

## Scope

This tech note presents a framework for creation of an oligonucleotide calibration sample for a variety of applications on the nCounter platform, including:

- 1) Calibration of nCounter gene expression data across different batches or manufacturing lots of CodeSet or TagSet reagents
- 2) Calibration of nCounter PlexSet data

An in-depth discussion of lot-to-lot variability and methods for data normalization with calibration samples is beyond the scope of this document. Please refer to [Best Practices for Clinical Research Biomarker Studies Using the nCounter® Platform: Strategies to Control for Variability](#) and [Panel Standard and Calibration Sample Usage](#) for more information. Calibration of PlexSet data is discussed further in the [PlexSet user manual](#).

Lot-to-lot calibration is a feature offered in NanoString's data analysis software called nSolver, and in Rosalind (<https://www.rosalind.bio/nanostring>), a cloud-based analysis package accessible through a web browser. Contact [support@nanostring.com](mailto:support@nanostring.com) or [info@rosalind.bio](mailto:info@rosalind.bio) to learn more.

## Types of Calibration Samples

For the purposes of this document and most applications, it is recommended to create a calibration sample with synthetic DNA oligonucleotide targets. They are the most economical option that offers near optimal results. In-vitro transcribed RNA is the optimal choice, but is high cost and labor-intensive, and not recommended for most applications.

For additional information about different types of calibration samples, refer to NanoString's White Paper titled: [Best Practices for Clinical Research Biomarker Studies Using the nCounter® Platform: Strategies to Control for Variability](#).

## Guidelines for Creation of a Synthetic DNA Calibration Sample

A framework for creation of a calibration sample from a pool of synthetic DNA oligonucleotides is presented below.

- 1) **For each target in the assay, purchase a synthetic DNA oligonucleotide corresponding to the 100-nucleotide probe target sequence.**

### Target sequence

Depending on the type of chemistry and panel being used, the correct target sequences can be found in a Gene List, CodeSet Design Report, or Design Summary

spreadsheet. Please contact the NanoString Bioinformatics team ([bioinformatics@nanostring.com](mailto:bioinformatics@nanostring.com)) with the name of your panel or custom design, and the team will assist you with identifying the correct target sequences. Probe pairs for nCounter chemistry span 75-100 nucleotides, but the Bioinformatics team will always disclose and recommend ordering a full 100 nt oligo regardless of the exact length of the real target.

### Purchase of oligonucleotides

Oligos can be purchased from various vendors, such as Integrated DNA Technologies (IDT) or Eurofins, in two different configurations:

- Individual oligos in separate tubes or wells
- A pool of multiple oligos

For a relatively large number of targets, it is recommended to order the oligos as a pool to mitigate the risk of pipetting errors that can occur during manual pooling in one's own lab. For calibration samples consisting of a relatively small number of targets, individual oligos can be ordered and pooled manually by the researcher. Purchasing individual oligos also adds the flexibility to remake a new pool if it is desired to remove or add targets.

Please note that oligo vendors utilize robotic pooling mechanisms that are imperfect. This may lead to occasional dropout of individual targets. Ideally, if the targets are ordered as a pool, the pool should be tested to ensure it contains all targets by running it with the CodeSet or TagSet. Even if the oligos are ordered as a pool, the original plate can typically be requested from the vendor. Targets missed by robotic pooling can usually be reclaimed by visiting the original plate well or requesting a re-synthesis run from the vendor.

If individual oligonucleotides are the preferred format, it is recommended to order them with the following specifications:

- DNA (single-stranded)
- Lyophilized
- Minimum scale
  - The minimum varies between vendors and storage format (plate vs. tube) but typically ranges from 25 nmole - 250 nmole. A minimum scale order is usually sufficient to run hundreds to thousands of reactions.

- Standard desalted purification
  - Standard desalted is appropriate for most applications, including lot-to-lot calibration and PlexSet calibration. For the most stringent applications, PAGE-purified oligos may provide increased performance.
  - Some oligonucleotide vendors may recommend PAGE purification during the ordering process, but this notification can be ignored if desalted is sufficient for your application.

If DNA oligonucleotides are ordered as a pool directly from a vendor, please skip steps #2 and #3 below, and proceed directly to step #4.

**2) For individual oligonucleotides, redissolve the lyophilized oligos at a nominal 100  $\mu$ M concentration in TE buffer (10 mM Tris pH 8.0; 1 mM EDTA).**

After redissolution, it is recommended to create and freeze aliquots at -80C or as recommended by the supplier for long-term storage.

**Caution:** The DNA oligonucleotides represent highly concentrated targets for each probe in the nCounter hybridization assay. Handle the oligos with great care to prevent contamination of equipment and the lab space. It is strongly recommended to dilute the oligonucleotides with dedicated pipettes using filtered tips, in a separate location or room from where the NanoString hybridization will be performed. It is also important to follow other best practices such as changing gloves frequently, cleaning surfaces and equipment with a reagent that eliminates DNA oligonucleotides (e.g., DNAZap™ or DNA AWAY™), and changing lab coats after completion.

**3) Create an equimolar\*, master stock pool from the individual oligonucleotides created in step #2.**

Two examples of how to create the master stock pool are presented below. Please note that it is possible to make the pool with an increased final concentration, if preferred, by adjusting the volume of each individual oligo added to the pool, adjusting the final volume of the pool, and/or adjusting the concentration of the redissolved lyophilized oligos in step #2. If pipetting less than 5 $\mu$ L per oligo, it is important to consider that it is generally recommended to pipette larger rather than smaller volumes in order to minimize variability that occurs with smaller volumes.

For a pool of less than or equal to 200 DNA targets:

- Pipette 5  $\mu$ L of each 100  $\mu$ M DNA oligo into a 1.7 mL nuclease-free microfuge tube.
- If necessary, add TE (10 mM Tris pH 8.0; 1 mM EDTA) to a final volume of 1 mL.

- The final concentration of each DNA oligo in the pool will be 500 nM.
- Store in aliquots at -20C or -80C, or as recommended by the supplier for long-term storage.

For a pool of 201 to 800 DNA targets:

- Pipette 5  $\mu$ L of each 100  $\mu$ M DNA oligo into a 5 or 15 mL nuclease-free centrifuge or conical tube.
- Add TE (10 mM Tris pH 8.0; 1 mM EDTA) to a final volume of 5 mL.
- The final concentration of each DNA oligo in the pool will be 100 nM.
- Store in aliquots at -20C or -80C, or as recommended by the supplier for long-term storage.

**\*Note:** For the most stringent applications, it is possible to quantify the resuspended oligos from step #2 in triplicate via spectrophotometry or fluorometry. The average concentration of each oligo could then be used to more precisely create an equimolar pool.

**Caution:** The DNA oligonucleotides represent highly concentrated targets for each probe in the nCounter hybridization assay. Handle the oligos with great care to prevent contamination of equipment and the lab space. It is strongly recommended to dilute the oligonucleotides with dedicated pipettes using filtered tips, in a separate location or room from where the NanoString hybridization will be performed. It is also important to follow other best practices such as changing gloves frequently, cleaning surfaces and equipment with a reagent that eliminates DNA oligonucleotides (e.g., DNAZap™ or DNA AWAY™), and changing lab coats after completion.

**4) Using serial dilutions of the master stock pool, create two separate working pools with concentrations of 150 fM and 50 fM. Dilutions should be performed with TE (10 mM Tris pH 8.0; 1 mM EDTA).**

**Note:** It is recommended to perform the serial dilutions with large volumes as this increases the accuracy of the dilutions and the linearity across inputs in a titration experiment. For example, it is preferable to dilute in 100-fold increments by combining 10  $\mu$ L of an oligo pool with 990  $\mu$ L of TE, instead of diluting in 1000-fold increments by combining 1  $\mu$ L of an oligo pool with 999  $\mu$ L of TE.

**Caution:** Due to the dilute nature of the 150 fM and 50 fM working pools, they should not be reused or refrozen. If additional calibration samples need to be run in the future, prepare a fresh dilution from frozen aliquots of master stock pools.

5) Using the nCounter gene expression assay of interest (off-the-shelf panel, custom panel, Elements TagSet, or PlexSet) perform a 12-sample input titration using the oligonucleotide working pools.

This titration will inform selection of an appropriate concentration for the calibration sample and will confirm that all oligonucleotides have been diluted and pooled correctly.

An example design for this 12-sample titration experiment is:

- 50 fM pool (n=3 technical replicates)

- 25 fM pool (n=3 technical replicates)
- 10 fM pool (n=3 technical replicates)
- 5 fM pool (n=3 technical replicates)

The concentrations listed above represent the oligonucleotide pool in the 15 µL final reaction volume of the nCounter hybridization. The maximum volume of oligonucleotide pool that can be added into the hybridization is 5 µL for CodeSet chemistry (i.e., off-the-shelf panels or custom panels) and 7 µL for TagSet chemistry (i.e., Elements or PlexSet titration).

An example of a titration using CodeSet chemistry, starting from 150 fM and 50 fM working pools is shown below:

Working concentration of oligo pool	Desired concentration of pool in the hybridization	Required dilution factor	Total volume of the hybridization	Volume of working pool to use in the hybridization	Water	Total sample volume (working pool + water) to add in the hybridization
150 fM	50 fM	3	15 µL	5.0 µL	0 µL	5 µL
150 fM	25 fM	6	15 µL	2.5 µL	2.5 µL	5 µL
50 fM	10 fM	5	15 µL	3.0 µL	2.0 µL	5 µL
50 fM	5 fM	10	15 µL	1.5 µL	3.5 µL	5 µL

An example of a titration using TagSet chemistry, starting from 150 fM and 50 fM working pools is shown below:

Working concentration of oligo pool	Desired concentration of pool in the hybridization	Required dilution factor	Total volume of the hybridization	Volume of working pool to use in the hybridization	Water	Total sample volume (working pool + water) to add in the hybridization
150 fM	50 fM	3	15 µL	5.0 µL	2.0 µL	7 µL
150 fM	25 fM	6	15 µL	2.5 µL	4.5 µL	7 µL
50 fM	10 fM	5	15 µL	3.0 µL	4.0 µL	7 µL
50 fM	5 fM	10	15 µL	1.5 µL	5.5 µL	7 µL

## 6) Analyze the titration data to select an appropriate concentration for the calibration sample.

Based on the results of the titration, select the appropriate concentration of oligonucleotide pool for use as a calibration sample. Since the assay is highly linear, it is not a requirement to select a concentration that was specifically included in the titration (e.g., 50, 25, 10, or 5 fM). The titration data form a linear dilution.

The Binding Density QC metrics generated in the titration experiment can be used to gauge saturation potential across the range of concentrations tested. On the MAX instrument, the upper limit of binding density is 2.25. On the SPRINT instrument, the binding density upper limit is 1.8.

Given that nCounter experiments can span a range of approximately 12-800 targets, there are no strict guidelines for total raw counts. For optimal results, however, all DNA oligonucleotide targets should be at a high enough concentration to yield robust counts for data calibration. In general, raw counts for each target should be greater than 100 and ideally more than 1000, while avoiding QC flags for

high binding density. If using PlexSet chemistry to multiplex 8 samples in a single lane on a cartridge, it is important to consider the increased risk for saturation. Refer to the PlexSet assay manual for more information.

There can be differences in the fraction of full-length oligonucleotide that is present for the various DNA targets in the calibration pool. These differences are introduced during oligonucleotide synthesis and purification. Due to this factor and the slight differences in hybridization efficiency from probe to probe, the range of raw counts generated by the nominally equimolar pool will vary for each DNA oligonucleotide target.

Since the nCounter assay is highly linear, it is straightforward to increase the counts of specific targets during the pooling process. For example, after examining the titration data, if it were desired to double the raw counts of a specific target, this can be accomplished by remaking the pool with double the amount of that target. In other words, if 5  $\mu$ L of a specific target were added into the initial equimolar pool, then remake the pool using 10  $\mu$ L of that target to double its counts.

For more information, please visit [nanosttring.com](https://nanosttring.com)

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