All About PlexSet Technology Data Analysis in nSolver Software

PlexSet is a multiplexed gene expression technology which allows pooling of up to 8 samples per nCounter cartridge lane, enabling users to run up to 96 samples in one run. It is based on molecular barcoding and digital quantification of target sequences through the use of uniquely-designed PlexSet reagents and the corresponding oligonucleotide probes (designed by NanoString and procured by the user) that recognize the targets of interest (Figure 1). Users can modify targets of interest by adding additional targets or by ordering new oligonucleotide probes that link Reporter Tags in the existing PlexSet with new targets. To do this, the user simply orders the new probes and re-pools them with the new configuration.

This guide steps through nSolver™ software (version 4.0 or later) analysis of titration data, as well as the analysis of data from three different potential plate configurations. Topics include data import, experiment creation, calibration, and data export and gene expression visualizations. For laboratory procedures, see the PlexSet Reagents Manual (MAN-10040). For more information on topics covered in this guide, see the nSolver User Manual (MAN-C0019).

Consider your Study Design and the impact that different plate configurations may have on your data. See pages 7-8 of this manual.

Sample Run
See the PlexSet Reagents Manual (MAN-10040) for this laboratory procedure.

Processing PlexSet Data in nSolver
See pages 9-15 of this manual.

Sample Analysis in nSolver or Excel
Export the raw or normalized data from nSolver as a .csv file. Process the data in a spreadsheet program (such as Microsoft Excel). You can also use Analysis to create visualizations, if desired. See page 15 of this manual.

Tritiation Workflow

Assess the need for a Tritiation Run
Prepare a Tritiation Run at least once per study.

Tritiation Run
See the PlexSet Reagents Manual (MAN-10040) for this laboratory procedure. The Tritiation Kit contains nCounter XT TagSet reagents that correspond to the PlexSet assay (e.g. PlexSet-48 will use nCounter XT TagSet-48).

Processing Tritiation Data in nSolver
See pages 2-5 of this manual.

Tritiation Analysis in Excel
Export the Normalized data from nSolver as a .csv file. Process the data in a spreadsheet program (such as Microsoft Excel). See pages 5-6 of this manual.

Figure 2: Tritiation and PlexSet workflows

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Processing Titration Data in nSolver

The following pages take you through Import, QC assessment, Exploring Raw Data, and Creating an Experiment with Titration data in nSolver.

Titration Data Import

Open your data folder and unzip RCC data files using right click and Extract All. Note: most operating systems have built-in unzipping functions but a freeware program (e.g. 7-Zip) can achieve the same thing.

Open nSolver 4.0 and select:

- **Import RLF.** Navigate to the RLF for your dataset and select Open. You should have received your Titration kit RLF in an email from NanoString Bioinformatics with your Design Summary file.
- **Import RCC Files.** Navigate to your unzipped data folder and select your RCC files. Select Next. You will be taken automatically to the QC page.

Titration Data QC

Choose the RLF (if not auto-filled), then review the QC parameters (see Figure 3). If hidden, select the double arrow at the right side of the screen to reveal the System QC parameters. nSolver displays and applies the QC parameters recommended by NanoString; it is usually not necessary to adjust the default settings. See the nSolver User Manual (MAN-C0019) QC section for more detailed information.

The titration kit contains 12 controls: 6 positive controls A-F (and corresponding targets, each at one of the following concentrations: 128 fM, 32 fM, 8 fM, 2 fM, 0.5 fM, and 0.125 fM) and 6 negative controls. The QC parameters listed below will be measured.

- The **Imaging QC** is a measure of the percentage of requested fields of view (FOV) successfully scanned in each cartridge lane.
- The **Binding Density QC** is a measure of reporter probe density on the cartridge surface within each sample lane.
- The **Positive Control Linearity QC** is a measure of correlation between the counts observed for the Positive ERCC probes and the concentrations of the corresponding targets (spike-in synthetic nucleic acids).
- The **Positive Control Limit of Detection QC** indicates whether the counts for the POS_E control probe with target sequence, spiked in at 0.5fM (assumed to be the system’s limit of detection) are greater than 2 SD above the counts of the Negative control probes.

Select Import.

Figure 3: QC window
Exploring Raw Titration Data

Imported RCC files and RLFs should be visible on the Raw Data tab (see Figure 6). Expanding the navigation tree (by clicking on the + sign) reveals the list of RCC files in the set and generates a central table of information. In this table, you can check all samples for QC flags (you may need to scroll right to see these columns, see Figure 5) and add descriptions to samples that will be informative when you later export your data.

Add a description to each titration sample by typing in the cells in the Description column. You may also Copy and Paste this information from another source.

Click on column headings to sort; click and drag them to move columns. Use the Column Options icon (see Figure 4) to reveal hidden columns, which contain QC metrics.

To investigate QC-flagged data, you may export results as a .csv file at this point by selecting the Export button above the central table (see Figure 6). Alternatively or in addition, you may continue with Experiment creation and assess exported data at a later point (see page 5). See the nSolver User Manual (MAN-C0019) QC section for more detailed information on QC flags.
Creating an Experiment with Titration Data

Within nSolver, any studies and experiments you create will be visible on the Experiments tab (see Figure 6).

A Study is an organizational folder used to store experiments; select the New Study button to create a study.

An Experiment is a collection of samples that have been analyzed together to allow comparisons between samples or samples grouped in conditions; select the New Experiment button to create an experiment under your study of choice. Follow the prompts to select the samples to include in your experiment.

Annotations to define sample groups should be assigned for experiments in which fold-change estimates and their statistical significance will be studied (see Figure 7).

Background Subtraction/Thresholding: leave this window de-selected, as you should skip this step.

Normalization: by default, both Positive Control and CodeSet Content Normalization boxes will be selected (See Figure 8).

- Confirm that the Positive Control Normalization is set to the geomean of the Top 3 POS counts.
- De-select CodeSet Content Normalization, as you should skip this step.
- Select Next.

Ratios: De-select the Build Ratios checkbox in the upper left, as you should skip this step. Select Finish.

See the nSolver User Manual (MAN-C0019) Background, Normalization, or Ratio sections for more information.
Titration Data Export

Your experiment will now be visible under your study on the Experiments tab. Expand the navigation tree (Figure 9).

- **Raw Data** table contains unprocessed data for all samples in this experiment.
- **Normalized Data** table contains the processed data for all samples. From this level, Export your Titration data (next section).
- **Grouped Data** table contains the geometric mean of expression levels for all samples.
- **Ratio Data** level will contain the fold-change results, as well as any statistical inferences surrounding those estimates.
- **Analysis Data** table contains any analysis you have run.

Figure 9: Experiment data hierarchy

Titration Data Analysis

Running a Titration Kit provides information on the optimal sample input amounts for the PlexSet assay. This can be used to test probe attenuation strategies, if needed, but should not be used to combine samples or for final data analysis. Serial dilutions of control and experimental samples should have been tested across lanes 1-12 of the cartridge.

Highlight relevant lanes of **Normalized Data**, select Export (see Figure 10), then use default settings of **RCC Collector Tool Format Export**. Save the resulting .csv file and open it in the spreadsheet program of your choice.

Samples are listed along the top row, organized in columns, and probes are listed as you scroll down, organized in rows (see Figure 11). Scroll to view your Positive and Negative Controls.

Figure 10: Exporting Titration data

Figure 11: Exported Titration Data
Titration Data Analysis (continued)

Copy the rows containing gene names (all rows except for the POS and NEG controls); this will select the counts for each titration category (see Figure 12).

Paste them into another spreadsheet tab and label them appropriately.

Calculate the total normalized gene counts per lane by using the SUM function, excluding POS and NEG controls (see Figure 13).

Average the total counts for technical replicates.

Highlight the summed counts and the sample input amount and Insert a Line Graph.

The titration categories (sample input) should be set as the x-axis, and Total Normalized Counts should be set as the y-axis (see Figure 14).

View the equation for the line in the format:

\[ y = mx + b \]

Copy this into a new cell and set y to the applicable value:

- \( y = 150,000 \) for MAX/FLEX platforms
- \( y = 400,000 \) for SPRINT platforms

Solve for \( x \). This is your optimum input amount for the sample.
PlexSet Sample Data Analysis

Recommended PlexSet Sample Plate Setup for Effective Multiplexing

You may:
- Run all 96 samples on a plate (Figure 15).
- 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 17 and 19, respectively).
- Run all 96 samples on a plate, but split the plate across multiple experiments/probe sets by columns (Figure 20).

At this time, PlexSet is NOT designed to:
- Run all 96 samples on a plate, but split the plate across multiple experiments/probe sets by rows.
- Exclude data from selected wells.

A reference sample for calibration is essential for accurate data analysis across PlexSets (Figure 15). The same RLF is used for these scenarios. Reserve lane 1 of the first plate for your Calibration Sample. Subsequent PlexSet cartridges using the same lot of probe sets will not need an additional reference sample lane (Figure 15). With our current nSolver data analysis software, your experiments should be organized down columns (Figure 16).

Less than 96 samples can be run per cartridge; the same RLF is used for these scenarios (first and second run, Figure 17). 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 PlexSets A through H (e.g., all PlexSets), a reference sample should be re-run across all PlexSets for calibration (third run, Figure 18).
Recommended PlexSet Sample Plate Setup for Effective Multiplexing (continued)

PlexSet kits can be used with different probe sets, but specific configurations are required for downstream analysis (Figure 19). 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 19).

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 20). For more information on setting up plates in this manner, see the PlexSet Reagents Manual (MAN-10040).
Processing PlexSet Data in nSolver

The following pages take you through Import, QC assessment, Exploring Raw Data, and Creating an Experiment with PlexSet data in nSolver. In addition, you have the option to use nSolver basic Analysis visualizations (see the Analysis section).

Data Import

Open your data folder and unzip RCC data files using right click and Extract All. Note: most operating systems have built-in unzipping functions but a freeware program (e.g. 7-Zip) can achieve the same thing.

Open nSolver 4.0 and select:

- Import RLFs. Navigate to the RLF for your dataset and select Open. You will need this for SNV data, creating a multiRLF experiment, or using Advanced Analysis. If you are not doing any of those, you may skip RLF import.
- Import RCC Files. Navigate to your unzipped data folder and select your RCC files. Select Next. You will be taken automatically to the QC page.

QC

Choose the RLF (if not auto-filled), then review the QC parameters (see Figure 21). If hidden, select the double arrow at the right side of the screen to reveal the System QC parameters. nSolver displays and applies the QC parameters recommended by NanoString; it is usually not necessary to adjust the default settings. See the nSolver User Manual (MAN-C0019) QC section for more detailed information.

PlexSet Quality Control uses only the Binding Density and Imaging in PlexSet assays. Neither Positive QC parameter is measured (will appear greyed out). Instead, identical positive controls are included in each PlexSet set (one for each row A-H). These eight POS controls are listed with each sample’s counts, acting collectively as lane controls (see below). One negative control is included in each PlexSet set (one for each row A-H), as well.

The Imaging QC is a measure of the percentage of requested fields of view successfully scanned in each cartridge lane.

The Binding Density QC is a measure of reporter probe density on the cartridge surface within each sample lane.

Select Import.

Figure 21: QC window
Exploring Raw PlexSet Data

Your RCC data files will now be stored under the corresponding RLF CodeSet(s) on the **Raw Data tab** (see Figure 24). Selecting the RLF name allows you to view all RCC files in a table format. Note that the main raw data table columns are labeled as Set A, Set B, etc. through Set H (see Figure 22). Rows are labeled with RCC file names, which correspond to the different cartridge lanes 1-12. Scroll to the right to check for QC flags (see Figure 22). You can right-click on any column header or select the Column Options icon to view the QC metric columns and the Sample Name/SampleID or Comments information entered during run creation. See the *nSolver User Manual* (MAN-C0019) QC section for more detailed information on QC flags.

![Figure 22: Initial PlexSet raw data table format with QC flags](image)

Highlighting your samples of interest and selecting the **Table** button (see Figure 24) allows you to view the individual counts of each sample (see Figure 23); column headers are sorted by well number (A1, B1, etc.). Note that all eight POS and NEG controls are listed for each sample. Collectively, these act as lane controls. The example in Figure 23 is from a partial plate; note that POS control counts for the wells that were not loaded are present (Rows 109-112), but their calls can be disregarded.

You may export results as a .csv file at this point by selecting the **Export** button above the central table (see Figure 24). Alternatively or in addition, you may continue with Experiment creation and assess exported data at a later point (see pages 13-15).

![Figure 23: PlexSet raw data table output](image)

![Figure 24: nSolver dashboard – experiments tab](image)
Creating Experiments with PlexSet Data

Within nSolver, any studies and experiments you create will be visible on the **Experiments tab** (see Figure 24).

A **Study** is an organizational folder used to store experiments; select the **New Study** button to create a study.

An **Experiment** is a collection of samples that have been analyzed together to allow comparisons between samples or samples grouped in conditions; select the **New Experiment** button to create an experiment under your study of choice. Follow the prompts to select the samples to include in your experiment.

**Annotations** to define sample groups should be assigned for experiments in which fold-change estimates and their statistical significance will be studied. These annotations can be used in ratio creation and visualizations during Analysis (see Figure 25).

The recommended method for adjusting for **Background** noise in data will appear by default. Confirm/select an option below (see wizard steps) and select **Next**.

- **No background calculation** (option clicked off or greyed out).
- **Background thresholding**, which uses a user-defined threshold count value; all raw counts below this value will be adjusted to it. If applying background correction to your sample data, this is recommended over subtraction; set to a count value of **20**.
- **Background subtraction** is not recommended for PlexSet data.

**Positive Control Normalization** can be accomplished by using the **geomean** of the Top 3 POS counts (See Figure 26). Sample data can be additionally normalized by selecting normalization genes in the CodeSet Content. User dictates which housekeeper genes are used for CodeSet Content Normalization. In the example below, all CodeSet Content are moved to Normalization Codes for normalization. Review defaults, set preferences, and select **Next**.

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**Figure 25:** Annotation window

**Figure 26:** Normalization window
Experiments with PlexSet (continued) – Calibration & Ratios

For **calibration**, a reference sample should have been loaded in all corresponding wells of one column of the 96 well hybridization plate so that at least one known sample is run across the entire PlexSet (see example setups, Figures 14-19).

Select the **Sample Reference Normalization** checkbox to activate the options in the window. Select the lane in which you loaded your reference sample in the **Subcode Samples** window (on the left). In most setup configurations, this is lane 1 (lanes are listed at the end of the file name – see arrow in Figure 27). Use the arrows to move the desired lane to the **Selected Samples** window (on the right of "Subcode Samples" window). Select one of the rows in which your PlexSet was loaded in the **Use as Reference** dropdown at the bottom of the window. The default is **Set A**; if running a partial plate in rows E-H (as in Cartridge 2 of Figure 16, above), you must change this default to **Set E, F, G, or H**. **The selection is arbitrary, as long as you choose a Set that was run.**

Ensure that the box **Warn if count of genes is less: ...** is checked and selecting the value **500** from the dropdown will elicit a popup, warning you of the genes whose counts fall below that value. Readings from empty wells in partial plates may trigger this warning. Select **Next**.

Fold Changes (**Ratios**) can be calculated by specifying the sample(s) that represent the baseline of your experiment. **All pairwise ratios** will compare all groups to one another, while **Partitioning by** allows you to choose a group as the reference. Use the **Calculate False Discovery Rate** box (if active) to calculate False Discovery Rate (**FDR**); output for this will be in the Ratio Table. Select **Next**, confirm the ratios you wish to calculate, and select **Finish**.

See the **nSolver User Manual (MAN-C0019)** Background, Normalization, or Ratio sections for more information.

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**Data Export**

Your experiment will now be visible under your study on the **Experiments tab**. Expand the navigation tree.

- **Raw Data** table contains unprocessed data for all samples in this experiment.
- **Normalized Data** table contains the processed data for all samples.
- **Grouped Data** table contains the geometric mean of expression levels for all samples.
- **Ratio Data** level will contain the fold-change results, as well as any statistical inferences surrounding those estimates.
- **Analysis Data** table contains any analysis you have run.
PlexSet Data Analysis – a Note About POS & NEG Controls

There is one POS and one NEG control associated with each PlexSet A through H (see Figure 30). Note that all eight POS and NEG controls are listed for each sample even though only one POS and one NEG control is present per PlexSet. Collectively, these act as lane controls because each lane is a pool of up to 8 PlexSets A through H.

PlexSet A is associated with NEG_1 and POS_1.
PlexSet B is associated with NEG_2 and POS_2, and so on.

In Figure 29, even though only PlexSet E through H was used in this experiment, the central table in nSolver will list A-H. Once you export your data in Custom Text Format, you can sort PlexSet A through H and scroll down to the POS and NEG controls (see next page for instructions).

The example in Figure 30 is the exported data from a partial plate containing PlexSet E-H. The positive controls for PlexSet A-D are listed in the spreadsheet, but were not present in the reaction, so the data from PlexSets A through D should be ignored.

Note that on a per-lane basis (see columns labeled Lane 3 in Figure 30, for example), the same NEG and POS counts are replicated across different PlexSets. This is because all eight NEG controls and POS controls from PlexSets A through H act as lane controls. As such, the same counts are replicated per lane across all PlexSets A through H.

To figure out which positive or negative control is associated with which sample, array the NEG and POS controls into a 96-well format.

Figure 29: Partial plate data in the central table

Figure 30: POS and NEG controls in exported data from partial plate
PlexSet Data Analysis and Exporting Data

Select only the PlexSets (A through H) that were used in the experiment. Data in PlexSets that were not run may appear over-normalized, meaning you may see high counts in these fields; these fields should be disregarded.

In Figure 31, a partial plate was run with PlexSets E-H. Since A-D appear in the central table anyway, we must manually, specifically highlight Sets E-H for Export.

Highlight your relevant lanes of Raw or Normalized Data, select Export, then use default settings of Custom Text Format Export. Save the resulting .csv file and open it in your spreadsheet program of choice.

Note that the eight identical NEG controls and eight identical POS controls are listed for each sample, even though only one POS and one NEG were run in each lane. Recall that at the Normalization step in nSolver, we accepted the default setting to use the Top 3 POS counts (See Figure 26) for normalization.

Note, also, if you entered multiple sample names into each of your lanes when creating the run (in the Control Center on the Sprint or via a CDF (Cartridge Definition File) on the Max/Flex) you can now separate them into individual samples here. Each lane is now represented by 8 columns in the data set. For the row you entered your Sample Names into (Sample Name/SampleID or Comments) you will see all 8 names repeated in all 8 columns for that lane. You can now go through and remove all the names, except the one for that sample, to give each sample a unique name.
PlexSet Data Analysis (continued)

To create visualizations, highlight your data table and select Analysis. Select the plot desired, then select Next. Select the samples, then the probes you would like included in your analysis and select Next/Finish.

![Figure 33: Creating an Analysis](image)

Creating visualizations in nSolver follows the same guidelines as standard sample assays. Once your data is plotted, you can fine tune the settings. In each plot window, File allows you to save and print the plot image.

If creating a heatmap, you will be asked to set Clustering Parameters. Select Finish. The Heatmap is interactive and provides several customization options.

The options to the left of the Scatter Plot allow you to select the sample(s) you would like included in the plot, as well as the color designations of the data points. Additional customization (such as the axis and legend settings) is possible in Settings.

The tables to the left of the Violin, Box, and Histogram plots allow you to select the probes you would like included in the plots.

![Figure 34: Sample visualizations in nSolver](image)