NANOSTRING USER MANUAL

PlexSet™ Reagents for Gene Expression

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nanoString™

PlexSet Reagents for Gene Expression User Manual

Contents

Introduction

PlexSet reagents enable digital, multiplexed, high-throughput detection of gene expression levels from a wide variety of sample types, including purified total RNA from fresh, frozen, or FFPE samples, as well as whole cell lysates. PlexSet reagents use fluorescent barcoded reporters that recognize a sequence-specific tag, allowing up to 96 samples to be analyzed per nCounter run and detect the expression levels of up to 96 gene targets. With this sample multiplexing capability, PlexSet reagents allow for processing of hundreds of samples per day on a single nCounter instrument.

nCounter TagSet Technology

NanoString's nCounter PlexSet technology is an expansion of the nCounter TagSet technology. Specific Reporter Tags, comprised of unique fluorescent barcodes linked to specific nucleotide Tags, and a biotinylated Universal Capture Tag hybridize to a pair of target-specific oligonucleotide probes (Probes A and B), which in turn hybridize directly to the single-stranded RNA target (Figure 1).

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 initially anchors each target to the surface of a streptavidin-coated lane.

Probe A hybridizes to a specific Reporter Tag and to the 5' region of the target nucleic acid sequence. Probe B hybridizes to the Universal Capture Tag and to the 3' region of the target nucleic acid sequence. Each complete structure—containing the target RNA, two oligonucleotide probes, and the Reporter and Capture Tags—is referred to as a Tag Complex.

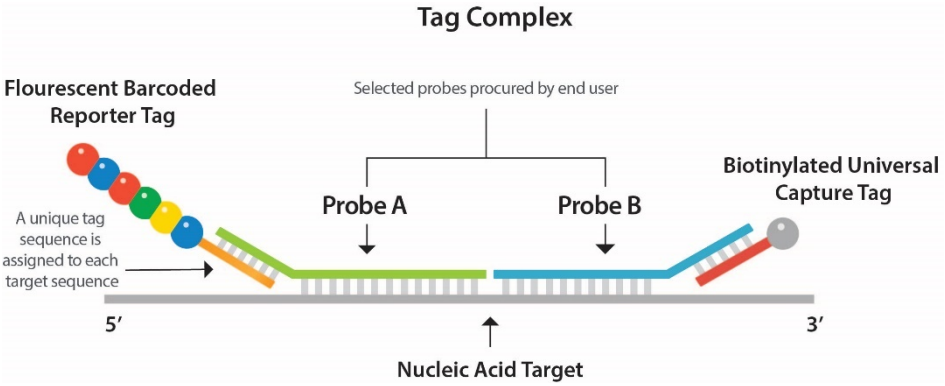


Figure 1. A Tag Complex: Customized oligonucleotide probes (A and B) hybridized with general purpose Reporter and Capture Tags and the target nucleic acid.

In TagSet, each gene of interest is paired with a Reporter (which is comprised of a Barcode and a Tag). The Probe A that is created for Gene 1 will contain the complementary sequence for hybridizing to Tag T001 (Figure 2). This is represented by the overlapping yellow and green section in Figure 1.

Figure 2. Examples of TagSet Reporter Tags with unique fluorescent barcodes and recognition sequences (Tags) for each gene of interest.

PlexSet expands on the use of TagSet barcode and tag combinations by pairing *different* barcodes to the same tag to enable up to 8 samples to be multiplexed in each lane of a nCounter run. This allows a gene of interest to be paired with different barcodes but all on the same tag (Figure 3).

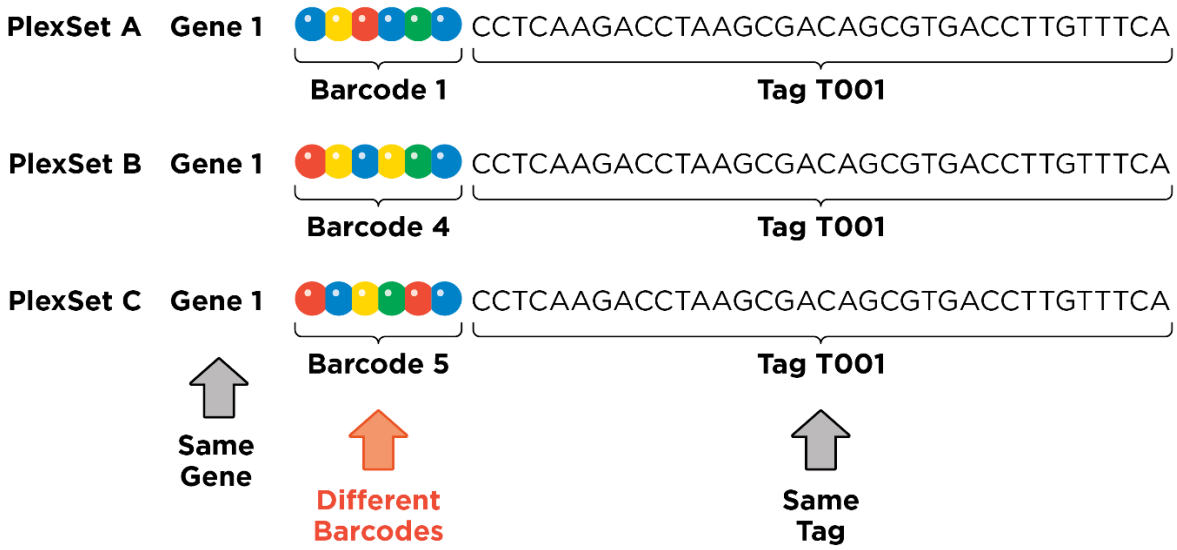
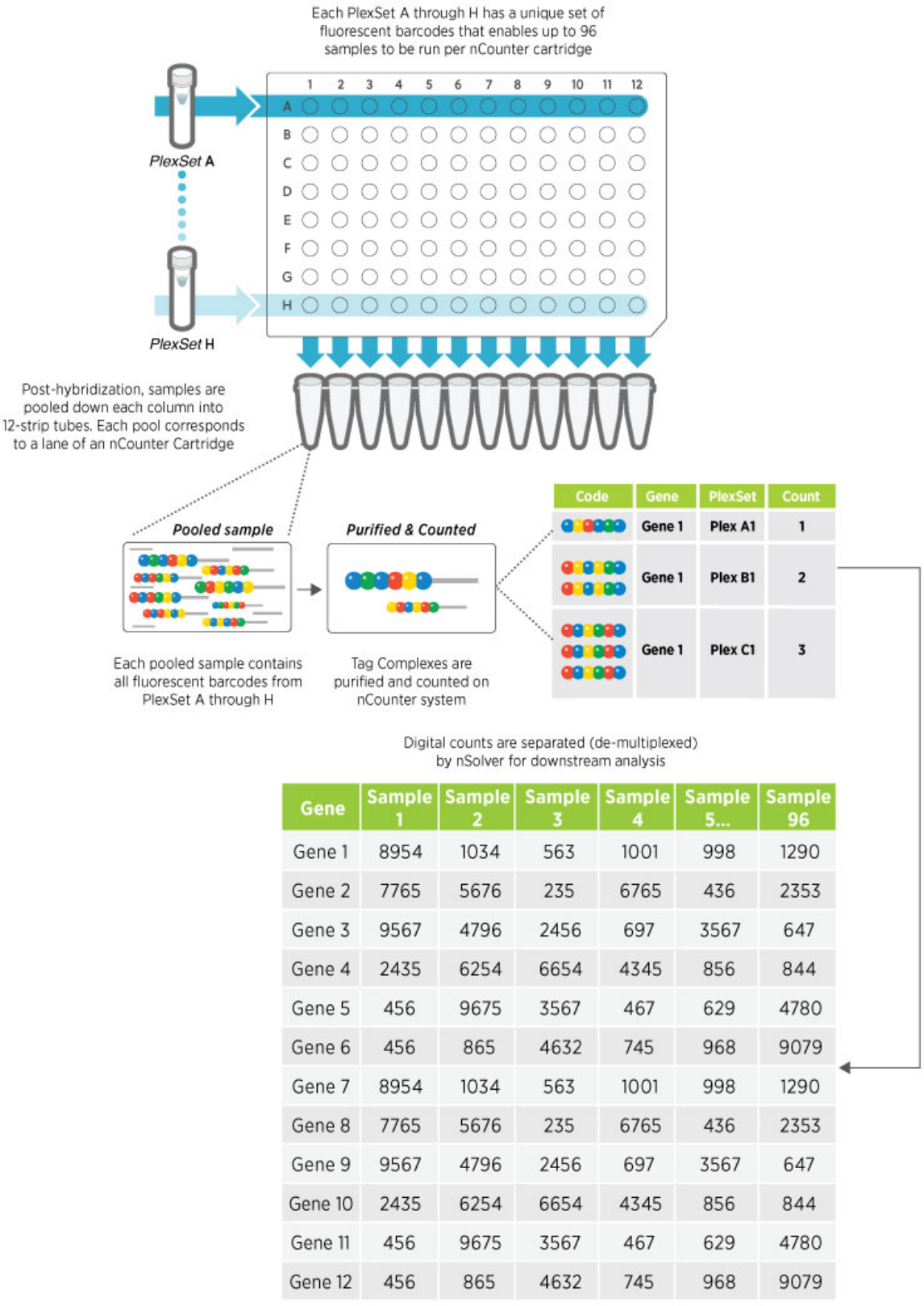


Figure 3. Examples of PlexSet Reporter Tags with unique fluorescent barcodes and the same recognition sequence (Tag) for each gene of interest.

One full PlexSet contains 8 sets of reagents, called PlexSet A through PlexSet H. The Probe A that was created for Gene 1 will hybridize to that Tag in all the PlexSets, **so it's very important to hybridize each PlexSet (A-H) separately.** *After* the samples have been hybridized overnight, the different PlexSets can be combined for processing, and the instrument can sort which sample was hybridized with which PlexSet based on the unique barcode.

The PlexSet reagents and probes are present in the reaction in large excess to the target molecules to ensure that each target undergoes complete hybridization. After hybridization, samples are pooled, and the Tag Complex is purified on the nCounter Prep Station or SPRINT using a two-step magnetic bead-based purification process. Data collection is performed on the nCounter Digital Analyzer or SPRINT, where each fluorescent barcode is individually resolved and counted (Please review detailed instructions in the appropriate manuals). Lastly, counts are separated (de-multiplexed) by nSolver for downstream data analysis (Figure 4).



Gene	Sample 1	Sample 2	Sample 3	Sample 4	Sample 5...	Sample 96
Gene 1	8954	1034	563	1001	998	1290
Gene 2	7765	5676	235	6765	436	2353
Gene 3	9567	4796	2456	697	3567	647
Gene 4	2435	6254	6654	4345	856	844
Gene 5	456	9675	3567	467	629	4780
Gene 6	456	865	4632	745	968	9079
Gene 7	8954	1034	563	1001	998	1290
Gene 8	7765	5676	235	6765	436	2353
Gene 9	9567	4796	2456	697	3567	647
Gene 10	2435	6254	6654	4345	856	844
Gene 11	456	9675	3567	467	629	4780
Gene 12	456	865	4632	745	968	9079

Figure 4. Multiplexing with PlexSet

nCounter PlexSet Configurations

The nCounter PlexSet assays are comprised of 8 PlexSets, A through H, and the corresponding oligonucleotide Probe A and Probe B pairs (Designed by NanoString's bioinformatics team and supplied by the user) that recognizes up to 96 RNA targets of interest (Table 1). Five different PlexSet configurations are available for 12, 24, 48, 72, or 96 tags. It is important to remember that larger PlexSet configurations include all tags found in smaller PlexSet configurations. For example, a PlexSet of 12 tags (i.e., PlexSet-12) includes tags T001 through T012, and a PlexSet of 24 tags (i.e., PlexSet-24) includes tags T001 through T024.

Table 1. Available configurations of the PlexSet assay and the Tags utilized for each.

PlexSet Configurations		
Genes of Interest	**PlexSet**	**Tags used**
Up to 12	PlexSet-12	T001–T012
13–24	PlexSet-24	T001–T024
25–48	PlexSet-48	T001–T048
49–72	PlexSet-72	T001–T072
73–96	PlexSet-96	T001–T096

PlexSets with different configurations cannot be combined to increase the number of targets in an experiment, as their Reporter probes will overlap. For example, PlexSet-12 and PlexSet-24 cannot be combined to create "PlexSet-36" because the reporter barcodes for tags T001 to T012 are identical between the respective PlexSet tubes and it will be impossible to separate the counts. For 36 genes of interest, PlexSet-48 should be used, as shown in Table 1.

Assigning housekeeping genes and other frequently analyzed targets to Reporter Tags with the lowest tag number will make the most efficient use of assay 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 in an assay: (1) order a larger PlexSet configuration or (2) order new Probe A and Probe B oligonucleotide pairs that link Reporter Tags in the existing PlexSet with new targets. Some examples are shown in Table 2.

NOTE: If you are not reassigning probes, proceed to PlexSet Workflow.

In Experiment 1, existing PlexSet-12 probe pairs can be modified by reassigning Reporter Tags to new targets in Experiment 2 (Table 2). Targets that have been added or replaced are indicated in blue. Genes in blue will require the creation of a new Probe A and Probe B oligonucleotide pairs; the remaining genes can use the oligos previously created in Experiment 1. The additions of Gene S and Gene T require the use of the next larger PlexSet configuration, PlexSet-24, because the tag assignments go above tags T001–T012. Always select the PlexSet configuration one size larger than the tag assignments, up to PlexSet-96.

Table 2. Reassigning Tags.

Experiment 1		Experiment 2	
Existing PlexSet		Tags Reassigned	
PlexSet-12		PlexSet-24	
Tag	**Target**	**Tag**	**Target**
T001	Gene A	T001	Gene A
T002	Gene B	T002	Gene B
T003	Gene C	T003	Gene M
T004	Gene D	T004	Gene N
T005	Gene E	T005	Gene E
T006	Gene F	T006	Gene F
T007	Gene G	T007	Gene G
T008	Gene H	T008	Gene O
T009	Gene I	T009	Gene P
T010	Gene J	T010	Gene Q
T011	Gene K	T011	Gene R
T012	Gene L	T012	Gene L
		T013	Gene S
		T014	Gene T
		…	…
		T024	Gene 24

PlexSet Workflow

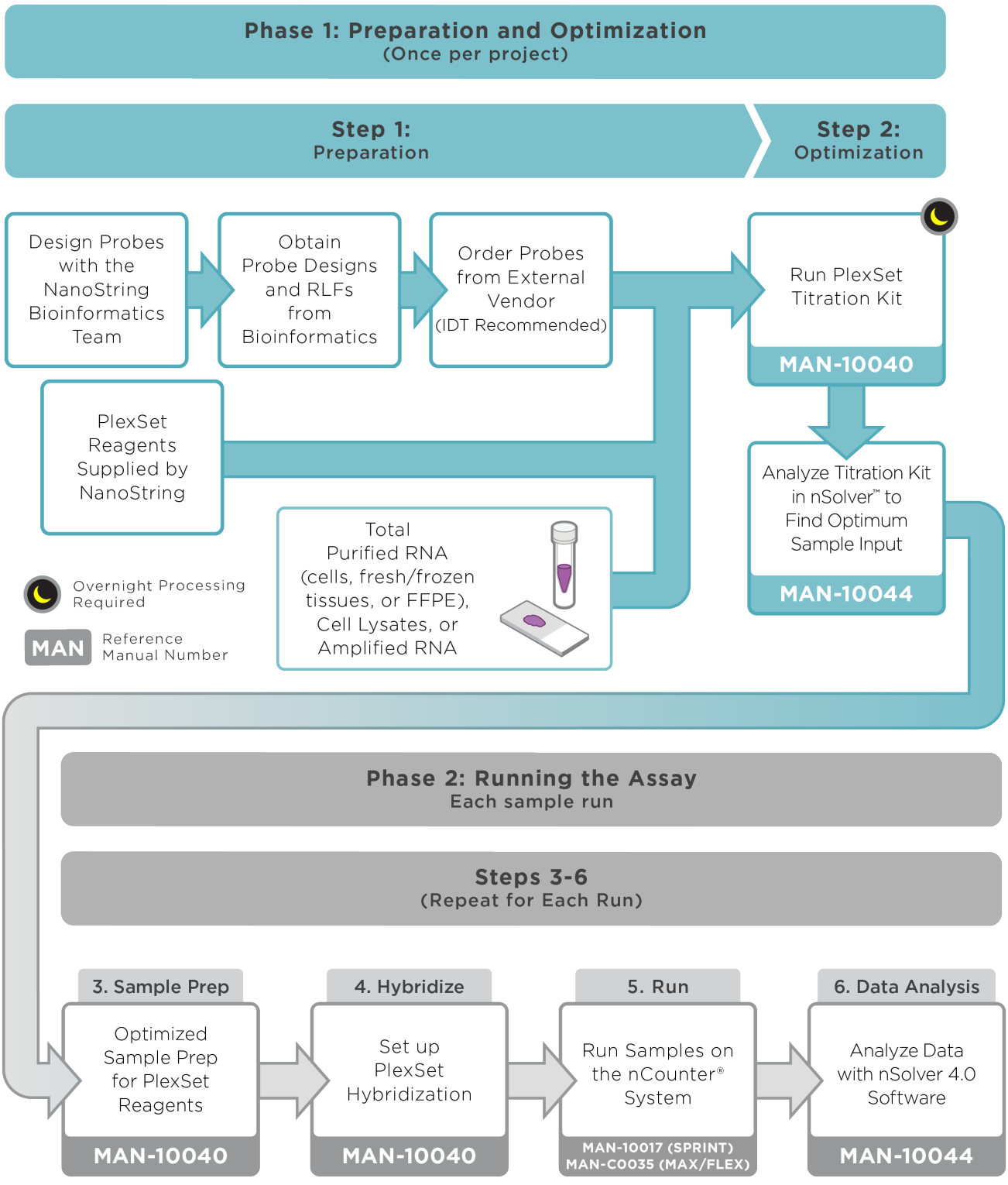


Figure 5. Workflow for PlexSet Reagents, including the Titration Kit.

Phase 1: Preparation and Optimization (once per project):

Step 1: Preparation—Create the probes

1. Design the probe(s) with the Bioinformatics team at NanoString.
2. Obtain probe designs and RLFs from Bioinformatics.
3. Order primers from an any external oligo vendor (preferred vendor—IDT, as NanoString validations have been performed on IDT and pre-filled forms for ordering can be provided when ordering from the Bioinformatics Dept.)
4. Order PlexSet and Titration Reagents from NanoString.

Step 2: Optimization—Titration & Selection of Calibration Sample

1. Perform the Titration run to find a Calibrator sample and ensure proper input levels so the cartridge lane is not saturated.
2. Analyze the results with All About PlexSet Data Analysis with nSolver (MAN-10044); optimize and attenuate as needed.

Phase 2: Running the Assay (each sample run)

Step 3: Prepare the samples

Prepare your optimized sample with the appropriate Calibrator.

Step 4: Hybridize the samples

Set up and run the PlexSet Hybridization Protocol.

Step 5: Run the PlexSet assay

Run the hybridized PlexSet assay on the SPRINT or MAX/FLEX system.

Step 6: Analyze the results

Analyze the results with All About PlexSet Data Analysis with nSolver (MAN-10044).

Materials and Equipment

Table 3. Materials provided in the PlexSet Reagent Packs/Titration Kits.

Item	Catalog Number	Reagents	Storage
nCounter PlexSet-12 Reagent Pack	PS-012-GX-192S (CSO)	2 sets of PlexSets-12 (A–H) 2 sets of Hybridization Buffer	At or below -80°C RT (15–25°C)
nCounter PlexSet Titration Kit-12	PS-GX-PTK-12 (CSO)	2 sets of nCounter XT Tagset-12	At or below -80°C
nCounter PlexSet-24 Reagent Pack	PS-024-GX-192S (CSO)	2 sets of PlexSets-24 (A–H) 2 sets of Hybridization Buffer	At or below -80°C RT (15–25°C)
nCounter PlexSet Titration Kit-24	PS-GX-PTK-24 (CSO)	2 sets of nCounter XT Tagset-24	At or below -80°C
nCounter PlexSet-48 Reagent Pack	PS-048-GX-96S (CSO)	1 set of PlexSets-48 (A–H) 1 set of Hybridization Buffer	At or below -80°C RT (15–25°C)
nCounter PlexSet Titration Kit-48	PS-GX-PTK-48 (CSO)	2 sets of nCounter XT Tagset-48	At or below -80°C
nCounter PlexSet-72 Reagent Pack	PS-072-GX-96S (CSO)	1 set of PlexSets-72 (A–H) 1 set of Hybridization Buffer	At or below -80°C RT (15–25°C)
nCounter PlexSet Titration Kit-72	PS-GX-PTK-72 (CSO)	2 sets of nCounter XT Tagset-72	At or below -80°C
nCounter PlexSet-96 Reagent Pack	PS-096-GX-96S (CSO)	1 set of PlexSets-96 (A–H) 1 set of Hybridization Buffer	At or below -80°C RT (15–25°C)
nCounter PlexSet Titration Kit-96	PS-GX-PTK-96 (CSO)	2 sets of nCounter XT Tagset-96	At or below -80°C

Table 4. Additional materials required (not provided).

Material and Reagent	Recommended Supplier	Catalog number
Probe A†	IDT	n/a
Probe B†	IDT	n/a
Master Kits (for MAX and FLEX)	NanoString	NAA-AKIT-012
SPRINT Reagent Pack (for SPRINT)	NanoString	SPRINT-REAG-KIT
SPRINT Cartridges (for SPRINT)	NanoString	SPRINT-CAR-1.0
Pipettes for 10–1,000 µL*	Various	Various
Manual multi-channel pipette for 20 µL*	Rainin	L12-20XLS+
12-strip standard tubes*	Bioexpress	T-3034-1
15 mL conical tubes*	FisherBrand	S50712
Low-Binding Microfuge Tubes*	FisherBrand	02-681-320
TE-Tween (10mM Tris-HCl, 1 mM disodium EDTA (pH 8.0) and 0.1% Tween 20)	See below	See below
Tris-EDTA buffer solution*	Sigma	93283
Tween 20*	Sigma	P9416
96-well plates with strip caps (optional)*	Applied Biosystems	N8010560 (96-well plate) N8010534 (cap strip)

† Information on designing and ordering Probe A and Probe B oligonucleotides can be found below.

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

Table 5. Additional materials required for whole cell lysis (not provided).

Material and Reagent	Recommended Supplier	Catalog number
iScript™ RT-qPCR Sample Preparation Reagent (10 mL)*	Bio-Rad	1708898
Buffer RLT (220 mL)**	Qiagen	79216
Proteinase K Solution (20 mg/mL)	ThermoFisher	AM2546

**For suspension or adherent cell line whole cell lysis*

***For primary cell lysis*

Table 6. Equipment required for use of the PlexSet Reagents.

Instruments
NanoString nCounter SPRINT, MAX, or FLEX analysis system
Microfuge or picofuge
Calibrated thermal cycler with a heated lid*
Fluorometer**

** See Thermal Cycler Guidelines below.*

*** NanoString recommends using a Qubit™ Fluorometer for RNA and DNA quantification.*

Thermal Cycler Guidelines

Please note that a thermal cycler **with a heated lid** is required for this protocol. 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.

PlexSet Flexibility: Preselected Pathway Panels and Custom Probe Sets

The largest PlexSet configuration currently available can detect up to 96 targets (see Table 3 for all available configurations) and multiplex 96 samples on one nCounter cartridge. Researchers are also able to run more than one experiment on a 96-well plate using different probe pairs targeting a different set of targets. They can also run less than 96 samples on different days or with different gene targets.

In addition to any user-defined custom target list, panels covering ~140 biological pathways and fields of interest for human, mouse, and rat samples are available. Each panel contains 96 genes including housekeeping genes chosen to comprehensively cover each pathway involved in the topic. All necessary controls and reference genes are included in each panel. You can customize pre-selected panels by exchanging genes of interest or omitting genes that are not of interest. By utilizing PlexSet reagents with these panels, up to 96 samples can be run in a single nCounter run. Browse the list of PlexSet Pathway panels, click on the specific panel of interest to browse the genes included and then click Submit for a quote on the right-hand side of the panel webpage. A specialist will contact you regarding the design of the panel. Researchers can also request panel design for other species than human, mouse, and rat, simply by clicking the “Request Other Species” tab on the panel table.

Ordering Oligonucleotide Probes

Prior to PlexSet assay setup, oligonucleotide probes should be ordered separately for each target RNA.

Researchers can provide their custom target gene list or choose from one of our Preselected Pathway panels. Contact NanoString Bioinformatics Ordering (orders@nanostring.com) to discuss probe design and oligonucleotide ordering.

Researchers can buy preformulated Probe A and Probe B Master Stock pools from an external manufacturer. For manually creating Probe A and Probe B Master Stock pools from individual probes, please refer to Appendix 1: Creating Oligonucleotide Master Stock.

Oligo Submission Process

1. Customer submits a gene/target list to orders@nanostring.com.
2. NanoString Bioinformatics team designs probe pairs to the requested targets.
3. Customer receives 5 files from Bioinformatics team once their probes are created.
   - Design summary file—Gives a summary of their probes
   - IDT order form—Pre-populated with customer's probe designs
   - CDF (Cartridge Definition File) template—Can be used to define Sample IDs and info to be loaded onto the Digital Analyzer
   - Titration RLF (Reporter Library File)—This RLF file gets loaded onto the Digital Analyzer or the SPRINT at the time of the run. It pairs the barcodes with the customer's gene. Titration RLFs will always be in the format "*Titration_customer-specific name.rlf*"
   - PlexSet RLF—This RLF file gets loaded onto the Digital Analyzer or the SPRINT at the time of the run. It pairs the barcodes with the customer's genes. PlexSet RLFs will always be in the format "*PS_customer-specific name.rlf*"

Sample Input and Experimental Design Considerations

A Titration Kit should be used to optimize sample input concentrations (ng/µl) for the PlexSet assay. Each assay can accommodate up to **7 µL** of purified total RNA, **7 µL** of cells lysed with iScript buffer, or **4.5 µL** of cells lysed with diluted Buffer RLT. The amount of material (RNA or lysate) can vary slightly depending on the experiment. Prior to performing the PlexSet or Titration hybridization reaction, it is useful to prepare samples in **≥10 µL** volume in a 96-well plate to minimize the hybridization setup time.

Purified Total RNA

For sample input amount, NanoString recommends the following starting points for samples from most biological systems in Table 7. The minimum recommended sample input for the SPRINT platform is increased as a result of sample volume limitations on the SPRINT. Post-hybridization, when 8 samples are pooled per lane of a cartridge, only **35 µl** out of each **120 µl** pool will be loaded onto a SPRINT cartridge. Therefore, the minimum recommended starting input is increased for the SPRINT.

Table 7. Recommended minimum total RNA input per hybridization.

nCounter System	Total RNA (ng)	FFPE-derived RNA (ng)
MAX/FLEX	50	150
SPRINT	100	300

NOTE: Highly degraded samples will give lower counts, and input should be adjusted accordingly. While degradation is common for RNA purified from formalin-fixed, paraffin-embedded (FFPE) samples, these sample types can still provide high-quality results due to NanoString's enzyme-free chemistry and probe design targeting a relatively short region of sequence. If using FFPE or other highly degraded sample types, evaluate RNA quality using an Agilent Bioanalyzer® to measure nucleic acid fragmentation; best results will be achieved with RNA that averages 300 nucleotides in length or greater. For additional information based on

your sample type, please 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.

Whole Cell Lysates

For most cell types, NanoString recommends a minimum of 5,000 to 20,000 cells (or cell equivalents) per hybridization reaction for measuring gene expression. Some cell types, such as freshly isolated immune cells, may require 20,000 to 30,000 cells per hybridization reaction for adequate input. 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 and should be determined empirically by the end user for their biological system. Furthermore, the maximum sample input volume when using cell lysates depends on the type of lysis buffer used. Cell type plays an important role when choosing which buffer to use.

Detergent-based lysis buffers, i.e., those that do not contain chaotropic salts, are fully compatible with nCounter hybridization reagents; as much as **7 µl** may be added to each hybridization reaction. Other lysis buffers that contain chaotropic salts may alter nucleic acid hybridization thermodynamics and are compatible with nCounter reagents with some modifications to the 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 hybridization reaction. To determine the optimal whole cell lysate input, use a Titration Kit that corresponds to the PlexSet configuration (Table 3).

Three lysis protocols have been developed for adherent cell lines, suspension cell lines, and primary cells. These protocols are in a 96-well plate lysis format for direct input of crude cell lysates into the PlexSet assay without RNA purification and all protocols have a lysis volume of **30 µL**. Adherent and suspension cell lines are lysed with a detergent-based lysis buffer, iScript buffer (Bio-Rad, 1708898), and primary cells are lysed with a chaotropic buffer, Buffer RLT (Qiagen, 79216), that has been diluted to 1/3 concentration in water. For hybridizations containing whole cell lysate, the **addition of Proteinase K to the hybridization is essential** to improve counts and data quality. These cell lysis protocols can be found in Appendix 2: 96-well Plate Whole Cell Lysis Protocols.

Sample Input Optimization

The PlexSet Titration Kit has two distinct purposes:

1. Optimizing Sample Input: Determine the optimal sample input for use with PlexSet reagents to avoid saturation and potential data loss.
2. Finding a Calibration Sample: Test possible samples (or mixtures of samples) to be used as a calibrator for the PlexSet runs. Finding a suitable calibrator is part of the optimization.

Optimizing Sample Input

When overall reporter probe density exceeds optimal guidelines, some data loss may occur. As up to 8 samples are multiplexed in a single lane with the nCounter PlexSet assays, high sample inputs for one or all samples in a lane can lead to saturation, and, in extreme cases, to loss of data for ALL samples in that lane. You should perform a Titration of the input of representative samples before running a full 96-sample PlexSet assay. Representative samples at a range of input amount should be run with this kit. The samples chosen should represent expected extremes—e.g., untreated and most heavily treated

samples—should be analyzed. If some targets lead to extremely high levels of counts, it may be necessary to attenuate these signals. Counts obtained from these Titration experiments can be used to approximate the ideal input for the full assay, as well as to determine if any of the selected targets should be attenuated or replaced (see Attenuation of Highly Expressed Genes). If a previous nCounter assay has been performed on the same or similar sample type, counts from these experiments can be used to estimate the ideal sample input. A PlexSet Titration Calculator can be obtained from Technical Services (support@nanostring.com) or your Field Application Scientist to assist with Titration analysis. See Table 9 and Table 10 for sample Titration setups.

Finding a Calibration Sample

Calibration of the PlexSet reagents is an important part of the process for obtaining the cleanest data possible. Even though all 8 PlexSets A–H are used to find the same gene set (via Probes A and B), the fluorescent barcodes differ between sets. So, for every lot of PlexSet Reagent purchased, there are *actually* 8 lots (A–H). The calibrator normalizes the lot-to-lot variation that we normally deem necessary between lots of CodeSet, to ensure that fold change data is not skewed by differences in the barcode efficacy.

Ideally, you will want to use a sample to calibrate the PlexSets that has >200 counts for every gene. There might be some genes in your set that do not get above background for any of your samples. In cases like this, it's not crucial that your calibrator sample cover those genes, since the samples in your study are too low to provide any meaningful fold change data.

Housekeeper Control Selection

The accuracy and reliability of gene expression results are dependent upon the proper normalization of the data against internal reference genes. Usually 3 to 5 "housekeeping" genes are chosen as reference, since they often display uniform expression during various phases of development, across different tissue types, and under different environmental and experimental conditions. For all experiments, NanoString recommends the analysis of at least 3 housekeepers. If possible, avoid targets that typically have very high levels of RNA expression (e.g., GAPDH, ACTB, or B2M).

Table 8 lists 14 potential housekeepers that may be considered for human samples. These genes typically exhibit moderate expression and low variability across tissues and thus may be suitable housekeepers for a variety of biological experiments. Note that expression levels will vary depending on a variety of factors, and some users may need to use alternative housekeepers depending on sample type and experimental conditions. Housekeeper selection for human samples is thoroughly outlined in the Reference Genes for Normalization of Expression Data Technical Note. For non-human samples, contact Bioinformatics at orders@nanostring.com.

Table 8. Potential Housekeeping Genes for Human Samples.

ABCF1	G6PD	LDHA	POLR2A	SDHA	ALAS1	GUSB
PGK1	RPL19	TBP	CLTC	HPRT1	POLR1B	RPLP0

Attenuation of Highly Expressed Genes

Information about expression levels for genes within a biological system may be available from published data. Highly expressed genes may be identified early using the nCounter PlexSet Titration Kit before they interfere with the results from the nCounter PlexSet Assay. In some cases, a highly expressed gene target can be replaced with a more moderately expressing alternate target (for example, housekeeper genes). However, in situations where it is critical to measure an abundant gene, an attenuation strategy utilizing competitive inhibition can be used to maintain high quality data even in the presence of highly expressed transcripts.

Attenuation involves adding excess inactive probes for the target(s) of interest to the hybridization reaction in proportion to the desired level of attenuation. The inactive probe is the region of the Probe A sequence that hybridizes directly to the target. For example, the standard hybridization reaction contains 20 pM of each Probe A. To attenuate reporter counts for a particular gene by 90%—that is, to count only 10% of its endogenous expression level—it is necessary that 90% of the final Probe A concentration for that target in the hybridization mixture be replaced with an inactive oligonucleotide probe. Therefore, 180 pM of inactive probe can be added to the existing 20 pM of active probe, bringing the total concentration to 200 pM. Higher or lower attenuation levels can be achieved by making similar adjustments to the ratio of active to inactive probes. The “true” expression level of the target can then be calculated during data analysis.

This attenuation strategy is thoroughly outlined in the NanoString whitepaper Strategies for Successful Gene Expression Assays. For help in designing attenuation probes, please contact NanoString Bioinformatics (orders@nanostring.com).

PlexSet Titration Kit Hybridization Protocol

Overview

The objective of running the Titration Kit Hybridization Protocol ahead of the experiment is to enable the user to optimize the sample input to ensure they will be within the dynamic range of the assay and avoid experimental failure. Furthermore, a candidate calibration sample should also be run to verify that there are abundant counts for all gene targets (see PlexSet Calibration). Accordingly, this protocol should be run under the same conditions (e.g., hybridization time) and with the same nCounter MAX/FLEX, or SPRINT, that will be used for the PlexSet assay protocol.

The Titration Kit contains an nCounter TagSet reagent that is compatible with the Probe A and Probe B Master Stocks used in the PlexSet assay. Each Titration Kit comes with two nCounter TagSet reagents in order to enable multiple rounds of sample input optimization and possible target attenuation to prevent signal saturation in the PlexSet assay. The data generated with the Titration Kit should not be used in the final PlexSet data analysis. Samples of interest run with the Titration Kit should be included again in the final PlexSet assay setup.

Due to sample input limitations between a MAX/FLEX system and a SPRINT system, the optimal sample input into the PlexSet assay will vary depending on which system is used. For assays run on an nCounter MAX/FLEX system, the goal of the Titration run is to determine the sample input amount for the PlexSet assay so that the sum of all gene targets in a sample is a maximum of ~150,000 counts, excluding positive and negative controls. For assays run on an nCounter SPRINT system, the goal of the Titration run is to optimize sample input amount so that the sum of all gene targets has a maximum of ~400,000 counts. The differences in gene target counts between the MAX/FLEX and SPRINT systems is due to sample volume limitations between the two systems. Post-hybridization, the entire pooled sample is loaded on a MAX/FLEX but only 30% of each pool is loaded onto a SPRINT cartridge. As a result, the recommended sample input amount in the PlexSet assay is higher for SPRINT.

Titration Kit hybridization setups are shown below for purified RNA (Table 9) and cell lysate (Table 10). These are just examples—one can include more samples or more Titration data points. The samples chosen for the Titration should be representative of the sample groups in the experiment. If you have a control and 3 experimental groups, at least one sample from each group should be used in the Titration. Initial RNA inputs should be estimated from bioanalyzer data or cover a generic spread of quantities that are expected (**50 ng**, **100 ng**, **150 ng** is a good starting point). The assay results are highly linear, and thus it may be most informative to include fewer Titration points and more experimental samples.

Targets that are in high abundance should be attenuated. Please refer to NanoString whitepaper Strategies for Successful Gene Expression Assays. For help in attenuation probe design, please contact NanoString Bioinformatics (orders@nanostring.com).

Table 9. Example Setup for Total RNA.

Lane	Sample	RNA Input
1	Sample A	50 ng
2	Sample A	100 ng
3	Sample A	150 ng
4	Sample B	25 ng
5	Sample B	75 ng
6	Sample B	150 ng
7	Sample C	100 ng
8	Sample C	200 ng
9	Sample C	300 ng
10	Sample D	10 ng
11	Sample D	50 ng
12	Sample D	100 ng

Table 10. Example Setup for whole cell lysates.

Lane	Sample	Cell Input
1	Lysate A	5,000 cells
2	Lysate A	10,000 cells
3	Lysate A	20,000 cells
4	Lysate B	20,000 cells
5	Lysate B	30,000 cells
6	Lysate B	40,000 cells
7	Lysate C	2,000 cells
8	Lysate C	5,000 cells
9	Lysate C	10,000 cells
10	Lysate D	10,000 cells
11	Lysate D	15,000 cells
12	Lysate D	20,000 cells

Titration Kit Checklist

Prior to running the PlexSet Titration Kit, please make sure the following supplies are procured from the following sources to streamline sample processing and data analysis:

- ☐ PlexSet Titration Kit and corresponding PlexSet assay configuration.
- ☐ Master Kit(s) or SPRINT cartridge(s) & Reagent pack from NanoString (Table 4).
- ☐ Custom RLF file for the Titration run. The nomenclature for the Titration run RLF is Titration_*customer-specific Design Name*.rlf. Please contact orders@nanostring.com to receive the custom Titration run RLF for every unique set of Probe A and Probe B pools used.
- ☐ Purified RNA samples or whole cell lysates supplied by the user.
- ☐ Custom Master Stock pools of Probe A (**5 nM** each) and Probe B (**25 nM** each). Contact orders@nanostring.com to help with the ordering of Probe A and Probe B Master Stock pools. Please refer to Appendix 1: Creating Oligonucleotide Master Stock for creating custom Master Stock pools and the PlexSet Titration Kit Hybridization Protocol for the Master Stock pool dilutions.

PlexSet Titration Kit Protocol using nCounter XT TagSet Chemistry

Each final hybridization reaction will have a volume of **15 µL**. To set up 12 assays, Master Mix is created for 14 reactions to allow for dead volume in pipetting.

IMPORTANT: No vortexing at any time.

1. Remove an aliquot of nCounter XT TagSet from the freezer and thaw at room temperature. Invert several times to mix well, and briefly spin down the reagent on a small picofuge (probes should always be spun at less than 1,000 RCF).
2. Create Probe A and Probe B Working Pool dilutions.
    a. Add **3 µL** of the Probe A Master Stock to **22 µL** of TE-Tween (10 mM Tris pH 8, 1 mM EDTA, 0.1% Tween-20). Mix well. This is the diluted **0.6 nM** each Probe A Working Pool.
    b. Add 3 µL of the Probe B Master Stock to **22 µL** of TE-Tween (10 mM Tris pH 8, 1 mM EDTA, 0.1% Tween-20). Mix well. This is the diluted **3 nM** each Probe B Working Pool.
3. Each nCounter XT TagSet tube contains **28 µL** of reagent. Create a Master Mix by adding reagents directly into the TagSet tube.
    a. Add **70 µL** of hybridization buffer.
    b. Add **7 µL** of the **0.6 nM** each Probe A Working Pool. Mix well by flicking the tube and briefly spin down in a picofuge.
    c. Add **7 µL** of the **3 nM** each Probe B Working Pool. Mix and spin again.

Table 11. Titration Kit hybridization and Master Mix components

Reagent	Volume per reaction (µL)	Volume for 14x Master Mix (µL)
Hybridization Buffer	5	70
nCounter XT TagSet	2	28
30X Working Probe A Pool (0.6 nM each Probe A)	0.5	7
30X Working Probe B Pool (3 nM each Probe B)	0.5	7
Proteinase K (20 mg/mL)**	—	2.1**
Master Mix Total Volume	8	112 (114.1**)
RNA sample or crude whole cell lysate	up to 7 µL*	—
Nuclease-free water	7 µL minus [Sample volume]	—
Final Hybridization Volume	**15 µL**	—

**The maximum input for cells lysed with 1/3 Diluted RLT is 4.5 µL (see Appendix 2: 96-well Plate Whole Cell Lysis Protocols).*

***If using crude whole cell lysates as input, add Proteinase K to Master Mix. Addition of Proteinase K does not affect assay results for purified total RNA sample.*

4. Label a 12-tube strip and add **8 µL** of Master Mix to each of the 12 tubes.

   NOTE: For MAX/FLEX users, check the orientation of the strip tubes provided with the Master Kit, to ensure proper direction for the Prep Station run. The notches in the strip tubes should be between Tubes 1 & 2 and Tubes 8 & 9, as shown in Figure 6 below.

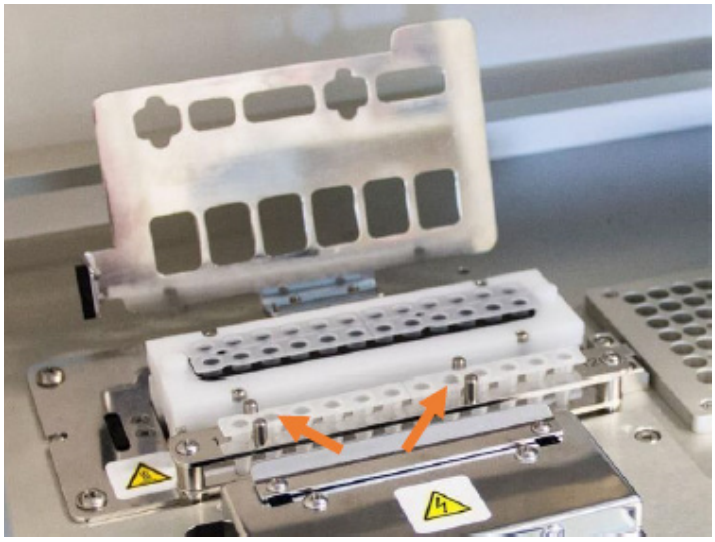


Figure 6. Hybridization sample strip tubes with notched orientation guides for use in the Prep Station.

5. Add sample to each of the 12 tubes to each tube.

   If less than **7 µL** of sample was used, add nuclease-free water to bring the final hyb volume to **15 µL**.

6. 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 in a picofuge and immediately place the strip tubes in the thermal cycler.

7. Incubate reactions for at least 16 hours. Hybridization times should match the PlexSet assay hybridization time. 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: An RLF specific to the Titration Kit must be used. This RLF is distinct from the RLF used with PlexSet reagents. Customers should have received this RLF from the bioinformatics team in an email along with the PlexSet RLF, design files, CDF (Cartridge Definition File) and ordering information. The Titration RLF should start with the prefix “Titration_”.

Post-Hybridization Processing

nCounter MAX/FLEX system

See the nCounter Analysis System User Manual (MAN-C0035) and Quick Start Guide for detailed instructions on running the instrument.

1. Briefly spin down 12-tube strip with hybridization in a picofuge.
2. Set up a standard nCounter Prep Station run with the strip tube containing the reactions.
3. Scan the cartridge on the Digital Analyzer using the custom Titration Kit RLF emailed from the Bioinformatics team.

nCounter SPRINT system

See the nCounter SPRINT User Manual (MAN-10017) and Quick Start Guide for detailed instructions on running the instrument.

1. Briefly spin down 12-tube strip with hybridization in a picofuge.
2. Add **15 µL** nuclease-free water to each hybridization.
3. Load all **30 µL** of each hybridization into the SPRINT cartridge and run using the custom Titration Kit RLF emailed from the Bioinformatics team.

Titration Kit Data Analysis and PlexSet Sample Input Calculation

Analyze the data with All About PlexSet Data Analysis with nSolver (MAN-10044) **before** running the PlexSet experiment.

> **IMPORTANT:** You MUST obtain an optimized sample input amount by analyzing the Titration data in nSolver before proceeding.

PlexSet Calibration

Calibration is required for accurate results. A single calibration sample must be run across all 8 PlexSets (A through H) in a single lane at least once per set of Probe A and Probe B reagents or for each new PlexSet reagent lot. This calibration sample lane is used to calibrate inter-PlexSet variability.

Running the PlexSet Titration Kit will help provide options for possible calibration samples. User-specified calibration samples must be performed with samples that give robust counts for **all targets** of interest (ideally, over 200 counts). If there are targets that are low, perfect DNA targets can be ordered from an oligo manufacturer (sequence design provided by Bioinformatics) to be spiked into the assay for calibration. This sample can be a control sample related to the study, artificial targets (e.g., DNA oligos), or a combination of the two. A "study" is defined as a set of experiments using the same Probe A and Probe B Master Stocks and the same lot of PlexSet reagents. If the Master Probe Stocks and/or PlexSet reagent lots change, it is required to run another calibration lane for accurate results. Failure to run a calibration may result in sample-unrelated but PlexSet-specific over- or underestimation of target expression levels and potentially inadequate conclusions in data interpretation.

A calibration lane consisting of all PlexSets hybridized with an identical calibration sample should be run on the first plate used for the study. Figure 7 shows a plate setup with the calibration sample run in the first column. All other hybridization reactions on this plate can contain unique samples. Additional experimental samples do not need to be related to the calibration sample. After hybridization, pooling, and counting, calibration factors will be obtained for all barcodes and these calibration factors will be used throughout the study. Therefore, all subsequent plates run with the assay in the same study need contain only experimental samples.

> **IMPORTANT**: If a new lot of PlexSet reagents or Master Probe Stocks is introduced to an ongoing project, run a new calibration lane.

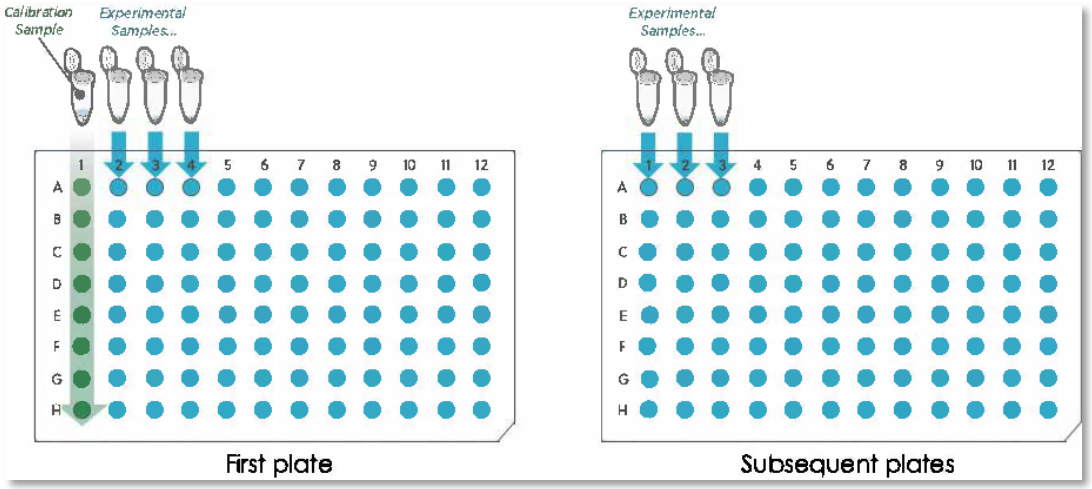


Figure 7. Calibration samples are required on the initial plate for accurate results.

Analyze the results with All About PlexSet Data Analysis with nSolver (MAN-10044).

You may:

- Run all 96 samples on a plate (Figure 8). Refer to PlexSet Hybridization Protocol.
- Run all 96 samples on a plate but split the plate across multiple experiments/probe sets by columns (Figure 9). Refer to PlexSet Hybridization Protocol for Plates with More Than One Probe Set.
- Run less than 96 samples on a plate, with PlexSet tubes A–D (for example) on one plate (by rows) and E–H on another plate using the same or different probe sets (Figure 10 and Figure 12, respectively). Refer to PlexSet Titration Kit Hybridization Protocol.

You may not:

- Run all 96 samples on a plate but split the plate across multiple experiments/probe sets **by rows**.
- Exclude data from selected wells.

A calibration sample is essential for accurate data analysis across PlexSets (Figure 8). The same RLF is used for these scenarios. Reserve column 1 of the first plate for your Calibration sample. Subsequent PlexSet cartridges using the same lot of probe sets will not need an additional calibration lane (Figure 8). With the current nSolver data analysis software, your experiments should be organized down the columns (Figure 9).

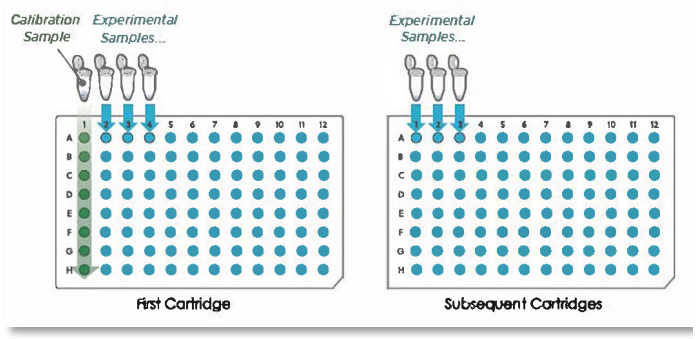


Figure 8. PlexSet layout—full plates

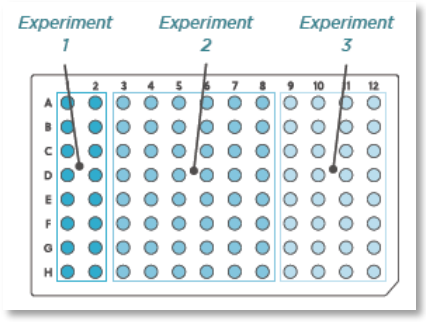


Figure 9. PlexSet samples organized in columns

Less than 96 samples can be run per cartridge; **the same RLF** is used for these scenarios (first and second run, Figure 10). PlexSets A–D can be run on one cartridge (first run), and PlexSets E–H can be run on another (second run). If you subsequently run a full plate with a different combination of PlexSet A through H (e.g., all PlexSets), a calibration sample should be re-run across all PlexSets for calibration (third run, Figure 11).

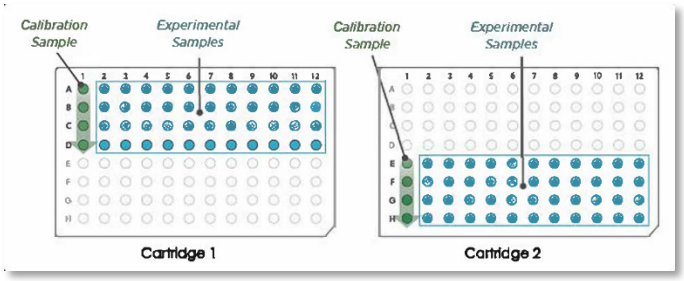


Figure 10. First and second run—partial PlexSet plates

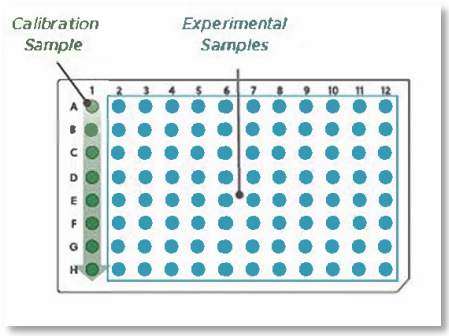


Figure 11. Third run—Full PlexSet plate

PlexSet kits can be used with different probe sets, but specific configurations are required for downstream analysis. **Two RLFs** are used for these scenarios (one for each probe set). Running partial plates with different probe sets is possible, but the considerations above apply when running subsequent plates (Figure 12).

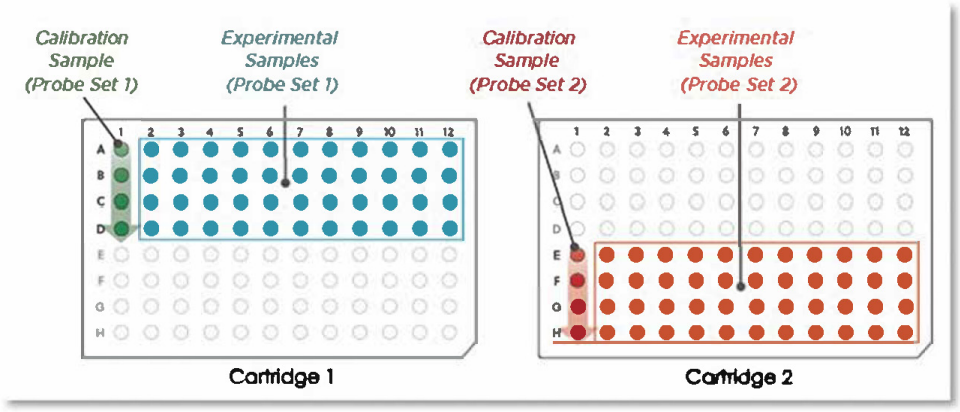


Figure 12. Partial PlexSet plates, different Probe Sets

If multiple probe sets are run on the same plate, probe sets should be organized down columns to allow downstream analysis with nSolver software (Figure 13).

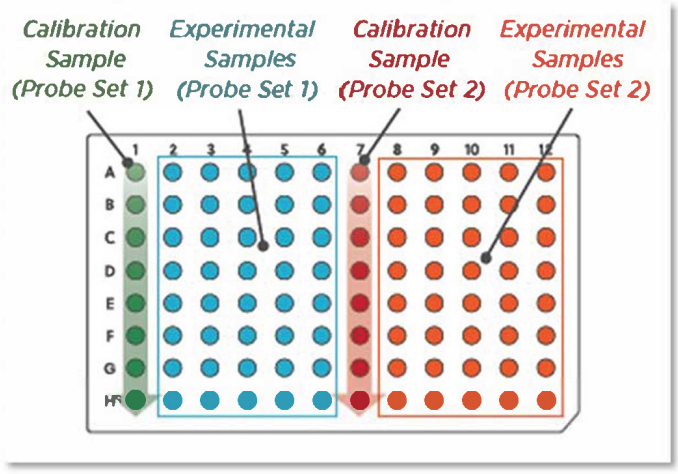


Figure 13. Different Probe Sets organized down the columns

PlexSet Assay Checklist

Prior to running the PlexSet assay, please make sure the following supplies are procured from the following sources to streamline sample processing and data analysis.

- ☐ PlexSet reagents (PlexSet-12, -24, -48, -72, or -96).
- ☐ Master Kits or SPRINT cartridges and Reagent Pack from NanoString (Table 4). When splitting PlexSet reagents across multiple cartridges, additional Master Kits will be required.
- ☐ Custom RLF file for the specific Probe A and Probe B Master Stocks. Please contact orders@nanostring.com to receive the custom PlexSet RLF for every unique set of Probe A and Probe B pools used.
- ☐ Purified RNA samples or whole cell lysates supplied by the user. For quick assay setup, these samples should be diluted to correct concentrations in a 96-well PCR plate with a sample volume **≥10 µL**. A multi-channel pipette can then be used to transfer samples into PlexSet Master Mix in Step 5: Add Samples of the PlexSet protocol below.
- ☐ Custom Master Stock Pools of Probe A (**5 nM** each) and Probe B (**25 nM** each). Contact orders@nanostring.com to help with the ordering of Probe A and Probe B Master Stock Pools. Please reference Appendix 1: Creating Oligonucleotide Master Stock for creating custom Master Stock Pools and the Titration Kit protocol for the Master Stock Pool dilutions.

PlexSet Hybridization Protocol

The protocol describes the PlexSet assay setup for 12 to 96 samples using one set of Probe A and Probe B pools. Any combination of PlexSets A through H may be used. However, the same PlexSet tube (i.e., two tubes of PlexSet A) may not be used in combination because the fluorescent barcodes are the same. Each tube of PlexSet A through H is enough for 12 samples each. Plan the experiment accordingly in multiples of 12 samples to prevent repeated cycles of freeze-thaw of the PlexSet reagents.

IMPORTANT: The Master Stocks of Probe A and Probe B oligos **must be diluted** to the Working Pool concentration before being added to the assay mix. Use of undiluted Master Stocks will result in assay failure. See Appendix 1: Creating Oligonucleotide Master Stock for instructions on how to create Master Stocks of Probe A and Probe B.

Table 12. PlexSet Assay hybridization components for each well.

Reagent	Volume per reaction (µL)
Hybridization Buffer	5
PlexSet Reagent (PlexSet A through H)	2
Probe A Working Pool (0.6 nM each Probe A)	0.5
Probe B Working Pool (3 nM each Probe B)	0.5
RNA sample or crude whole cell lysate	Max 7 µL*
Nuclease-free water	7 µL minus [Sample volume]
Proteinase K (20 mg/mL)	**
Final Hybridization Volume	**15 µL**

**The maximum input for cells lysed with 1/3 Diluted RLT is 4.5 µL (Appendix 2: 96-well Plate Whole Cell Lysis Protocols)*

***If using crude whole cell lysates as input, proteinase K **MUST BE ADDED** to Probe-Buffer Master Mix (Table 14). Addition of Proteinase K does not affect assay results for purified total RNA sample.*

NOTE: The following steps are for running one experiment with up to 96 samples using the **same** Probe Sets. If you are using **more than one** Probe Set for splitting the PlexSet reagents, follow the guidelines in PlexSet Hybridization Protocol for Plates with More Than One Probe Set.

Step 1: Dilute Probe A and Probe B Master Stock Pools into 30X Working Stocks

Probe A and Probe B Master Stocks should be at a concentration of **5 nM** and **25 nM** for each probe, respectively.

1. Dilute Probe A Master Stock to a Working Pool concentration of **0.6 nM** for each probe using Table 13 based on the number of PlexSet tubes used in the experimental setup. Flick/invert tubes to mix and briefly spin in picofuge.
2. Dilute Probe B Master Stock to a Working Pool concentration of **3 nM** for each probe using Table 13 based on the number of PlexSet tubes used in experimental setup. Flick/invert tubes to mix and briefly spin in picofuge.

Table 13. Guide to diluting Master Probe Stocks to generate Working Probe Pools based on the number of PlexSets, A through H, used in the experimental setup. Any combination of PlexSet A through H can be used. 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. The green highlighted row is for using all 8 PlexSets at once.

Number of PlexSet Tubes (A–H) in Experimental Setup	Total number of Samples	Aliquot from Probe A or B Master Stock (µL)	TE-Tween (µL)	Final Volume (µL)
1	12	4	29	33
2	24	4	29	33
3	36	4	29	33
4	48	6	44	50
5	60	6	44	50
6	72	8	59	67
7	84	8	59	67
8	96	10	73	83

Step 2: Create Buffer-Probe Mix

Volumes of Buffer/Probe mix will vary depending on the number of PlexSet tubes used in experimental setup.

Table 14. Volumes to mix of hybridization buffer, Probe A Working Pool, and Probe B Working Pool are dependent on the number of PlexSet tubes in experimental setup. If using crude whole cell lysates, proteinase K (**20 mg/mL** stock solution) **MUST** be added to the buffer/probe mix.The green highlighted row is for using all 8 PlexSets at once.

Number of PlexSet Tubes (A–H) in Experimental Setup	Volume Hybridization Buffer (µL)	Volume 0.6 nM each Probe A Working Pool (µL)	Volume 3 nM each Probe B Working Pool (µL)	Proteinase K @ 20 mg/mL*
1	80	8	8	2.4
2	150	15	15	4.5
3	230	23	23	6.9
4	300	30	30	9.0
5	380	38	38	11.4
6	450	45	45	13.5
7	530	53	53	15.9
8	600	60	60	18.0

If using crude whole cell lysates as sample input, proteinase K **MUST BE ADDED to Buffer/Probe Master Mix. Addition of Proteinase K does not affect assay results for purified total RNA sample.*

Based on the number of PlexSet tubes used in experiment, pipette the following reagent volumes from Table 14 into a 1.5 mL microfuge tube.

1. Pipette appropriate volume of Hybridization Buffer into a 1.5 mL microfuge tube.
2. Add appropriate volume of diluted Probe A Working Pool from Step 1: Dilute Probe A Pool and B Pool Master Stocks into the Hybridization buffer. Flick/invert tubes to mix and briefly spin in picofuge. Solution should be well mixed before adding diluted Probe B Working Pool.
3. Add appropriate volume of diluted Probe B Working Pool from Step 1 into the Hybridization buffer/Probe A mixture. Flick/invert tubes to mix and briefly spin in picofuge.
4. If using crude whole cell lysates as sample input, add appropriate volume of Proteinase K into the Hybridization buffer/Probe A/Probe B mixture. Flick/invert tubes to mix and briefly spin in picofuge.

These are the Buffer/Probe Mixes. Diluted Buffer/Probe Mixes should not be stored for re-use. Store on ice.

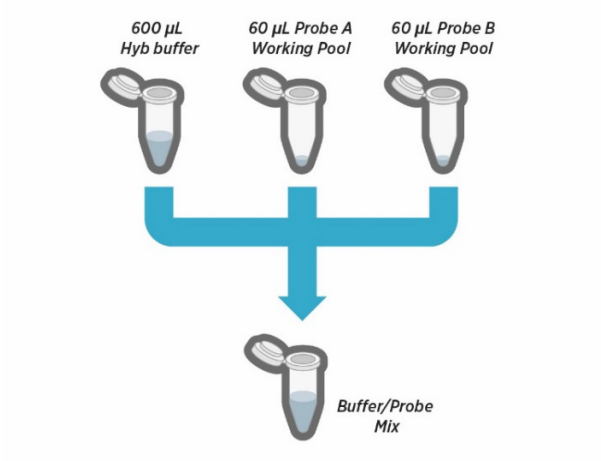


Figure 14. Buffer/Probe Mix. The volumes shown in this figure are specific to 8 PlexSets as shown in Table 14

Step 3: Create PlexSet A through H Master Mixes

1. Remove the appropriate number of PlexSet tubes from the freezer and thaw.
2. Invert several times to mix well, and briefly spin down in the picofuge. Store on ice.

   IMPORTANT: Avoid prolonged exposure of PlexSets to light during thawing and storing.
3. Add **84 µL** of the Buffer/Probe Mix from Step 2: Create Buffer-Probe Mix to each of the PlexSet tube. Mix by flicking tubes. Spin briefly in picofuge. These are the individual PlexSet Master Mixes. Store on ice.

Step 4: Set Up Hybridization

1. Set up the hybridization reactions in a 96-well plate with a tight seal that does not allow for evaporation in an overnight incubation at 67°C.

   NOTE: NanoString recommends using plates with caps to seal the wells, such as Applied Biosystems MicroAmp Optical 96-well Reaction Plate (Catalog No. N8010560) and MicroAmp 12-Cap Strip (Catalog No. N8010534), or any similar product. Eight 12-well strip tubes can also be used in place of a 96-well plate. Strips should be well labeled to maintain order. **DO NOT USE sticky foil or plastic seals** because of sample evaporation during overnight hybridization**.** A heat sealer can be used, such as the ALPS 50V heat sealer from Thermo Scientific to ensure the integrity of a sealing system, NanoString recommends testing a plate in an overnight incubation at 67°C with **15 µL** of water per well to verify that there is no significant evaporation. Carefully monitor the volume in all wells following incubation (uneven volume loss across the plate is common). The loss of 1–3 microliters per well will not significantly affect the assay outcome, but larger volume loss will negatively impact the results.
2. Pipette **8 µL** of each PlexSet Mix across the plate into each of the 12 wells of the appropriate row (Figure 15). Pipette directly into the bottom or spin down briefly after adding PlexSet reagents. For example, PlexSet A should go into row A and PlexSet B should go into row B. The use of a repeater pipette will help speed up this process.

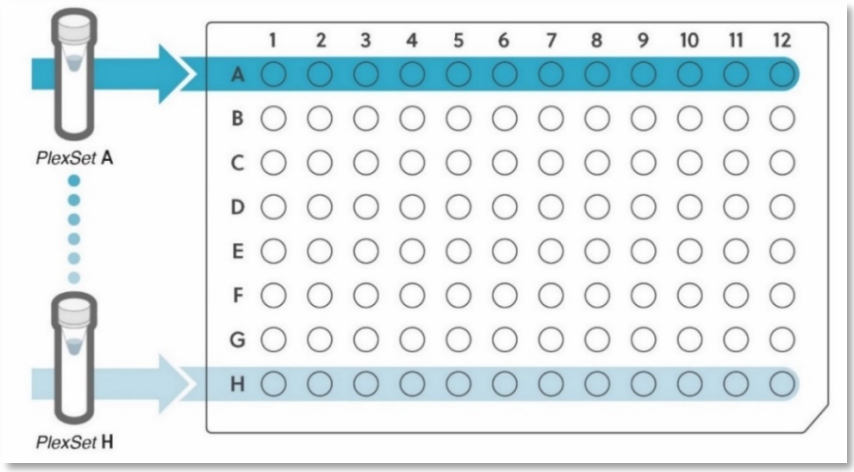


Figure 15. Hybridization Setup

Step 5: Add Samples

1. Based on the PlexSet Titration Kit results, prepare samples at the desired input concentration in a 96-well PCR plate with a sample volume **≥ 10 µL** for faster assay setup. A multi-channel pipette can then be used to transfer samples into the 96-well plate prepared in Step 4: Set Up Hybridization.

   NOTE: Samples do not have to be at the same quantities. Some samples might be higher quality or more concentrated than others. It is not important to add the same number of ng for each sample. Differences in the input will be normalized during analysis.

   NOTE: For lysates, thoroughly mix lysate samples and do NOT centrifuge them before adding to the reaction plate.

2. Add one sample to each well and mix by pipetting up and down 5 times. The final volume of each hybridization reaction will be **15 µL** (Figure 16).

   NOTE: For sample preparation and input guidelines, see Sample Input and Experimental Design Considerations. A calibration sample for PlexSet calibration should be included in Column 1 of the first plate for each new study. **If using a different number combination, or number, of PlexSet tubes each time, a new calibration must be run.**

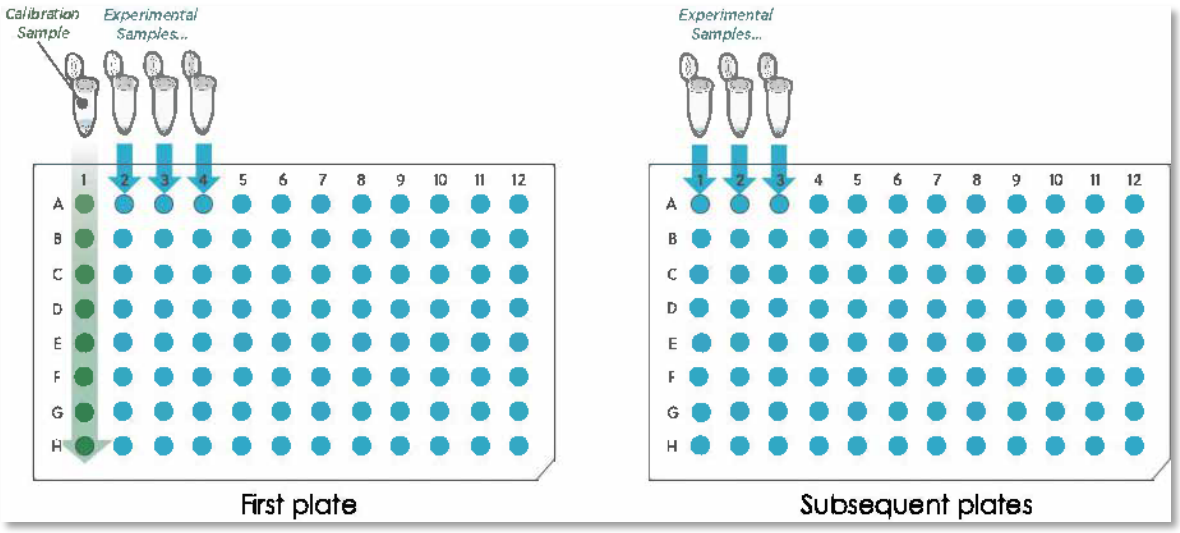


Figure 16. Adding Samples. PlexSet calibration sample should only be run on the first plate of a study.

Step 6: Incubate Reactions

1. Seal the plate carefully.

   IMPORTANT: See Note on sealing plate in Step 4: Set Up Hybridization.

   a. Quick spin the plate, spinning just long enough to reach 2,000*g* and then allowing to stop.

   b. Use a thermocycler with a heated lid set to 5°C above the temperature of the block.

   c. Program the thermocycler to incubate the plate at 67°C for 16–24 hours, selecting a time that is compatible with the workflow the following day, and then ramp down to 4°C indefinitely.

   NOTE: Do not leave the reactions at 4°C for more than 24 hours or increased background may result. For a series of related experiments, 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. 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.

Step 7: Pool the Samples

1. Quick spin the plate, spinning just long enough to reach 2,000*g* and then allowing to stop.

   NOTE: The overnight incubation at 67°C may cause small, variable amounts of evaporative loss from the wells. **Pool each complete assay regardless of volume.** Volume loss of greater than 3 µL may negatively affect the results of the assay from that well. See Step 4: Set Up Hybridization for plate sealing recommendations.

2. Pool the samples into a strip tube (Figure 17).

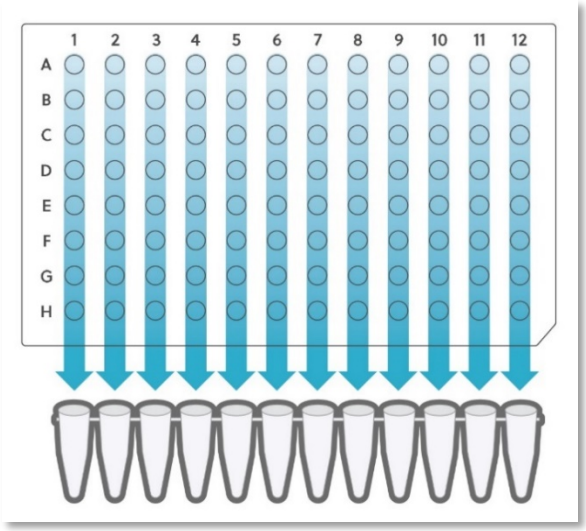


Figure 17. Pool the samples

3. Mix the final pool by gently pipetting up and down.
4. Load the samples on the workstation. Reference your Instrument's manual for how to run the nCounter system (MAN-10017 for the SPRINT Profiler or MAN-C0035 for the MAX/FLEX).

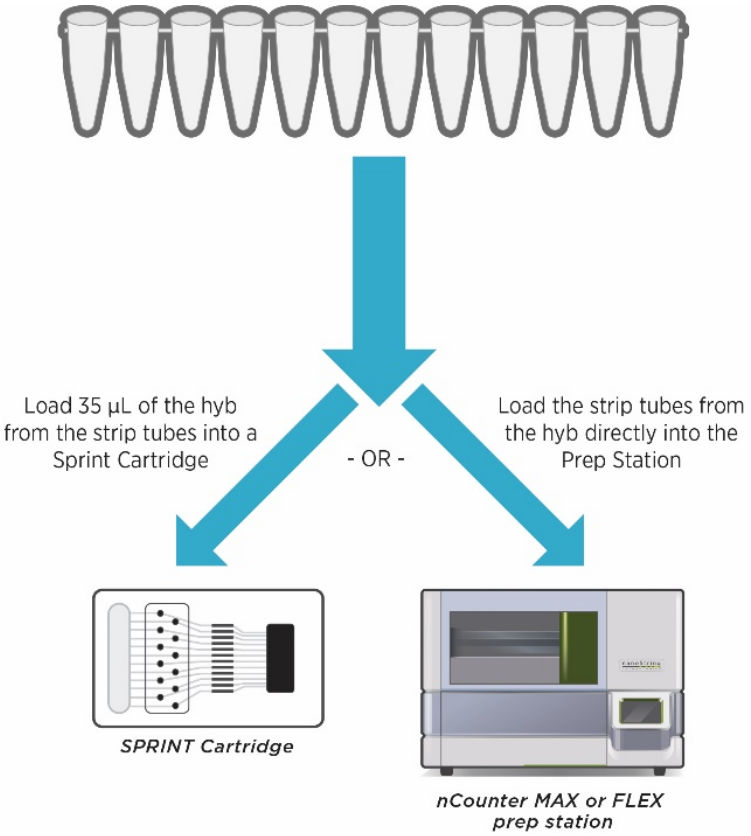


Figure 18. Load the samples on the workstation

5. Proceed to Post-Hybridization Processing.

PlexSet Hybridization Protocol for Plates with More Than One Probe Set

The protocol describes the setup for a single 96-well plate of samples run with multiple Probe Pairs (targeting different sets of genes). Volumes can be scaled for setting up more than one plate. For additional guidance, please refer to PlexSet Calibration if fewer than 96 samples are being run.

> **IMPORTANT:** The Master Stocks of Probe A and Probe B oligos **must be diluted** to the Working Pool concentration before being added to the assay mix. Use of undiluted Master Stocks will result in assay failure.

Step 1: Dilute Probe A Pool and B Pool Master Stocks

Probe A and Probe B Master Stocks should be at a concentration of **5 nM** and **25 nM** for each probe, respectively.

1. Dilute Probe A Master Stock to a Working Pool concentration of **0.6 nM** for each probe (see Appendix 1: Creating Oligonucleotide Master Stock to create Master Stocks).

   Add **10 µL** of the Probe A Master Stock to **73 µL** of TE-Tween (10 mM Tris pH 8, 1 mM EDTA, 0.1% Tween-20). Mix well and briefly spin in picofuge. This is the **diluted Probe A Working Pool**.

2. Dilute Probe B Master Stock to a Working Pool concentration of **3 nM** for each probe.

   Add **10 µL** of the Probe B Master Stock to **73 µL** of TE-Tween (10 mM Tris pH 8, 1 mM EDTA, 0.1% Tween-20). Mix well and briefly spin in picofuge. This is the **diluted Probe B Working Pool.**

Step 2: Create Buffer/Probe Mix for each Probe Pair

A different Buffer/Probe mix must be made for each Probe Pair (unique Probe A and Probe B pools). Volumes of Buffer/Probe mixes will vary depending on the number of columns run for each Probe Pair. If using crude whole cell lysates as sample input, Proteinase K (**20 mg/mL** stock solution) **MUST** be added to the buffer/probe mix (Appendix 2: 96-well Plate Whole Cell Lysis Protocols). Refer to Table 15 for necessary volumes for each Buffer/Probe Mix.

Table 15. Guide to creating the Buffer/Probe mix for each Probe Set based on the number of columns used.

Number of Columns	Volume Hybridization Buffer (µL)	Volume 0.6 nM each Probe A Working Pool (µL)	Volume 3 nM each Probe B Working Pool (µL)	Proteinase K @ 20 mg/mL*
1	30	5	5	1.5
2	60	10	10	3.0
3	90	15	15	4.5
4	120	20	20	6.0
5	150	25	25	7.5
6	180	30	30	9.0
7	210	35	35	10.5
8	240	40	40	12.0
9	270	45	45	13.5
10	300	50	50	15.0
11	330	55	55	16.5

If using crude whole cell lysates as sample input, Proteinase K **MUST BE ADDED to Probe-Buffer Master Mix. Addition of Proteinase K does not affect assay results for purified total RNA sample.*

For **EACH** Probe Pair, create a new Buffer/Probe mix in a **1.5 mL** microfuge tube by combining the appropriate volumes of hybridization buffer, diluted Probe A Working Pool, and diluted Probe B Working Pool from Table 15.

1. Pipette the appropriate volume of hybridization buffer to a **1.5 mL** microfuge tube.
2. Add the appropriate volume of diluted Probe A Working Pool from Step 1: Dilute Probe A Pool and B Pool Master Stocks into the Hybridization buffer and mix. Flick/invert tubes to mix and briefly spin in a picofuge. Solution should be well mixed before adding diluted Probe B Working pool.
3. Add the appropriate volume of diluted Probe B Working Pool from Step 1 into the Hybridization buffer/Probe A mixture. Flick/invert tubes to mix and briefly spin in a picofuge. This the Buffer/Probe mix for one Probe Pair.
4. Repeat Steps 2–3 for each Probe Pair.

These are the **Buffer/Probe Mixes** (Figure 19). Diluted Buffer/Probe Mixes should not be stored for re-use. Store on ice until use.

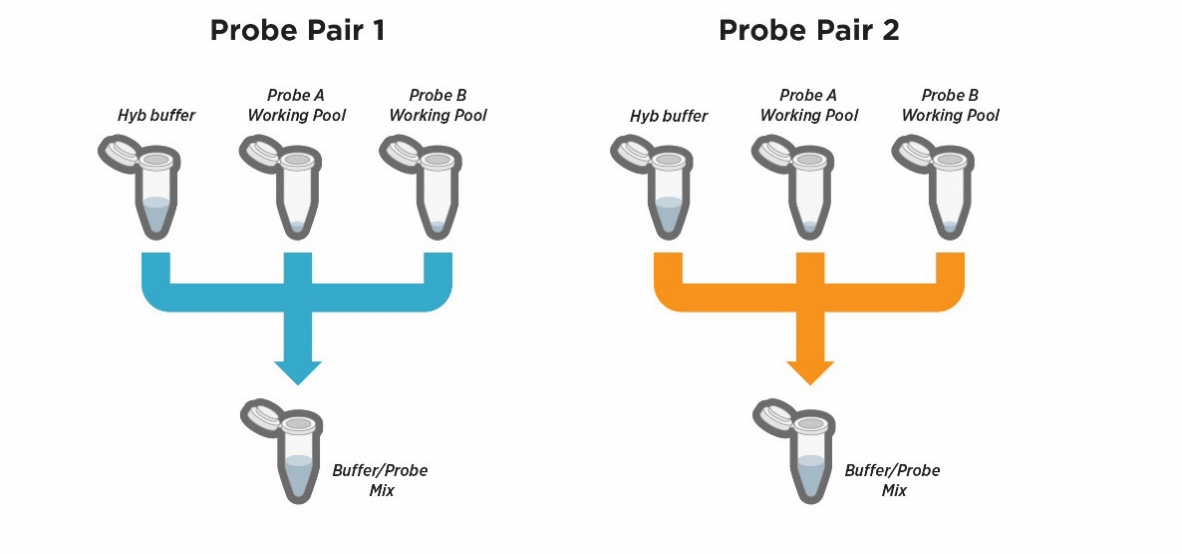


Figure 19. Buffer/Probe Mixes (number may vary based on Table 15)

Step 3: Create PlexSet A through H Master Mixes

1. Remove PlexSets A–H from the freezer and thaw at room temperature.

   NOTE: Avoid prolonged exposure of PlexSets to light during thawing and storing.

2. Invert several times to mix well, and briefly spin down in a picofuge. Store on ice.
3. Add **28 µL** of the provided Hybridization Buffer to each of the 8 PlexSet tubes (A–H) and mix by flicking or pipetting gently up and down. Spin briefly. These are the PlexSet Mixes (A–H). Store on ice.

Step 4: Set Up Hybridization

1. Set up the hybridization reactions in a 96-well plate with a tight seal that does not allow for evaporation in an overnight incubation at 67°C.

   NOTE: NanoString recommends using plates with caps to seal the wells, such as Applied Biosystems MicroAmp Optical 96-well Reaction Plate (Catalog No. N8010560) and MicroAmp 12-Cap Strip (Catalog No. N8010534), or any similar product. Eight 12-well strip tubes can also be used in place of a 96-well plate. Strips should be well labeled to maintain order.

   IMPORTANT: DO NOT USE sticky foil or plastic seals because of sample evaporation during overnight hybridization**.** A heat sealer can be used, such as the ALPS 50V heat sealer from Thermo Scientific to ensure integrity of a sealing system, NanoString recommends testing a plate in an overnight incubation at 67°C with **15 µL** of water per well to verify that there is no significant evaporation. Carefully monitor the volume in all wells following incubation (uneven volume loss across the plate is common). The loss of 1–3 microliters per well will not significantly affect the assay outcome, but larger volume loss will negatively impact the results.

2. Pipette **4 µL** of each PlexSet Mix across the plate into each of the 12 wells of the appropriate row (Figure 20). Pipette directly into the bottom or spin down briefly after adding PlexSet reagents. For example, PlexSet A should go into row A and PlexSet B should go into row B. The use of a repeater pipette will help speed up this process.

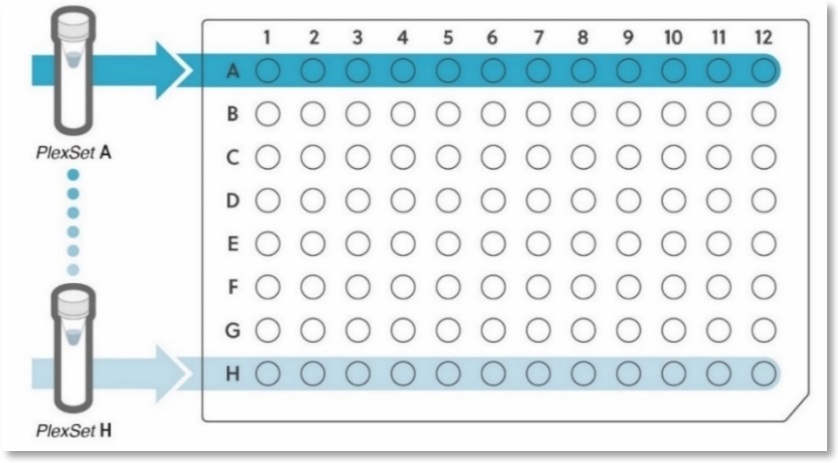


Figure 20. Hybridization Setup

Step 5: Add Diluted Buffer/Probe Mixes

1. Pipette **4 µL** of each Buffer/Probe Mix from Step 2: Create Buffer-Probe Mix **down the plate** into each of the 8 wells of the appropriate column (Figure 21).

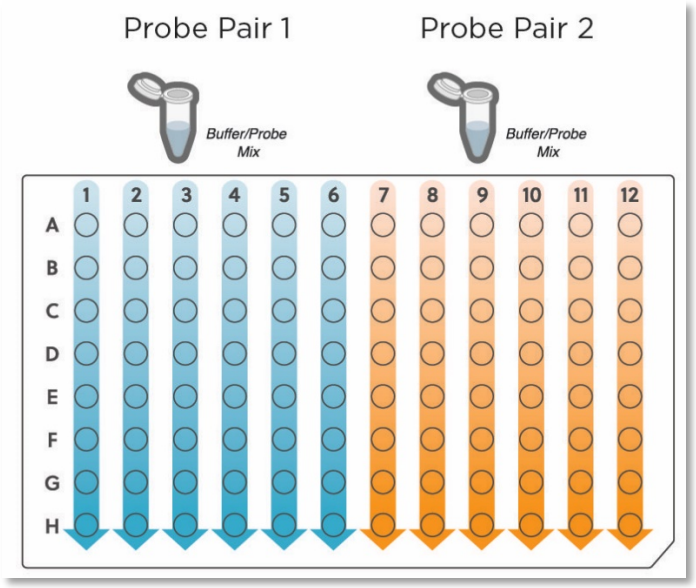


Figure 21. Adding Buffer/Probe Mixes (using 6 columns of Pair 1 and 6 columns of Pair 2 as an example)

Step 6: Add Samples

1. Prepare samples at the correct input concentration in a 96-well PCR plate with a sample volume **≥ 10 µL** for a faster assay setup. A multi-channel pipette can then be used to transfer samples into the 96-well plate.
2. Add one sample to each well and mix by pipetting up and down 5 times. The final volume of each hybridization reaction will be **15 µL** (Figure 22).

   NOTE: For sample preparation and input guidelines, see MAN-10050, Preparing Nucleic Acid from FFPE Samples or MAN-10051, Preparing Nucleic Acid from Fresh/Frozen Samples. **A calibration sample for PlexSet calibration should be included down one column for each pair of Probe A and Probe B used.**

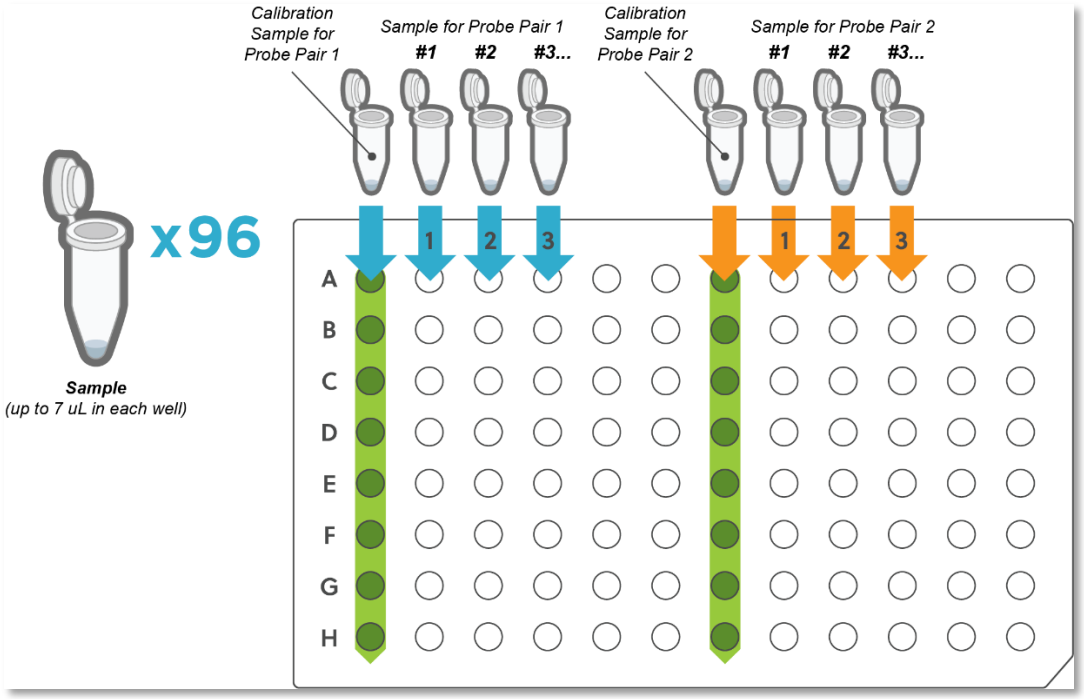


Figure 22. Adding Samples

Step 7: Incubate Reactions

1. Seal the plate carefully.

   IMPORTANT: See Note on sealing plate in Step 4: Set Up Hybridization

2. Spin down samples at 2,000*g* for 2 minutes.
3. Use a thermocycler with a heated lid set to 5°C above the temperature of the block.
4. Program the thermocycler to incubate the plate at 67°C for 16–24 hours, selecting a time that is compatible with the workflow the following day, and then ramp down to 4°C.

   NOTE: Do not leave the reactions at 4°C for more than 24 hours or increased background may result. For a series of related experiments, 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. 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.

Step 8: Pool Assays

1. Quick spin the plate, spinning just long enough to reach 2,000*g* and then allowing to stop.

   NOTE: The overnight incubation at 67°C may cause small, variable amounts of evaporative loss from the wells. **Pool each complete assay regardless of volume.** Volume loss of greater than **3 µL** may negatively affect the results of the assay from that well. See Step 4: Set Up Hybridization for plate sealing recommendations.

2. Pool the assays by columns down the plate, into a strip tube (Figure 23).

   IMPORTANT: If using the Prep Station, make sure the notches in the tubes are correctly oriented (see Figure 6).

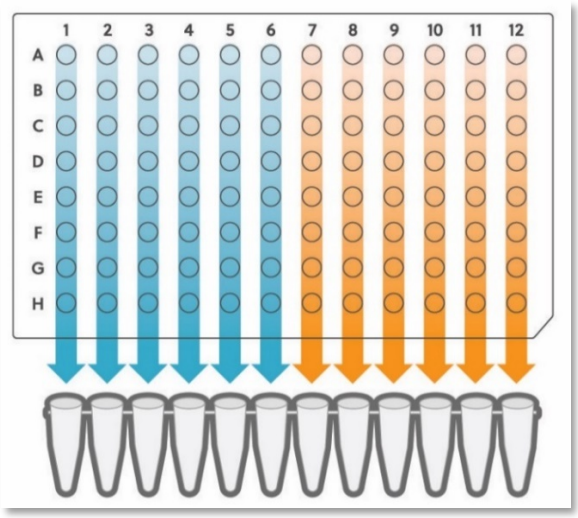


Figure 23. Pool the samples

3. Mix the final pool by gently pipetting up and down.
4. Load the samples on the workstation. Reference your Instrument's manual for how to run the nCounter system (MAN-10017 for the SPRINT Profiler or MAN-C0035 for the MAX/FLEX).

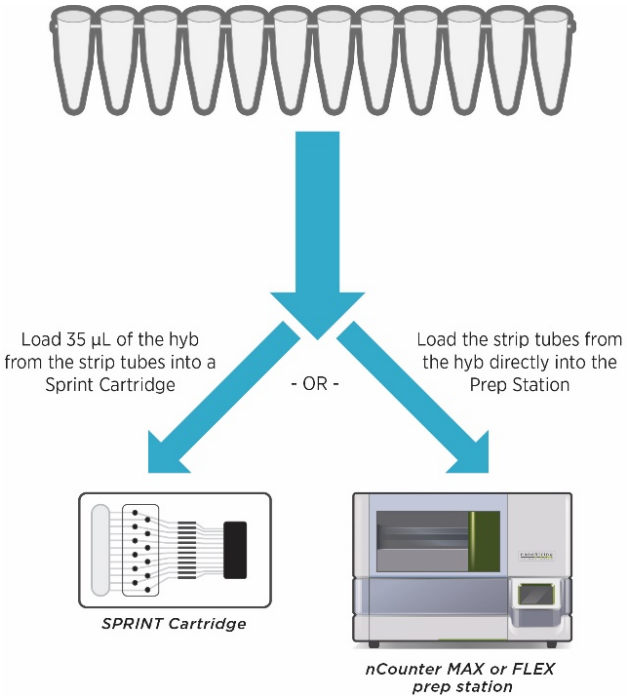


Figure 24. Load the samples on the workstation.

5. Proceed to Post-Hybridization Processing.

Post-Hybridization Processing

- **nCounter MAX/FLEX system**: Set up a standard nCounter Prep Station run with the strip tube containing the **120 µL** pooled reactions. Load your custom RLF (and optional CDF—see Naming Your Samples During Run Creation) onto the Digital Analyzer and scan the cartridge.
- **nCounter SPRINT system**: Create a run and upload your custom RLF from the SPRINT Control Center—see Naming Your Samples During Run Creation, or use the Default Run setting from the touchscreen (which will not allow you to enter any sample-specific data) and upload your custom RLF to the SPRINT via USB. Load **35 µL** of each pool into the SPRINT cartridge and run.

Naming Your Samples During Run Creation

The SPRINT and the Digital Analyzer recognize only the 12 lanes, not multiple samples within a lane (multiple samples within a lane are separated later by the nSolver software). This means that when creating a run (in the Control Center on the SPRINT or via a CDF (Cartridge Definition File) or on the touch screen on the MAX/FLEX), you cannot enter sample-specific information individually, as the instrument is only resolving down to the level of the lane. You are limited to entering information for the entire lane (up to 8 samples). nSolver will parse the data for you by plate coordinates, so it is not *required* to have sample identifiers in the run, but with no sample identifiers in the run, you are relying on an external source, such as a plate map, to use as a key.

If you would like sample identifiers to be associated with the run, you will need to enter the sample names for the entire column (which all end up in one lane) into the Sample Name/SampleID or Comments line of the Control Center New Run page (SPRINT) or via CDF or Run Creation on the touch screen (for the MAX/FLEX).

For a more extensive explanation of the CDF, please see the nCounter Analysis System User Manual (MAN-C0035) section on Cartridge Definition Files. A CDF template with a PlexSet example is included in the e-mail from Bioinformatics that contained your RLFs.

SPRINT Run Creation Using the Control Center

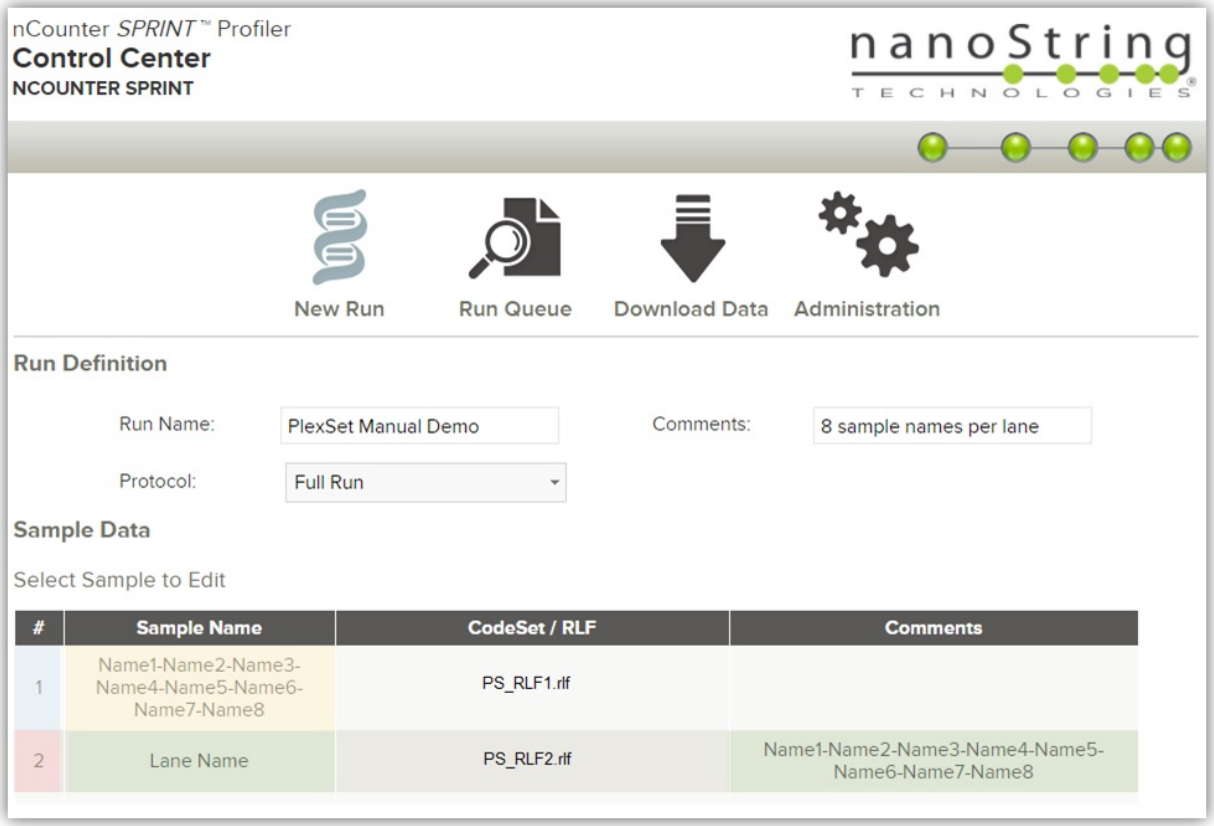
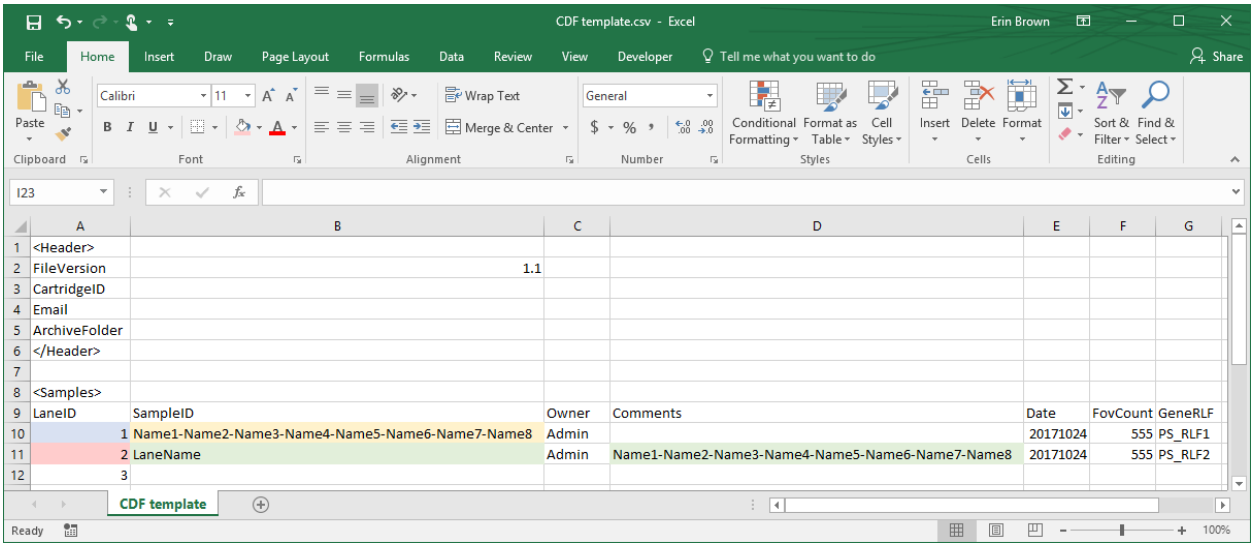


Figure 25. Choices for Sample Naming in the Sprint Control Center. Up to eight sample names can be listed in either the Sample Name column (Lane 1 in yellow) or into the Comments column with the Lane Name remaining generic (Lane 2 in green). The Sample Name field is limited to 64 characters and the Comments field is limited to 255 characters. See Figure 28 to see how these two options look on the exported data.

MAX/FLEX Run Creation using a CDF

Cartridge Definition Files (CDFs) can be modified in two ways. Open the file as an Excel-editable .csv file or as a .txt file. Both examples are shown below; please see the nCounter Analysis System User Manual (MAN-C0035) section 'Cartridge Definition Files' for more details on the modification. The file must be saved with the .cdf extension before being loaded onto a USB for uploading to the Digital Analyzer. Place the file in a folder named "CDFData" or the Digital Analyzer will not recognize it.



	A	B	C	D	E	F	G
1	<Header>						
2	FileVersion	1.1					
3	CartridgeID						
4	Email						
5	ArchiveFolder						
6	</Header>						
7							
8	<Samples>						
9	LaneID	SampleID	Owner	Comments	Date	FovCount	GeneRLF
10	1	Name1-Name2-Name3-Name4-Name5-Name6-Name7-Name8	Admin		20171024	555	PS_RLF1
11	2	LaneName	Admin	Name1-Name2-Name3-Name4-Name5-Name6-Name7-Name8	20171024	555	PS_RLF2
12	3						

Figure 26. Choices for Sample Naming in the CDF file (Excel .csv format). Up to eight sample names can be listed in either the SampleID column (Lane 1 in yellow) or into the Comments column with the Lane Name remaining generic (Lane 2 in green). The Sample Name field is limited to 64 characters and the Comments field is limited to 255 characters. See Figure 28 to see how these two options look on the exported data.

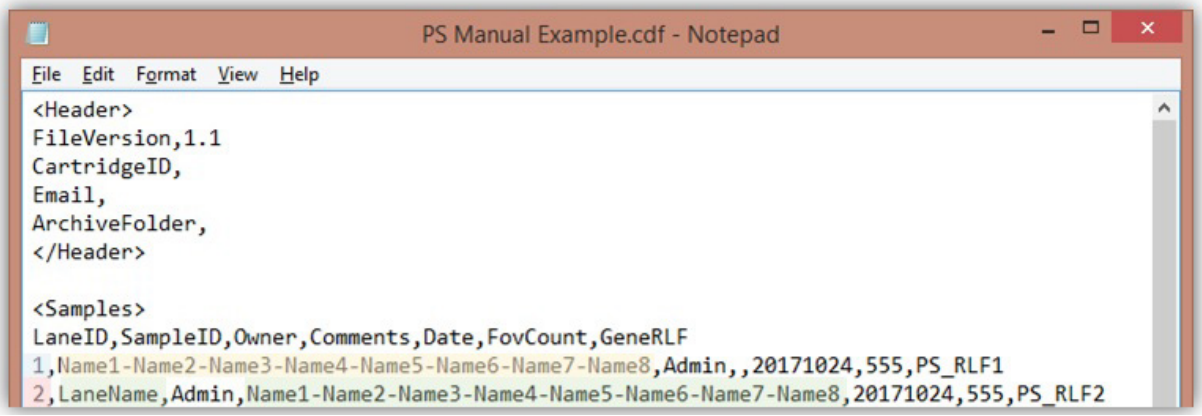


Figure 27. Choices for Sample Naming in the CDF file (.txt format). Up to eight sample names can be listed in either the SampleID column (Lane 1 in yellow) or into the Comments column with the Lane Name remaining generic (Lane 2 in green). The Sample Name field is limited to 64 characters and the Comments field is limited to 255 characters. See Figure 28 to see how these two options look on the exported data.

Exported Data for MAX/FLEX and the SPRINT

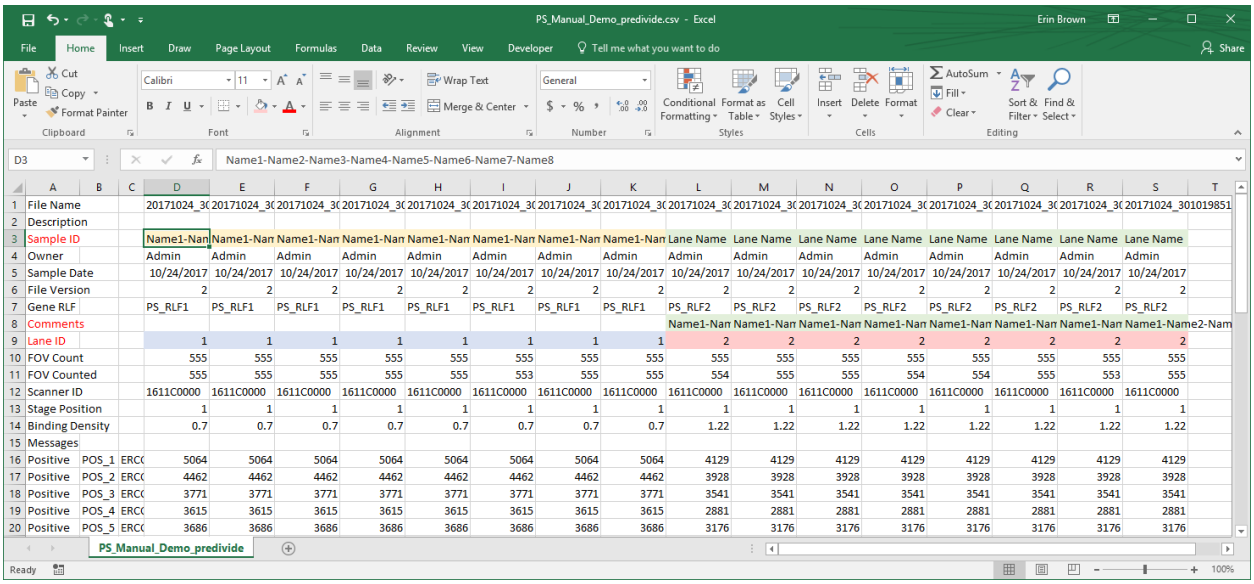


Figure 28. Exported Data. Sample Names input into the Sample Name/SampleID field are shown in yellow. Sample Names entered into the Comments field are shown in green in row 8. Lane 1 (blue) is now expanded to 8 columns, one for each sample in the Lane. Lane 2 is shown in red. All 8 names entered for the lane are repeated in all 8 columns for that lane.

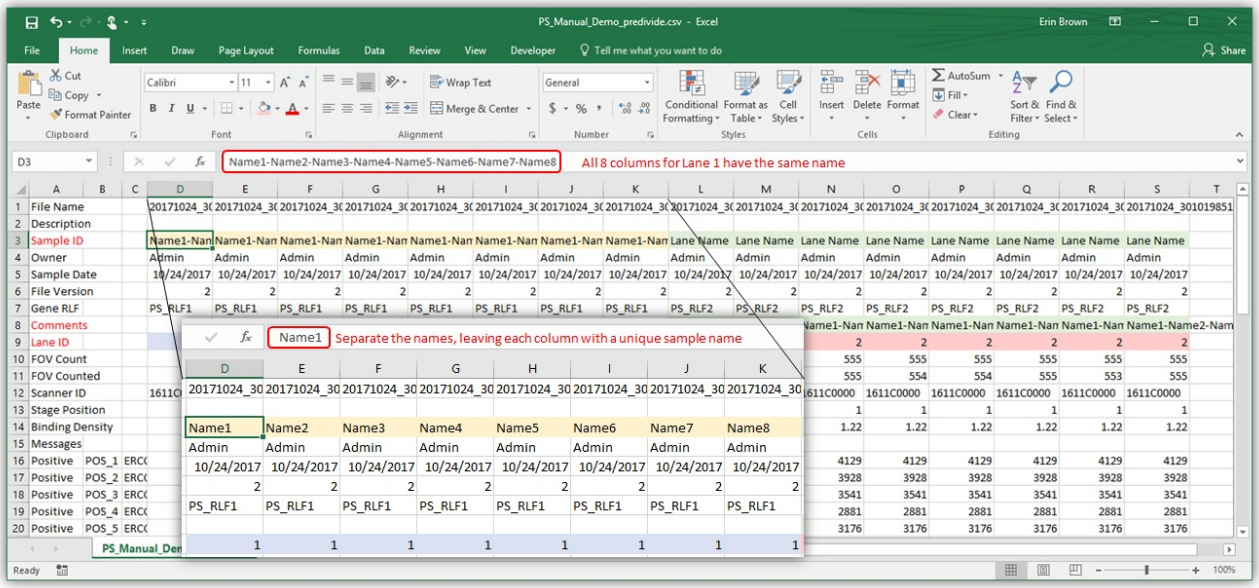


Figure 29. Dividing out the Sample Names. You can now go through and remove all the names, except the one for that sample, to give each sample a unique name.

To analyze the data, refer to All About PlexSet Data Analysis in nSolver (MAN-10044).

Appendix 1: Creating Oligonucleotide Master Stock

Overview

The oligonucleotide probes must be formatted into two separate pools, one containing all A Probes (Master Probe A Stock), and one containing all B Probes (Master Probe B Stock). If stored in aliquots at -20°C to -80°C, the Master Probe Stocks can be used for many experiments. Please refer to your oligonucleotide supplier for specific storage recommendations and shelf-life information.

Before setting up the hybridization reactions, 30X Working Probe Pools must be generated by diluting each Master Probe Stock. The final concentration of the oligonucleotides in 30X Working Probe A Pool will be 0.6 nM each, and the final concentration of the oligonucleotides in 30X Working Probe B Pool will be 3 nM each. The absolute concentration of the pool will vary depending on the plex of the experiment; due to the dilute DNA concentrations of many 30X Working Probe Pools, long-term storage and reuse of these pools is not recommended.

The protocols below provide an example of how to generate the Master Probe Stocks and 30X Working Pools for Probe A and Probe B. Depending on the oligonucleotide format obtained from the supplier, different pipetting volumes and dilutions may be necessary to achieve the required final concentrations.

> **IMPORTANT:** The concentrations of Probe A and Probe B in the hybridization reaction are critical for maximizing the sensitivity of the assay. Be sure to follow appropriate pooling and dilution protocols carefully to create accurate 30X Working Probe Pools.

Creating Master Probe Stocks

> **NOTE:** Some oligo suppliers will provide pooled oligos which can be used in place of creating your own Master Probe Stocks. If utilizing this service, specify a pool of Probe A at a final concentration of **5 nM** per oligo, and a separate pool of Probe B at a final concentration of **25 nM** per oligo. Pooled probes provided by oligo supplies at the recommended concentrations must still be further diluted to create 30X Working Probe Pools (see next page) before addition to the assay.
>
> **IMPORTANT:** Always create separate Probe A and Probe B stocks. Do NOT create a combined Master Probe Stock containing Probe A and Probe B in the same tube; elevated background and lowered assay sensitivity may result.

1. NanoString recommends resuspending individual Probe A oligonucleotides at a **1 µM** concentration, and individual Probe B oligonucleotides at a **5 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 supplier. To prepare Master Probe Stocks, begin by removing the appropriate Probe A and Probe B oligonucleotides from the freezer and thawing on ice.

2. Create Master Probe A Stock
    a. Pipette **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 Master Probe A Stock will be **5nM**.
    d. Store in aliquots at -20°C or -80°C as recommended by supplier.
3. Create Master Probe B Stock
    a. Pipette **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 Master Probe B Stock will be **25 nM**
    d. Store in aliquots at -20°C or -80°C as recommended by supplier.

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

    IMPORTANT: Minimize freeze-thaw cycles by storing Master Probe 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 30X Working Probe Pools. A suitable aliquot size for your workflow can be calculated from Table 13 and Table 14 based on your expected assay throughput. Follow supplier's guidance on stability of the oligonucleotide stocks at 4°C.

Appendix 2: 96-well Plate Whole Cell Lysis Protocols

Overview

Three lysis protocols have been developed for use with adherent cell lines, suspension cell lines, and primary cells. These protocols are in a 96-well plate lysis format for direct input of crude cell lysates into the PlexSet assay without RNA purification and all protocols have a lysis volume of **30 µL**.

- Primary cells are lysed with a chaotropic buffer, Buffer RLT (Qiagen, 79216), that has been diluted to 1/3 concentration in water.
- Adherent and suspension cell lines are lysed with a detergent-based lysis buffer, iScript buffer. (Bio-Rad, 1708898)

For hybridizations containing whole cell lysate, the addition of Proteinase K to the hybridization is essential to improve counts and data quality. To minimize the number of pipetting steps, add Proteinase K (**20 mg/mL** stock) to the Buffer-Probe Master Mix shown in Table 14. Recommended starting cell numbers based on cell type and nCounter system are summarized in Table 16**.**

Table 16. Recommended starting cell numbers to seed into 96-well plate.

		Viable cells seeded into 96-well plate		Max lysate input volume into hybridization
Cell source	Lysis Buffer	MAX/FLEX	SPRINT	
Adherent cell lines	iScript Buffer	5,000	5,000	7 µL
Suspension cell lines	iScript Buffer	43,000	86,000	7 µL
Primary cells	1/3 Diluted RLT Buffer	35,000	70,000	4.5 µL

IMPORTANT: Proteinase K **MUST** be added to the hybridization Master Mix (Table 14) to improve data quality.

See Figure 30 for the workflow for each cell type.

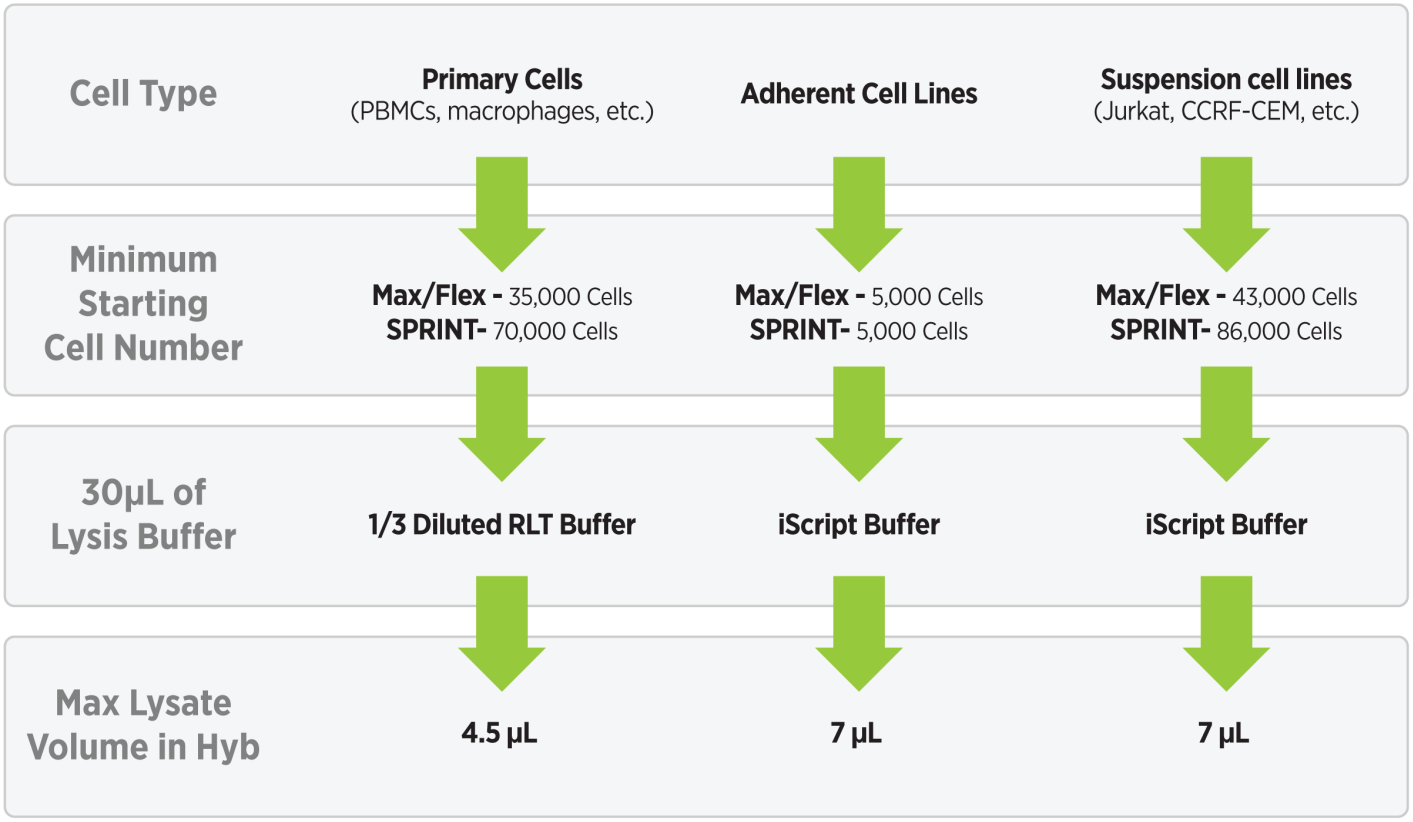


Figure 30. Whole Cell Lysis workflow

Suspension Cell Line Lysis

The following steps involve cell lysis with iScript Buffer

1. Centrifuge 96-well round bottom plate containing cells at 500*g* for 5 minutes at 4°C.
2. Remove media by tilting plate and carefully aspirating media with a vacuum. Make sure to aspirate as much of the media as possible without disturbing the cell pellet.
3. Add at least **30 µL** volume of lysis buffer to each well with a multichannel pipette.

   IMPORTANT: To prevent bubbles, which could lead to incomplete lysis, **DO NOT** go to second pipette stop and **DO NOT** pipette up and down at this step.
4. Set pipette to half the volume of lysis buffer added. Return pipette to the same wells with the lysis buffer and pipette up and down 10 times per sample while avoiding bubble formation. Change pipette tips with each row of cells to prevent cross-contamination.
5. Incubate lysates at room temperature for 5 minutes.
6. Transfer lysates to a PCR plate and place lysates on ice to use immediately or freeze at -80°C for long-term storage.
7. Use up to 7 µL of lysate in each NanoString hybridization reaction. Add proteinase K to hybridization at a final concentration of **200 µg/mL** to the Buffer/Probe Master Mix (Table 14).

Adherent Cell Line Lysis

The following steps involve cell lysis with iScript Buffer

1. Remove media by tilting plate and carefully aspirating media with a vacuum. Make sure to aspirate as much of the media as possible without disturbing the cells.
2. Add at least **30 µL** of lysis buffer to each well with a multichannel pipette.

   IMPORTANT: To prevent bubbles, which could lead to incomplete lysis, **DO NOT** go to second pipette stop and **DO NOT** pipette up and down at this step.
3. Incubate lysates at room temperature for 5 minutes.
4. Carefully collect the lysis supernatant without disturbing the cells. Avoid collecting any cell debris.
5. Transfer lysates to a PCR plate and place lysates on ice to use immediately or freeze at -80°C for long term storage.
6. Use up to **7 µL** of lysate in each NanoString hybridization reaction. Add Proteinase K to hybridization at a final concentration of **200 µg/mL** to the Buffer/Probe Master Mix (Table 14).

Primary Cell Lysis

1. Make fresh 1/3 Diluted RLT Buffer by combining **1 part Buffer RLT** with **2 parts nuclease-free water**. For example, 2 mL Buffer RLT with 4 mL nuclease-free water.

The following steps involve cell lysis with 1/3 Diluted RLT Buffer

2. Centrifuge 96-well round bottom plate containing cells at 400*g* for 8 minutes @ 4°C.
3. Remove media by tilting plate and carefully aspirating media with a vacuum. Make sure to aspirate as much of the media as possible without disturbing the cell pellet.
4. Add at least **30 µL** volume of 1/3 Diluted RLT Buffer to each well with a multichannel pipette.

   IMPORTANT: To prevent bubbles, which could lead to incomplete lysis, **DO NOT** go to second pipette stop and **DO NOT** pipette up and down at this step.
5. Set pipette to half the volume of lysis buffer added. Return pipette to the same wells with the lysis buffer and pipette up and down **15 times per sample** while avoiding bubble formation. Change pipette tips with each row of cells to prevent cross-contamination.
6. Transfer lysates to a PCR Plate and place lysates on ice to use immediately or freeze at -80°C for long term storage.

   IMPORTANT: If lysates are frozen at -80°C, thaw samples on ice, spin down, then remix the lysates prior to adding to hybridization.
7. Use up to **4.5 µL of primary cell lysate** in each NanoString hybridization reaction. Add proteinase K to hybridization at a final concentration of **200 µg/mL** to the Buffer/Probe Master Mix (Table 14).

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