# Gene Expression Data Analysis Guidelines

## Purpose

This document describes recommended procedures for the analysis of nCounter® gene expression assays. It can serve either as a stand-alone guide for analyzing nCounter datasets using a third-party software package, or it may be utilized as a companion guide to our own nSolver™ Analysis Software (https://www.nanostring.com/products/analysis-software/nsolver, *Getting Started* tab). The following content mirrors the available tools and options within nSolver, and contains brief "break-out boxes" illustrating how to implement a subset of these options in this Analysis Software. For a shorter overview of workflow without any accompanying recommendations, refer to the *Quick Start Guide to nSolver*, or, for a more comprehensive guide on the execution of all nSolver features, see the full *nSolver Analysis Software User Manual* (https://www.nanostring.com/products/analysis-software/nsolver, *Support Documents* tab).

## Introduction

Gene expression assays on the nCounter system provide reliable, sensitive, and highly-multiplexed detection of mRNA targets. These assays are usually performed without the use of enzymes or amplification protocols, and do not rely on degree of fluorescence intensity to determine target abundance. These characteristics, combined with the highly-automated nature of barcoded sample processing, result in a platform that yields data which is both precise and reproducible. Nevertheless, it is prudent to follow procedures outlined herein to ensure that you are generating the highest data quality possible.

Figure 1 shows a general workflow for analyzing an nCounter gene expression experiment. In an iterative process, data quality is assessed after every stage of analysis. We start by evaluating the general assay performance using **Quality Control (QC)** metrics recorded in the nCounter data files (RCC files). Afterwards, we perform **Background** correction and data **Normalization** followed by another round of data QC. Finally, we assess the resulting **Ratios, Fold-Changes and Differential Expression**, and these results, too, should be carefully checked for quality control characteristics.

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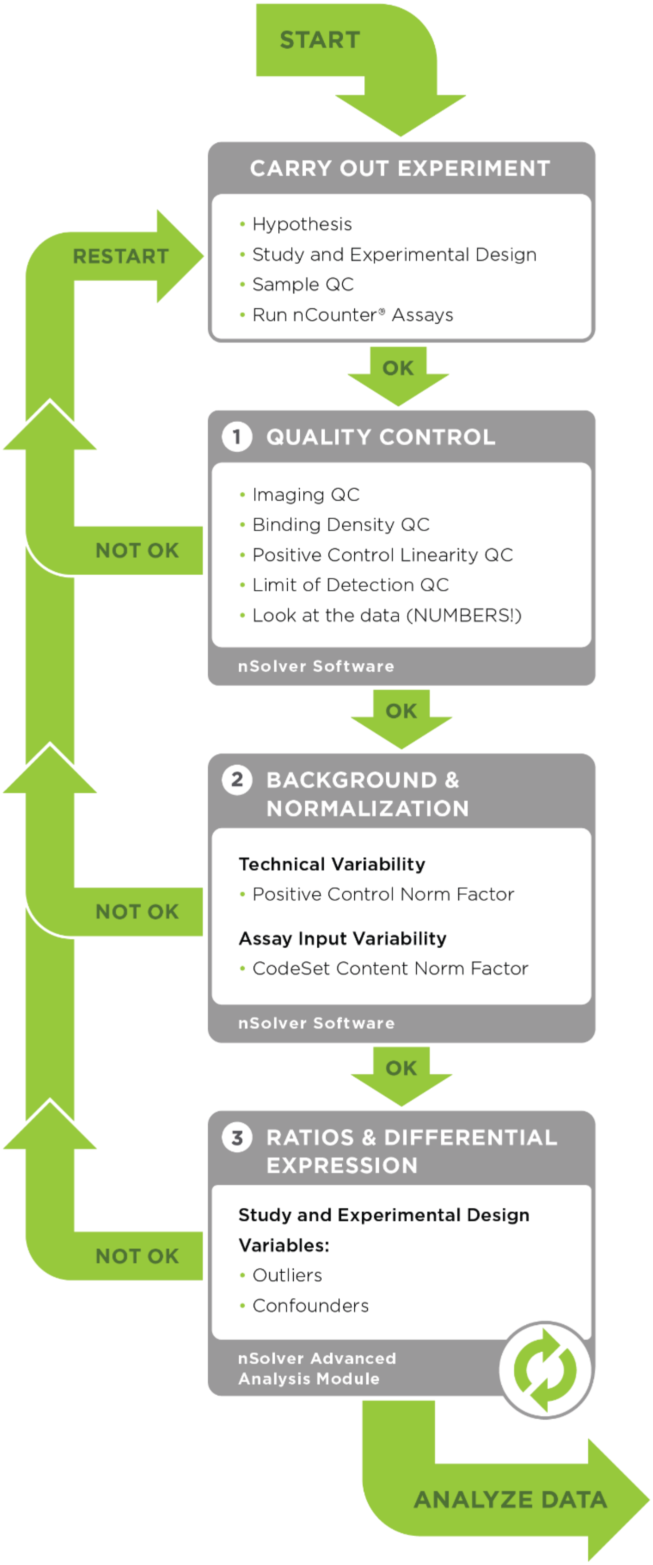


Figure 1: Quality Control workflow for NanoString nCounter® Gene Expression experiments

## Quality Control

To ensure that data resulting from an nCounter Gene Expression experiment on the nCounter analysis system is of substantial quality to be used in a subsequent in-depth statistical analysis, it is necessary to perform a thorough quality control check on that data. In this section, we address Quality Control (QC) for General Assay Performance.

We start checking the overall performance of the nCounter assay by evaluating the **Imaging** and **Binding Density** QC metrics. Next, we assess the performance of the positive controls using the **Positive Control Linearity** and **Limit of Detection** parameters. Finally, we do an overall visual inspection of the data and assess the severity of any **QC flags**.

### Imaging QC

Each individual lane scanned on an nCounter system is divided into a few hundred imaging sections, called Fields of View (FOV), the exact number of which will depend on the system being used (i.e., MAX/FLEX or SPRINT), and the scanner settings selected by the user. The system images these FOVs separately, and sums the barcode counts of all FOVs from a single lane to form the final raw data count for each unique barcode target. Finally, the system reports the number of FOVs successfully imaged as **FOV Counted**.

Significant discrepancy between the number of FOV for which imaging was attempted (FOV Count) and for which imaging was successful (FOV Counted) may indicate an issue with imaging performance. Recommended percentage of registered FOVs (i.e. FOV Counted over FOV Count) is 75%. Lanes will be flagged if this percentage is lower.

*Factors Affecting Imaging*

- *Cartridge surface:* fingerprints or dust particles on the cartridge should be wiped off.
- *Cartridge placement:* for MAX/FLEX instruments, a slightly tilted cartridge can lead to low FOV registration. Rescan the cartridge after ensuring that it is firmly seated in the imaging slot.

$$\frac{\text{FOV Counted (Fields of View successfully counted)}}{\text{FOV Count (Fields of View attempted)}} = \text{at least } 75\%$$

## Binding Density QC

The imaging unit only counts the codes that are unambiguously distinguishable. It simply will not count codes that overlap within an image. This provides increased confidence that the molecular counts you receive are from truly recognizable codes. Under most conditions, forgoing the few barcodes that do overlap will not impact your data. Too many overlapping codes in the image, however, will create a condition called **image saturation** in which significant data loss could occur (*critical* data loss from saturation is uncommon; see *Assessing QC Flags*, below).

To determine the level of image saturation, the nCounter instrument calculates the number of optical features per square micron for each lane as it processes the images. This is called the **Binding Density**. The Binding Density is useful for determining whether data collection has been compromised due to image saturation. With nSolver 4.0 (if you use a version other than 4.0, consult the user manual for that version), the acceptable range for Binding Density is:

- 0.1 - 2.25 for MAX/FLEX instruments
- 0.1 - 1.8 for SPRINT instruments

Within these ranges, relatively few reporters on the slide surface will overlap, enabling the instrument to accurately tabulate counts for each reporter species. A Binding Density significantly greater than the upper limit in either range is indicative of overlapping reporters on the slide surface. The counts observed in lanes with a Binding Density at this level may have had significant numbers of codes ignored, which could potentially affect quantification and linearity of the assay. Some of the factors that may contribute to increased Binding Density are listed in the *Factors affecting Binding Density* box.

### *Factors Affecting Binding Density*

- *Assay input quantity:* the higher the amount of input used for the assay, the higher the Binding Density will be. The relationship between input amount and Binding Density is linear until the point of assay saturation.
- *Expression level of genes:* if the target genes have high expression levels, there will be more molecules on the lane surface which will increase the Binding Density value.
- *Size of the CodeSet:* a large CodeSet with probes for many targets is more likely to have high Binding Density values than a CodeSet with probes for significantly fewer targets.

## Positive Control Linearity QC

Six synthetic DNA control targets are included with every nCounter Gene Expression assay. Their concentrations range linearly from 128 fM to 0.125 fM, and they are referred to as ***POS_A*** to ***POS_F***, respectively. These positive controls are typically used to measure the efficiency of the hybridization reaction, and their step-wise concentrations also make them useful in checking the linearity performance of the assay. An $R^2$ value is calculated from the regression between the known concentration of each of the positive controls and the resulting counts from them (this calculation is performed using $\log_2$-transformed values).

$$R^2 = \text{linear regression of } \frac{\text{Log}_2 \text{ Known [positive controls]}}{\text{Log}_2 \text{ Measured [positive controls]}} > 0.95$$

Since the known concentrations of the positive controls increase in a linear fashion, the resulting counts should, as well. Therefore, $R^2$ values should be higher than 0.95.

Note that because *POS_F* has a known concentration of 0.125 fM, which is considered below the limit of detection of the system, it should be excluded from this calculation (although you will see that *POS_F* counts are significantly higher than the negative control counts in most cases).

### *Factors Affecting POS Control Linearity*

- *Probe vs sample interactions*: Uncommonly, a single positive control probe will show unusually high or low counts, usually because of an interaction between this probe and a sample, which will not manifest during NanoString manufacturing QC. This will usually have little to no effect on data quality.
- *Very low assay efficiency*: If the counts of all the positive controls are very low (less than ~500 even for *POS_A*), linearity will be compromised. This is usually the result of a critical assay failure and data will likely be compromised.

## Limit of Detection QC

The limit of detection is determined by measuring the ability to detect *POS_E*, the 0.5 fM positive control probe, which corresponds to about 10,000 copies of this target within each sample tube. On a FLEX/MAX system, the standard input of 100 ng of total RNA will roughly correspond to about 10,000 cell equivalents (assuming one cell contains 10 pg total RNA on average). An nCounter assay run on the FLEX/MAX system should thus conservatively be able to detect roughly 1 transcript copy per cell for each target (or 10,000 total transcript copies). In most assays, you will observe that even the *POS_F* probe (equivalent to 0.25 copies per cell) is detectable above background.

To determine whether *POS_E* is detectable, it can be compared to the counts for the negative control probes. Every nCounter Gene Expression assay is manufactured with eight negative control probes that should not hybridize to any targets within the sample. Counts from these will approximate general non-specific binding of probes within the samples being run. The counts of *POS_E* should be higher than two times the standard deviation above the mean of the negative control.

### *Factors Affecting Limit of Detection*

*Too high background may be due to:*

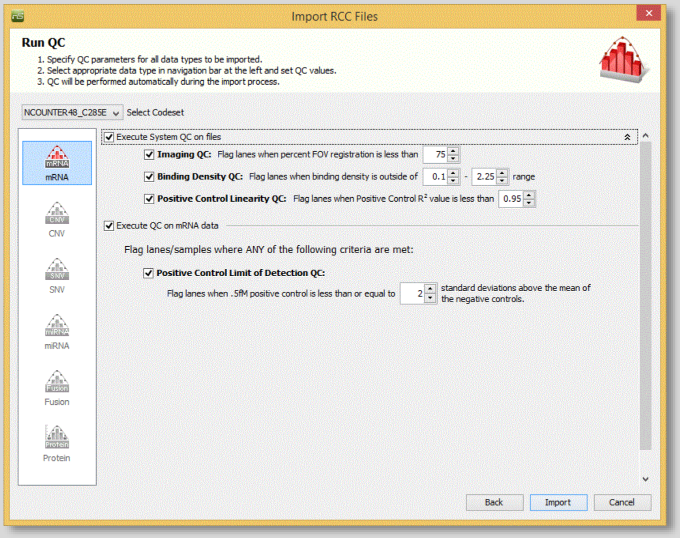
- Premature mixing of reporter and capture probes in hybridization buffer master mix.
- Too much time elapsed between adding capture probe and loading in thermal cycler.
- High counts in one of the negative controls – NEG controls could be elevated due to cross hybridization with targets in the sample.

*Very low POS counts may be due to:*

- Sub-optimal hybridization – check thermal cycler temperature and consider whether sample impurities (chaotropic salts, for example) may have been introduced.

### *How to Do It In nSolver: General Assay Performance QC*

- Import unzipped data files using the **Import RCC Files** button.
- Follow the prompts of the RCC Import Wizard to the **Run QC** window.
- Use the double arrow to view the System QC settings.
- The RCC Import Wizard will assign the default settings for System QC as well as mRNA QC as shown in Figure 2.



Import RCC Files

**Run QC**

1. Specify QC parameters for all data types to be imported.
2. Select appropriate data type in navigation bar at the left and set QC values.
3. QC will be performed automatically during the import process.

NCOUNTER48_C285E Select Codeset

☑ Execute System QC on files

☑ **Imaging QC:** Flag lanes when percent FOV registration is less than 75

☑ **Binding Density QC:** Flag lanes when binding density is outside of 0.1 - 2.25 range

☑ **Positive Control Linearity QC:** Flag lanes when Positive Control $R^2$ value is less than 0.95

☑ Execute QC on mRNA data

Flag lanes/samples where ANY of the following criteria are met:

☑ **Positive Control Limit of Detection QC:**

Flag lanes when .5fM positive control is less than or equal to 2 standard deviations above the mean of the negative controls.

Figure 2: nSolver GX QC window

## Assessing QC Flags

The recommended quality control specifications for NanoString data are designed to highlight samples that have moderate-to-severe reductions in assay efficiency or data quality. Some samples that do not pass quality control may still provide robust count data, but they are singled out to highlight a potential cause of poor assay efficiency that could result in data loss in future assays. Even if the data from these samples is usable, it is a good practice to determine the root cause of all such detectable changes in assay efficiency. For tips on how to assess these root causes, see the *Factors Affecting* … boxes for each QC parameter section, above. NanoString Technical Services (support@nanostring.com) and/or your Field Application Scientist can also assist you with troubleshooting, if necessary.

Samples with lowered assay efficiency (or with reduced quality or quantity of RNA) will also exhibit lowered raw counts for probes in the NanoString CodeSet. Depending on how background correction and normalization is performed, low expressing targets in these samples will either fail to be detected (false negatives) or will have higher-than-expected counts (false positives). To determine whether it would be necessary to discard the data from flagged samples, see the sections below for some rough guidelines to use.

### Examine the Positive and Negative Controls

- If samples were flagged because a **single** positive or negative control probe was much different than expected (leading to positive control linearity flag or Limit of Detection flag), it is very likely the data from that sample is still usable.
- If all the positive controls are extremely low, such that most (if not all) are close to the background (negative control probe counts), assay linearity and/or sensitivity are likely compromised and such samples may not provide robust data.

### Examine the Calculated QC Metrics

- If the QC metrics fail the flag threshold by a very small margin (i.e., FOV registration was 72%, just below the 75% recommended cut off), the data is likely robust and usable.
- Conversely, samples with QC metrics which miss the cutoff by a wide margin (i.e., FOV registration was 11% instead of >75%), will usually not provide robust data. NOTE: Binding density flags seldom result in critical data loss; use the *Visually Inspect the Data* and the *Identify Outlier Samples* sections below, especially when the Binding Density threshold has been exceeded by a considerable margin.

## Visually Inspect the Data

- A simple inspection of the data may either identify problematic samples or give greater confidence that data for the sample in question is sufficiently robust for downstream inclusion.
- Samples may need to be excluded if an inspection of raw counts indicates that substantially fewer genes are being detected above the background (negative control) probes compared to other samples from a similar treatment group.
- Samples may need to be excluded if an inspection of normalized counts indicates that substantially more genes are being detected above the background probes compared to other samples from the same treatment group. NOTE: The **Background Thresholding** option in nSolver will prevent these false positives from showing up; this approach is not recommended if Background Thresholding has been performed on the data.

## Identify Outlier Samples

- Instead of a visual inspection, various methods may be used to identify outlier samples, which may be indicative of assay- or sample-level problems which necessitate removal of such data from downstream analyses.
- One way to identify outlier samples is to generate a heat map of normalized data from all samples. If the flagged samples in question are strongly divergent from other samples with similar pathology, they may need to be removed from further analyses. In nSolver, you will need to proceed through the steps to create an experiment before doing a heat map Analysis, at which point you can refer to the *Agglomerative Cluster (Heat Map)* section in the *nSolver User Manual*.

### *How to Do It In nSolver: Assessing QC flags*

- Highlight your CodeSet on the **Raw Data** tab and scroll across the central table to view various **QC flag** columns. Right-click on the column headers or select the **Column Options** icon to reveal hidden columns, as the QC metric columns may be initially hidden.
- Alternatively, or in addition, once you have created an experiment, you can select it on the **Experiments** tab and select the **Experiment Report** button to generate a report.

## Background Correction

Despite the high specificity of nCounter probes, very low levels of non-specific counting are still inherent to any NanoString assay. Thus, a small number of counts for each of the targets in a CodeSet will represent false positives. Typically, we do not recommend correcting for non-specific counting since true counts for most mRNA targets will far outnumber false positives, and thus the effects of the latter on fold-change estimates will be negligible. However, for those projects where low expressing targets are common, or when the presence or absence of a transcript has an important research implication, it may be useful to more precisely delineate which counts are false positives. For these reasons, we introduce two methods of background correction: **Background Thresholding** and **Background Subtraction**. We also describe three different approaches in establishing a value to use in conjunction with thresholding or subtraction: using the **Negative Control Probes**, a **Fixed Background**, or a **Negative Control Sample**.

### Background Threshold

Background thresholding is a process whereby a threshold value is set and all counts which fall below that value are adjusted to match it. When data is background subtracted (see next section), in contrast, non-specific counts are subtracted from the raw counts to obtain a new estimate of counts above background.

For most studies of gene expression where fold-change estimations are an important result, background thresholding is preferred over background subtraction. This is because the latter tends to substantially overestimate fold-changes in low-expressing targets; background subtracted counts for low-expressing targets can be close to zero, potentially setting either the numerator or denominator of the fold-change calculation to a very small number. The biological importance of large absolute fold-changes in background subtracted data may be overestimated when the expression level of the gene is close to the background.

### Background Subtraction

If, instead of fold-changes, estimates of counted transcripts above background noise are an important result, then you may consider using background subtraction. Consider, however, the following cautionary notes about background subtraction values in nSolver:

- Because the nSolver math engine operates in $\log_2$ space, any counts below 1 (including negative counts) will be reset to 1.0. Thus, counts which appear as 1.0 in nSolver output will frequently represent counts less than 1.
- Background subtracted values may subsequently be multiplied by normalization scaling factors. Particularly for samples with normalization flags, some targets below background will be normalized to potentially large values depending on the size of the scaling factors, and thus may appear to be much higher than background. Counts in nSolver which have been set to the background threshold will not be altered further during normalization.

## Negative Control Probes

Every nCounter CodeSet or TagSet is manufactured to contain 6 or 8 **negative control probes**, designated by the probe name, Negative A (*NEG_A*), Negative B (*NEG_B*), etc. These control probes are designed against **engineered RNA sequences (ERCC RNA controls)** which are not present in biological samples. Negative control probes are commonly used to set background thresholds.

Every probe in an nCounter assay will exhibit some variability in non-specific counting. By observing this variability among the negative control probe targets, we can predict the level of variability in baseline non-specific counts that we expect to see for the endogenous and housekeeping targets.

We can use the mean, mean plus standard deviation, median, geometric mean, or maximum of the negative control counts. The level of stringency used in setting this threshold will affect the balance between false positive and false negative target occurrences. Examples of metrics you can use and their potential implications include:

- You can minimize the number of false positives by setting the threshold to the mean plus two standard deviations or to the maximum of the negative control counts; note, however, that although false positives will be rare, false negatives may be relatively abundant.
- Conversely, you can set a more liberal threshold, such as the geometric mean of the negative controls. This will increase the number of false positives, but simultaneously decrease the number of false negatives.

*Rare Negative Control Deviations*

Rarely, unique and difficult-to-predict probe-to-probe interactions may occur, resulting in one or two negative control probes being more than 3-fold higher than all the other negative control probes. It is acceptable to remove these outliers for purposes of background threshold or background subtraction.

## Fixed Value Background

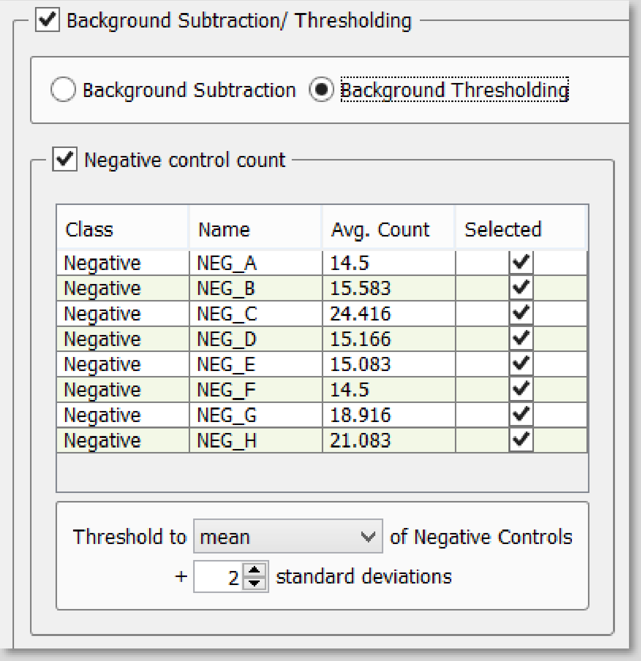
Another approach to setting the background is to simply define a fixed value. This would only be appropriate if previous experiments had identified a robust measure of probe non-specific binding that met the project qualifications for target exclusion. Used in conjunction with background thresholding, all targets with raw counts equal to or less than this defined value would be set to this value. Used with background subtraction, this defined value would be subtracted from the raw counts of all targets.

## Negative Control Sample

One limitation of the previously-described approaches (negative controls or a fixed value) is that some proportion of false positives or false negatives are expected, given the variance in non-specific counting across probes. One approach to defining background more precisely would be to run samples which contain no target transcripts, such as an RNA-free water sample. This would require using one or more sample lanes per project (not per cartridge) for these **negative control** samples in lieu of experimental samples. Ideally, three negative control samples would be run for each build of a CodeSet, and a *t*-test performed for each target transcript. These three negative control replicates would then be compared to the biological replicates run within each of the other treatment groups.

### *How to Do It In nSolver: Background Thresholding*

- Create an experiment and follow the Experiment Wizard prompts to the **Background Subtraction/Thresholding** screen.
- Check the box for **Background Subtraction/Thresholding.**
- Check the box for **Negative control count.**
- Set the **Threshold** to *mean +2* standard deviations above the mean.



☑ Background Subtraction/ Thresholding

○ Background Subtraction ● Background Thresholding

☑ Negative control count

| Class | Name | Avg. Count | Selected |
|---|---|---|---|
| Negative | NEG_A | 14.5 | ☑ |
| Negative | NEG_B | 15.583 | ☑ |
| Negative | NEG_C | 24.416 | ☑ |
| Negative | NEG_D | 15.166 | ☑ |
| Negative | NEG_E | 15.083 | ☑ |
| Negative | NEG_F | 14.5 | ☑ |
| Negative | NEG_G | 18.916 | ☑ |
| Negative | NEG_H | 21.083 | ☑ |

Threshold to mean of Negative Controls

+ 2 standard deviations

Figure 3: nSolver background settings window

# Normalization

Data normalization is designed to remove sources of technical variability from an experiment, so that the remaining variance can be attributed to the underlying biology of the system under study. The precision and accuracy of nCounter Gene Expression assays are dependent upon robust methods of normalization to allow direct comparison between samples. There are many sources of technical variability that can potentially be introduced into nCounter assays. The largest and most common categories of variability originate from either the platform or the sample. We describe how both types of variability can be normalized using standard normalization procedures for Gene Expression assays.

Standard normalization uses a combination of **Positive Control Normalization**, which uses synthetic positive control targets, and **CodeSet Content Normalization**, which uses housekeeping genes, to apply a sample-specific correction factor to all the target probes within that sample lane. These correction factors will control for all those technical sources of variability which should affect all the probes equally, compensating for sources of error including pipetting errors, instrument scan resolution, lot-to-lot variation in Prep Plates and Cartridges, and sample input variability. These procedures are effective as long as the CodeSets or probes themselves are derived from the same manufacturing lot (if not, see the *Lot to Lot Variation in Probes* box).

Note that positive control normalization will not correct for sample input variability, and thus should usually be used in combination with CodeSet Content (housekeeping gene) Normalization. Performing such a two-step normalization will usually not differ mathematically from Content Normalization alone, and thus is mathematically somewhat redundant. Nevertheless, normalizing to both target classes will provide a good indicator of how technical variability is partitioned between the two major sources of assay noise (platform and sample), and thus may provide a good tool for troubleshooting low assay performance. Below, we describe both types of normalization, as well as **Normalization QC**.

### *Lot to Lot Variation in Probes*

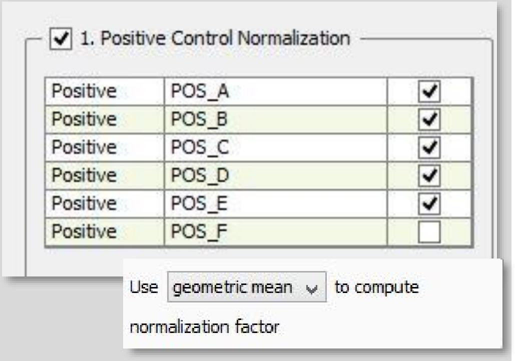
Due to the level of precision and sensitivity of the nCounter platform, it will likely detect any differences in the counting efficiency between lots of nCounter probes manufactured at different times. However, unlike batch effects for other reagents, the probe efficiencies from new lots of probes will potentially differ in magnitude and direction for each probe. Although the size of this change is generally small (<30% for each probe), a sample-wide normalization will not correct for this extra noise. The optimal solution for correcting lot to lot variation in CodeSets is to run reference samples within each lot of CodeSet, and use the counts from these to mathematically correct the probe efficiency changes. Please consult with your Applications Scientist or support@nanostring.com for help in optimizing this and other approaches for controlling for this variance.

## Positive Control Normalization

nCounter Reporter probe (or TagSet) tubes are manufactured to contain **six synthetic ssDNA control targets**. The counts from these targets may be used to normalize all **platform-associated** sources of variation (e.g., automated purification, hybridization conditions, etc.).

### *How to Do It In nSolver: Positive Control Normalization*

- Check the **Positive Control Normalization** box within the Experiment Wizard.
- (optional) If the counts for the ***POS_F*** control are close to background, then exclude this probe from normalization (Uncheck the box as shown on the right).
- Select the **geometric mean** to compute normalization factors. This will weigh the targets approximately equally in the ensuing calculations regardless of their absolute counts.



☑ 1. Positive Control Normalization

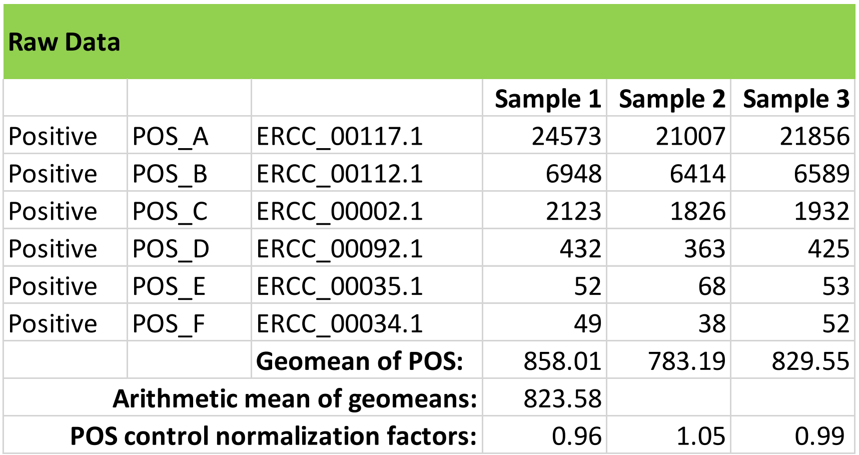
| | | |
|---|---|---|
| Positive | POS_A | ☑ |
| Positive | POS_B | ☑ |
| Positive | POS_C | ☑ |
| Positive | POS_D | ☑ |
| Positive | POS_E | ☑ |
| Positive | POS_F | ☐ |

Use geometric mean to compute normalization factor

Figure 4: nSolver Positive Control Normalization settings

Although the calculations are performed automatically in nSolver, the procedure is as follows (see Figure 5):

1. Calculate the geometric mean (**Geomean**) of the positive controls for each lane.
2. Calculate the **arithmetic mean** of these geometric means for all sample lanes.
3. Divide this arithmetic mean by the geometric mean of each lane to generate a lane-specific **normalization factor**.
4. Multiply the counts for every gene by its lane-specific normalization factor.



| **Raw Data** | | | | | |
|---|---|---|---|---|---|
| | | | **Sample 1** | **Sample 2** | **Sample 3** |
| Positive | POS_A | ERCC_00117.1 | 24573 | 21007 | 21856 |
| Positive | POS_B | ERCC_00112.1 | 6948 | 6414 | 6589 |
| Positive | POS_C | ERCC_00002.1 | 2123 | 1826 | 1932 |
| Positive | POS_D | ERCC_00092.1 | 432 | 363 | 425 |
| Positive | POS_E | ERCC_00035.1 | 52 | 68 | 53 |
| Positive | POS_F | ERCC_00034.1 | 49 | 38 | 52 |
| | | **Geomean of POS:** | 858.01 | 783.19 | 829.55 |
| **Arithmetic mean of geomeans:** | | | 823.58 | | |
| **POS control normalization factors:** | | | 0.96 | 1.05 | 0.99 |

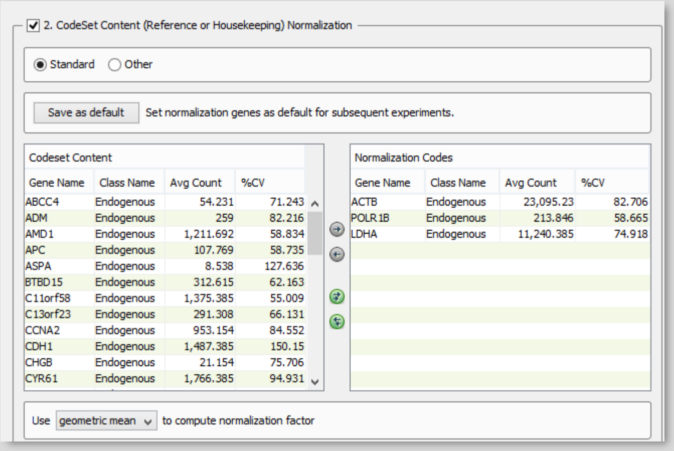
Figure 5: spreadsheet calculations for positive control normalization

## CodeSet Content or Housekeeping Gene Normalization

It is expected that some noise will invariably be introduced into the nCounter assay due to variability in sample input. Since it is not always practical to measure real world samples with the precision and accuracy necessary to ensure that each lane contains the same amount and quality of RNA, it is desirable to correct for this variability. We can do this by normalizing to a surrogate measure of total RNA input into the assay. For most experiments run on the nCounter platform, this is most effectively done using so-called **housekeeping genes**; these are mRNA targets included in a CodeSet which are known to or suspected to show little-to-no variability in expression across all treatment conditions in the experiment. Because of this, these targets will ideally vary only according to how much sample RNA was loaded.

### *How to Do It In nSolver: CodeSet Content Normalization*

- Check the **CodeSet Content Normalization** box within the Experiment Wizard.
- Check the **Standard** option. If housekeeping genes have been identified for the CodeSet being analyzed, these will automatically be moved into the table **Normalization Codes**. If housekeeping genes were not identified during CodeSet design, they will be classified as Endogenous genes and will need to be manually added to the **Normalization Codes** table via the green arrow buttons.
- Using the **geometric mean** of **at least three housekeeping genes** to calculate normalization factors is recommended to minimize the noise from individual genes as well as to ensure that the calculations are not weighted towards the highest expression housekeeping targets. It is important to note that some previously-identified housekeeping genes may, in fact, behave poorly as normalizing targets in the current experiment, and may therefore need to be excluded from normalization (see section below, *Selecting Housekeeping Genes*).



☑ 2. CodeSet Content (Reference or Housekeeping) Normalization

● Standard ○ Other

Save as default — Set normalization genes as default for subsequent experiments.

Codeset Content

| Gene Name | Class Name | Avg Count | %CV |
|---|---|---|---|
| ABCC4 | Endogenous | 54.231 | 71.243 |
| ADM | Endogenous | 259 | 82.216 |
| AMD1 | Endogenous | 1,211.692 | 58.834 |
| APC | Endogenous | 107.769 | 58.735 |
| ASPA | Endogenous | 8.538 | 127.636 |
| BTBD15 | Endogenous | 312.615 | 62.163 |
| C11orf58 | Endogenous | 1,375.385 | 55.009 |
| C13orf23 | Endogenous | 291.308 | 66.131 |
| CCNA2 | Endogenous | 953.154 | 84.552 |
| CDH1 | Endogenous | 1,487.385 | 150.15 |
| CHGB | Endogenous | 21.154 | 75.706 |
| CYR61 | Endogenous | 1,766.385 | 94.931 |

Normalization Codes

| Gene Name | Class Name | Avg Count | %CV |
|---|---|---|---|
| ACTB | Endogenous | 23,095.23 | 82.706 |
| POLR1B | Endogenous | 213.846 | 58.665 |
| LDHA | Endogenous | 11,240.385 | 74.918 |

Use geometric mean to compute normalization factor

Figure 6: nSolver CodeSet Content Normalization settings

The calculations are performed automatically in nSolver, which follows this procedure (for an example of this procedure, see the *Positive Control Normalization* section and Figure 5, above):

1. Calculate the geometric mean of the selected housekeeping genes for each lane.
2. Calculate the arithmetic mean of these geometric means for all sample lanes.
3. Divide this arithmetic mean by the geometric mean of each lane to generate a lane-specific normalization factor.
4. Multiply the counts for every gene by its lane-specific normalization factor.

## Normalization QC

It is important to perform some Quality Control on normalized nCounter data to ensure that normalization has not inadvertently introduced variability into the data set. The **scaling factors** generated **by Positive Control** and **Housekeeping (CodeSet Content)** Normalization can be used as surrogate measures for potential bias which may be introduced by these procedures.

A **Positive Control scaling factor** for a sample that exceeds 3-fold (that is, it is less than 0.3 or greater than 3.0), may skew results from the samples, particularly for those low expression targets which are close to the background noise. Scaling factors in these extreme ranges most commonly result from samples with positive control counts that are much lower than normal. Even if these samples appear otherwise normal, positive controls that are lower than expected may indicate a reduction in assay efficiency.

A **CodeSet Content normalization scaling factor** that exceeds 10-fold (less than 0.1 or greater than 10.0) indicates that housekeeping genes have counts that are much lower than average for that sample. The safest course would be to exclude samples with Normalization factors outside the expected range from further analysis, although samples that yielded marginal QC specs (i.e., a '12.0' for CodeSet Content Normalization) are often still usable. Even for samples with normalization flags, data from moderate- to highly-expressed targets will often show little bias. More concerning are the data from low count targets; these can become biased upwards for samples with normalization flags, and their inclusion may potentially skew the results of a study. The use of background thresholding in nSolver will minimize this upward bias in low count genes, but samples with normalization flags will still have a reduced ability to detect low expression genes. See the *Assessing QC Flags* section to determine if these samples are outliers in the normalized dataset.

### Selecting Housekeeping Genes

Not every pre-selected housekeeping gene will necessarily correlate well with sample input. Even well-validated housekeepers may show increased variability when tested under novel experimental conditions, new pathologies, or new tissue types. Therefore, it is good practice to critically examine the data from putative housekeeping genes and exclude any targets with notable instability in expression. Indications that you may need to discard a housekeeper include:

- A housekeeping target that is expressed at or near the background; this will likely introduce noise into the normalized data.
- A housekeeping target with a higher variability than other targets (as measured by %CV; this column can be seen in Figure 6 above, *How to Do It In nSolver: CodeSet Content Normalization*).
- A housekeeping target whose expression does not correlate with other housekeepers when compared across samples. The nSolver Advanced Analysis module includes a published methodology and algorithm (geNorm) to formally assess these characteristics and identify the optimal housekeepers for the current data set. See the *Advanced Analysis User Manual* for more information on optimizing normalization.

### Assessing a Normalization QC Flag

- **POS Control Flag**: Samples with a POS Control Normalization flag have lower than typical POS control counts, and may be experiencing a reduced level of assay level efficiency. These samples will also sometimes receive an Assay Performance QC flag, due to factors such as: failed imaging QC, high binding density, a failed pipetting step, or low hybridization efficiency, which should aid in determining the root cause of the lowered efficiency. See the *Assessing QC Flags* section, above, to determine how to treat these samples.

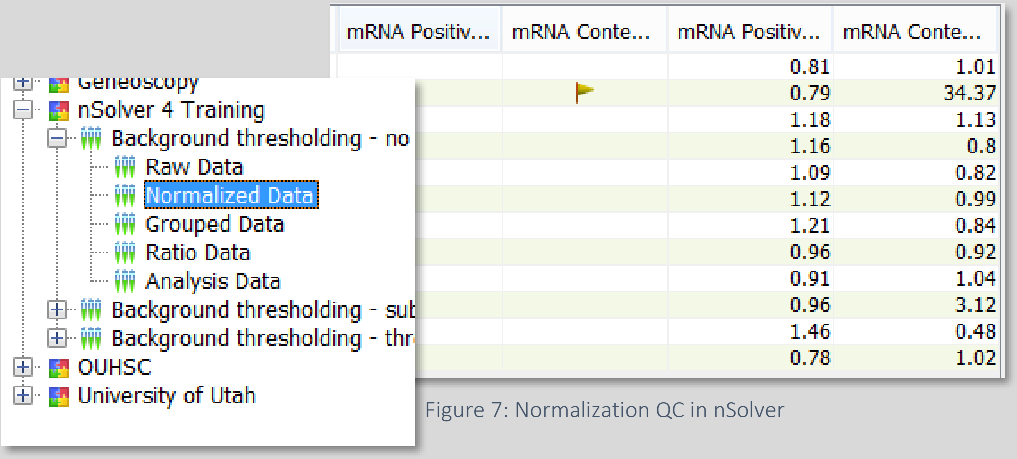
- **Content (Housekeeping) Normalization Flag:** Samples with a Content Normalization flag typically have very low housekeeping gene counts, and thus likely have very low amounts of intact RNA. This may result from pipetting errors, inaccurate quantification, or sample degradation. For help in determining the root cause of low RNA counts, consult with NanoString support (support@nanostring.com), but also refer to the *Assessing QC Flags* section, above, to determine if the data from these samples should still be included in further analyses.

*How to Do It In nSolver: Normalization QC*

nSolver will automatically compare normalization factors for POS controls and Housekeeping genes to the default cutoffs mentioned in the text (3-fold and 10-fold, respectively). To determine whether samples received normalization flags:

- Select the **Normalized Data** table in the finished experiment (see Figure 7).

- Scroll right in the central table viewer: there are two columns reserved for potential normalization flags, then two more for the normalization correction factors.



| mRNA Positiv... | mRNA Conte... | mRNA Positiv... | mRNA Conte... |
|---|---|---|---|
| | | 0.81 | 1.01 |
| | ⚑ | 0.79 | 34.37 |
| | | 1.18 | 1.13 |
| | | 1.16 | 0.8 |
| | | 1.09 | 0.82 |
| | | 1.12 | 0.99 |
| | | 1.21 | 0.84 |
| | | 0.96 | 0.92 |
| | | 0.91 | 1.04 |
| | | 0.96 | 3.12 |
| | | 1.46 | 0.48 |
| | | 0.78 | 1.02 |

Figure 7: Normalization QC in nSolver

# Ratios, Fold-Changes, and Differential Expression

For many experiments that quantify mRNA expression levels, a primary end goal is to characterize changes in gene expression across samples that differ in some key characteristic (i.e., different pathology or treatment). Here, we describe the process of differential expression analysis in a NanoString gene expression dataset. We will also highlight important considerations in experimental design and how they may impact the ability to determine differential expression.

An experiment that is designed to include biological replicates is amenable to formal statistical testing, as it maximizes biological inference of expression differences between sample groups. In contrast, experiments with one replicate per condition are limited in terms of both statistical testing options and biological interpretation. Technical replicates are not required, since the enzyme-free, digital nature of the assay generates exceptionally precise and reproducible measurements. Biological replicates, therefore, are strongly recommended.

In the following sections, we discuss creating **ratios and fold-change** estimates, and for those experiments with sufficient biological replicates, we discuss appropriate **statistical tests** and **multiple testing correction.**

## Ratio and Fold-Change Calculations

Normalized NanoString gene expression data is most commonly analyzed in terms of ratios or fold-changes. These calculations are used to determine relative over- or under-expression of each gene across different sample groups in an experiment. These expressions can be calculated in either **linear** or **log$_2$** scale.

Here, we illustrate how to calculate ratios using a hypothetical experiment with three groups of samples: those in a control group, those in group 1, and those in group 2. Calculating linear ratios for a single gene in this data set is shown in Figure 8. Overexpressed genes will have ratios >1, but under-expressed genes will have a ratio which ranges in value from 0 – 1 and are therefore compressed in the linear scale.

| Comparison | Linear Ratio Calculation | Interpretation |
|---|---|---|
| Group #1 vs. Control | $Ratio = \frac{\textit{geometric mean of gene ‘A’ from group \#1}}{\textit{geometric mean of gene ‘A’ from control group}}$ | Relative over- or under-expression of gene ‘A’ in group #1 vs. control |
| Group #2 vs. Control | $Ratio = \frac{\textit{geometric mean of gene ‘A’ from group \#2}}{\textit{geometric mean of gene ‘A’ from control group}}$ | Relative over- or under-expression of gene ‘A’ in group #2 vs. control |
| Group #1 vs. Group #2 | $Ratio = \frac{\textit{geometric mean of gene ‘A’ from group \#1}}{\textit{geometric mean of gene ‘A’ from group \#2}}$ | Relative over- or under-expression of gene ‘A’ in group #1 vs. group #2 |

Figure 8: Linear ratio calculations

Although ratios are relatively easy to calculate, these are commonly converted into $\log_2$ ratios, **fold-changes, or $\log_2$ fold-changes.** These transformed metrics are generally more intuitive to interpret since their distributions are symmetrical. Calculations for ratios in **$\log_2$ scale** are shown in the table below, and are mathematically identical to $\log_2$ fold-changes.

| Comparison | $\text{Log}_2$ Ratio (= $\log_2$ fold-change) | Interpretation |
|---|---|---|
| Group #1 vs. Control | (arithmetic mean of $\log_2$ counts of gene 'A' in group #1) – (arithmetic mean of $\log_2$ counts of gene 'A' in control group) | Relative over- or under-expression of gene 'A' in group #1 vs. control |
| Group #2 vs. Control | (arithmetic mean of $\log_2$ counts of gene 'A' in group #2) – (arithmetic mean of $\log_2$ counts of gene 'A' in control group) | Relative over- or under-expression of gene 'A' in group #2 vs. control |
| Group #1 vs. Group #2 | (arithmetic mean of $\log_2$ counts of gene 'A' in group #1) – (arithmetic mean of $\log_2$ counts of gene 'A' in group #2) | Relative over- or under-expression of 'gene 'A' in group #1 vs. group #2 |

Figure 9: $\text{Log}_2$ ratio calculations

Unlike in linear data, these $\log_2$ metrics are determined by subtracting, rather than dividing, group means. They are moreover both symmetrical around zero. Thus, for example, a $\log_2$ ratio of 0.8 has the same magnitude as a $\log_2$ ratio of -0.8. It is important to note that ratios calculated using the **geometric mean of linear data** correspond to ratios calculated using the **arithmetic mean of $\log_2$-transformed data**. A simple mathematical formula can be applied to convert between the two types of ratios.

To convert **$\log_2$ ratio *y*** to **linear ratio *x*,** use the antilog function such that:
$x = \text{antilog}_2(y)$, which is equivalent to $x = 2^y$.

Conversely, **linear ratio** *x* can be converted to **$\log_2$ ratio *y*** with the following formula:
$y = \log_2(x)$.

Fold-changes are probably the most commonly displayed metric for measures of differential expression, and are readily calculated from linear ratio data. If the ratio numerator is greater than the ratio denominator (i.e., ratio > 1), then fold-change is equal to the ratio. If the ratio numerator is less than the ratio denominator (i.e., ratio < 1), then fold-change equals the negative inverse of the ratio:

If ratio > 1, then:

$$Fold\ change = Ratio$$

If ratio < 1, then:

$$Fold\ change = -1 * \frac{1}{Ratio}$$

The distribution of fold-change **is symmetrical around zero**, with all overexpressed genes being represented as values > 1 and all under-expressed genes being represented as values <-1 (thus there are no fold-changes possible between -1 and 1).

## Statistical Tests

In this section, we will cover a few basic recommendations for statistical testing of differential expression in nCounter data sets. It is beyond the scope of this document to exhaustively cover the various approaches that are appropriate for making statistical inferences from gene expression studies, so we will focus primarily on the options for testing which can be found in the nSolver analysis software.

As stated previously, a minimum of three biological replicates are required from each experimental group to obtain reasonably reliable statistical information about the underlying populations from which those samples were drawn. If the magnitude of between-group differences is small, or if variability within groups is high, then more replicates per group may still be required to obtain statistical significance.

### *T*-test

A ***t*-test** can be performed on $\log_2$-transformed count data if there are only two groups of samples to compare. An example of this would be a simple experiment consisting of control group samples that you would like to compare to samples in a single treatment group. The $\log_2$ transformation will generally satisfy the *t*-test's requirement of normally distributed data, and the use of a heteroscedastic *t*-test (Welch's) also relaxes the assumption that variance is equally distributed among groups. The output of a *t*-test is a *p*-value, and standard convention is to assign significance to all genes with a nominal *p*-value of less than 0.05. The lower the *p*-value, the stronger the evidence that a gene has different expression levels in two different groups. It is important to note that a *p*-value is the probability of obtaining an effect at least as extreme as the one observed, assuming the null hypothesis of no difference between sample groups is true. The *p*-value addresses the question, "how likely is the data?", assuming the null hypothesis is true. It is a common mistake for a specific *p*-value to be incorrectly interpreted as the probability of a false positive; this is called a Type I error. If desired, post-hoc corrections can be applied to adjust *p*-values to account for multiple testing (see below).

### Multivariate Linear Regression

Normalized and $\log_2$-transformed nCounter data may also be analyzed using multivariate linear regression. This is a powerful approach for dealing with more complex experimental designs than a two-group experiment. It allows one to isolate the independent effect of each covariate on gene expression levels, permitting comparisons between multiple experimental groups and variables, including potentially confounding variables. Though the details are beyond the scope of this document, guidance on how to perform these tests in the nSolver Advanced Analysis module may be found in the *Advanced Analysis User Manual.*

## False Discovery Rate and Correcting for Multiple Comparisons

In large, multiplexed gene expression datasets, the large number of statistical tests performed may inadvertently increase the chances of obtaining false-positive results. This is because, all else being equal, 1 out of every 20 statistical tests is expected by chance alone to result in a *p*-value of approximately 0.05, the traditional threshold for obtaining statistical significance. The frequency of these potential false positives is expected to go up with increasing numbers of tested genes, so for larger NanoString CodeSets, this may become a potential concern.

Despite this possibility, we do not necessarily recommend a universal application of multiple comparison corrections for NanoString datasets. First, CodeSets generally comprise collections of genes known or suspected to be affected by the biology under study, and the resulting correlations in expression levels violates the assumption of independence of significance levels between tests, which forms the basis of the more conservative Bonferroni multiple comparisons tests.

As a corollary, if the purpose of the NanoString study is to validate the results of a transcriptome-wide survey, the CodeSet will, just as above, comprise a highly non-random set of genes. For such validation work, it may not be desirable at this stage to limit the number of false positives. Arguably, instead of statistical adjustments to significance levels, one might consider cross-platform validation as a better standard towards determining the statistical robustness of any expression level differences.

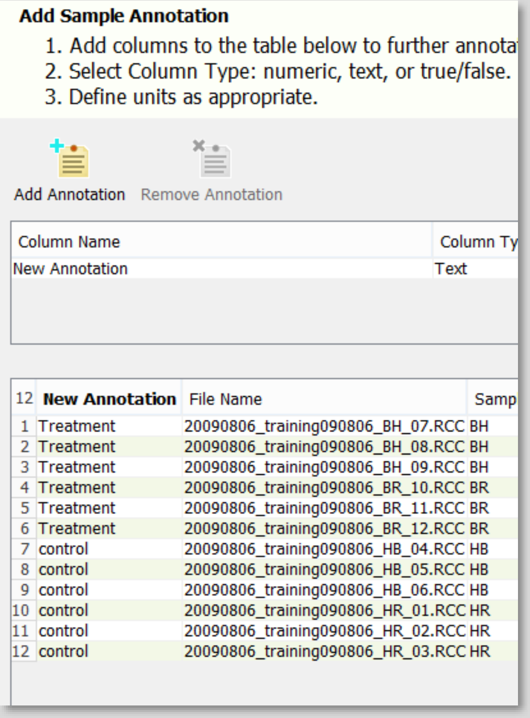
Lastly, the use of multiple comparisons corrections may simply be too conservative for experiments where the tolerance for false negatives is low. Statistical methods designed to reduce the number of false positives will invariably result in an increase in the number of false negatives, and for some experimental goals, this extra level of stringency may not be an acceptable tradeoff.

In general, when multiple comparison corrections are performed on NanoString data, using a less conservative method which allows gene significance levels to be either positively or negatively correlated is preferred (i.e., when probing for correlated and interdependent expression pathways). The **Benjamini-Yekutieli False Discovery Rate** method[1] accounts for this expectation that significant changes in genes may be correlated with or dependent on each other, and the resulting FDR adjusted *p*-value provides a middle ground between the more conservative tests which control for family-wise error rates (epitomized by tests like the Bonferroni correction) and more permissive uncorrected *p*-values.

[1] The Benjamini-Yekutieli adjustment was originally described in the following publication:
Benjamini, Y, and Yekutieli, D. (2001) "The control of the false discovery rate in multiple testing under dependency." Annals of Statistics. 29(4):1165-1188.

### *How to do it in nSolver: Defining Experimental Groups*

Differential expression testing in nSolver can be configured during the Experiment wizard setup. In the **Add Sample Annotation** window (see Figure 10), replicates within an experimental group should be given an identical annotation. In this example, a control and treatment group have both been created with 6 samples in each. Fold-changes will be calculated between groups of samples with identical annotations, and *t*-tests performed if there are two or more replicates within each group. Note that three replicates per group is the recommended minimum sample size, and having more than two experimental groups will require other statistical testing options (i.e., ANOVA or Multivariate Linear Regression) to provide unbiased statistical results.



**Add Sample Annotation**

1. Add columns to the table below to further annota
2. Select Column Type: numeric, text, or true/false.
3. Define units as appropriate.

| Column Name | Column Ty |
|---|---|
| New Annotation | Text |

| 12 | **New Annotation** | File Name | Samp |
|---|---|---|---|
| 1 | Treatment | 20090806_training090806_BH_07.RCC | BH |
| 2 | Treatment | 20090806_training090806_BH_08.RCC | BH |
| 3 | Treatment | 20090806_training090806_BH_09.RCC | BH |
| 4 | Treatment | 20090806_training090806_BR_10.RCC | BR |
| 5 | Treatment | 20090806_training090806_BR_11.RCC | BR |
| 6 | Treatment | 20090806_training090806_BR_12.RCC | BR |
| 7 | control | 20090806_training090806_HB_04.RCC | HB |
| 8 | control | 20090806_training090806_HB_05.RCC | HB |
| 9 | control | 20090806_training090806_HB_06.RCC | HB |
| 10 | control | 20090806_training090806_HR_01.RCC | HR |
| 11 | control | 20090806_training090806_HR_02.RCC | HR |
| 12 | control | 20090806_training090806_HR_03.RCC | HR |

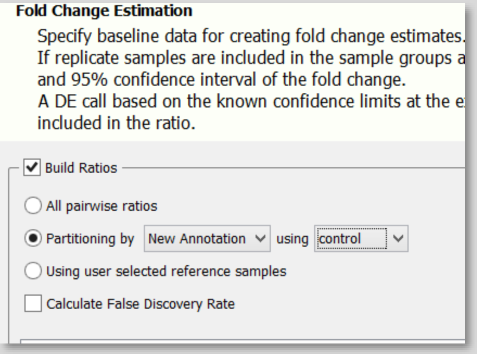
Figure 10: Creating sample annotations in nSolver

*Advanced Analysis Module Tip:*

Annotations added in the basic nSolver package can be used when setting up an experiment in the Advanced Analysis module. Thus, even if an experimental design outstrips the power of a *t*-test in basic nSolver, it can simplify workflow to include these annotations in the Advanced Analysis module when setting up Differential Expression testing, which utilizes multivariate linear regression for statistical testing.

### *How to do it in nSolver: T-test and FDR*

In the **Fold-Change Estimation** window (see Figure 11), select the appropriate option for building ratios. For differential expression using a *t*-test, this option will usually be **Partitioning by,** defining the reference group (usually a control group) in the drop-down menu. A *t*-test will be performed if there are two or more replicates in each group and fold-changes will be calculated regardless of sample sizes. A check-box is also provided to have the software generate **False Discovery Rate** thresholds (Benjamini-Yekutieli FDR).



**Fold Change Estimation**

Specify baseline data for creating fold change estimates.
If replicate samples are included in the sample groups a
and 95% confidence interval of the fold change.
A DE call based on the known confidence limits at the e
included in the ratio.

☑ Build Ratios

○ All pairwise ratios

● Partitioning by New Annotation using control

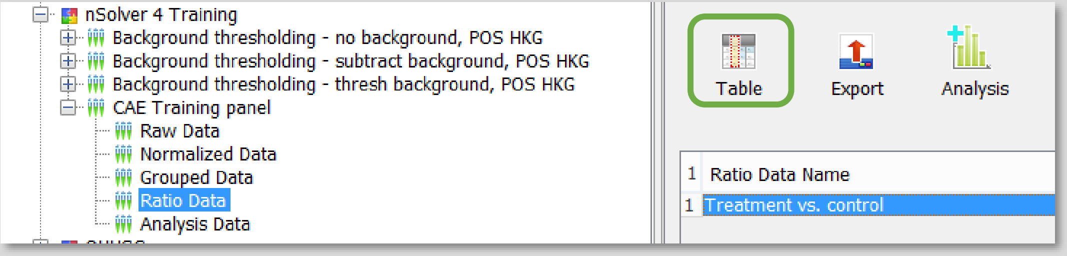
○ Using user selected reference samples

□ Calculate False Discovery Rate

Figure 11: Fold change options in nSolver

*Note:* If the experiment does not contain sufficient replicates to perform a *t*-test, the software will instead automatically generate a Differential Expression (DE) call which can be found in the Experiment **Ratio Data** table. The **DE call** is a test developed for nCounter data which determines whether observed count differences between individual samples are larger than can be explained by platform technical noise, alone. For exploratory studies with only a single biological replicate per treatment, the DE call can provide a more robust measure of expression differences than merely calculating fold-changes between individual samples. See the *nSolver User Manual* for more information on the use of this metric.

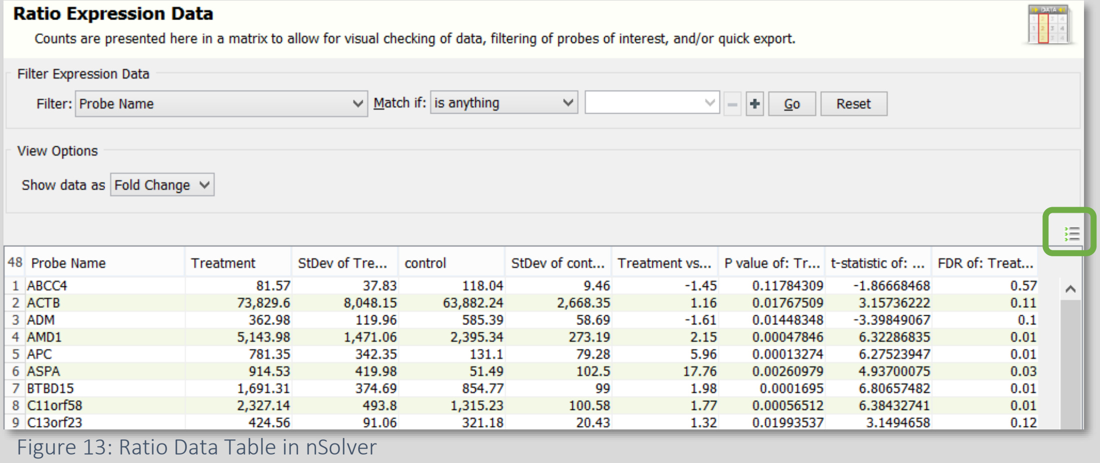
Once you have created an experiment (including annotations and fold-change settings), select the **Ratio Data** level on the Experiments tab. Highlight the ratio you'd like to examine in the central table and select the **Table** button (see Figure 12).


nSolver 4 Training
Background thresholding - no background, POS HKG
Background thresholding - subtract background, POS HKG
Background thresholding - thresh background, POS HKG
CAE Training panel
Raw Data
Normalized Data
Grouped Data
Ratio Data
Analysis Data
Table
Export
Analysis

| 1 | Ratio Data Name |
|---|---|
| 1 | Treatment vs. control |

Figure 12: Creating a Ratio Table in nSolver

By default, only fold-changes are shown in the Ratio Data Table (see Figure 13), but clicking on the Column Options icon (highlighted below) will allow viewing of additional hidden data fields. Note that the column titled FDR contains FDR adjusted *p*-values rather than FDR thresholds.



**Ratio Expression Data**

Counts are presented here in a matrix to allow for visual checking of data, filtering of probes of interest, and/or quick export.

Filter Expression Data

Filter: Probe Name Match if: is anything Go Reset

View Options

Show data as Fold Change

| 48 | Probe Name | Treatment | StDev of Tre... | control | StDev of cont... | Treatment vs... | P value of: Tr... | t-statistic of: ... | FDR of: Treat... |
|---|---|---|---|---|---|---|---|---|---|
| 1 | ABCC4 | 81.57 | 37.83 | 118.04 | 9.46 | -1.45 | 0.11784309 | -1.86668468 | 0.57 |
| 2 | ACTB | 73,829.6 | 8,048.15 | 63,882.24 | 2,668.35 | 1.16 | 0.01767509 | 3.15736222 | 0.11 |
| 3 | ADM | 362.98 | 119.96 | 585.39 | 58.69 | -1.61 | 0.01448348 | -3.39849067 | 0.1 |
| 4 | AMD1 | 5,143.98 | 1,471.06 | 2,395.34 | 273.19 | 2.15 | 0.00047846 | 6.32286835 | 0.01 |
| 5 | APC | 781.35 | 342.35 | 131.1 | 79.28 | 5.96 | 0.00013274 | 6.27523947 | 0.01 |
| 6 | ASPA | 914.53 | 419.98 | 51.49 | 102.5 | 17.76 | 0.00260979 | 4.93700075 | 0.03 |
| 7 | BTBD15 | 1,691.31 | 374.69 | 854.77 | 99 | 1.98 | 0.0001695 | 6.80657482 | 0.01 |
| 8 | C11orf58 | 2,327.14 | 493.8 | 1,315.23 | 100.58 | 1.77 | 0.00056512 | 6.38432741 | 0.01 |
| 9 | C13orf23 | 424.56 | 91.06 | 321.18 | 20.43 | 1.32 | 0.01993537 | 3.1494658 | 0.12 |

Figure 13: Ratio Data Table in nSolver