TECH NOTE:
Panel Standard and Calibration Sample Usage

nanoString

Panel Standards

Introduction

Panel Standards contain a pool of synthetic DNA oligonucleotides that correspond to the target sequence of each of the unique probes in the associated panel. They enable for correction of technical variability not related to sample quality or sample input, which is critical when comparing data within many study designs. Panel standards are just one type of calibration sample. If a panel standard is not available for your CodeSet, please refer to WP_MK3415 for other calibration sample options. Analysis and recommendations for calibration samples are the same as for panel standard.

NOTE: Panel standards 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 panel standard with dedicated pipettes using filtered tips, in a separate location or room from where the 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. Instructions for Panel Standard usage can be found in MAN-10056, CodeSet Hybridization Setup manual.

Panel Standard Usage

Recommendations for Use

In ranked order of most to least optimal experimental design, the options for use of the panel standard are:

1) Once per cartridge

2) Once per batch, where a batch is defined by a unique combination of the factors below. If any one of these variables change, we recommend you consider that run a new "batch".

   a. Lot of codeset (found on the tube vial)

   b. User

   c. Instrument; Note: we recognize that some high-throughput users will employ multiple prep stations and 1 Digital Analyzer (DA) with MAX/FLEX instrument setups. It is not necessary to control for the slight amount of variation introduced by the Prep Station but depending on the data application, it may be desirable to use a panel standard to control for variability across digital analyzers (DA). If a customer were using different versions of the nCounter instrument (i.e. a MAX and a Sprint), this would be a crucial variable to control for with panel standard. While different MAX/FLEX DAs may provide slightly different results, the variability due to this factor is smaller than the variability between MAX/FLEX and SPRINT instruments.

   d. Date of the run; if the runs were all made in sequence (one right after another), these can be considered a batch. However, if there was a span of time between them (e.g. weeks to a month), it would be prudent to consider these runs separate batches.

Application-Specific Recommendations

Usage of any calibration sample is based on the application intended for the study. In this section, panel standard could be replaced by any calibration sample if one is not available for your CodeSet (see previous note). Reference sample is specifically defined later in this document. Please refer to that definition if it is relevant to your application. The following are the recommendations based on the application for the data in question:

1) Any RUO clinical uses, or data to be used for development of a diagnostic predictor or signature: recommend once per cartridge, not less than once per batch

2) Data Analysis Service (DAS) for PAM50 or TIS calculation, regardless of panel/codeset utilized: recommend once per cartridge, not less than once per batch

3) TCR Diversity or other panels DAS: recommend once per cartridge, not less than once per batch

4) CRO nAlgorithm Program

   a. Without PanCancer IO 360™ or Breast Cancer 360™ Data Analysis Report: reference sample only, as directed (once per cartridge)

   b. With PanCancer IO 360 or Breast Cancer 360: reference sample as directed (once per cartridge), panel standard at least once per batch

   c. Note: Specific signature assays have associated reference samples covering the probes in the signature (see section on Reference Samples later in this document). Some panels contain these signatures and many additional

genes. Please be aware of when choosing a calibration sample which genes have corresponding targets in that sample. This guidance also applies to customers using Panel Plus with an existing panel. As the targets of the Panel Plus probes are not in the existing panel standard, these genes will require their own calibration sample.

5) RUO Panels use, no DAS: recommend once per batch

Lot Control and Longitudinal Studies

Panel Standard lots have comparable shelf life to CodeSet (or panel) lots, thus the consistency amongst lots of panel standard should be considered in longitudinal studies. If the lot of panel standard changes, bridging is required. A bridging experiment requires 2 lots of panel standard, one lot of CodeSet, and 3 or more samples that are run on each lot to be bridged. This should be run on a single cartridge with a single lot of CodeSet, containing 1 lane of panel standard lot A, 1 lane of panel standard lot B, and 3+ samples.

It is ideal to use the same lot of codeset, and at a minimum the same lot of panel standard, across the whole study. Please consider the design of your experiment and obtain the required amounts of panel standard and CodeSet to complete your study. It's also prudent to plan for the potential of failed runs and the necessity to rerun specific samples. Note: the panel standard has a 2 year expiration, and CodeSets have a 3 year expiration from date of manufacture. We recommend you discuss your study design with your account manager or NanoString Support, who can assist you with creating a plan for your research.

Reference Sample and Differences with Panel Standard

A panel standard is a mixture of 100 base pair DNA oligos representing the target sequences for each probe pair in a gene expression panel. They enable calibration of data across different batches of samples. The process of creating the panel standard DNA oligo pool does not provide for the sequences to be present at precise equimolar concentration across oligos, however the sequences are generally present at relatively similar concentrations across oligos. Thus, each lot of panel standard requires bridging, even if the utility of a panel standard is very similar to that of a reference sample within a lot of panel standard. A reference sample is a highly controlled mixture of in vitro transcribed RNA molecules that represent the targets for each probe pair in a signature calculation. These tend to be longer than the 100 base target sequence, and are produced in such a way as to be identical in performance to all previous lots. Thus, reference samples are more absolute and do not require bridging. However, they only cover the probes found in our signature assays (PAM50, LST, and TIS). Reference sample is only available when purchased with certain assays, and is not necessary for calculation of the signatures.

Panel Standard Normalization

Background

Using the Panel Standard addresses all types of variability within the assay. For longitudinal studies across multiple panel lots, it is critical to include at least one lane of Panel Standard at each time point and for each lot to calibrate counts across different time points and panel lots. Panel Standard Normalization refers to the process of using the Panel Standard to calibrate counts across biological samples from multiple cartridges and/or panel lots. Samples associated with the same Panel Standard, either from the same cartridge or the same study, are termed one "set" of samples.

Normalization Procedures

Here, we explain three methods of normalization using two vials of the same lot of Panel Standard (Panel Standard A and B) with two sets of samples (Sample Set A and Sample Set B), representing separate batches of samples for calibration. The first two procedures begin with Panel Standard Normalization, followed by Housekeeping Normalization, which accounts for sample input variability, while the third normalization procedure reverses the normalization order.

Procedures 1-3 will each be illustrated using this example dataset:

Target	Sample Set A			Sample Set B		
	Sample 1	Sample 2	Panel Standard A	Sample 3	Sample 4	Panel Standard B
Housekeeping Gene 1	54249	63731	3278	56776	71673	2778
Housekeeping Gene 2	412	461	2640	431	555	2351
Housekeeping Gene 3	1642	1936	1842	1690	2163	1500
Gene 1	724	806	2053	532	676	1320
Gene 2	54	57	2607	48	44	2099
Gene 3	1639	2015	2924	1658	2078	2406
Gene n	...	...	...	...	...	...

Procedure 1 - Use one Panel Standard as an absolute reference and calculate housekeeping normalization based on the average across all samples

Step 1. Create Panel Standard normalization factors for Set B

For each target, divide the raw count of Panel Standard B by the corresponding raw count of Panel Standard A to create a normalization factor for Set B.

Target	Panel Standard A	Panel Standard B	Panel Standard Normalization factor B to A (B/A)
Housekeeping Gene 1	3278	2778	0.847
Housekeeping Gene 2	2640	2351	0.891
Housekeeping Gene 3	1842	1500	0.814
Gene 1	2053	1320	0.643
Gene 2	2607	2099	0.805
Gene 3	2924	2406	0.823
Gene n	...	...	...

Step 2. Panel Standard Normalization

For the samples in Set B, divide the raw counts for each target by the corresponding Panel Standard normalization factors. Combine biological samples from Set A and Set B into one data set.

Target	Sample Set A		Sample Set B			Panel Standard normalized Set A		Panel Standard normalized Set B	
	Sample 1	Sample 2	Sample 3	Sample 4	Normalization factor B to A	Sample 1	Sample 2	Sample 3	Sample 4
Housekeeping Gene 1	54249	63731	56776	71673	0.847	54249	63731	66995	84573
Housekeeping Gene 2	412	461	431	555	0.891	412	461	484	623
Housekeeping Gene 3	1642	1936	1690	2163	0.814	1642	1936	2075	2656
Gene 1	724	806	532	676	0.643	724	806	827	1051
Gene 2	54	57	48	44	0.805	54	57	60	55
Gene 3	1639	2015	1658	2078	0.823	1639	2015	2015	2525
Gene n	...	...	...	...	...	...	...	...	...

Step 3. Create Housekeeping normalization factors for each sample

Calculate the geometric mean of the housekeeping genes only for each biological sample. Calculate the average of these geometric means across all biological samples in the combined data set. Create a normalization factor for each sample by dividing the sample's geometric mean by the average geometric mean.

Target	Panel Standard A	Panel Standard B	Panel Standard Normalization factor B to A (B/A)	Panel Standard Normalization factor B to A (B/A)
Housekeeping Gene 1	54249	63731	66995	84573
Housekeeping Gene 2	412	461	484	623
Housekeeping Gene 3	1642	1936	2075	2656
Gene 1	724	806	827	1051
Gene 2	54	57	60	55
Gene 3	1639	2015	2015	2525
Gene n	...	...	...	...
Geometric mean of Housekeeping genes	3323	3846	4067	5193
Average of HK geomean	4017			
Normalization factor	0.81	0.94	0.99	1.26

Step 4. Housekeeping normalization

Divide the panel standard normalized counts in a given biological sample by the corresponding housekeeping normalization factor generated in Step 3.

Target	Sample 1	Sample 2	Sample 3	Sample 4
Housekeeping Gene 1	67048	69063	67651	66897
Housekeeping Gene 2	509	492	489	493
Housekeeping Gene 3	2029	2068	2096	2101
Gene 1	895	861	836	832
Gene 2	67	61	60	43
Gene 3	2026	2152	2035	1998
Gene n	...	...	...	...

Procedure 2 - Calculate a Panel Standard within each set, then calculate housekeeping normalization based on each sample

Step 1. Panel Standard Normalization

For the biological panel samples in Set A, divide the raw count for each target by its corresponding raw count of Panel Standard A; for the biological panel samples in Set B, divide the raw count for each target by its corresponding raw count of Panel Standard B.

Target	Sample Set A		Sample Set B	
	Sample 1	Sample 2	Sample 3	Sample 4
Housekeeping Gene 1	16.549	19.442	20.438	25.8
Housekeeping Gene 2	0.156	0.175	0.183	0.236
Housekeeping Gene 3	0.891	1.051	1.127	1.442
Gene 1	0.353	0.393	0.403	0.512
Gene 2	0.021	0.022	0.023	0.021
Gene 3	0.561	0.689	0.689	0.864
Gene n	...	...	...	...

Step 2. Create Housekeeping normalization factors for each sample

Calculate the geometric mean of the housekeeping genes only for each biological sample.

Target	Sample Set A		Sample Set B	
	Sample 1	Sample 2	Sample 3	Sample 4
Housekeeping Gene 1	16.549	19.442	20.438	25.8
Housekeeping Gene 2	0.156	0.175	0.183	0.236
Housekeeping Gene 3	0.891	1.051	1.127	1.442
Gene 1	0.353	0.393	0.403	0.512
Gene 2	0.021	0.022	0.023	0.021
Gene 3	0.561	0.689	0.689	0.864
Gene n	...	...	...	...
Geometric mean of Housekeeping genes	1.32	1.528	1.616	2.063

Step 3. Housekeeping normalization

Divide the panel standard normalized counts in a given biological sample by the corresponding housekeeping geometric mean.

Target	Sample Set A		Sample Set B	
	Sample 1	Sample 2	Sample 3	Sample 4
Housekeeping Gene 1	12.533	12.724	12.647	12.506
Housekeeping Gene 2	0.118	0.175	0.183	0.236
Housekeeping Gene 3	0.675	1.051	1.127	1.442
Gene 1	0.267	0.393	0.403	0.512
Gene 2	0.016	0.022	0.023	0.021
Gene 3	0.425	0.689	0.689	0.864
Gene n	...	...	...	...

Procedure 3 - Calculate housekeeping normalization based on each sample, then Panel Standard normalization factors within each set

Step 1. Create Housekeeping normalization factors for each sample

Calculate the geometric mean of the housekeeping genes for both biological samples and Panel Standard.

Target	Sample Set A			Sample Set B		
	Sample 1	Sample 2	Panel Standard A	Sample 3	Sample 4	Panel Standard B
Housekeeping Gene 1	54249	63731	3278	56776	71673	2778
Housekeeping Gene 2	412	461	2640	431	555	2351
Housekeeping Gene 3	1642	1936	1842	1690	2163	1500
Gene 1	724	806	2053	532	676	1320
Gene 2	54	57	2607	48	44	2099
Gene 3	1639	2015	2924	1658	2078	2406
Gene n	...	...	...	...	...	...
Geometric mean of Housekeeping genes	3323	3846	2517	3458	4415	2140

Step 2. Housekeeping normalization

Divide the panel standard normalized counts in a given sample by the corresponding housekeeping geometric mean.

Target	Sample Set A			Sample Set B		
	Sample 1	Sample 2	Panel Standard A	Sample 3	Sample 4	Panel Standard B
Housekeeping Gene 1	16.324	16.572	1.302	16.418	16.235	1.298
Housekeeping Gene 2	0.124	0.12	1.049	0.125	0.126	1.099
Housekeeping Gene 3	0.494	0.503	0.732	0.489	0.49	0.701
Gene 1	0.218	0.21	0.816	0.154	0.153	0.617
Gene 2	0.016	0.015	1.036	0.014	0.01	0.981
Gene 3	0.493	0.524	1.162	0.479	0.471	1.124
Gene n	...	...	...	...	...	...

Step 3. Panel Standard Normalization

For the biological panel samples in Set A, divide the housekeeping normalized counts for each target by its corresponding housekeeping normalized counts of Panel Standard A; for the biological panel samples in Set B, divide the housekeeping normalized counts for each target by its corresponding housekeeping normalized counts of Panel Standard B.

Target	Sample Set A		Sample Set B	
	Sample 1	Sample 2	Sample 3	Sample 4
Housekeeping Gene 1	12.533	12.723	12.646	12.505
Housekeeping Gene 2	0.118	0.114	0.113	0.114
Housekeeping Gene 3	0.675	0.688	0.697	0.699
Gene 1	0.267	0.257	0.249	0.248
Gene 2	0.016	0.014	0.014	0.01
Gene 3	0.425	0.451	0.426	0.419
Gene n	...	...	...	...

Other Considerations for Panel Standard Normalization

The methods described all result in calibration of batches of samples, but there are considerations for the use of each method. The first procedure uses one of the Panel Standards as an absolute reference and calculates the housekeeping normalization factor based on the average across all samples. However, this procedure is not sample-intrinsic, i.e., the normalized counts for one biological sample depend on all other samples normalized at the same time, and the full dataset requires re-normalization upon the addition of each new sample set. This adds undesirable complexity to longitudinal or aggregate studies. In contrast, the second procedure is sample-specific, and defines the Panel Standard normalization factor within each set and housekeeping normalization factor within each sample, which makes each normalized value more challenging to interpret but simpler to implement and adapt to longitudinal or aggregate studies. Shifting the order of Panel Standard normalization and housekeeping normalization in Procedure 2, making it Procedure 3, does not change the results. It is noted that the results from Procedure 1 versus Procedures 2 or 3 will only differ by a scaling factor. We recommend Procedure 2 or 3 but acknowledge that Procedure 1 is also reasonable with some caveats.

nSolver™ has a batch calibration option that is similar to Procedures 2 and 3, in that normalization is done in a manner useful for longitudinal studies. All normalizations in nSolver are performed per set, i.e., the normalizations are based on factors calibrated within each set of samples registered to each panel standard. Normalization is first applied to the positive controls, followed by the housekeeping genes, and then per gene to the sample chosen as the calibration sample, in this case, the panel standard. In order to use this option, the sets are annotated as separate batches in nSolver (Figure 1), and the panel standard in each set of samples is identified as your calibration sample (Figure 2). Please refer to the section on batch calibration in MAN- C0019, nSolver 4.0 Analysis Software User Manual for more detailed instructions.

	File Name	Description	Batch ID
1	20191030_208513901220_01_07.RCC	BRCA_AU565	1
2	20191030_208513891220_01_05.RCC	BRCA_BT-549	1
3	20191030_208513891220_01_07.RCC	BRCA_CAL51	2
4	20191107_208513881220_01_09.RCC	BRCA_CAMA1	2

FIGURE 1: Annotate sets with batch ID

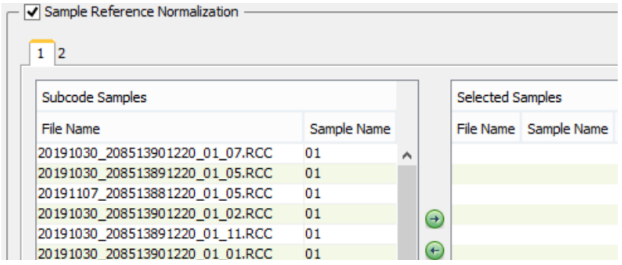

FIGURE 2: Select panel standard in each set as a calibration sample

For more information, please visit nanostring.com

NanoString Technologies, Inc.
530 Fairview Avenue North
Seattle, Washington 98109
T (888) 358-6266
F (206) 378-6288
nanostring.com
info@nanostring.com

Sales Contacts
United States **us.sales@nanostring.com**
EMEA: **europe.sales@nanostring.com**
Asia Pacific & Japan **apac.sales@nanostring.com**
Other Regions **info@nanostring.com**

JUL 2021 MK3415