nanoString TECHNOLOGIES®

nCounter® Gene Fusion Panels
User Manual

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nCounter Gene Fusion Panels

Overview

This manual 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 a variety of clinical sample types including total RNA, RNA extracted from FFPE samples, blood lysates and blood fractions such as PBMCs, cell lysates and cell extracts for clinical or non-clinical samples.

nCounter Gene Fusion Panels have been created with nCounter Elements™ TagSet chemistry that uses biotinylated universal Capture Tags and fluorescently-labeled specific Reporter Tags, with the addition of oligonucleotide probes (Probe A and Probe B for each target) that complement these tags and the targets of interest. Each Probe A recognizes a unique Reporter Tag and the 5' region of the target, while each Probe B recognizes the 3' region of the target and the common Capture Tag. 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.

Gene Fusion Panels incorporate a third oligonucleotide, the Protector (Probe P), that is only required when detecting exon-exon junctions. Probe P is complimentary to a portion of Probe A, which recognizes both halves of the exon-exon junction. To minimize off-target hybridization, Probe A is made partially double stranded by the addition of Probe P to the hybridization mix, allowing for a new level of target specificity to be created.

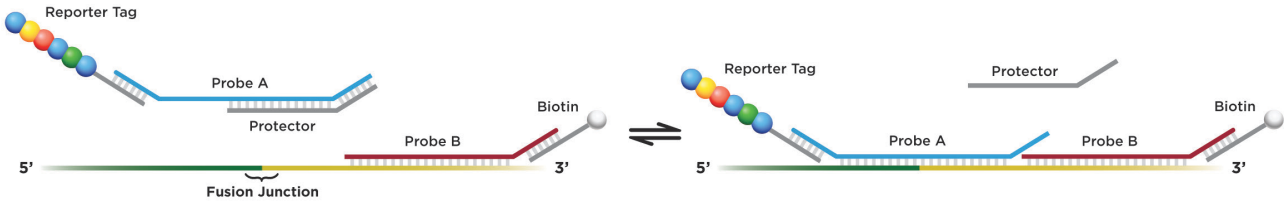


FIGURE 1 Fusions Junction probe design.

Required Materials

Gene Fusion Panels are provided with TagSet and all required oligonucleotide probes for 12 hybridization reactions. Core TagSets are pre-mixed with a comprehensive set of controls for data analysis. Extension TagSets, provided without controls, can be added to any core TagSet to expand the multiplexing capability by up to 24 targets (see Appendix for more information). nCounter Elements XT Master Kits must be purchased separately.

Sample Input Recommendations

Sample input recommendations were developed using purified total RNA from a variety of tissues, of which mRNA typically composes 5–10%. Nanostring recommends evaluating RNA quality using a NanoDrop™ or other spectrophotometer and an Agilent Bioanalyzer®. For optimal performance, RNA should exhibit an A260/A280 ratio of 1.7–2.3 and an A260/A230 ratio of 1.8–2.3 with a majority of the sample greater than 300 nucleotides respectively.

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. if you have any questions about how to ensure the best results from your experiment, please consult with your Field Applications Scientist or contact NanoString Support at **support@nanostring.com**.

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

TABLE 1 Recommended Sample Input

Sample Type	nCounter *MAX/FLEX* Analysis System	nCounter *SPRINT* Profiler
FFPE-derived RNA	300 ng	150 ng
Total RNA	100 ng	50 ng

NOTE: nCounter Analysis System in MAX, FLEX or SPRINT configurations are for **Research Use Only**. Not for use in diagnostic procedures.

Using Whole Cell Lysates

NanoString recommends a minimum of 5,000 to 10,000 cell equivalents per reaction (half for the *SPRINT* Profiler). The required number of cells will ultimately be dependent on the abundance of mRNA targets of interest in the sample and 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 Gene Fusion Panel hybridization reaction. Other lysis buffers that contain chaotropic salts may alter nucleic acid hybridization thermodynamics and are compatible with Gene Fusion Panel reagents at reduced volume. 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 reaction. Follow the guidance provided by the lysis buffer supplier to prepare cell lysates.

TABLE 2 Recommended Sample Input for Cell Lysates

Initial Number of Cells	Recommended Lysis Buffer	nCounter *MAX/FLEX* Analysis System	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

Hybridization Protocol for nCounter Gene Fusion Panels

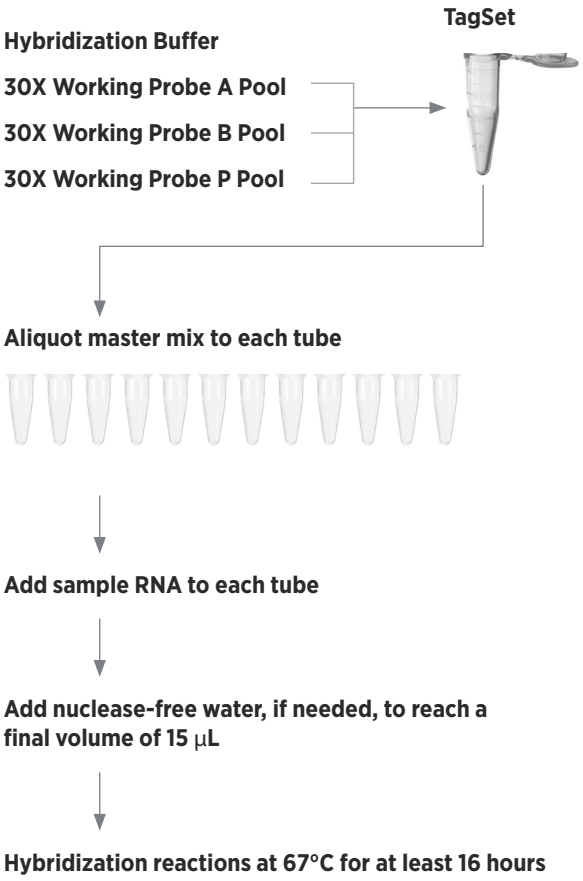
To set up 12 assays, a master mix is created for 14 reactions to allow for dead volume in pipetting. Each final hybridization reaction will have a total volume of 15 µL and contain the following components: 5 µL of hybridization buffer, 2 µL of TagSet, 0.5 µL of 30X Working Probe A Pool, 0.5 µL of 30X Working Probe B Pool, 0.5 µL of 30X Working Probe P Pool and up to 6.5 µL of sample RNA.

NOTE: If using XT Extension Probes, refer to the *Appendix: Designing and Preparing Extension Probes* 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.

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 (core TagSet only) or 12 µL (core TagSet plus Extension TagSet) of master mix to each of the 12 tubes using a fresh pipette tip for each tube.
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.



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.*

Appendix: Designing and Preparing Extension Probes

Overview

nCounter Gene Fusion Panels are provided with all components required to run an assay, 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.

To prepare Extension Master Pool Stocks (one for each probe type) as outlined in **TABLE A.1**, combine 5 µL of each individual oligo and bring the total volume to 1 mL with TE. For each tube of 30X Working Probe Pool, add 4 µL of Master Stock Pool to 29 µL of TE-Tween.

TABLE A.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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