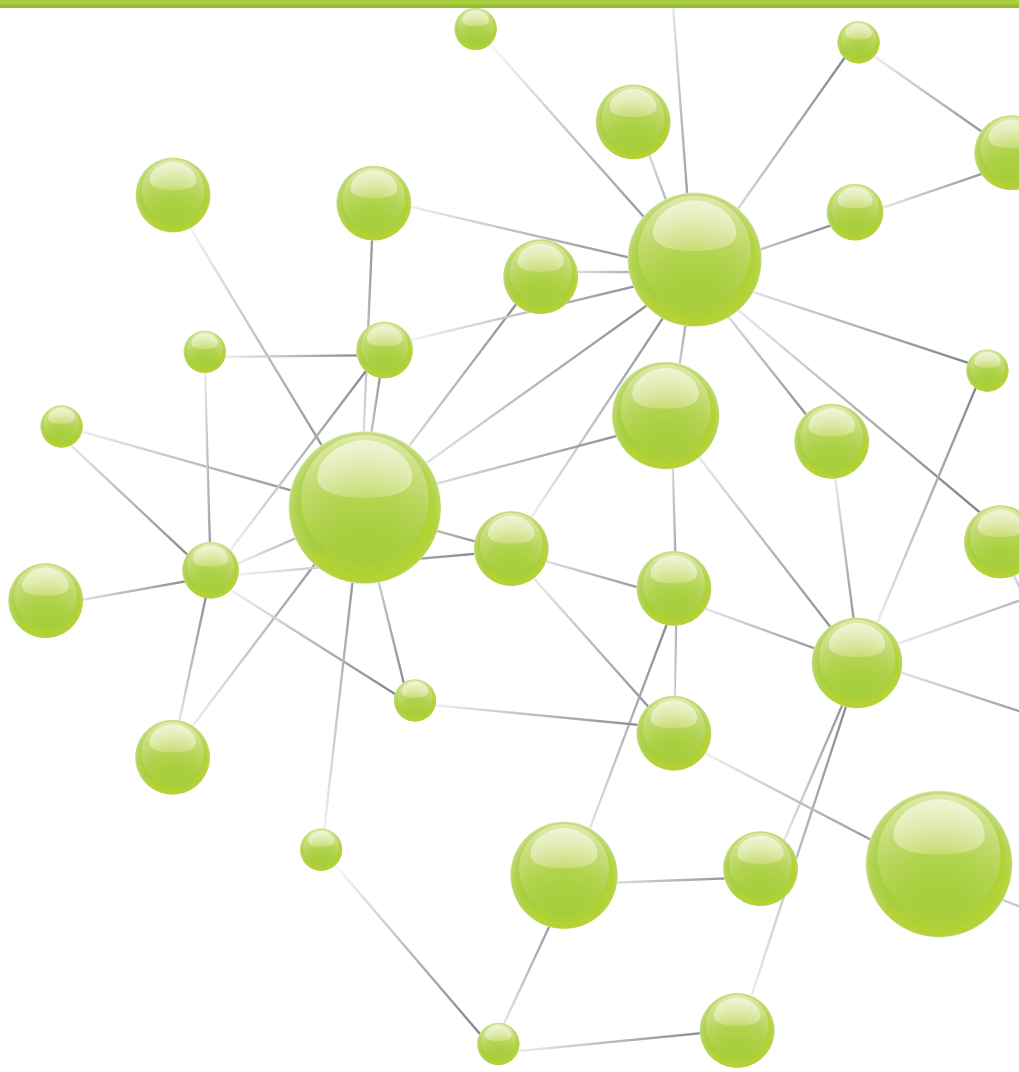
nanoString™



# nCounter® Elements™ XT Reagents
## USER MANUAL

**For Research Use Only. Not for Use in Diagnostic Procedures.**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## Versions for MAN-10086

| Version Number | Description |
|---|---|
| 01 | Initial release |

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# 1 Introduction

## A. Overview

This manual describes the recommended procedures for setting up hybridization reactions that use nCounter Elements™ TagSets reagents. nCounter Elements TagSets are flexible, off-the-shelf reagents that can be used for measuring digital counts of nucleic acid targets when used in conjunction with user-designed and -supplied target-specific oligonucleotide probes. Elements TagSets are compatible with a variety of sample sources including but not limited to: formalin-fixed paraffin-embedded (FFPE) tissue, fresh frozen tissue, blood products, fine-needle aspirates, cell lines, and immunoprecipitated nucleic acids.

All nCounter Elements TagSets are based on NanoString's core technology for measuring the abundance of nucleic acids via digital detection of fluorescent molecular barcodes. However, target recognition is decoupled from the molecular barcode through the use of intermediate probes, as discussed in the next section. These probes are designed and supplied by the user. The TagSets described in this manual allow for detection of up to 228 unique target sequences.

This manual describes in detail the recommended methods for setting up nCounter Elements TagSets protocols when working with RNA or DNA, as well as considerations for ordering target-specific oligonucleotide probes.

## B. nCounter Elements Technology

NanoString's nCounter Elements technology is based on molecular barcoding and digital quantification of target RNA or DNA sequences through the use of an nCounter Elements TagSet and target-specific oligonucleotide probe pairs (supplied by the user). The TagSet consists of fluorescently-labeled specific Reporter Tags and a biotinylated universal Capture Tag (**FIGURE 1.1** and **FIGURE 1.2**). The 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. The universal Capture Tag enables hybridized complexes to be captured on the imaging surface.

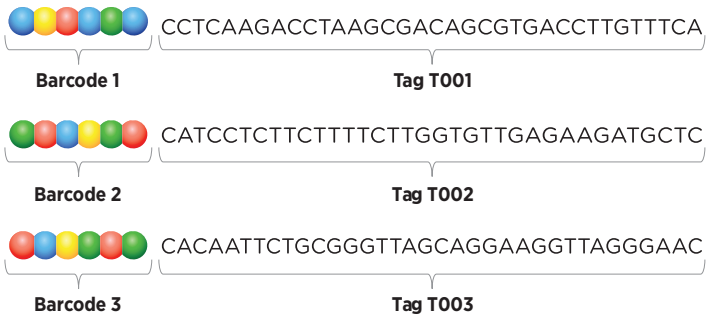



**FIGURE 1.1** Examples of Reporter Tags with unique fluorescent barcodes and recognition sequences.

During hybridization, 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 (FIGURE 1.2). 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.

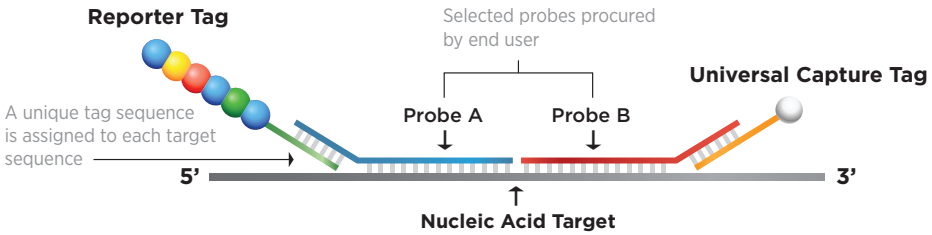



FIGURE 1.2 Customized oligonucleotide Probes A and B (supplied by the user, not provided by NanoString) hybridize with Reporter and Capture Tags, respectively, and the target nucleic acid to create a Tag Complex.

The TagSet and probes are present in the reaction in large excess to the target molecules to ensure that each target undergoes hybridization. After hybridization, excess probes and tags as well as non-target nucleic acids are washed away using a two-step magnetic bead-based purification process. The hybridized complex can then be captured via interaction of the biotinylated Capture Tag with streptavidin. The barcodes can then be imaged and counted.

Standard nCounter chemistry refers to the reporter and capture elements as a "CodeSet". NanoString differentiates nCounter Elements with the term "TagSet" in order to emphasize that the Reporter Tag and Capture Tag are distinct from Probe A and Probe B. CodeSets are created by combining a TagSet with the necessary probes (FIGURE 1.3).

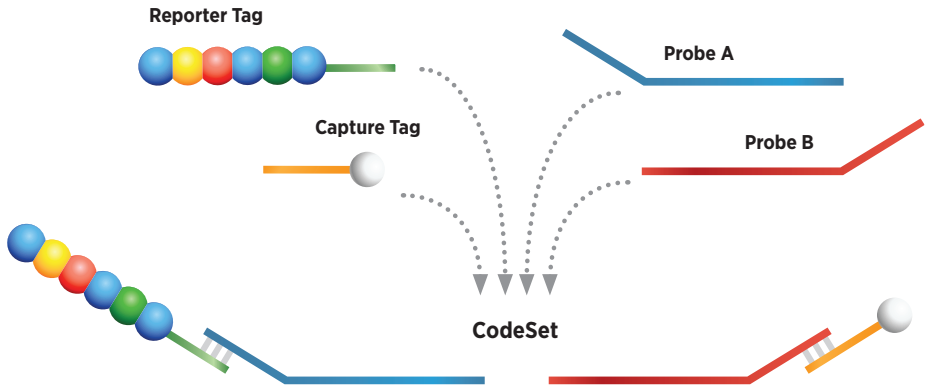



FIGURE 1.3 Combining an nCounter Elements TagSet with user-supplied oligonucleotide probes produces a custom CodeSet to detect a specific set of targets.

Ten different core TagSets are available. It is important to remember that larger TagSets include all tags found in smaller TagSets. For example, a core TagSet of 12 tags includes T001 through T012, and a core TagSet of 24 tags includes T001 through T024. The largest core TagSet currently available can detect up to 192 targets.

Two core TagSets cannot be combined to increase the number of targets in an experiment, as some of their Reporter Tags will overlap. To add additional targets to an ongoing experiment, use an Extension TagSet, which is available for 12, 24, or 36 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.

Assigning reference genes and other frequently analyzed targets to Reporter Tags with the lowest tag number will make the most efficient use of reagents in experiments where the target set is expected to evolve over the course of the project. In this way, the same oligonucleotide probes can be used for common targets in more than one experiment. There are multiple ways to examine additional targets: (1) order a larger TagSet, (2) order an Extension TagSet, or (3) order new oligonucleotide probes that link Reporter Tags in the existing TagSet with new targets.

**Existing TagSet**

| Core TagSet | |
|---|---|
| Tag | Target |
| T001 | Gene A |
| T002 | Gene B |
| T003 | Gene C |
| T004 | Gene D |
| T005 | Gene E |
| T006 | Gene F |
| T007 | Gene G |
| T008 | Gene H |
| T009 | Gene I |
| T010 | Gene J |
| T011 | Gene K |
| T012 | Gene L |

**Tags Reassigned**

| Core TagSet | |
|---|---|
| Tag | Target |
| T001 | Gene A |
| T002 | Gene B |
| T003 | Gene M |
| T004 | Gene N |
| T005 | Gene E |
| T006 | Gene F |
| T007 | Gene G |
| T008 | Gene O |
| T009 | Gene P |
| T010 | Gene Q |
| T011 | Gene R |
| T012 | Gene L |

**Extension TagSet Added**

| Core TagSet | | Extension TagSet | |
|---|---|---|---|
| Tag | Target | Tag | Target |
| T001 | Gene A | X001 | Gene M |
| T002 | Gene B | X002 | Gene N |
| T003 | Gene C | X003 | Gene O |
| T004 | Gene D | X004 | Gene P |
| T005 | Gene E | X005 | Gene Q |
| T006 | Gene F | X006 | Gene R |
| T007 | Gene G | X007 | Gene S |
| T008 | Gene H | X008 | Gene T |
| T009 | Gene I | X009 | Gene U |
| T010 | Gene J | X010 | Gene V |
| T011 | Gene K | X011 | Gene W |
| T012 | Gene L | X012 | Gene X |

**FIGURE 1.4** An existing TagSet can be modified by reassigning Reporter Tags to new targets or adding an extension TagSet. In this example, targets that have been added or replaced are indicated in blue.

## C. Sample Type Considerations

Sample input recommendations for nCounter reagents 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.

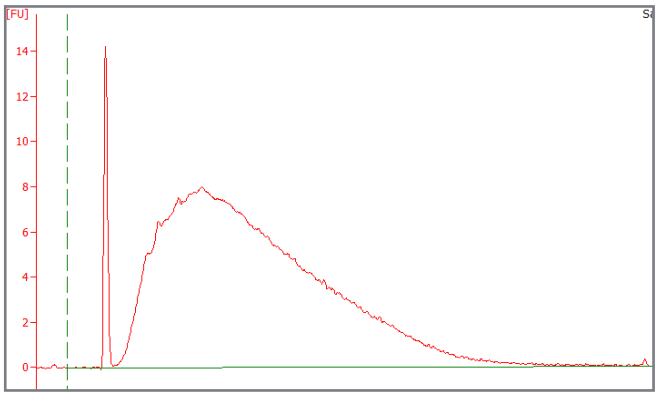



**FIGURE 1.5** Ideal fragmentation profile for nucleic acids. This fragmentation profile was obtained using an Agilent 2100 Bioanalyzer and an Agilent RNA 6000 Nano Kit to 300 ng of total RNA purified with a commercial FFPE extraction kit. The majority of the sample is greater than 300 nucleotides.

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,
- evaluating RNA quality using an Agilent Bioanalyzer® to measure nucleic acid fragmentation, and
- 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 used in Elements TagSets protocols 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.

**NOTE:** Recommendations for preparing DNA samples are provided in Chapter 5, *nCounter Elements XT for DNA*. For questions on additional sample types outside the scope of this section, contact NanoString Support at **support@nanostring.com**.

## D. 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 hybridizations 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 1.1** to determine the recommended sample input amount for most sample types included in this manual. These recommendations apply to sample *mass* only; sample *volume* does not vary between systems.

**TABLE 1.1** Recommended sample input mass for nCounter Elements XT reagents.

| Sample Type | nCounter Analysis System (MAX/FLEX) | nCounter *SPRINT* Profiler |
|---|---|---|
| mRNA | 100–300 ng | 50–100 ng |
| Fragmented DNA | 300 ng | 150 ng |
| ChIP DNA (unamplified) | 10 ng | 5 ng |
| ChIP DNA (whole genome amplification) | 100 ng | 50 ng |

### Using Whole Cell Lysates in Gene Expression

NanoString recommends a minimum of 5,000 to 10,000 cell equivalents per nCounter Elements 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 hybridized. Furthermore, the maximum sample input when using cell lysates depends on 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 Elements XT hybridization reaction. Other lysis buffers that contain chaotropic salts may alter nucleic acid hybridization thermodynamics and are compatible with nCounter Elements XT reagents with some modifications to protocol. These include RLT buffer 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 Elements XT hybridization reaction. For this reason, NanoString recommends the use of RLT buffer 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 *MAN-10051, Preparing Nucleic Acid from Fresh Frozen Samples for Use with nCounter Assays* for additional details.

## E. Provided Materials

TagSets are available for analysis of 12 to 228 targets. Core TagSets are pre-mixed with a comprehensive set of controls and enable analysis of 12 to 192 targets depending on the product ordered. Extension TagSets, provided without controls, can be added to any core TagSet to expand the multiplexing capability by 12, 24, or 36 targets. Each nCounter Elements TagSet is provided with sufficient material to perform 12 hybridization reactions.

**TABLE 1.4** Materials provided for nCounter Elements TagSets hybridization reactions

| Product Type | Description | Product Number |
|---|---|---|
| **nCounter Elements TagSet**<br>(Contains reagents for 12 reactions; includes controls) | 12 Tags<br>24 Tags<br>36 Tags<br>48 Tags<br>60 Tags<br>72 Tags<br>84 Tags<br>96 Tags<br>144 Tags<br>192 Tags | XT-ELE-P1TS-012-RUO<br>XT-ELE-P1TS-024-RUO<br>XT-ELE-P1TS-036-RUO<br>XT-ELE-P1TS-048-RUO<br>XT-ELE-P1TS-060-RUO<br>XT-ELE-P1TS-072-RUO<br>XT-ELE-P1TS-084-RUO<br>XT-ELE-P1TS-096-RUO<br>XT-ELE-P1TS-144-RUO<br>XT-ELE-P1TS-192-RUO |
| **nCounter Elements Extension TagSet**<br>(Contains reagents for 12 reactions; no controls provided) | 12 Tags<br>24 Tags<br>36 Tags | XT-ELE-P1EX-012-RUO<br>XT-ELE-P1EX-024-RUO<br>XT-ELE-P1EX-036-RUO |

### Contents Description

nCounter Elements TagSet: 12 Reaction Aliquot (1 x 28 µL) buffer, nucleic acids, nucleic acids with fluorescent dyes.
nCounter Elements Extension TagSet: 12 Reaction Aliquot (1 x 28 µL) buffer, nucleic acids, nucleic acids with fluorescent dyes.

### Storage and Handling

The expiration date for the nCounter Elements TagSet is listed on the outer box labeling. Reagents must be stored at -80°C.

# F. Recommended Materials

**TABLE 1.5** lists materials and instrumentation that are recommended or required for nCounter Elements XT protocols. Additional materials are recommended for RNA purification in **TABLE 1.6**. Information for cell lysates is provided in *Section C: Sample Input Recommendations*.

**TABLE 1.5** Materials recommended for all nCounter Elements XT protocols.

| 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 |

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

*†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 1.6** Additional materials recommended for gene expression using total RNA (standard protocol).

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

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

## 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.

# 2 Creating Oligonucleotide Probe Pools

## A. Overview

The oligonucleotide probes used with nCounter Elements must be formatted into two separate pools. One pool contains every Probe A, and the other pool contains every Probe B. Pools are initially created as Master Stocks, which are stored in aliquots at -20°C or -80°C. (Refer to the oligonucleotide supplier for specific storage recommendations and shelf-life information.) An aliquot of each Master Stock is then diluted immediately before the reaction to create Working Pools. These Working Pools are added to the hybridization master mix. **Never add a Master Stock directly to the hybridization master mix.**

The concentration of the oligonucleotides in a Working Pool is different for each type of probe. Each Probe A will be present at 0.6 nM, each Probe B will be present at 3 nM, and each Protector Probe (optional; for fusion gene reactions only) will be present at 1.2 nM. Probes will be diluted further when added to the hybridization reaction. Due to the dilute concentrations of many Working Pools, long-term storage and reuse are not recommended.

The protocols in this chapter provide an example of how to generate the Master Stocks and Working Pools for Probe A, Probe B, and the Protector Probes. Depending on the oligonucleotide format obtained from the supplier, different pipetting volumes and dilutions may be necessary to achieve the required concentrations.

**IMPORTANT:** The concentrations of each probe in the hybridization reaction are critical for maximizing the sensitivity of the reaction. Be sure to follow appropriate pooling and dilution protocols carefully to create accurate Working Pools.

**NOTE:** The following guidelines cover general steps for use with nCounter Elements TagSets. Each specific application will require optimization and validation using appropriate performance metrics defined by the end user.

## B. Creating Master Stocks

**NOTE:** Some oligo suppliers will provide pooled oligos which can be used in place of creating your own Master Stocks. If utilizing this service, specify a pool of Probe As at a final concentration of 5 nM per oligo and a separate pool of Probe Bs at a final concentration of 25 nM per oligo. Pooled probes provided by oligo suppliers at the recommended concentrations must still be diluted to create Working Pools (see next section) before addition to the hybridization reaction.

**IMPORTANT: Always create separate Master Stocks for Probe A, Probe B, and Protector Probes.** Do not create a combined Master Stock containing Probe A, Probe B, and Protector Probes in the same tube; elevated background and lowered reaction sensitivity may result.

1. NanoString recommends the following concentrations for resuspending individual oligonucleotides: Probe A oligonucleotides at a 1 µM concentration, Probe B oligonucleotides at a 5 µM concentration, and Protector probes at a 2 µM concentration. Oligonucleotides should be resuspended in TE (10 mM Tris pH 8, 1 mM EDTA) or a similar buffer and stored frozen under conditions recommended by the supplier. To prepare Master Probe Stocks, begin by removing the appropriate Probe A and Probe B oligonucleotides from storage and thawing them on ice.
2. Probe A Master Stock
   a. Pipet 5 µL of each Probe A (starting concentration 1 µM) into a 1.7 mL microfuge tube.
   b. Add TE to a final combined volume of 1 mL.
   c. The final concentration of each Probe A in the Probe A Master Stock will be 5 nM.
   d. Store in aliquots at -20°C or -80°C as recommended by the supplier.
3. Probe B Master Stock
   a. Pipet 5 µL of each Probe B (starting concentration 5 µM) into a 1.7 mL microfuge tube.
   b. Add TE to a final combined volume of 1 mL.
   c. The final concentration of each Probe B in the Probe B Master Stock will be 25 nM.
   d. Store in aliquots at -20°C or -80°C as recommended by the supplier.
4. Protector Probe Master Stock (optional; for fusion gene reactions only)
   a. Pipet 5 µL of each Protector Probe (starting concentration 2 µM) into a 1.7 mL microfuge tube.
   b. Add TE to a final combined volume of 1 mL.
   c. The final concentration of each Protector Probe in the Protector Probe Master Stock will be 10 nM.
   d. Store in aliquots at -20°C or -80°C as recommended by the supplier.

**NOTE:** The probes in the Master Stocks must be appropriate for the targets being queried. If reporter tags are reassigned to new targets, new Master Stocks containing the specific set of appropriate probes must be created. Do **NOT** add additional probes to existing Master Stocks. If using an Extension Tagset as well as a Core TagSet, create separate Master Stocks for the Extension Probes.

**IMPORTANT:** Minimize freeze-thaw cycles by storing Master Stocks in appropriate aliquots at -20°C or -80°C. Thaw each aliquot only once and then place at 4°C for use in creating multiple Working Pools. A suitable aliquot size for the workflow can be calculated from the information in TABLE 2.1 based on your expected reaction throughput. Follow the supplier's guidance on stability of the oligonucleotide stocks at 4°C.

## C. Creating Working Pools

**IMPORTANT: Always create separate Probe A and Probe B 30X Working Probe Pools.** Do **NOT** create a combined 30X Working Probe Pool containing Probes A and Probe B in the same tube; elevated background and lowered sensitivity may result.

1. Probe A Working Pool
   a. Determine the number of reactions to be performed. Starting with the Probe A Master Stock, follow the 8.3-fold dilution outlined in TABLE 2.1 to generate a Working Pool of all Probe As at the appropriate scale.
   b. Mix well and spin down contents to the bottom of the tube. The concentration of each Probe A will be 0.6 nM.
   c. Proceed to the hybridization protocol. The concentration of each Probe A in the hybridization reaction will be 20 pM.
2. Probe B Working Pool
   a. Determine the number of reactions to be performed. Starting with the Probe B Master Stock, follow the 8.3-fold dilution outlined in TABLE 2.1 to generate a Working Pool of all Probe Bs at the appropriate scale.
   b. Mix well and spin down contents to the bottom of the tube. The concentration of each Probe B will be 3 nM.
   c. Proceed to the hybridization protocol. The concentration of each Probe B in the hybridization reaction will be 100 pM.
3. Protector Probe Working Pool (optional; for fusion gene reactions only)
   a. Determine the number of reactions to be performed. Starting with the Protector Probe Master Stock, follow the 8.3-fold dilution outlined in TABLE 2.1 to generate a Working Pool of all Protector Probes at the appropriate scale.
   b. Mix well and spin down contents to the bottom of the tube. The concentration of each Protector Probe will be 1.2 nM.
   c. Proceed to the hybridization protocol. The concentration of each Protector Probe in the hybridization reaction will be 40 pM.

**IMPORTANT:** Due to the dilute DNA concentrations of many Working Pools, long-term storage and reuse is not recommended. A fresh dilution of each Master Stock should be made for subsequent hybridizations.

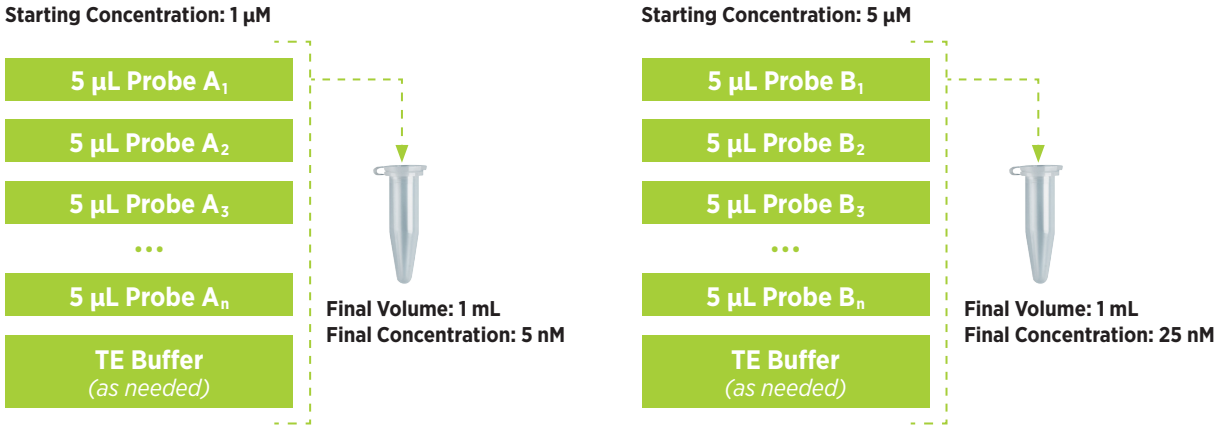
TABLE 2.1 Guide to diluting Master Stocks to generate Working Pools. Due to the dilute nature of the final pool, use of TE-Tween® (10 mM Tris pH 8, 1 mM EDTA, 0.1% Tween-20) is recommended.

| Number of Reactions | Aliquot from Master Probe Stock (µL) | TE-Tween (µL) | Final Volume (µL) |
|---|---|---|---|
| 12 | 4 | 29 | 33 |
| 24 | 4 | 29 | 33 |
| 36 | 5 | 37 | 42 |
| 48 | 7 | 51 | 58 |
| 60 | 8 | 59 | 67 |
| 72 | 10 | 73 | 83 |
| 84 | 11 | 81 | 92 |
| 96 | 13 | 95 | 108 |
| 144 | 19 | 139 | 158 |
| 192 | 26 | 191 | 217 |

# D. Workflow for Creating Probe Pools

## 1. Create Master Stocks

Each  requires one Probe A pool and one Probe B pool. These pools contain one probe for each target in the hybridization. (Hybridizations to detect fusion genes require a third pool of Protector Probes.) Dilute the pooled probes with TE buffer to obtain a final volume of 1 mL.

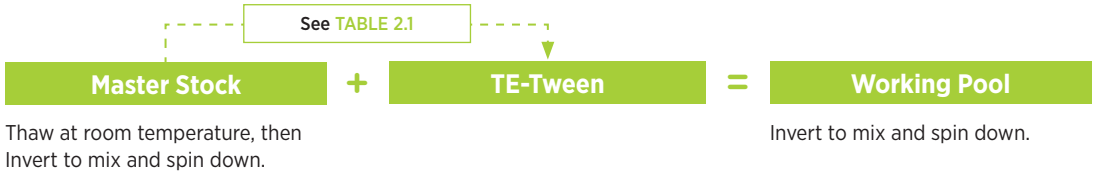



**Store the Master Stocks at -80°C and dilute immediately before use. Never add Master Stocks directly to the hybridization reaction.**

**IMPORTANT:** Store the Master Stocks in aliquots at -80°C and dilute immediately before use. Never add Master Stocks directly to the hybridization reaction. Avoid freeze-thaw cycles.

## 2. Dilute Master Stocks to Create Working Pools

Thaw each Master Stock and create a Working Pool by following the guidelines in TABLE 2.1 on the previous page. Repeat this step for each probe pool, including Probe A and Probe B. Protocols to detect fusion genes require a third pool of Protector Probes.




**IMPORTANT:** Master Stocks should be diluted immediately before use. Do not store Working Pools.

# 3 nCounter Elements XT for Gene Expression

## A. Overview

nCounter Elements TagSets technology can be used to detect RNA for measuring gene expression. These reagents are compatible with a wide variety of sample types, including whole cell lysate as well as RNA purified from FFPE tissue, fresh frozen tissue, blood products, fine-needle aspirates, cell lines, and others. See below for recommended instructions for hybridizing the Reporter and Capture Tags, oligonucleotide probes, and RNA sample.

**NOTE:** Each specific application using nCounter Elements TagSets will require optimization and validation using appropriate performance metrics defined by the end user.

## B. Setting Up 12 Hybridization Reactions for RNA

All reagents are provided for multiples of 12 reactions. Scale up the suggested volumes for additional reactions. Note that the instructions for 12 reactions use a multiplier of 14 to allow for dead volume in the master mix.

**IMPORTANT:** During setup, do not vortex or pipette vigorously to mix as it may shear the Reporter 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 rpm 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 use 15 µL volume. Set at 67°C for the selected hybridization time (typically 16–21 hours; see **Step 12** for guidance) and then ramp down to 4°C. To minimize the potential for evaporation, the thermal cycler lid should be set at 5° above the block temperature.

1. See the instructions in Chapter 2 to create the Working Pools for each probe. The accuracy of probe concentration in the hybridization reaction is important for maximizing sensitivity. Never add the Master Stock directly to the master mix.
2. If using total RNA, go to **Step 4**.
3. If using cell lysates, see *Using Whole Cell Lysates in Gene Expression* in Chapter 1, and the accompanying instructions for preparing cell lysates.
4. Remove an aliquot of TagSet from the freezer and thaw it at room temperature. Invert several times to mix well, and spin down the reagent.
5. Create a master mix by adding 70 µL of hybridization buffer and 7 µL of the Probe A Working Pool directly to the tube containing the TagSet. Invert repeatedly to mix and spin down master mix.
   a. If using an Extension TagSet, also add 28 µL of the Extension TagSet reagent and 7 µL of the Extension Probe A Working Pool before mixing.
6. Add 7 µL of the Probe B Working Pool to the master mix. RNAse-free water may also be added to this mix if the volume of the individual RNA samples is less than 7 µL and is constant. (Add enough water for 14 reactions to allow 2 reaction's worth of dead volume.) Invert repeatedly to mix and spin down master mix.
   a. If using an Extension TagSet, also add 7 µL of the Extension Probe B Working Pool before mixing.
7. Label the hybridization tubes. If using strip tubes, ensure they fit in a microfuge or picofuge and cut the strip in half if necessary.
8. Add 8 µL (core TagSet only) or 11 µL (core TagSet plus Extension TagSet) 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.
9. Add the RNA sample to each tube (up to 7 µL for core TagSet only, or 4 µL for core TagSet plus Extension TagSet).
10. If necessary, add RNAse-free water to each tube to bring the volume of each reaction to 15 µL.
11. Cap tubes and mix the reagents by inverting the strip tubes several times and flicking with a finger to ensure complete mixing. Briefly spin and immediately place the tubes in the pre-heated 67°C thermal cycler.
12. Incubate reactions for at least 16 hours but no more than 48 hours. Ramp reactions down to 4°C when complete and process the following day. Do not leave the reactions at 4°C for more than 24 hours or increased background may result.

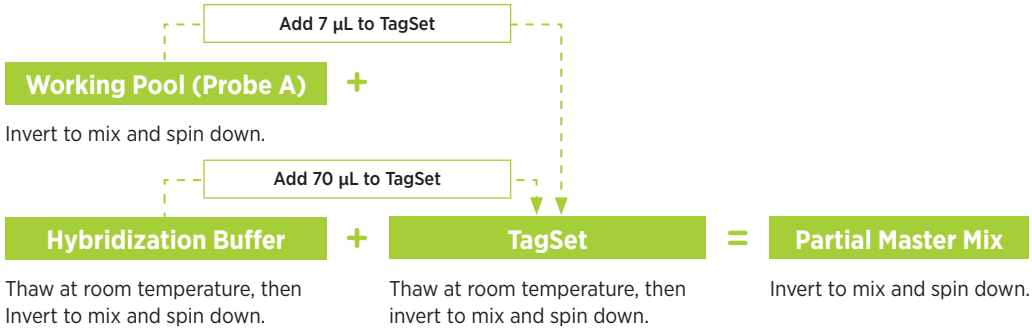
**NOTE:** The purpose of selecting a fixed hybridization time followed by a ramp down to 4°C is to ensure equivalent hybridization times of all reactions being directly compared in the same series of experiments. Hybridization efficiency improves with time, and target counts may increase 5% per hour between 16 and 24 hours. Although a 16-hour incubation is adequate for most purposes, a longer incubation will typically increase counts.

# C. Workflow for nCounter Elements XT for Gene Expression

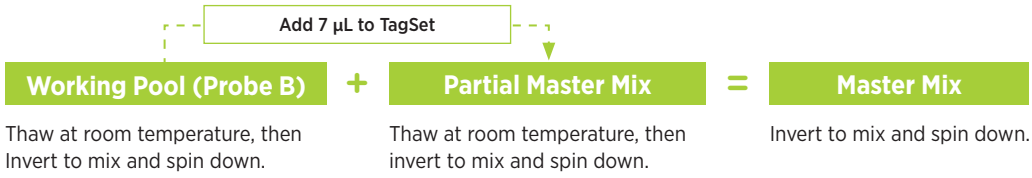
## 1. Prepare 30X Working Probe Pools

1. See instructions in Chapter 2 to prepare working pools. Never add master stocks directly to the hybridization reaction.
2. Do not store working pools. Prepare each working pool using a new aliquot of the master stock. Exact volumes depend on the number of hybridizations as indicated in TABLE 2.1.

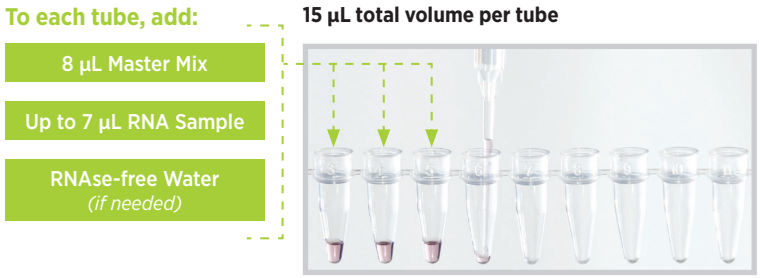
## 2. Create Partial Master Mix




## 3. Complete Master Mix




## 4. Set up Hybridization Reactions




## 5. Begin Hybridization

1. Cap tubes.
2. Mix by inverting tubes.
3. Briefly spin down.
4. Incubate at 67°C for minimum of 16 hours.

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# 4 nCounter Elements XT for Fusion Genes

## A. Overview

nCounter Elements TagSets can be used to detect RNA as a measure of gene fusion. These reagents are compatible with a wide variety of sample types, including whole cell lysates, RNA purified from FFPE tissue, fresh frozen tissue, blood products, fine-needle aspirates, cell lines, and others. These are recommended instructions for hybridizing the TagSet with oligonucleotide probes and the sample RNA.

**NOTE:** Each specific application using nCounter Elements TagSets will require optimization and validation using appropriate performance metrics defined by the end user.

## B. Hybridization Protocol for Fusion Genes

All reagents are provided for multiples of 12 reactions. Scale up the suggested volumes for additional reactions. Note that the instructions for 12 reactions use a multiplier of 14 to allow for dead volume in the master mix.

**GENERAL PROBE HANDLING WARNING:** During setup of the reaction, do not vortex or pipette vigorously to mix as it may shear the 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 rpm 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 using 15 µL volume. Set at 67°C for the selected hybridization time (typically 16–21 hours; see **Step 10** for guidance) and then ramp down to 4°C. To minimize the potential for evaporation, the thermal cycler lid should be set at 5° above the block temperature.

1. See the directions in Chapter 2 to create Working Pools for each probe. The accuracy of probe concentration in the hybridization reaction is important for maximizing sensitivity. Never add the Master Stock directly to the master mix.
2. Remove an aliquot of the TagSet from the freezer and thaw at room temperature. Invert several times to mix well and spin down the reagent.
3. Create a master mix by adding 70 µL of hybridization buffer, 7 µL of the Probe A Working Pool, and 7 µL of the Protector Probe Working Pool directly to the tube containing the TagSet. Invert repeatedly to mix and spin down master mix.
   a. If using an Extension TagSet, also add 28 µL of the Extension TagSet reagent, 7 µL of the Extension Probe A Working Pool and 7 µL of the Extension Protector Probe Working Pool before mixing.
4. Add 7 µL of the Probe B Working Pool to the master mix. RNAse-free water may also be added to this mix if the volume of the individual RNA samples is less than 7 µL and is constant. (Add enough water for 14 reactions to allow 2 reactions' worth of dead volume.) Invert repeatedly to mix and spin down master mix.
   a. If using an Extension TagSet, also add 7 µL of the Extension Probe B Working Pool before mixing.
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 8.5 µL (core TagSet only) or 12 µL (core TagSet plus Extension TagSet) 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 the RNA sample to each tube (up to 6.5 µL for core TagSet only, or 3 µL for core TagSet plus Extension TagSet).
8. If necessary, add RNAse-free water to each tube to bring the volume of each reaction to 15 µL.
9. 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 and immediately place the tubes in the pre-heated 67°C thermal cycler.
10. Incubate reactions for at least 16 hours but no more than 48 hours. Ramp reactions down to 4°C when complete and process the following day. Do not leave the reactions at 4°C for more than 24 hours or increased background may result.

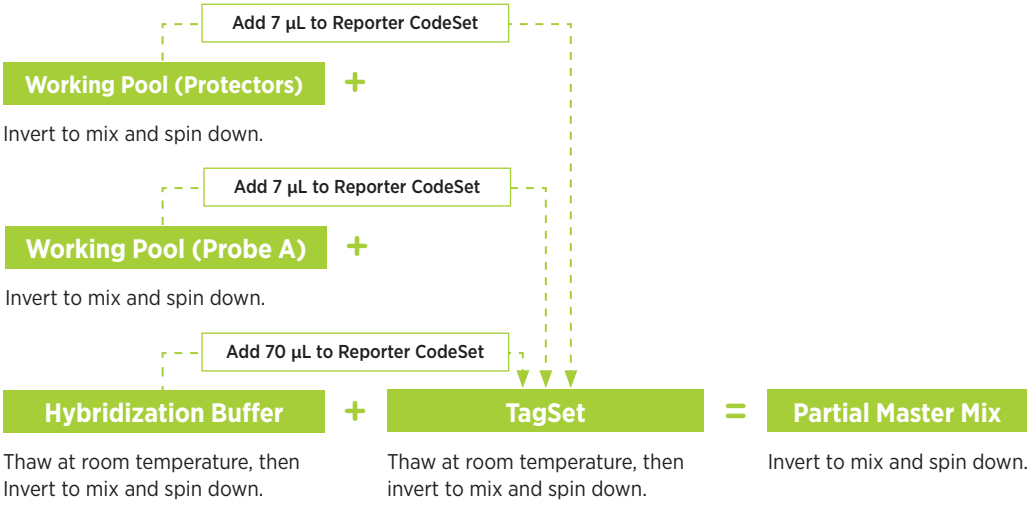
**NOTE:** NanoString suggests selecting a fixed hybridization time followed by a ramp down to 4°C to ensure equivalent hybridization times of all reactions that will be directly compared in the same series of experiments. Hybridization efficiency improves with time, and target counts may increase 5% per hour between 16 and 24 hours. Although a 16-hour incubation is adequate for most purposes, a longer incubation will typically increase counts.

# C. Workflow for nCounter Elements XT for Fusion Genes

## 1. Prepare 30X Working Probe Pools

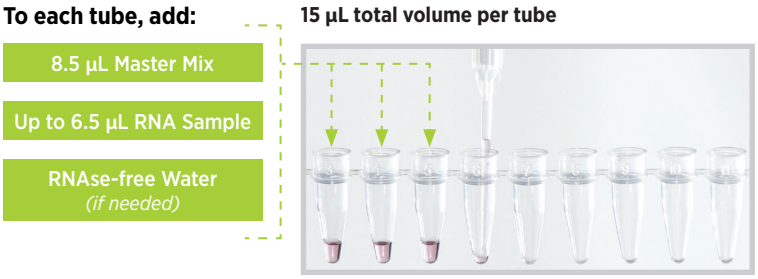
1. See instructions in Chapter 2 to prepare working pools. Never add master stocks directly to the hybridization reaction.
2. Do not store working pools. Prepare each working pool using a new aliquot of the master stock. Exact volumes depend on the number of hybridizations as indicated in **TABLE 2.1**.

## 2. Create Partial Master Mix




## 3. Complete Master Mix


## 4. Set up Hybridization Reactions




## 5. Begin Hybridization

1. Cap tubes, and mix by inverting tubes.
2. Briefly spin down.
3. Incubate at 67°C for minimum of 16 hours.

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# 5 nCounter Elements XT for DNA

## A. Overview

nCounter Elements TagSet technology can be used to detect DNA for purposes such as determining copy number variation (CNV) and performing counts of genetic loci in enriched DNA. Reagents are compatible with a wide variety of DNA sample types, including formalin-fixed paraffin-embedded (FFPE) tissue, fresh frozen tissue, blood products, fine-needle aspirates, cell lines, and immunoprecipitated DNA. However, additional sample processing steps are required for most types of DNA samples. Read these important considerations before performing the hybridization protocol if using nCounter Elements TagSet reagents with DNA samples.

### Fragmentation

For guidance regarding DNA fragmentation based on your sample type, see:

- *MAN-10050, Preparing Nucleic Acid from FFPE Samples for Use with nCounter Assays* **or**
- *MAN-10051, Preparing Nucleic Acid from Fresh Frozen Samples for Use with nCounter Assays*

### CNV Reference Sample Considerations

Genomic DNA from a reference sample may be used to determine copy number with nCounter Elements TagSets. Reference samples should not contain copy number variations in the test regions. NanoString recommends selecting one or more genomic DNA reference samples that are of the same sample type as the test samples (e.g., FFPE, cell line, or blood) and have been purified and fragmented in the same way as the test samples. The reference DNA(s) should be run at least once whenever the content of the CodeSet is changed (i.e., different loci are being measured).

## Denaturation

DNA is typically double-stranded in its biological conformation, whereas RNA is single-stranded. Double-stranded DNA must be denatured prior to hybridization.

**IMPORTANT:** NanoString recommends a denaturation temperature of 95°C for 5 minutes, followed by an immediate ramp down to 4°C or quick cooling on ice for 2 minutes. Incomplete denaturation results in decreased sensitivity.

## DNA Input Amount

The recommended mass of input genomic DNA for most hybridization reactions is 150–300 ng, three times greater than the mass of input RNA recommended as a starting point when measuring gene expression. This is due to the fact that most non-repetitive genomic DNA sequences are present at two copies per cell in normal diploid samples. In contrast, the amount of a given RNA can range from one to many thousands of copies per cell. The maximum sample input volume for DNA hybridization is 7 µL using a core TagSet, and 4 µL if an Extension TagSet is added. Thus, the sample DNA concentration must be high enough to provide an adequate mass.

If the available sample is not a limiting factor, increasing the DNA input amount to 600 ng may provide better resolution for small variations in CNV reactions and increase accuracy for highly-degraded FFPE samples.

For DNA that has been enriched (*e.g.*, via immunoprecipitation, exome capture, or target enrichment) the input requirements are typically lower than those required for standard genomic DNA. For these sample types, recommended starting input amounts are 5-50 ng depending on the level of enrichment. Input amounts may need to be optimized for some experimental conditions.

## DNA Quality

DNA should be free of contaminating RNA for accurate copy number analysis. NanoString strongly recommends that DNA preparations be RNAse-treated as described by the extraction kit manufacturer. RNA contamination may negatively impact the quality of the data in two ways:

1. Even when probes are selected to non-coding sequences, the presence of unprocessed RNA may result in artificially increased signal and copy number determinations.
2. RNA may result in over-estimation of DNA concentration when measured by UV absorbance, leading to lower than recommended DNA input amounts and lower counts. For pure genomic DNA preparations, NanoString recommends A260/280 ratios between 1.7 and 1.9 and A260/230 ratios between 1.3 and 2.0. Fluorescence-based reactions using dyes specific for DNA may provide the most accurate concentration measurements if RNA contamination is suspected.

DNA extracted from FFPE samples is typically degraded due to the fixation and storage process. NanoString recommends that the average size of extracted DNA be greater than 1 kb prior to fragmentation.

## B. DNA Hybridization Protocol

**GENERAL PROBE HANDLING WARNING:** During setup of the hybridization, do not vortex or pipette vigorously to mix as it may shear the Reporter 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 rpm 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 using 15 µL volume. Set at 67°C for the selected hybridization time (typically 16-21 hours; see **Step 11** for guidance) and then ramp down to 4°C. To minimize the potential for evaporation, the thermal cycler lid should be set at 5° above the block temperature.

1. See the directions in Chapter 2 to create Working Pools for each probe. The accuracy of probe concentration in the hybridization reaction is important for maximizing sensitivity. Never add the Master Stock directly to the master mix.
2. Remove an aliquot of the TagSet from the freezer and thaw at room temperature. Invert several times to mix well and spin down the reagent.
3. Create a master mix by adding 70 µL of hybridization buffer and 7 µL of the Probe A Working Pool directly to the tube containing the TagSet. Invert repeatedly to mix and spin down master mix.
    a. If using an Extension TagSet, also add 28 µL of the Extension TagSet reagent and 7 µL of the Extension Probe A Working Pool before mixing.
4. Add 7 µL of the Probe B Working Pool to the master mix. RNAse-free water may also be added to this mix if the volume of the individual RNA samples is less than 7 µL and is constant. (Add enough water for 14 hybridizations to allow two hybridizations' worth of dead volume.) Invert repeatedly to mix and spin down master mix.
    a. If using an Extension TagSet, also add 7 µL of the Extension Probe B Working Pool before mixing.
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 (core TagSet only) or 11 µL (core TagSet plus Extension TagSet) 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. Denature DNA samples or restriction digest at 95°C for 5 minutes, followed by an immediate ramp down to 4°C or quick cooling on ice for 2 minutes.
8. Add the denatured DNA sample or restriction digest to each tube (up to 7 µL for core TagSet only, or 4 µL for core TagSet plus Extension TagSet).
9. If necessary, add RNAse-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.
11. Incubate reactions for at least 16 hours but no more than 48 hours. Ramp reactions down to 4°C when complete and process the following day. Do not leave the reactions at 4°C for more than 24 hours or increased background may result.

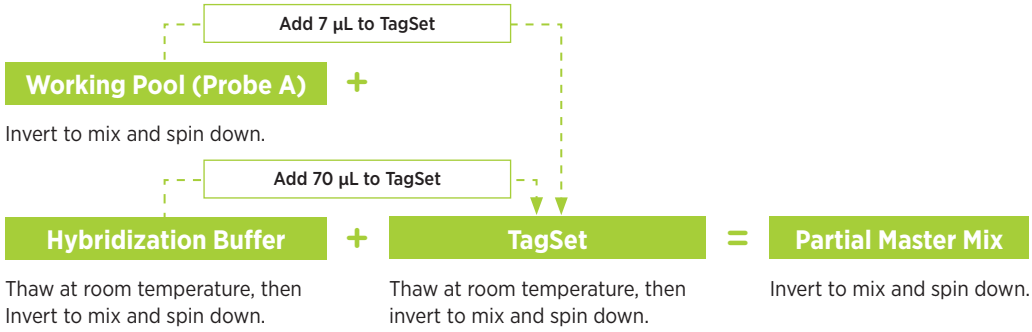
**NOTE:** NanoString suggests selecting a fixed hybridization time followed by a ramp down to 4°C to ensure equivalent times of all hybridizations that will be directly compared in the same series of experiments. Hybridization efficiency improves with time, and target counts may increase 5% per hour between 16 and 24 hours. Although a 16-hour incubation is adequate for most purposes, a longer incubation will typically increase counts.

# C. Workflow for nCounter Elements XT for DNA

## 1. Prepare Working Pools

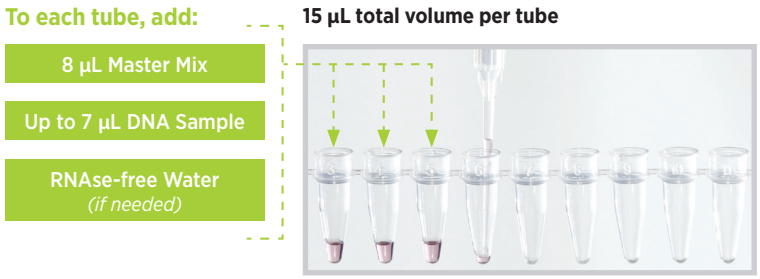
1. See Chapter 2 to prepare working pools for each probe. Never add master stocks directly to the hybridization reaction.
2. Do not store working pools. Prepare each working pool using a new aliquot of the master stock. Exact volumes depend on the number of hybridizations as indicated in TABLE 2.1.

## 2. Create Partial Master Mix




## 3. Complete Master Mix


## 4. Set up Hybridization Reactions




## 5. Begin Hybridization

1. Cap tubes, and mix by inverting tubes.
2. Briefly spin down.
3. Incubate at 67°C for minimum of 16 hours.

# 6 Evaluating Experimental Test Characteristics

Each individual laboratory should evaluate the performance of nCounter Elements to ensure that it is adequate for the specific application for which the reagents are being used. Appropriate performance metrics should be defined based on the desired attributes of the final user-designed reaction. Below are examples of analyses that may be performed to evaluate reproducibility, compare responses to changing sample input concentration, and correct for lot-to-lot variability.

**NOTE:** Note that these examples are not meant to imply specific performance characteristics of nCounter Elements reagents; results may vary depending on experiment design, sample input, or other factors.

## A. Technical Replicates

In order to determine the reproducibility of a reaction utilizing nCounter Elements reagents, two replicates testing the same purified RNA sample can be hybridized to an nCounter Elements TagSet and associated target-specific probes (Probe A and Probe B). Replicates can be plotted in linear space with the first replicate on the *y*-axis and the second replicate on the *x*-axis. Linear regression can then be performed to generate an $R^2$ value indicating the degree of correlation between the two replicates. In the example shown in FIGURE 6.1, technical replicates on 100 ng of target RNA were run in a 192-Plex hybridization and compared as described above.

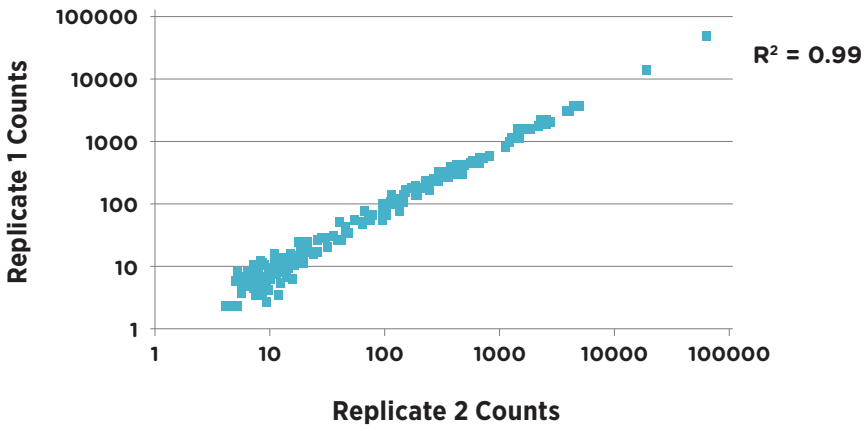



FIGURE 6.1 Correlation between two hybridization reactions using the same RNA sample.

## B. Sample Input Titration

In order to evaluate the response of reactions utilizing nCounter Elements reagents to varying amounts of sample input, comparisons can be made by titrating sample inputs. Data from one sample can be defined as the baseline value to which the others are compared. Linear regression can then be applied to determine a slope value that should correspond to the concentration of the test sample relative to the baseline sample. In the example shown in **FIGURE 6.2**, normalized counts from total RNA input amounts of 100, 200, 300, and 500 ng of the same sample are plotted. The 100 ng is used as the baseline value on the *x*-axis against which the others are plotted. The slopes correlate closely with the expected values of 2, 3, and 5 with 99% correlation, indicating a linear response to increasing target concentration.

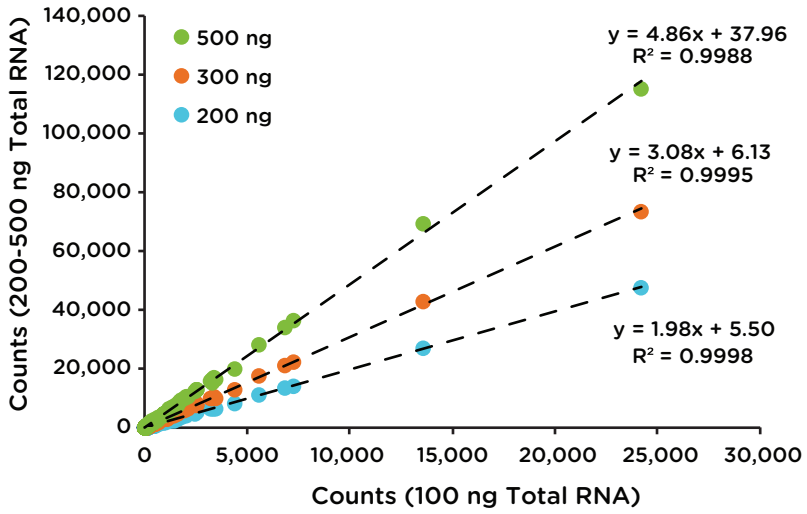


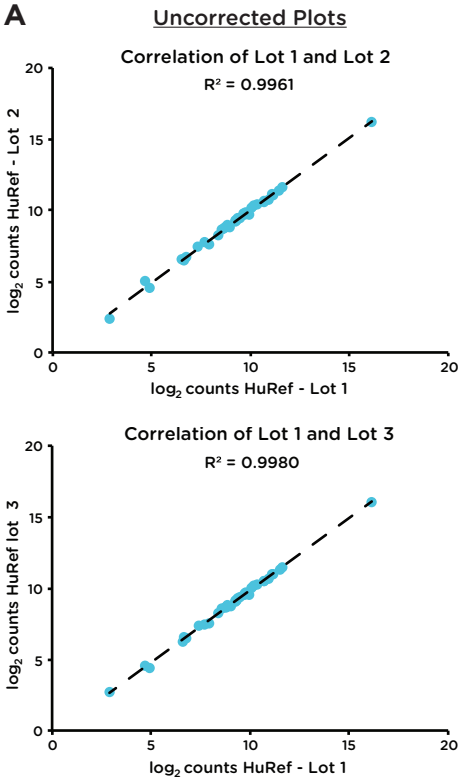

**FIGURE 6.2** Linear regression analyses of a sample titration.

## C. Calculating Correction Factors Between Lots

In order to evaluate variation between lots of nCounter Elements TagSets reagents and calculate correction factors, comparisons can be made between one or more lots of Elements reagents run on the same reference sample. Results from new lots can be plotted against older lots and linear regression applied to determine $R^2$ values quantifying the correlation between lots. For each individual gene or target being analyzed, a correction factor to enable normalization between lots can be calculated by dividing the value (number of counts) obtained for each target using Elements Lot 1 (reference lot) with the value obtained using Lot 2. When Lot 2 is run on an experimental sample, each data point can be multiplied by its correction factor to create a data set normalized to Lot 1.

In the **FIGURE 6.3**, three lots (Lots 1–3) of 36-plex nCounter Elements reagents were compared (**FIGURE 6.3A**) and correction factors were created using Lot 1 as the reference lot (**FIGURE 6.3B**) and applied to experimental data generated with Lots 2 and 3 (**FIGURE 6.3C**) as described above.

**A**




**B**

| Target | Correction Factor | |
|---|---|---|
| | Lot 2 | Lot 3 |
| Gene 1 | 0.88 | 0.98 |
| Gene 2 | 0.90 | 0.99 |
| Gene 3 | 0.86 | 0.97 |
| Gene 4 | 0.86 | 0.98 |
| Gene 5 | 0.91 | 1.04 |
| Gene 6 | 0.83 | 0.95 |
| Gene 7 | 0.88 | 1.00 |
| Gene 8 | 0.89 | 0.99 |
| Gene 9 | 0.92 | 1.00 |
| Gene 10 | 0.86 | 0.97 |
| Gene 11 | 0.95 | 1.04 |
| Gene 12 | 0.86 | 0.96 |
| Gene 13 | 0.91 | 1.02 |
| Gene 14 | 0.87 | 1.00 |
| Gene 15 | 0.87 | 0.96 |
| Gene 16 | 0.84 | 0.96 |
| Gene 17 | 1.03 | 1.19 |
| Gene 18 | 0.86 | 0.96 |
| Gene 19 | 0.90 | 1.02 |
| Gene 20 | 0.85 | 0.95 |
| Gene 21 | 0.96 | 1.08 |
| Gene 22 | 0.87 | 0.98 |
| Gene 23 | 0.88 | 0.98 |
| Gene 24 | 0.91 | 1.03 |
| Gene 25 | 1.02 | 1.15 |
| Gene 26 | 0.88 | 0.99 |
| Gene 27 | 0.87 | 0.97 |
| Gene 28 | 0.85 | 0.97 |
| Gene 29 | 0.92 | 1.02 |
| Gene 30 | 0.89 | 1.02 |
| Gene 31 | 0.89 | 0.98 |
| Gene 32 | 0.98 | 1.11 |
| Gene 33 | 0.92 | 1.03 |
| Gene 34 | 0.92 | 1.02 |
| Gene 35 | 0.83 | 0.94 |
| Gene 36 | 0.81 | 0.95 |

**C**

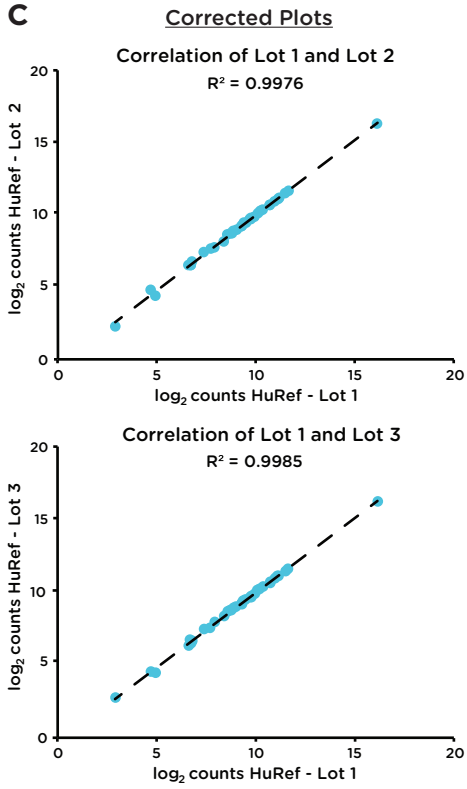



**FIGURE 6.3** **(A)** Uncorrected plots comparing Lot 1 with Lots 2 and 3. **(B)** Correction factors. **(C)** Corrected plots that compare Lot 1 with Lots 2 and 3.

## SYMBOLS AND DEFINITIONS


Manufacturer


Consult Instructions for Use

Catalogue or Reference Number

Batch Code / Lot Number


Temperature Range Storage Conditions


Lower Limit of Temperature Storage Conditions


Upper Limit of Temperature Storage Conditions


For Use By / Expiry Date


Date of Manufacture

NanoString Technologies, Inc.

530 Fairview Avenue North
Seattle, Washington 98109 USA

CONTACT US

support@nanostring.com
**Tel:** (888) 358-6266
**Fax:** (206) 378-6288
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