desired degree of fragmentation. Successful sonication should produce a single peak centered between 200–300 bp with an average mass between 250–450 bp. The presence of multiple peaks or peak heights greater than 300 bp will indicate less-than-optimal sonication.
7. Isolate the fragmented DNA via ethanol precipitation using linear acrylamide as the carrier† (other non-nucleic acid-based carriers such as glycogen can also be used).
   a. Add the following reagents to 130 µL of the sonicated sample:
      - 2 µL linear acrylamide (Ambion®; 5 mg/ml)
      - 14.7 µL sodium acetate (3M; pH 5.5)
      - 367 µL ethanol
   b. Cool at -20°C for at least 2 hours.
   c. Spin down at 4°C at max speed (16,000 RCF) for 30 minutes using a microcentrifuge.
   d. Carefully remove the supernatant.
   e. Add 250 µL 70% ethanol.
   f. Spin down at 4°C at max speed for 5 minutes.
   g. Carefully remove the ethanol, being careful not to disturb the pellet.
8. Resuspend the precipitated DNA in 11.5 µL of 10 mM Tris. Repeat Step 3 to confirm DNA concentration.
9. When fragmentation is complete, denature samples at 95°C for 5 minutes. Immediately cool samples on ice for 2 minutes. Keep on ice until ready to set up the hybridization reaction.
10. Proceed to the hybridization protocol. Any remainder of the fragmented DNA sample can be stored at -20°C for future use. Remember to denature the sample (Step 9) prior to use.

**The yield of DNA from a given FFPE sample is highly variable. In general, NanoString has found that three 10 µM slices (100 ng of DNA per slice, or 300 ng total) will yield a sufficient amount of input material for most assays. Accuracy of results may increase with greater DNA input.*

†Gaillard C and Strauss F. (1990) Ethanol precipitation of DNA with linear polyacrylamide as carrier. *Nucleic Acids Res* (18)2:378.

Special Considerations for ChIP-String Assays

Additional fragmentation beyond that performed during the chromatin immunoprecipitation (ChIP) protocol is not required prior to performing the nCounter ChIP-String DNA assay. However, there are other factors that should be considered prior to beginning the nCounter assay set-up:

- A minimum of 5–10 ng (1–2 ng/µL) of unamplified ChIP'd DNA input is recommended. The input amount is highly dependent on the level of enrichment of the target molecules (see below).
- Direct detection (without amplification) of target molecules requires that the ChIP'd DNA has been significantly enriched. For example, 1 ng of human genomic DNA has approximately 330 copies of any non-repetitive sequence (i.e., 2 copies per genome). Thus, 5–10 ng of un-enriched genomic DNA sample would contain approximately 1,600 to 3,300 molecules, roughly equal to the limit of detection for the nCounter system. In order to confidently measure the sequences associated with any given ChIP assay, NanoString recommends an enrichment of at least 10-fold over un-enriched genomic DNA (between 16,000 and 33,000 molecules, and with the same sample mass of 5–10 ng). Reporter Probe counts increase linearly with an increasing amount of sample input. If the DNA sample is not limiting, increasing the input amount will result in higher counts and higher confidence in the measurements.
- The amount of enrichment is highly dependent on the success of the ChIP protocol, the affinity of the antibody used, and whether the protein of interest is directly bound to genomic DNA or associated with other proteins that are bound to DNA. If a new procedure or ChIP reagents are used, a positive control sample is highly recommended. If ChIP'd DNA is amplified via whole-genome amplification, then a sample input of 50–100 ng is recommended.

DNA Fragmentation Quality Control

Ideal Fragmentation Profile of Alu1-Digested DNA

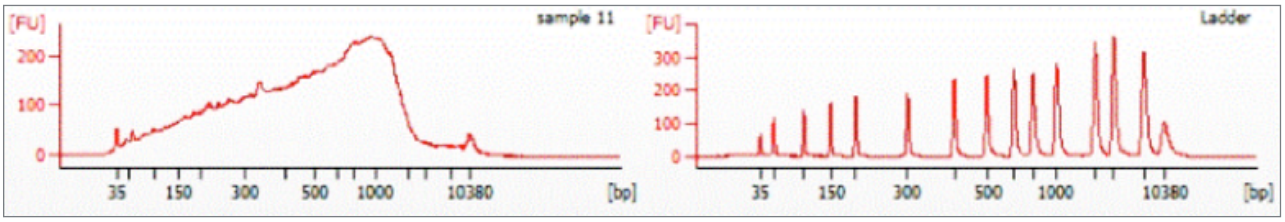


FIGURE 6.1 Agilent Bioanalyzer High-Sensitivity DNA assay profile of 20 ng human genomic DNA digested with Alu1 enzyme for 2 hours at 37°C.

Ideal Fragmentation Profile of Sonicated Genomic DNA

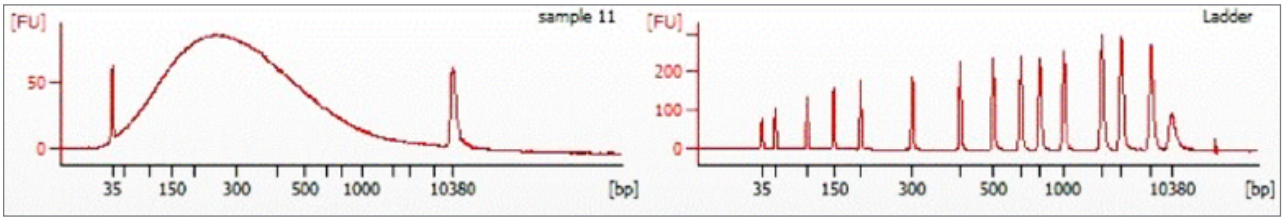


FIGURE 6.2 Agilent Bioanalyzer High-Sensitivity DNA assay profile of 20 ng human genomic DNA fragmented with Covaris-AFA technology using manufacturer's recommended settings for 200 bp peak.

Incomplete Sonication

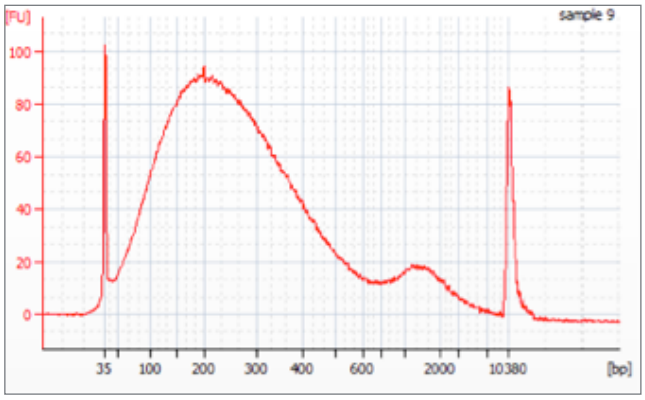


FIGURE 6.3 A Bioanalyzer High-Sensitivity DNA assay profile of 4 ng human genomic DNA fragmented with Covaris AFA technology. While the majority of the DNA is the correct size (~200 bp) the presence of a high molecular weight peak (1,000-1,500 bp) indicates that the fragmentation was not complete.

FFPE Gel Examples

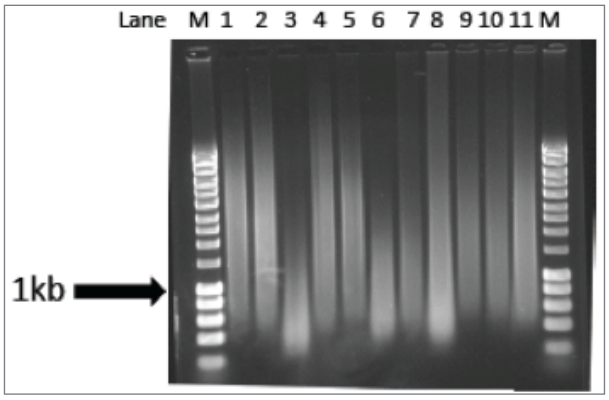


FIGURE 6.4 Analysis of genomic DNA size from FFPE tissues. DNA was extracted from three 10 µm slices of 11 different FFPE tissues using the DNeasy FFPE Kit (QIAGEN). To assess DNA size, 150 ng of extracted DNA was run on a 0.8% agarose gel. HyperLadder™ 1 kb (Bioline) was used as a marker. Samples 3, 6, and 7 have average sizes below 1 kb and should be considered "lower quality."

Summary

TABLE 6.2 Methods for performing quality control on fragmented DNA

Sample Type	Recommended Assay Input	Recommended Concentration	QC Method
Genomic DNA (un-enriched)	150–600 ng	> 30 ng/µL	Agarose gel; confirm complete digestion with nCounter® restriction digest controls
Target-enriched NGS library or DNA prepared by ChIP	5–50 ng	2-10 ng/µL	Agilent® BioAnalyzer

E. Hybridization Protocol for nCounter XT DNA Assays

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

Set up a hybridization reaction at room temperature for each sample with the following components: 3 µL of Reporter CodeSet, 5 µL of hybridization buffer, up to 10 µL of denatured sample DNA, and 2 µL of Capture ProbeSet. See **TABLE 1.3** in Chapter 1 for the sample input recommendations specific to each instrument.

1. Remove aliquots of both Reporter CodeSet and Capture ProbeSet reagent from the freezer to thaw at room temperature. Invert several times to mix well and spin down reagent.

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 at room temperature before using.

2. Create a master mix by adding 70 µL of hybridization buffer to the tube of Reporter Probes. Do not remove the Reporter Probes from the tube. RNase-free water may also be added to this mix if the volume of the individual DNA samples is less than 5 µL and is constant. (Add enough water for 14 assays to allow two assays' worth of dead volume.) **Do not add the Capture ProbeSet to the master mix.** Invert repeatedly to mix and spin down master mix.
3. Label the hybridization tubes. If using strip tubes, ensure they fit in a microfuge or picofuge and cut the strip in half if necessary.
4. Add 8 µL of master mix to each of the 12 tubes. NanoString recommends using a fresh tip for each pipetting step. The CodeSet has components that can wick up into the tip, and you may not dispense the correct amount if you use the same tip.

IMPORTANT: If not already done, denature DNA samples at 95°C for 5 minutes. Immediately place DNA samples on ice for 2 minutes to minimize re-annealing. Denaturation of DNA samples is critical for optimal assay performance.

5. Briefly spin down denatured DNA samples in a picofuge.
6. Add up to 10 µL of sample to each tube.
7. If necessary, add RNase-free water to bring the volume of each assay to 18 µL.
8. Invert the Capture ProbeSet tube to mix and spin down the contents. Add 2 µL of Capture ProbeSet to each tube immediately before placing at 65°C. Cap tubes and mix the reagents by inverting the tubes several times and flicking with a finger to ensure complete mixing. Briefly spin down and immediately place the tubes in the pre-set 65°C thermal cycler.

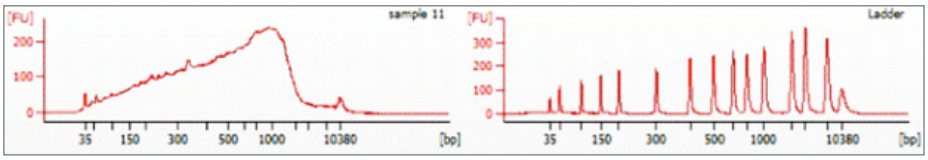
NOTE: Minimizing the time between addition of the Capture ProbeSet and incubation at 65°C will increase assay sensitivity.

9. Incubate reactions for at least 16 hours. Maximum hybridization time should not exceed 48 hours. 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: The purpose of selecting a fixed hybridization time followed by a ramp down to 4°C is to ensure equivalent hybridization times of all assays being directly compared in the same series of experiments. Counts continue to accumulate with time, 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 will increase sensitivity by increasing counts without significantly increasing background.

F. Workflow for nCounter XT DNA Assays

1. Fragment DNA



Follow guidelines to fragment DNA by using restriction digest with AluI or using sonication. Confirm fragmentation profile (~50% of sample > 300 bases). **NOTE:** Fragmentation is not required for ChIP-String assays.

2. Create Master Mix

Add 70 µL to Reporter CodeSet

Hybridization Buffer + **Reporter CodeSet** = **Master Mix**

Invert to mix and spin down.

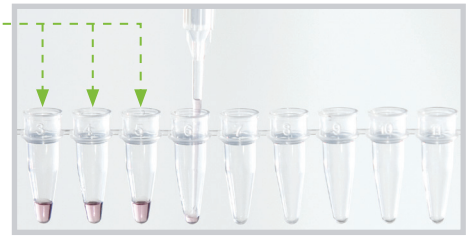
Thaw at room temperature, then invert to mix and spin down.

Invert to mix and spin down.

3. Set up Hybridization Reactions

To each tube, add:

8 µL Master Mix



4. Denature DNA and Add to Hybridization Reaction

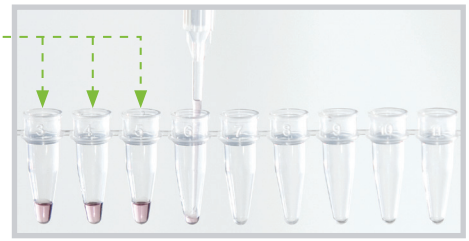
1. Denature DNA samples at 95°C for 5 minutes.
2. Immediately place samples on ice for 2 minutes.
3. Add 10 µL of sample to each tube.

5. Complete Hybridization Reactions

To each tube, add:

2 µL Capture ProbeSet

20 µL final volume after addition of Capture ProbeSet



1. Cap tubes.
2. Mix by inverting tubes.
3. Briefly spin down.
4. Incubate at 65°C.

G. Using Plus Reagents with nCounter XT DNA Assays

GENERAL PROBE HANDLING WARNING: During setup of the assay, do not vortex or pipette vigorously to mix as it may shear the Reporter CodeSet. Mixing should be done by flicking or inverting the tubes. If using a microfuge to spin down tubes, do not spin any faster than 1,000 RCF for more than 30 seconds. Do not "pulse" to spin because that will cause the centrifuge to go to maximum speed and may spin the CodeSet out of solution.

IMPORTANT: Pre-heat the thermal cycler to 65°C. Program the thermal cycler using 23 µL volume, calculated temperature, and heated lid.

Set up a hybridization reaction at room temperature for each sample with the following components: 3 µL of Reporter CodeSet, 2 µL of Reporter Plus, 5 µL of hybridization buffer, 10 µL of denatured sample DNA, 2 µL of Capture ProbeSet, and 1 µL of Capture Plus. See TABLE 1.3 in Chapter 1 for the sample input recommendations specific to each instrument.

1. Remove aliquots of Reporter CodeSet, Capture ProbeSet, and Plus reagents from the freezer to thaw at room temperature. Invert several times to mix well and 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 at room temperature before using.

2. Add 28 µL of Reporter Plus reagent to the thawed Reporter CodeSet. Invert several times to mix well, and spin down reagents.
3. Create a master mix by adding 70 µL of hybridization buffer to the tube containing the Reporter CodeSet and Reporter Plus reagents. Do not remove the Reporter CodeSet from this tube. RNase-free water may also be added to this mix if the volume of the individual DNA samples is less than 10 µL and is constant. (Add enough water for 14 assays to allow two assays' worth of dead volume.) **Do not add the Capture ProbeSet or Capture Plus reagent to the master mix.** Invert repeatedly to mix and spin down master mix.
4. Label the hybridization tubes. If using strip tubes, ensure they fit in a microfuge or picofuge and cut the strip in half if necessary.
5. Add 10 µL of master mix to each of the tubes. (If water was added to this mix, increase this volume as necessary.) Use a fresh tip for each pipetting step to accurately pipette the correct volume.

IMPORTANT: If not already done, denature DNA samples at 95°C for 5 minutes. Immediately place DNA samples on ice for 2 minutes to minimize re-annealing. Denaturation of DNA samples is critical for optimal assay performance.

6. Add up to 10 µL of sample to each tube.
7. If necessary, add RNase-free water to bring the volume of each assay to 20 µL.
8. Add 14 µL of Capture Plus reagent to the thawed Capture ProbeSet. Invert several times to mix well, and spin down reagents.
9. Add 3 µL of Capture ProbeSet and Capture Plus reagent mix to each tube immediately before placing at 65°C. Cap tubes and mix the reagents by inverting the tubes several times and flicking with a finger to ensure complete mixing. Briefly spin down.

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

10. Incubate reactions for at least 16 hours. Maximum hybridization time should not exceed 48 hours. 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.
11. Once removed from the thermal cycler, proceed immediately to post-hybridization processing as described in your instrument manual. Do not store hybridizations at 4°C.

NOTE: The purpose of selecting a fixed hybridization time followed by a ramp down to 4°C is to ensure equivalent hybridization times of all assays being directly compared in the same series of experiments. Counts continue to accumulate with time, 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 will increase sensitivity by increasing counts without significantly increasing background.

7 nCounter XT Gene Expression Assays for Single Cells and Low Input Material

This chapter explains the procedures for setting up hybridizations for single cell and low input material gene expression assays with nCounter XT CodeSets. Some sample preparation is required.

Do not use these instructions with Legacy nCounter CodeSets. Instead, refer to the *nCounter Single Cell Gene Expression Protocols*.

The nCounter Single Cell Gene Expression Assay enables the highly reproducible and highly multiplexed nCounter technology to be applied to single sorted cells and small quantities of RNA input material. The assay requires of the following reagents:

- Multiplexed Target Enrichment (MTE) Primers
- nCounter Single Cell Gene Expression CodeSet
- nCounter Master Kit

When a custom nCounter Single Cell Gene Expression CodeSet is ordered, a set of Multiplexed Target Enrichment (MTE) primers will be designed for every gene sequence targeted by the CodeSet. These primers are required for the MTE process detailed in this protocol.

A. Overview

Multiplexed Target Enrichment (MTE) performs specific and linear enrichment of up to 800 targets in a single sample. After cDNA conversion, a limited number of PCR cycles are performed with a pool of MTE primers, resulting in small amounts of cDNA being enriched without bias. After MTE enrichment, samples can be directly analyzed with an nCounter CodeSet that assays gene sequences internal to the corresponding MTE primers. No clean-up or dilution of the enriched sample is required prior to analysis, simplifying the workflow and maximizing sensitivity.

Multiplexed Target Enrichment Primers

Multiplexed Target Enrichment (MTE) primers enable a limited number of amplification cycles to produce sufficient target for detection in an nCounter hybridization assay. Upon request, NanoString will provide MTE primer designs flanking the target sequences for the Reporter and Capture Probes. As part of the CodeSet design process, NanoString can also provide sufficient information to design alternate primers if desired. For additional information or questions about primer design, please contact NanoString Support at **support@nanostring.com**.

MTE primers should be pooled at a final concentration of 500 nM per oligo in TE Buffer (pH 7.5). NanoString has partnered with Integrated DNA Technologies (IDT) to provide pre-pooled MTE primer sets at the recommended concentration of 500 nM per oligo.

cDNA Conversion and Multiplexed Target Enrichment

The nCounter Single Cell protocol can be used with either sorted cells or purified total RNA and is compatible with samples in water, TE buffer, or Cells-to-Ct®. Samples in other lysis/storage buffers, such as CellsDirect®, RNALater®, or buffers than contain guanidinium salts are not compatible with the assay.

Multiplexed Target Enrichment is a two-step process: input RNA is converted to cDNA, which is then amplified with target-specific primers. cDNA conversion can be accomplished with a variety of reverse transcriptases. For single cells and/or total RNA input amounts less than 100 pg, NanoString recommends the use of SuperScript® VILO™. After cDNA conversion, a highly multiplexed enrichment of target sequences is performed using TaqMan® PreAmp MasterMix.

B. Sample Preparation for Total RNA

cDNA Conversion of Purified Total RNA

NOTE: If using samples stored in Cells-to-Ct®, ensure that Cells-to-Ct® Stop Solution has been added.

1. Combine 1–4 µL of total RNA (in H_2O, TE buffer, or Cells-to-Ct) and 1 µL of SuperScript VILO Master Mix in each sterile PCR tube while on ice. Add RNase-free H_2O if necessary to obtain a final volume of 5 µL.
2. Gently mix and incubate at 25°C for 10 minutes.
3. Incubate at 42°C for 60 minutes.
4. Stop the reaction with a 5-minute incubation at 85°C.

Multiplexed Target Enrichment

1. Create the MTE master mix that will be added to the converted cDNA. For each 12 samples, combine 65 µL TaqMan PreAmp Master Mix (2X) and 13 µL pooled MTE primers. (Enough master mix will be left over for 1 sample's worth of dead volume.)
2. Add 6 µL of MTE master mix to each converted cDNA sample.
3. Mix the reactions by briefly vortexing or pipetting up and down followed by centrifugation.
4. Use **TABLE 7.1** to determine the number of amplification cycles* necessary based on the amount of sample input. Experimental conditions may require further optimization.

TABLE 7.1 Amplification cycles for multiplexed target enrichment

Total RNA	Recommended # of Cycles*
Up to 10 pg	18–20+
10–100 pg	14–18
100–1,000 pg	8–14
1–10 ng	2–8

5. Place the samples in a thermal cycler and program the instrument as indicated in **TABLE 7.2**:

TABLE 7.2 MTE Thermal Cycler Protocol

Stage	Temperature	Duration
Denaturation	94°C	10 minutes
MTE cycles	94°C	15 seconds
(2–20+ cycles)	60°C	4 minutes
Hold	4°C	∞

IMPORTANT: After multiplexed target enrichment, incubate the prepared samples for 2 minutes at 94°C and then snap cool on ice prior to addition to the hybridization reaction.

C. Sample Preparation for Whole Sorted Cells

cDNA Conversion of Whole Sorted Cells

1. Sort cells into sterile PCR tubes or plate wells containing either 5.5 µL of RNase-free H_2O or 5 µL of Cells-to-Ct lysis solution.
2. Immediately after sorting, spin down tubes or plates at low speed for 2 minutes.

NOTE: 0.5 µL of Cells-to-Ct stop solution must be added to the Cells-to-Ct lysis solution prior to continuing with this protocol.

3. Add 1.5 µL of SuperScript VILO Master Mix to each PCR tube or plate well containing sorted cells on ice for each sample to be processed. Add RNase-free H_2O if necessary to obtain a final volume of 7 µL.
4. Gently mix and incubate at 25°C for 10 minutes.
5. Incubate at 42°C for 60 minutes.
6. Stop the reaction with a 5-minute incubation at 85°C.

Multiplexed Target Enrichment

1. Create the MTE master mix that will be added to the converted cDNA. For each 12 samples, combine 97.5 µL TaqMan PreAmp Master Mix (2X) and 13 µL pooled MTE primers. (Enough master mix will be left over for 1 sample's worth of dead volume.)
2. Add 8.5 µL of MTE master mix to each converted cDNA sample.
3. Mix the reactions by briefly vortexing or pipetting up and down followed by centrifugation.
4. Use **TABLE 7.3** to determine the number of amplification cycles* necessary based on the amount of sample input. Experimental conditions may require further optimization.

TABLE 7.3 Amplification cycles for multiplexed target enrichment

Total RNA	Recommended # of Cycles*
Single cell	16–20+
2–10 cells	12–16
10–100 cells	8–12
100–1,000 cells	2–8

5. Place the samples in a thermal cycler and program the instrument as indicated in **TABLE 7.4**:

TABLE 7.4 MTE Thermal Cycler Protocol

Stage	Temperature	Duration
Denaturation	94°C	10 minutes
MTE cycles	94°C	15 seconds
(2–20+ cycles)	60°C	4 minutes
Hold	4°C	∞

IMPORTANT: After multiplexed target enrichment, incubate the prepared samples for 2 minutes at 94°C and then snap cool on ice prior to addition to the hybridization reaction.

D. Hybridization Protocol for Prepared Samples

GENERAL PROBE HANDLING WARNING: During setup of the assay, do not vortex or pipette vigorously to mix as it may shear the Reporter CodeSet. Mixing should be done by flicking or inverting the tubes. If using a microfuge to spin down tubes, do not spin any faster than 1,000 RCF for more than 30 seconds. Do not "pulse" to spin because that will cause the centrifuge to go to maximum speed and may spin the CodeSet out of solution.

IMPORTANT: Pre-heat the thermal cycler to 65°C. Program the thermal cycler using 18 µL volume, calculated temperature, and heated lid.

Set up a hybridization reaction at room temperature for each sample with the following components: 3 µL of Reporter CodeSet, 5 µL of hybridization buffer, up to 8 µL of the prepared MTE reaction, and 2 µL of Capture ProbeSet. See **TABLE 1.3** in Chapter 1 for the sample input recommendations specific to each instrument.

1. Remove aliquots of both Reporter CodeSet and Capture ProbeSet reagent from the freezer and thaw at room temperature. Invert several times to mix well and spin down reagent.

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 at room temperature before using.

2. Create a master mix by adding 70 µL of hybridization buffer to the tube of Reporter Probes. Do not remove the Reporter Probes from the tube. RNase-free water may also be added to this mix if the volume of the individual samples is less than 8 µL and is constant. (Add enough water for 14 assays to allow two assays' worth of dead volume.) **Do not add the Capture ProbeSet to the master mix.** Invert to repeatedly to mix and spin down master mix.
3. Label the hybridization tubes. If using strip tubes, ensure they fit in a microfuge or picofuge and cut the strip in half if necessary.
4. Add 8 µL of master mix to each of the 12 tubes. (If water was added to the master mix, increase this volume as necessary.) Use a fresh tip for each pipetting step to accurately measure the correct volume.
5. Add up to 8 µL of the prepared MTE reaction to each tube.
6. If necessary, add RNase-free water to bring the volume of each assay to 16 µL.
7. Invert the Capture ProbeSet tube to mix and spin down the contents. Add 2 µL of Capture ProbeSet to each tube immediately before placing at 65°C. Cap tubes and mix the reagents by inverting the tubes several times and flicking with a finger to ensure complete mixing. Briefly spin down and immediately place the tubes in the pre-heated 65°C thermal cycler.

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

8. Incubate reactions for at least 16 hours. Maximum hybridization time should not exceed 48 hours. 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: The purpose of selecting a fixed hybridization time followed by a ramp down to 4°C is to ensure equivalent hybridization times of all assays being directly compared in the same series of experiments. Counts continue to accumulate with time, 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 will increase sensitivity by increasing counts without significantly increasing background.

E. Workflow for Single Cell and Low Input Material Assays

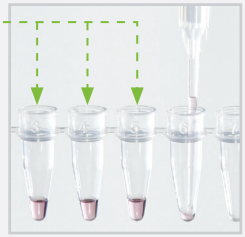
1. cDNA Conversion (Choose One)

Total RNA - Low Input Material

To each tube, add:

- 1–4 µL Total RNA
- 1 µL SuperScript VILO
- RNase-free Water *(if needed)*

5 µL total volume per tube

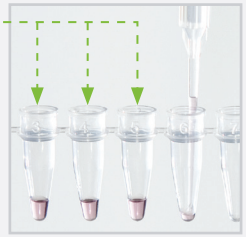


Sorted Cells

To each tube, add:

- 5–5.5 µL Sorted Cells
- 0.5 µL Cells-to-Ct Stop *(if needed)*
- 1.5 µL SuperScript VILO

7 µL total volume per tube



IMPORTANT: If using Cells-to-Ct for either protocol, add stop solution before continuing. cDNA conversion of sorted cells requires adding 0.5 µL of stop solution to 5 µL of sorted cells to maintain a final volume of 7 µL per tube. Stop solution for total RNA samples varies with sample volume.

2. Complete cDNA Conversion

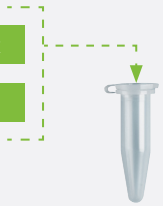
1. Cap tubes and mix gently.
2. Incubate at 25°C for 10 minutes.
3. Incubate at 42°C for 60 minutes.
4. Stop the reaction by incubating at 85°C for 5 minutes.

3. Create Master Mix for Multiplex Target Enrichment (Choose One)

Total RNA—Low Input Material

In a single tube, combine:

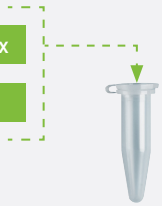
- 65 µL TaqMan PreAmp Master Mix
- 13 µL Pooled MTE Primers



Sorted Cells

In a single tube, combine:

- 97.5 µL TaqMan PreAmp Master Mix
- 13 µL Pooled MTE Primers



4. Complete Multiplex Target Enrichment

1. Distribute MTE Master Mix to each tube, using 6 µL for total RNA or 8.5 µL for sorted cells.
2. Cap tubes and mix gently.
3. Denature at 94°C for 10 minutes.
4. Incubate at 94°C for 15 seconds.
5. Incubate at 60°C for 4 minutes.
6. Repeat Steps 4 and 5 for 2-20 cycles per guidelines based on sample mass.
7. Hold indefinitely at 4°C when complete.

5. Create Master Mix for Hybridization

Add 70 µL to Reporter CodeSet

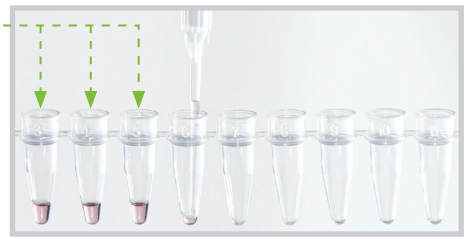
Hybridization Buffer	+	Reporter CodeSet	=	Master Mix
Invert to mix and spin down.		Thaw at room temperature, then invert to mix and spin down.		Invert to mix and spin down.

6. Set up Hybridization Reactions

To each tube, add:

- 8 µL Master Mix
- 5-8 µL MTE Reaction
- RNase-free Water *(if needed)*

16 µL total volume per tube



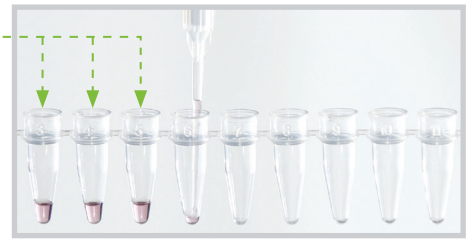
IMPORTANT: Incubate MTE samples at 95°C for 2 minutes and snap cool on ice immediately before adding them to the hybridization reaction. Add up to 8 ul of MTE sample to each hybridizationto reaction to maximize assay sensitivity.

7. Complete Hybridization Reactions

To each tube, add:

- 2 µL Capture ProbeSet

18 µL final volume after addition of Capture ProbeSet



1. Cap tubes.
2. Mix by inverting tubes.
3. Briefly spin down.
4. Incubate at 65°C.

F. Using Plus Reagents with Single Cell and Low Input Material Assays

NOTE: Prepare samples as instructed for low input material (Section B) or single sorted cells (Section C), and then return to this page to perform the sample hybridization.

GENERAL PROBE HANDLING WARNING: During setup of the assay, do not vortex or pipette vigorously to mix as it may shear the Reporter CodeSet. Mixing should be done by flicking or inverting the tubes. If using a microfuge to spin down tubes, do not spin any faster than 1,000 RCF for more than 30 seconds. Do not "pulse" to spin because that will cause the centrifuge to go to maximum speed and may spin the CodeSet out of solution.

IMPORTANT: Pre-heat the thermal cycler to 65°C. Program the thermal cycler using 21 µL volume, calculated temperature, and heated lid.

Set up a hybridization reaction at room temperature for each sample with the following components: 3 µL of Reporter CodeSet, 2 µL of Reporter Plus, 5 µL of Hybridization Buffer, up to 8 µL of the prepared MTE reaction, 2 µL of Capture ProbeSet, and 1 µL of Capture Plus. See TABLE 1.3 in Chapter 1 for the sample input recommendations specific to each instrument.

1. Remove aliquots of Reporter CodeSet, Capture ProbeSet, and Plus reagents from the freezer to thaw at room temperature. Invert several times to mix well and 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 at room temperature before using.

2. Add 26 µL of Reporter Plus reagent to the thawed aliquot of Reporter CodeSet (enough for 12 reactions). Invert several times to mix well, and spin down reagents.
3. Create a master mix by adding 70 µL of hybridization buffer to the tube containing the Reporter CodeSet and Reporter Plus reagent. Do not remove the Reporter CodeSet from this tube. **Do not add the Capture ProbeSet or Capture Plus reagent to the master mix.** Invert repeatedly to mix and spin down master mix.
4. Label the hybridization tubes. If using strip tubes, ensure they fit in a microfuge or picofuge and cut the strip in half if necessary.
5. Add 10 µL of master mix to each of the tubes. Use a fresh tip for each pipetting step to accurately pipette the correct volume.
6. Add up to 8 µL of prepared MTE reaction to each tube.
7. If necessary, add RNase-free water to bring the volume of each assay to 18 µL.
8. Add 14 µL of Capture-Plus reagent to the thawed aliquot of Capture ProbeSet. Invert several times to mix well, and spin down reagents.
9. Add 3 µL of Capture ProbeSet and Plus reagent mix to each tube immediately before placing at 65°C. Cap tubes and mix the reagents by inverting the tubes several times and flicking with a finger to ensure complete mixing. Briefly spin down and place the tubes in the pre-set 65°C thermal cycler.

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

10. Incubate reactions for at least 16 hours. Maximum hybridization time should not exceed 48 hours. 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: The purpose of selecting a fixed hybridization time followed by a ramp down to 4°C is to ensure equivalent hybridization times of all assays being directly compared in the same series of experiments. Counts continue to accumulate with time, 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 will increase sensitivity by increasing counts without significantly increasing background.

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8 nCounter Vantage Fusion Assays

This chapter describes the procedure for setting up RNA hybridization reactions for fusion gene analysis.

This method is designed to provide sensitive and specific detection of gene fusions from purified total RNA and RNA extracted from FFPE samples. nCounter Vantage Fusion assays utilize Nanostring's nCounter TagSet chemistry (see Chapter 1) with the addition of a Protector Probe, an oligonucleotide that is complimentary to a portion of Probe A, allowing for a new level of target specificity to be created (see FIGURE 8.1). Probe A, which links the unique reporter tag to a specific target sequence, recognizes both halves of the exon-exon junction. To minimize off-target hybridization, Probe A is made partially double-stranded by the addition of the Protector oligo to the hybridization mix. This combination of Probe A and the Protector Probe (also called Probe P) is referred to as a junction probe.

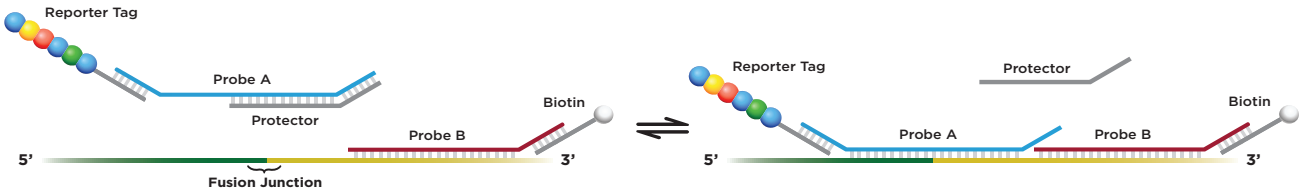


FIGURE 8.1 **Illustration of the nCounter Fusion assay.**

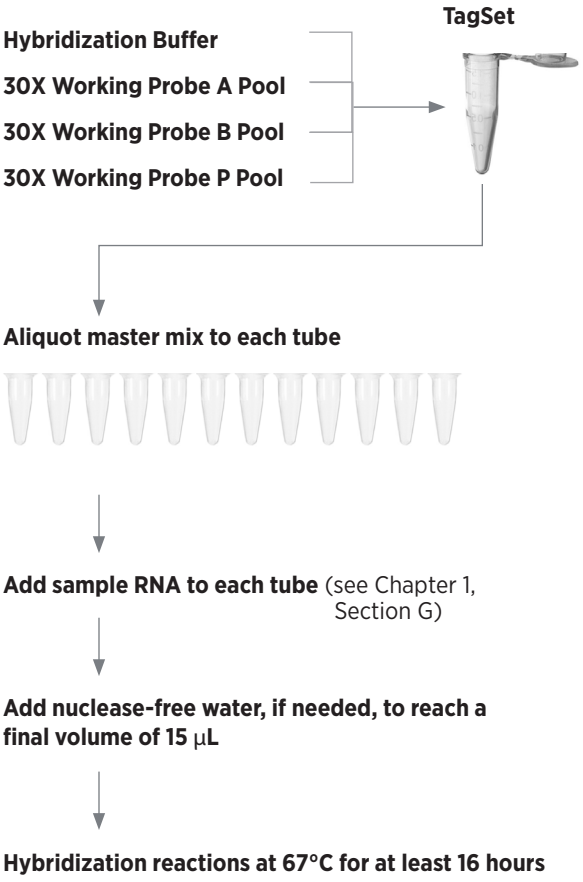
Hybridization Protocol for XT nCounter Vantage Fusion Assay

NOTE: If using XT Extension Probes, refer to the section Extension TagSets below prior to starting assay.

IMPORTANT: Do not vortex or pipette vigorously as this may shear the Reporter Tags. Mix only by flicking or inverting tubes. A picofuge is preferable when spinning down solutions due to its low speed. If using a microfuge, do not "pulse" the machine as it may reach maximum speed and spin the TagSet out of solution. Spin at less than 1,000 RCF for no more than 30 seconds.

To set up 12 assays, a master mix is created for 14 reactions to allow for dead volume in pipetting.

Each final hybridization reaction will have a volume of 15 µL and contain the following components: 5 µL of hybridization buffer, 2 µL of TagSet, 0.5 µL of 30X Working Probe A Pool, 0.5 µL of 30X Working Probe B Pool, 0.5 µL of 30X Working Probe P Pool and up to 6.5 µL of sample RNA.



Core TagSet Only

	Each Reaction	14 Reactions
Hybridization Buffer	5 µL	70 µL
TagSet	2 µL	28 µL
30X Working Probe A Pool	0.5 µL	7 µL
30X Working Probe B Pool	0.5 µL	7 µL
30X Working Probe P Pool	0.5 µL	7 µL
Sample*	Up to 6.5 µL	
Total Volume	15 µL	

Core TagSet + Extension TagSet

	Each Reaction	14 Reactions
Hybridization Buffer	5 µL	70 µL
TagSet	2 µL	28 µL
Extension TagSet	2 µL	28 µL
30X Working Probe A Pool	0.5 µL	7 µL
30X Working Probe B Pool	0.5 µL	7 µL
30X Working Probe P Pool	0.5 µL	7 µL
30X Extension Probe A Pool	0.5 µL	7 µL
30X Extension Probe B Pool	0.5 µL	7 µL
30X Extension Probe P Pool	0.5 µL	7 µL
Sample*	Up to 3 µL	
Total Volume	15 µL	

**NanoString recommends preparing samples with a high concentration to facilitate reducing the sample volume if an extension TagSet is necessary.*

FIGURE 8.2 Creating master mix and preparing RNA hybridization reactions.

1. Remove an aliquot of nCounter XT TagSet from the freezer and thaw it on ice. Invert several times to mix well, and briefly spin down the reagent at less than 1,000 RCF.
2. Create a 30X Working Probe A Pool by adding 22 µL of TE to the 3 µL Probe A Pool stock aliquot provided.
3. Create a 30X Working Probe B Pool by adding 22 µL of TE to the 3 µL Probe B Pool stock aliquot provided.
4. Create a 30X Working Probe P Pool by adding 22 µL of TE to the 3 µL Probe P Pool stock aliquot provided.
5. Each core TagSet tube contains 28 µL of reagent. Create a master mix by adding reagents directly into the TagSet tube as follows:
   a. Add 70 µL of Hybridization Buffer
   b. Add 7 µL of the 30X Working Probe A Pool
   c. Add 7 µL of the 30X Working Probe B Pool
   d. Add 7 µL of the 30X Working Probe P Pool
   e. If using an Extension TagSet, also add the following to the same master mix tube:
      - 28 µL of Extension TagSet
      - 7 µL of Extension 30x Working Probe A Pool (prepared in subsequent section)
      - 7 µL of Extension 30X Working Probe B Pool (prepared in subsequent section)
      - 7 µL of Extension 30X Working Probe P Pool (prepared in subsequent section)
6. Label a 12-tube strip.
7. Add 8.5 µL (or 12 µL if using an Extension TagSet) of master mix to each of the 12 tubes using a fresh pipette tip for each tube.

NOTE: See Chapter 1 for sample input recommendations

8. Add sample to each of the 12 tubes (maximum volume of 6.5 µL for core TagSet only, or 3 µL for core TagSet plus Extension TagSet) to each tube.
9. If necessary, add nuclease-free water to each tube to bring the volume of each reaction to 15 µL.
10. Cap tubes and mix the reagents by inverting the tubes several times and flicking with a finger to ensure complete mixing. Briefly spin down and immediately place the tubes in the pre-heated 67°C thermal cycler with a heated lid set at 75°C.
11. Incubate reactions for at least 16 hours. Maximum hybridization time should not exceed 48 hours. 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: The purpose of selecting a fixed hybridization time followed by a ramp down to 4°C is to ensure equivalent hybridization times of all assays being directly compared in the same series of experiments. Counts continue to accumulate with time, 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 will increase sensitivity by increasing counts without significantly increasing background.

Extension TagSets

Fusion panels are provided with all components required to prepare a hybridization, including TagSet and all required oligonucleotide probes. To add additional targets to an experiment, supplementary TagSet and probes should be ordered for each RNA target. The Extension TagSet, provided without ERCC controls, has unique Reporter Tags that do not coincide with any of those found in the core TagSets. Additional oligonucleotide probes should contain both Tag- and target-specific sequences that effectively link each target to a specific Reporter Tag and to the universal Capture Tag.

Designing and Ordering Probes

NanoString provides an online tool, nDesign™ Gateway, which enables users to design target-specific Extension Probe A and Probe B. Users may use nDesign Gateway to select specific probe sequences for human, mouse, and rat genes of interest and then establish associations between targets and Reporter Tags. Design data can then be downloaded and submitted to a third-party oligonucleotide manufacturer. See the instructions provided on the nDesign Gateway home page (http://store.nanostring.com) for additional information on the use of this tool.

Contact customer support (support@nanostring.com) to discuss options and tools available to facilitate the design of custom Protector Probes used in gene fusion analysis and for Extension TagSet ordering information.

Preparing Extension Probe Pools

Prior to hybridization, separate pools for Extension Probes A, B, and P need to be prepared.

NOTE: Some oligo suppliers will provide pooled oligos that can be used in place of creating your own Master Stocks. If utilizing this service, specify Master Stock pools at the concentrations outlined below. **These are Master Stocks and SHOULD NOT be used directly in the assay. Always dilute Master Stocks to create working pools before adding them to the assay.**

TE buffer (10 mM Tris pH 8, 1 mM EDTA) is recommended for oligo resuspension and to prepare Master Stock Pool aliquots. Store frozen oligos under conditions recommended by the oligonucleotide supplier.

Due to the low concentration of the final 30X working probe pools, NanoString suggests using TE-Tween® buffer (10 mM Tris pH 8, 1 mM EDTA, 0.1% Tween® 20) when preparing these dilutions. Long-term storage and reuse of the working probe pools is not recommended. A fresh dilution of the Master Stock should be made for subsequent assays.

IMPORTANT: Do not create a combined Master Stock containing Probe A, Probe B, and Probe P in the same tube; elevated background and lowered assay sensitivity may result.

the total volume to 1mL with TE. For each tube of 30X Working Probe Pool, add 4 µL of Master Stock Pool to 29 µL of TE-Tween.

TABLE 8.1 Final Concentrations for Probe Oligos and Pools

	Individual Oligo Resuspension	Master Stock Pool (200X dilution of resuspended oligo)	30X Working Probe Pool (8.3x dilution of Master Stock Pool)
Probe A	1 µM	5 nM	0.6 nM
Probe B	5 µM	25 nM	3 nM
Probe P	2 µM	10 nM	1.2 nM

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SYMBOLS AND DEFINITIONS

 — **Manufacturer**

— **Batch code / Lot number**

 — **Upper limit of temperature storage conditions**

 — **For Use by / Expiry Date**

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