

nCounter Gene Fusion RNA TagSet Hybridization Protocol User Manual

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Gene Fusion RNA TagSet Hybridization Protocol

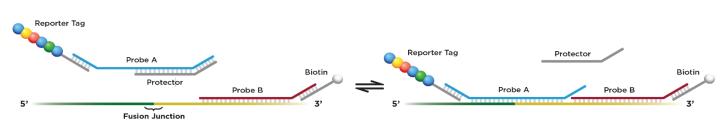
Contents

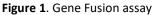
Overview	3
Materials and Reagents	4
Materials Supplied by NanoString	4
Additional Materials Required	4
Nucleic Acid Sample Input Recommendations	5
Important Probe Handling Instructions	5
Creating Oligonucleotide Probe Pools	6
Creating Master Stocks	7
Gene Fusion TagSet RNA Hybridization Protocol	9
Quick Reference for Gene Fusion TagSet RNA Hybridization	. 11
nCounter Extension Products	. 13
Preparing the RLF	. 13
Data Analysis Guidelines	. 13
Contact Information	. 14



Overview

nCounter Gene Fusion assays utilize NanoString's nCounter TagSet chemistry, with the addition of a third oligo (the Protector Probe, or Probe P) that is used simultaneously with the standard Probes A and B. The Protector Probe is an oligonucleotide that is complementary to a portion of Probe A, allowing for a new level of target specificity (Figure 1). Probe A, which links the unique reporter tag to a specific target sequence, recognizes both halves of the junction point in the fused target. When Probe A is a perfect match to the fusion junction, probe A binds to both the protector oligo and the target region in roughly equal proportions, whereas an imperfect match strongly favors the double stranded A and Protector configuration (which does not get counted in the assay).





nCounter Gene Fusion assays may be performed with pre-made, off-the-shelf content using Vantage RNA assays or with customized content using Elements XT TagSet reagents. The primary difference is that Vantage RNA Fusion kits from NanoString provide oligo pools at the correct Master Stock concentration, whereas Elements Fusion assays will use probes that will need to be acquired from a third-party oligo vendor. The NanoString bioinformatics team will generate the design of these custom probes, and for convenience will also provide a pre-populated order form for one oligo vendor. Oligos from a third-party vendor may require pooling at the correct Master Stock concentrations prior to use, and oligos from NanoString or other vendors will require dilution into working stock concentrations when setting up the assay. **This manual provides instructions for setting up either Vantage RNA or Elements XT Gene Fusion** Master Mix, and setting up nCounter hybridizations for gene fusions. If you are adding custom probes to either Vantage RNA panels or to any existing custom Elements design, refer to Creating Oligonucleotide Probe Pools, Creating Master Stocks, and nCounter Extension Products for information on Extension Probes.



Materials and Reagents

Materials Supplied by NanoString

The materials listed are supplied by NanoString for RNA:Fusion processing.

Table 1. Materials supplied by NanoString

Reagent	Description	Storage
Gene Fusions TagSet Reagents	Barcoded Reporter and Capture Probes	At or below -80°C
Probe Pools (A, B, and P)	Target-specific A, B and P (protector) oligonucleotide probes (only provided by NanoString for Vantage Fusion assays)	At or below -80°C
Extension Probe Pools (A, B, and P)	Optional; Vantage only	At or below -80°C
Hybridization Buffer	Supplied with nCounter Master Kits and SPRINT Reagent Packs	RT (15–25°C)

Additional Materials Required

The additional materials listed in are required to complete the hybridization setup.

Table 2. Additional materials required

Item	Manufacturer	Part #
Custom Elements XT Gene Fusion probes (optional)	IDT or other	Various
Thermal Cycler	Various	Various
Microfuge or picofuge	Various	Various
NanoDrop OR Qubit*	ThermoFisher	Various
Bioanalyzer 2100*	Agilent	G2939BA
12-tube PCR hybridization strip	Various	Various
Multi-channel pipettor	Various	Various
Pipettes for 0.5–10, 2–20, 20–200 μL*	Rainin	Various
96-well clear polystyrene round-bottom plates*	Corning	351177
Disposable gloves	Various	Various
RNeasy [®] Kit* (optional)	QIAGEN	74104, 74106
Proteinase K Solution (20mg/mL)**	Invitrogen	AM2546

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

**If using crude whole cell lysates as sample input, Proteinase K MUST BE ADDED to the CodeSet Master Mix.

IMPORTANT: NanoString highly recommends verifying the integrity of total RNA samples via a fragment analysis method (e.g., Bioanalyzer) before proceeding with hybridization. Recommended Total RNA sample: **50 ng to 300 ng** per hybridization assay. Recommended starting amount varies depending on your NanoString Instrument type and your sample quality (see Nucleic Acid Sample Input Recommendations).



Nucleic Acid Sample Input Recommendations

Table 3. Nucleic Acid Sample Input Recommendations

Analyte		MAX/FLEX	SPRINT
Unamplified Total RNA	Fresh Frozen	100 ng	50 ng
	FFPE	300 ng	150 ng

For additional information related to nucleic acid sample preparation based on sample type, see Preparing Nucleic Acid from FFPE Samples (MAN-10050) or Preparing RNA and Lysate from Fresh Frozen Samples (MAN-10051).

Important Probe Handling Instructions

- During setup, do not vortex or pipette vigorously to mix.
- 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 the maximum speed and may spin the probes out of solution.



Creating Oligonucleotide Probe Pools

The oligonucleotide probes used with nCounter customized fusion probes must be acquired from a thirdparty oligo vendor and pooled together with like oligos. The Vantage RNA fusion panels already come with the base oligo pools that are ready for dilution into 30X Working Pools, but the base probes for custom Fusion assays, as well as any Extension probes for Vantage or custom XT Elements Fusion assays, must be pooled into Master Stocks. Oligos are pooled only with like oligos, such that one pool contains every Probe A, another pool contains every Probe B, and another contains all the Probe P Protector oligos. Extension oligos (used to capture targets beyond the base limit of 192 transcripts) will need to be similarly pooled into Extension A, Extension B, and Extension P pools. Pools are initially created as Master Stocks, which are stable for several years when 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 30X 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 30X 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 Probe P will be present at 1.2 nM. Final probe concentrations in the hybridization will be lower after all the other components are added to the master mix. 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 Probe P. 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 Fusion Assays. Each specific application will require optimization and validation using appropriate performance metrics defined by the end user.



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 A at a final concentration of 5 nM per oligo**, a separate pool of **Probe B at a final concentration of 25 nM per oligo**, and a separate pool of **Probe P at a final concentration of 10 nM per oligo** (this also applies to custom Elements Fusion probes or Vantage Fusion Extension probes). 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 Probe P. Do not create a combined Master Stock containing A, B, and P probes in the same tube; elevated background and lowered reaction sensitivity may result.

1. Resuspend Individual Oligonucleotides at recommended concentration

NOTE: Oligo vendors may already provide individual probes at these NanoString-friendly concentrations, in which case Step 1 can be skipped.

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
- Probe P oligonucleotides 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.
- 2. Create Master Stocks of A and B Probes
 - a. Remove the appropriate Probe A and Probe B oligonucleotides from storage and thaw them on ice.
 - b. Create Probe A Master Stock (make extension Probe A pool separately)
 - i. Pipet 5 μ L of each Probe A (starting concentration 1 μ M) into a 1.7 mL microfuge tube.
 - ii. Add TE to a final combined volume of 1 mL.
 - iii. The final concentration of each Probe A in the Probe A Master Stock will be 5 nM.
 - iv. Store in aliquots at -20°C or -80°C as recommended by the supplier.
 - c. Create Probe B Master Stock (make extension Probe B pool separately)
 - i. Pipet 5 μ L of each Probe B (starting concentration 5 μ M) into a 1.7 mL microfuge tube.
 - ii. Add TE to a final combined volume of 1 mL.
 - iii. The final concentration of each Probe B in the Probe B Master Stock will be 25 nM.
 - iv. Store in aliquots at -20°C or -80°C as recommended by the supplier.



- 3. Create Probe P Master Stock (make extension Probe P pool separately)
 - a. Pipet 5 μ L of each Protector Probe (starting concentration 2 μ M) 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.

IMPORTANT: If using an Extension TagSet as well as a Core TagSet, create separate Master Stocks for the Extension Probes. Thus, for Fusion assays using Core and Extension Probes, you will generate 6 Master Stock tubes (probe A, extension probe A, probe B, extension probe B, probe P, and extension probe P).

IMPORTANT: Minimize freeze-thaw cycles by storing Master Stocks in appropriate aliquots at -20°C or -80°C. Thaw each aliquot only once and keep 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 3 based on your expected reaction throughput. Follow the supplier's guidance on stability of the oligonucleotide stocks at 4°C.



Gene Fusion RNA TagSet Hybridization Protocol

IMPORTANT: Check the reagent labels before you begin to ensure the correct reagents are being utilized.

NOTE: A thermal cycler with a heated lid is required for this protocol. NanoString recommends a thermal cycler with a programmable heated lid. Models without programmable lids may reach a high temperature that causes tubes to melt or deform during extended or overnight hybridization times, and if used, should be set to ensure that the heated lid does not exceed 110°C.

- 4. Pre-heat a thermal cycler to 67°C with a heated lid at 72°C.
- Remove Vantage or Elements TagSets (and custom Elements Fusion probes or Vantage Fusion Extension probes, if applicable) as well as the Master Stock Probe Pool tubes from the freezer and thaw at room temperature. Invert or flick the tubes several times to mix well and briefly spin down reagents.

NOTE: The pre-built Master Stock probe pools for Vantage RNA assays will come in the NanoString -80°C storage box with the TagSet probes; probes for all custom content will need to be acquired from a third-party oligo vendor.

NOTE: Working pools provide sufficient volume for at least 2 sets of 12 samples, but excess volumes of the 30X Probe Pool Working Dilutions should be discarded and not stored long term. For setting up more than 2 cartridges at a time, volumes can be scaled up proportionally.

NOTE: For probe working dilutions, RNase Free water can be substituted for TE as the diluent with minimal effects on assay efficiency.

- 6. Create working stocks:
 - For Vantage Fusion Assays, create a 30X working stock of each of the Probe Pools (A, B, and P) by adding 22 μL of nuclease-free water or TE buffer directly to each tube. Diluted probe pools should not be stored for long term use.

For Vantage Fusion Assays with Extension probes, create working stocks by adding 3 μ L of each Master Stock Extension Probe Pool into the respective Probe Pools included in the assay, and add 19 μ L of nuclease free water or TE buffer directly to each tube.

 For custom Elements Fusion Assays, create working stocks of all custom Probe Pools by combining 4 μL of each Probe master stock (A, B, and P) with 29 μL of nuclease-free water or TE buffer, and mix well by repeated finger flicking. The final volume is sufficient for up to 48 reactions.

For Elements Fusion Assays with Extension probes, create combined 30X working stocks of A, B, and P probes by mixing 4 μ L of base and extension Probe Pools (e.g., Probe A with Extension Probe A, etc.), and add 25 μ L of nuclease-free water to TE to bring the final volume to 33 μ L.

7. **Create a hybridization master mix** by adding the following reagents to the tube containing the Gene Fusions TagSet. **Do not remove the TagSet from the tube**, add components directly into the TagSet.

Component	Master Mix (µL)	Per Reaction (µL)
Gene Fusions TagSet	In tube (28)	2
Hybridization Buffer	70	5
30X Probe A Working Pool*	7	0.5
30X Probe B Working Pool*	7	0.5
30X Probe P Working Pool*	7	0.5
Water	21	1.5
Total Volume	119	8.5

Table 4. Fusions Master Mix for one nCounter assay (12 reactions + 2 reactions of dead volume)

*For custom Elements Fusion assays or Vantage Fusion Extension assays, this is the combined Probe base/Probe extension Working Pool

- 8. Flick or invert the tubes repeatedly to mix then briefly spin down the master mixes.
- 9. Label a 12-tube PCR hybridization strip. If necessary, ensure the strip will fit in a microfuge or picofuge by cutting both the strip tube and its lid in half prior to setting up the reactions, taking care not to crack the tubes.
- 10. Prepare hybridization reactions using a fresh tip for pipetting into each well:
 - a. Add 8.5 µL of Master Mix to each well of a strip tube.
 - b. Add 6.5 µL of sample to each tube containing master mix.
 - c. If less than 6.5 μL of sample was added, add RNAse-free water to each tube to bring the volume of each reaction to 15 μL.
 - d. **Cap the strip tubes tightly and mix** them by inverting the tubes several times and flicking to ensure complete mixing.
 - e. Spin briefly and immediately place the tubes in a pre-heated 67°C thermal cycler.
- 11. Incubate hybridization reactions for at least 16 hours. Maximum hybridization time should not exceed 30 hours.
- 12. Incubate at 4°C once desired hyb time is reached and process the following day. Do not leave the reactions at 4°C for more than 24 hours or increased background may result.

NOTE: Selecting a fixed hybridization time followed by a ramp down to 4°C ensures equivalent hybridization time for all assays being directly compared in the same series of experiments. Counts continue to accumulate with time at 67°C, 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 increases sensitivity by increasing counts without significantly increasing background.

13. Once the hybridization reactions have been removed from the thermal cycler, **proceed immediately to an nCounter Prep Station or SPRINT** as described in the nCounter Analysis System User Manual (MAN-C0035) or nCounter SPRINT User Manual (MAN-10017).



Please read through the full protocol before beginning. This illustrated workflow is intended for quick reference at the bench.

Create Master Stocks

□ See MAN-10058, Gene Fusion RNA Hybridization Protocol, for details.

Prepare for hybridization

- □ Preheat thermocycler to **67° C** with a heated lid at **72°C**.
- Thaw Elements or Vantage Gene Fusion probes

 (and custom Elements Fusion probes or Vantage
 Fusion Extension probes, if applicable) and A, B,
 and P Probe Pool tubes, and samples at room
 temperature



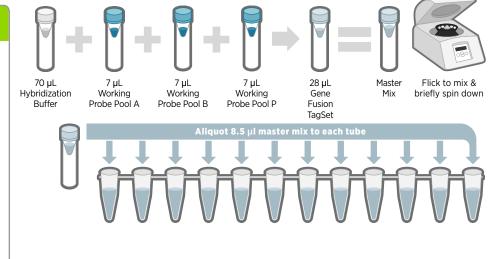
Preheat to 67°C with a heated lid at 72°C

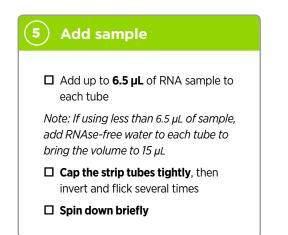
) Create working stocks:

- For Vantage Fusion Assays, create 30X working stocks of all the Probe Pools (A, B, and P) by adding 22 µL of nuclease-free water or TE buffer directly to each tube. Diluted probe pools should not be stored for long-term use.
- □ For Vantage Fusion Assays with Extension probes, create working stocks by adding 3 µL of each Master Stock Extension Probe Pool into the respective Probe Pools, and adding 19 µL of nuclease-free water or TE buffer directly to each tube.
- □ For custom Elements Fusion Assays, create working stocks of all custom Probe Pools by combining 4 μL of each Probe master stock with 29 μL of nuclease-free water or TE buffer, and mix well by repeated finger flicking. The final volume is sufficient for up to 48 reactions.
- □ For Elements Fusion Assays with Extension probes, create combined 30X working stocks of A, B, and P probes by mixing 4 µL of base and extension Probe Pools (e.g., Probe A with Extension Probe A, etc.), and add 25 µL of nuclease-free water to TE to bring the final volume to 33 µL.

Create & aliquot Master Mix

- □ Add the following to the Gene Fusion TagSet tube to create the Master Mix:
 - **70 µL** of Hybridization Buffer
 - **7 μL** of 30X Working Probe Pool A*
 - **7 μL** of 30X Working Probe Pool B*
 - **7 μL** of 30X Probe Pool P*
 - * For custom Elements Fusion assays or Vantage Fusion Extension assays, this the combined Probe base/Probe extension Working Pool
- □ Flick to mix, then briefly spin down contents
- □ Aliquot **8.5 µL** of Master Mix into each tube of a labeled 12-tube strip





6.5 µL of (each)

Flick to mix & briefly spin down

6 Hybridize

- Immediately place tubes in the thermocycler at 67° C for
 16-24 hours. Maximum hybridization time should not exceed 30 hours.
- Incubate at 4°C once desired hyb time is reached and process the following day. Do not leave the reactions at 4°C for more than 24 hours or increased background may result.





^{67°}C for 16 hours (30 hours maximum)

OPTION A: Use nCounter[®] MAX/FLEX



7a) Load nCounter MAX/FLEX

See MAN-CO035, nCounter Analysis System User Manual for details.



nCounter Extension Products

The nCounter Extension products and reagents are unique universal Reporter and Capture Probes that increase the flexibility of the nCounter platform. Probes for up to 24 additional targets are available in additional probe sets for 12, 24, or 36 genes. These are combined with an existing TagSet prior to overnight hybridization. Because the original TagSet contains the included POS and NEG controls, the Extension reagents do not have probes for these included, and therefore should not be utilized without a base panel.

- Extension products are not compatible with each other.
- Purchase of additional Master Kit reagents is not required since Extension probes are run in the same tube as a base panel.

Preparing the RLF

When creating nCounter Extension TagSet probes with the bioinformatics team, you will be provided with an RLF that merges the Core TagSet with your Extension TagSet. This new combined RLF should be used for scanning the full set of probes, and failure to use this merged RLF will result in no count information being collected for targets of the Extension reagents.

If you have questions, email NanoString at bioinformatics@nanostring.com.

Data Analysis Guidelines

Extension reagents do not include built-in ERCC standards and should only be used in conjunction with TagSets already containing these controls. Extension 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 TagSet and Extension reagents, NanoString recommends comparing the same samples using the TagSet alone vs. the TagSet and Extension reagents together. No interactions are expected.

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 User Manual for additional information on using the nSolver application.



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Contact Information

NanoString Technologies, Inc. 530 Fairview Avenue North Seattle, Washington 98109 USA

Tel: +1 888 358 NANO (+1 888.358.6266)

Email: support@nanostring.com Website: www.nanostring.com

