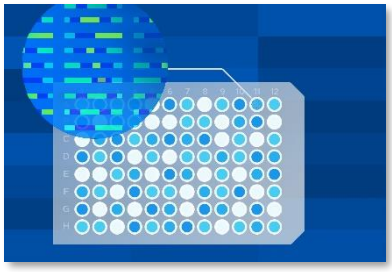
All About PlexSet Technology Data Analysis in nSolver Software



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*)*.

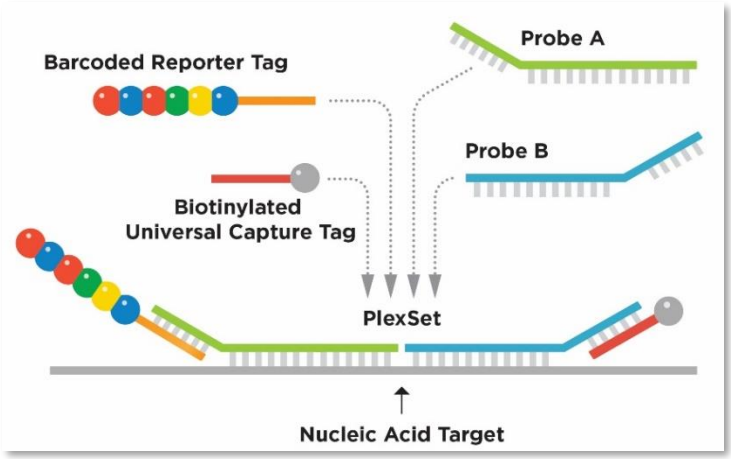


Figure 1: An existing PlexSet can be modified by reassigning Reporter Tags to new targets. By replacing Probes A and B with new oligos.

Titration Workflow

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

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

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

Titration 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.

Sample Workflow

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, if desired. You can also use Analysis to create visualizations, if desired. See page 15 of this manual.

Figure 2: Titration and PlexSet workflows

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.

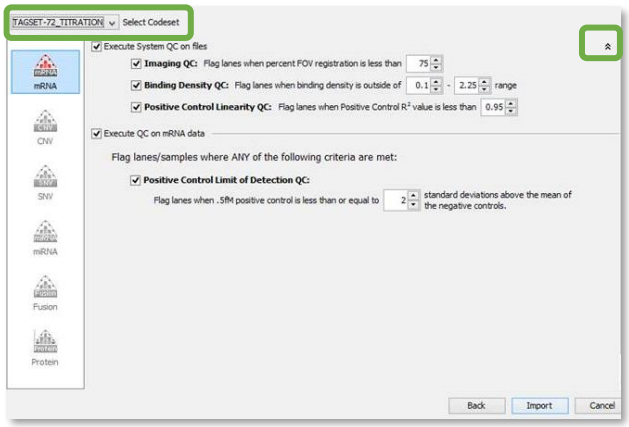


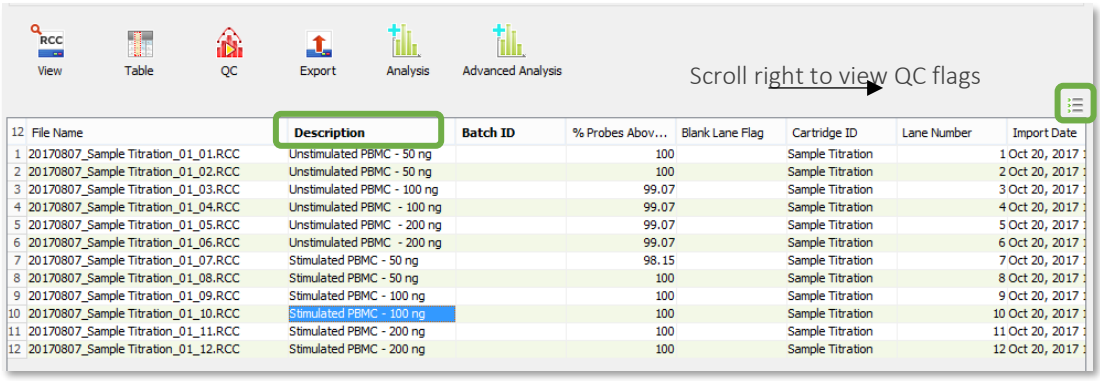
Figure 3: QC window

Select **Import**.

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.



	File Name	Description	Batch ID	% Probes Abov…	Blank Lane Flag	Cartridge ID	Lane Number	Import Date
1	20170807_Sample Titration_01_01.RCC	Unstimulated PBMC - 50 ng		100		Sample Titration	1	Oct 20, 2017
2	20170807_Sample Titration_01_02.RCC	Unstimulated PBMC - 50 ng		100		Sample Titration	2	Oct 20, 2017
3	20170807_Sample Titration_01_03.RCC	Unstimulated PBMC - 100 ng		99.07		Sample Titration	3	Oct 20, 2017
4	20170807_Sample Titration_01_04.RCC	Unstimulated PBMC - 100 ng		99.07		Sample Titration	4	Oct 20, 2017
5	20170807_Sample Titration_01_05.RCC	Unstimulated PBMC - 200 ng		99.07		Sample Titration	5	Oct 20, 2017
6	20170807_Sample Titration_01_06.RCC	Unstimulated PBMC - 200 ng		99.07		Sample Titration	6	Oct 20, 2017
7	20170807_Sample Titration_01_07.RCC	Stimulated PBMC - 50 ng		98.15		Sample Titration	7	Oct 20, 2017
8	20170807_Sample Titration_01_08.RCC	Stimulated PBMC - 50 ng		100		Sample Titration	8	Oct 20, 2017
9	20170807_Sample Titration_01_09.RCC	Stimulated PBMC - 100 ng		100		Sample Titration	9	Oct 20, 2017
10	20170807_Sample Titration_01_10.RCC	Stimulated PBMC - 100 ng		100		Sample Titration	10	Oct 20, 2017
11	20170807_Sample Titration_01_11.RCC	Stimulated PBMC - 200 ng		100		Sample Titration	11	Oct 20, 2017
12	20170807_Sample Titration_01_12.RCC	Stimulated PBMC - 200 ng		100		Sample Titration	12	Oct 20, 2017

Figure 4: Titration Kit raw data table format in nSolver with customizable Description column

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.

Figure 5: Titration Kit raw data table in nSolver; right side of central table contains QC flags

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.

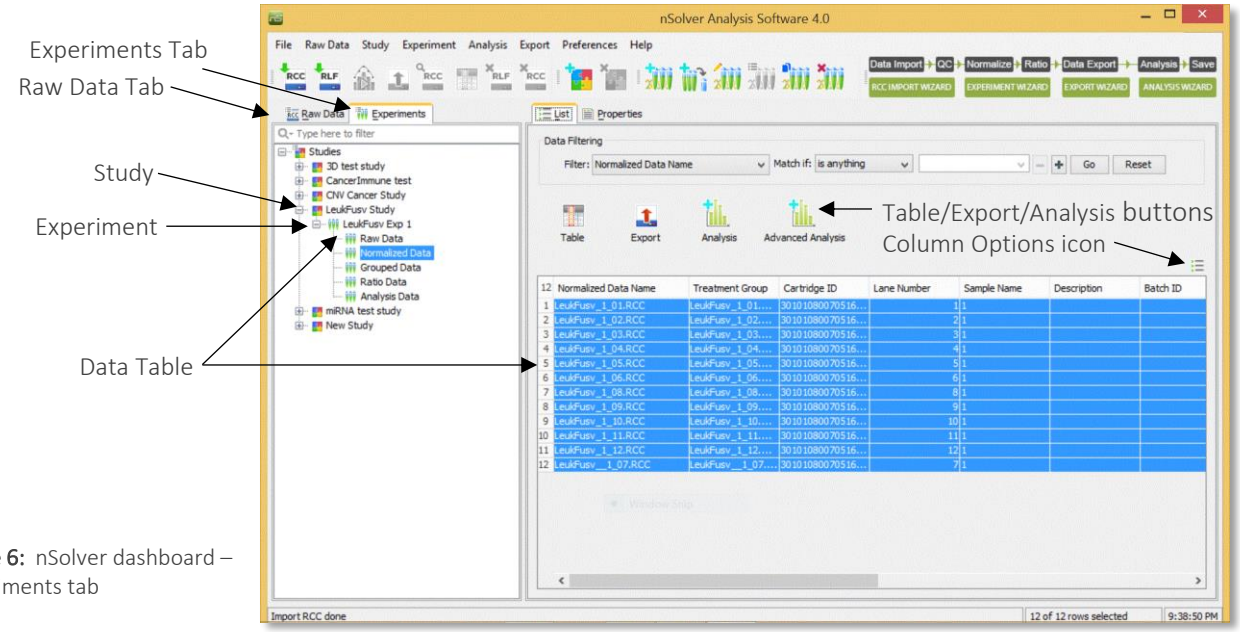


Figure 6: nSolver dashboard – experiments tab

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**.

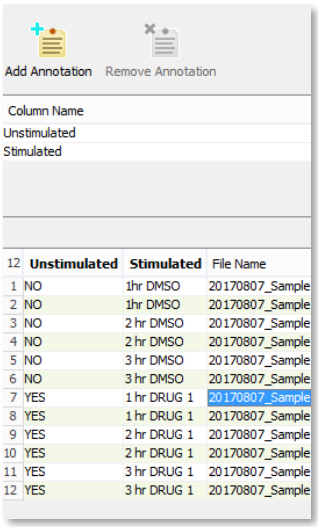


Figure 7: Annotation window

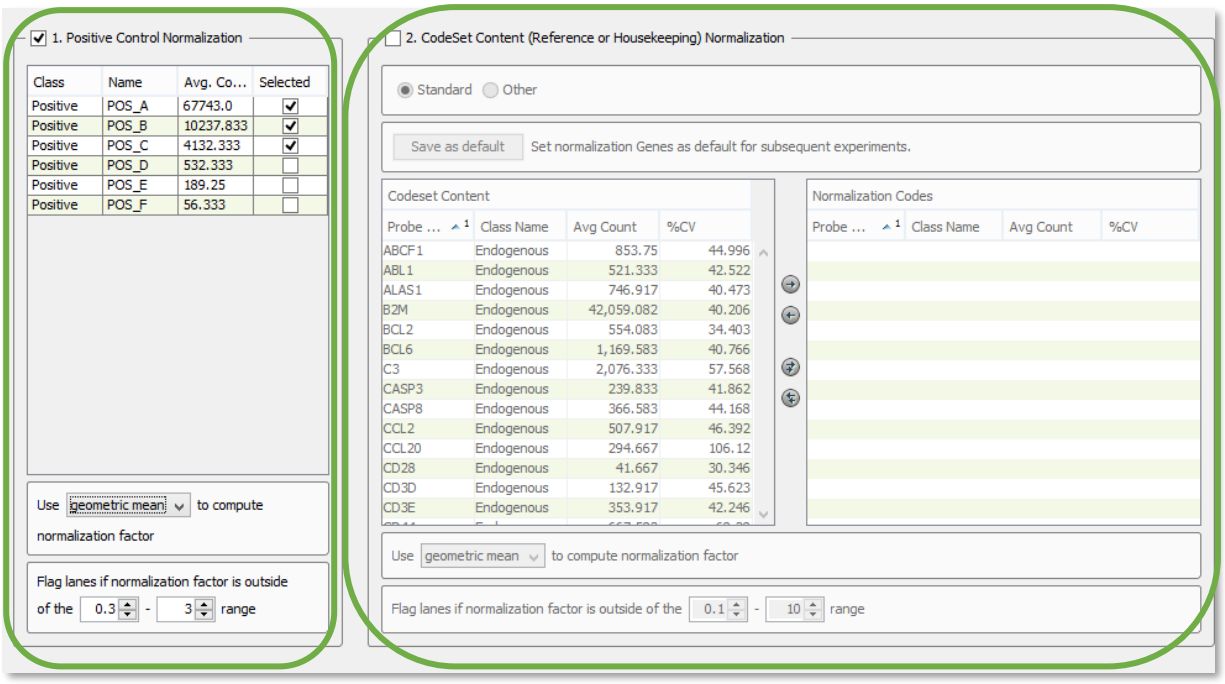


Figure 8: Normalization window

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).

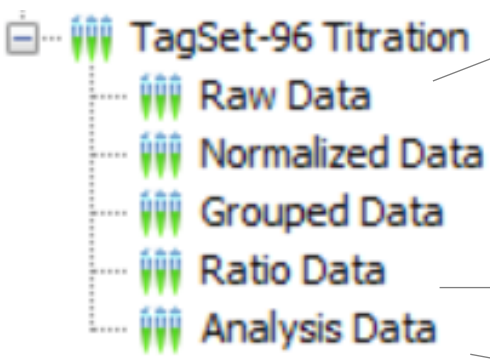


Figure 9: Experiment data hierarchy

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.

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**.

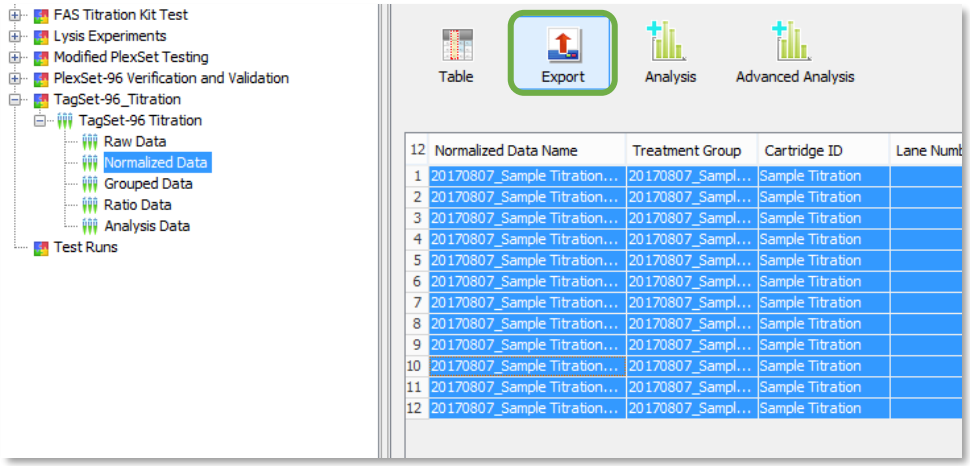


Figure 10: Exporting Titration data

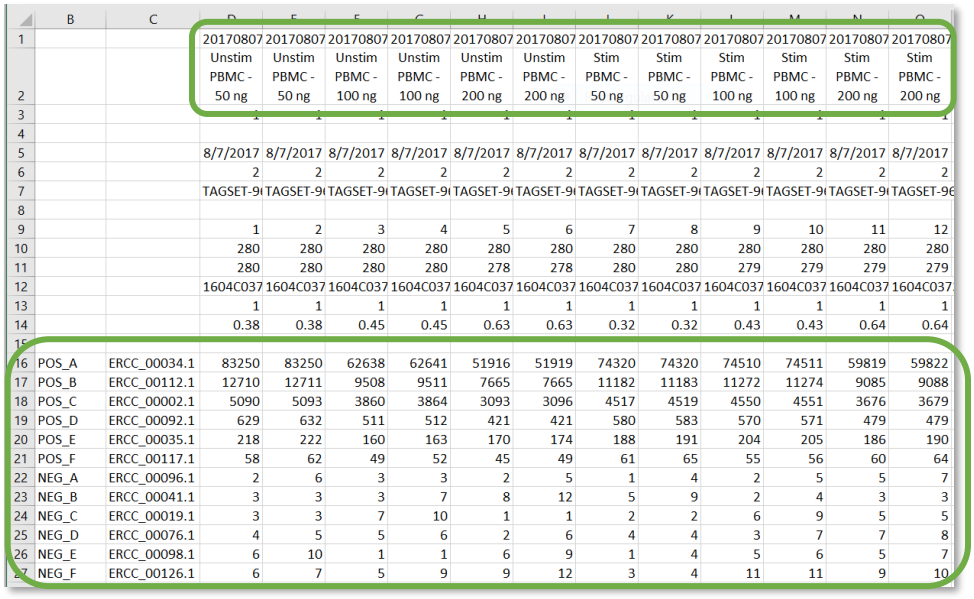


Figure 11: Exported Titration Data

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.

	B	C	D	E	F	G	H	I	J
21	POS_F	ERCC_00117.1	58	62	49	52	45	49	61
22	NEG_A	ERCC_00096.1	2	6	3	3	2	5	1
23	NEG_B	ERCC_00041.1	3	3	3	7	8	12	5
24	NEG_C	ERCC_00019.1	3	3	7	10	1	1	2
25	NEG_D	ERCC_00076.1	4	5	5	6	2	6	4
26	NEG_E	ERCC_00098.1	6	10	1	1	6	9	1
27	NEG_F	ERCC_00126.1	6	7	5	9	9	12	3
29	ABCF1	Tag-011.1	519	522	1063	1066	1986	1987	333
30	ABL1	Tag-058.1	233	234	474	477	909	914	271
31	ALAS1	Tag-010.1	350	352	702	702	1249	1254	370
32	B2M	Tag-005.1	22495	22493	43559	43549	83097	83084	18205
33	BCL2	Tag-012.1	327	330	568	570	999	1004	279
34	BCL6	Tag-038.1	592	595	1099	1103	2169	2169	543
35	C3	Tag-082.1	737	739	1363	1366	2537	2538	1250
36	CASP3	Tag-041.1	115	116	224	228	418	423	107
37	CASP8	Tag-062.1	142	145	288	292	556	558	234
38	CCL2	Tag-016.1	288	291	630	632	1169	1173	184
39	CCL20	Tag-032.1	313	315	513	514	1080	1081	21
40	CD28	Tag-065.1	31	33	33	33	75	77	23
41	CD3D	Tag-094.1	72	73	154	159	288	292	45

Figure 12: Titration data in spreadsheet

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**.

		UNSTIM					
		Unstim PBMC - 50 ng	Unstim PBMC - 50 ng	Unstim PBMC - 100 ng	Unstim PBMC - 100 ng	Unstim PBMC - 200 ng	Unstim PBMC - 200 ng
	Total Gene Target Counts	134433	134569	257683	257844	495730	495882
	Sample Input	50		100		200	
	Average of Replicate Total Counts	134501		257764		495806	
	Equation	y = 2404.7x + 15480					
	y-intercept	15480					
	Slope	2405					
	Correlation (R2)	0.99992					
MAX/FLEX	Input 150,000 counts	56					
SPRINT	Input 400,000	160					

Figure 13: Processing Titration data in spreadsheet

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.

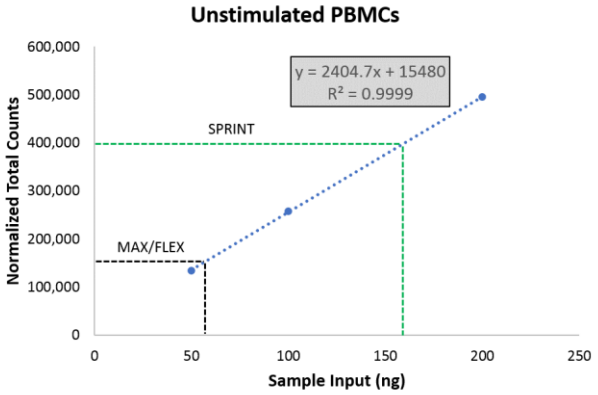


Figure 14: Line graph of Titration data

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).

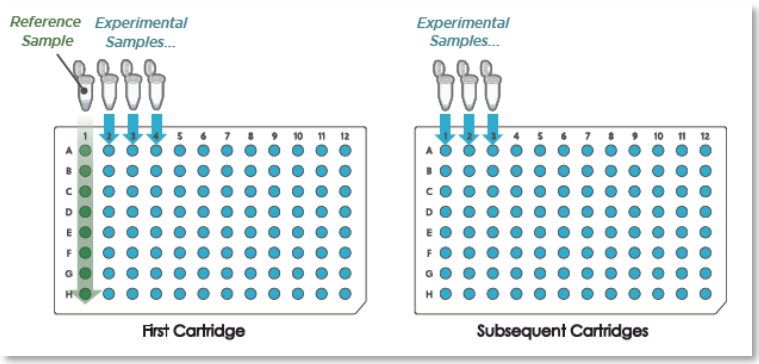


Figure 15: PlexSet layout – full plates

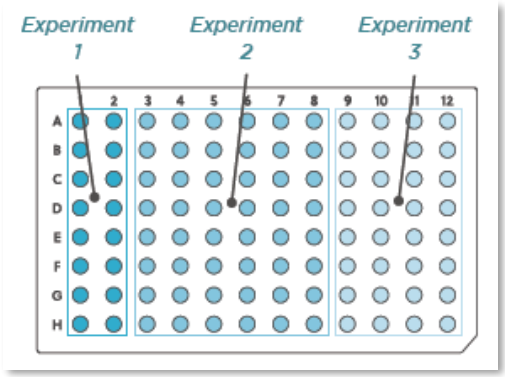


Figure 16: PlexSet samples organized in columns

Less than 96 samples can be run per cartridge; the same RLF is used for these scenarios (first and second run, Figure 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).

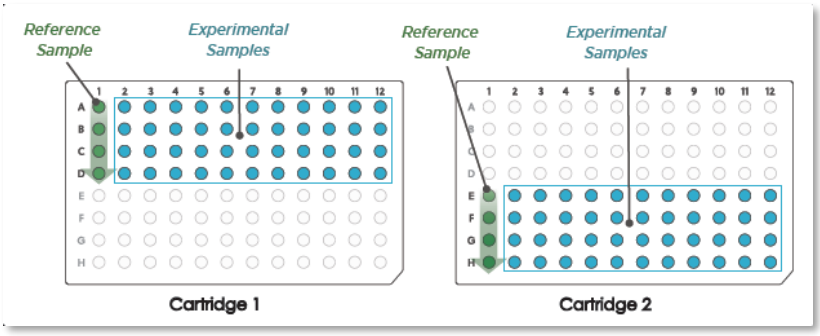


Figure 17: First and second run—partial PlexSet plates

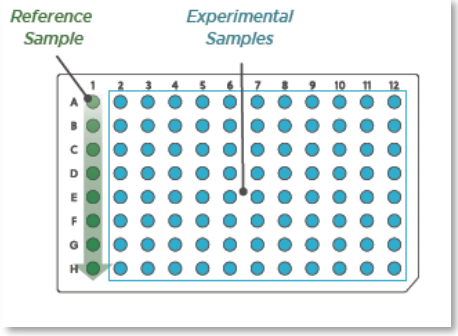


Figure 18: Third run—full PlexSet plate

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).

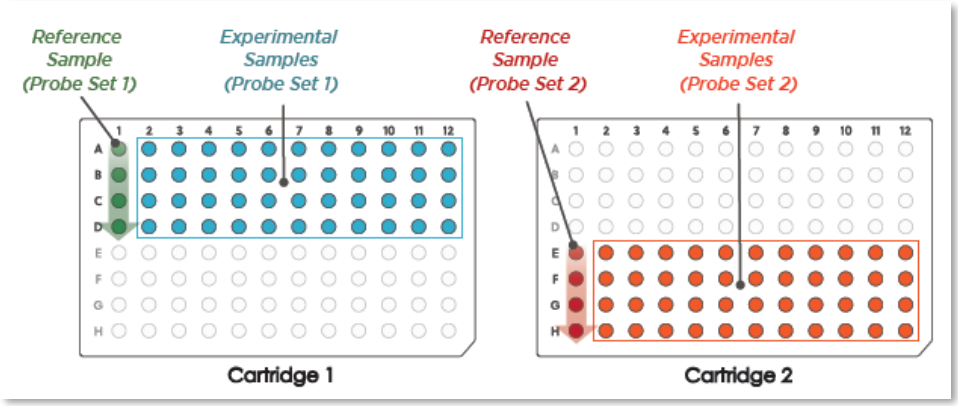


Figure 19: Partial PlexSet plates, different Probe Sets

If multiple probe sets are run on the same plate, probe sets should be organized down columns to allow downstream analysis with nSolver software (Figure 20). For more information on setting up plates in this manner, see the *PlexSet Reagents Manual (*MAN-10040*)*.

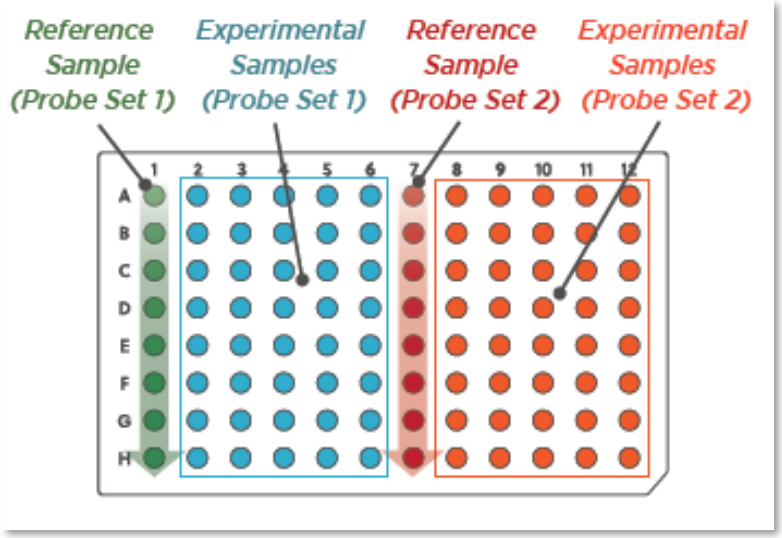


Figure 20: Different Probe Sets, organized down columns

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**.

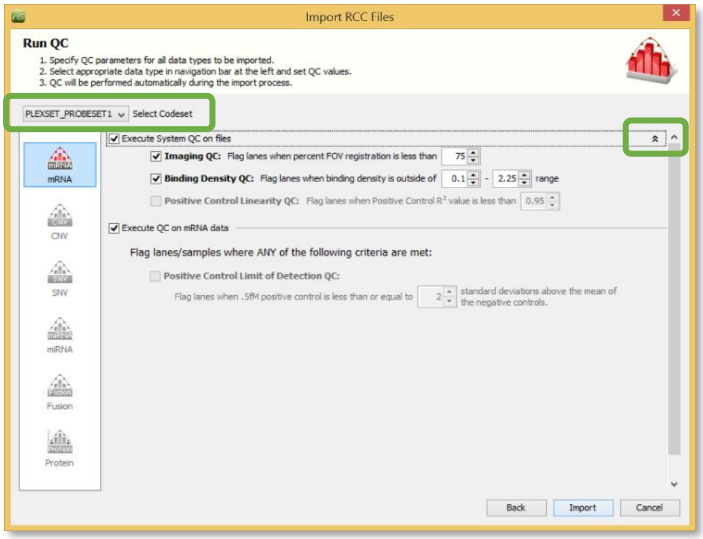
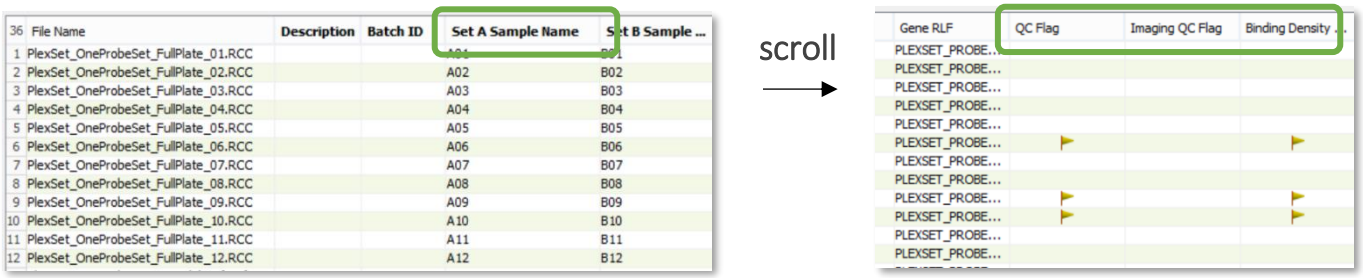


Figure 21: QC window

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.



36	File Name	Description	Batch ID	Set A Sample Name	Set B Sample ...
1	PlexSet_OneProbeSet_FullPlate_01.RCC				
2	PlexSet_OneProbeSet_FullPlate_02.RCC			A02	B02
3	PlexSet_OneProbeSet_FullPlate_03.RCC			A03	B03
4	PlexSet_OneProbeSet_FullPlate_04.RCC			A04	B04
5	PlexSet_OneProbeSet_FullPlate_05.RCC			A05	B05
6	PlexSet_OneProbeSet_FullPlate_06.RCC			A06	B06
7	PlexSet_OneProbeSet_FullPlate_07.RCC			A07	B07
8	PlexSet_OneProbeSet_FullPlate_08.RCC			A08	B08
9	PlexSet_OneProbeSet_FullPlate_09.RCC			A09	B09
10	PlexSet_OneProbeSet_FullPlate_10.RCC			A10	B10
11	PlexSet_OneProbeSet_FullPlate_11.RCC			A11	B11
12	PlexSet_OneProbeSet_FullPlate_12.RCC			A12	B12

Gene RLF	QC Flag	Imaging QC Flag	Binding Density ...
PLEXSET_PROBE...			
PLEXSET_PROBE...			
PLEXSET_PROBE...			
PLEXSET_PROBE...			
PLEXSET_PROBE...	⚑		⚑
PLEXSET_PROBE...			
PLEXSET_PROBE...			
PLEXSET_PROBE...	⚑		⚑
PLEXSET_PROBE...	⚑		⚑
PLEXSET_PROBE...			
PLEXSET_PROBE...			

Figure 22: Initial PlexSet raw data table format with QC flags

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).

112	Accession #	Class Name	Annotation	Set A (A01) PlexSet_OneProbeSet_PartialPlate(A-D)_01.RCC	Set B (B01) Plex...
96	NM_001128128.1	Endogenous		5,607	11,424
97	ERCC_00019.1	Negative		2	2
98	ERCC_00019.1	Negative		1	1
99	ERCC_00019.1	Negative		2	2
100	ERCC_00019.1	Negative		1	1
101	ERCC_00019.1	Negative		2	2
102	ERCC_00019.1	Negative		1	1
103	ERCC_00019.1	Negative		1	1
104	ERCC_00019.1	Negative		1	1
105	ERCC_00002.1	Positive		7,473	7,473
106	ERCC_00002.1	Positive		4,871	4,871
107	ERCC_00002.1	Positive		4,668	4,668
108	ERCC_00002.1	Positive		6,245	6,245
109	ERCC_00002.1	Positive		1	1
110	ERCC_00002.1	Positive		1	1
111	ERCC_00002.1	Positive		1	1
112	ERCC_00002.1	Positive		1	1

Figure 23: PlexSet raw data table output

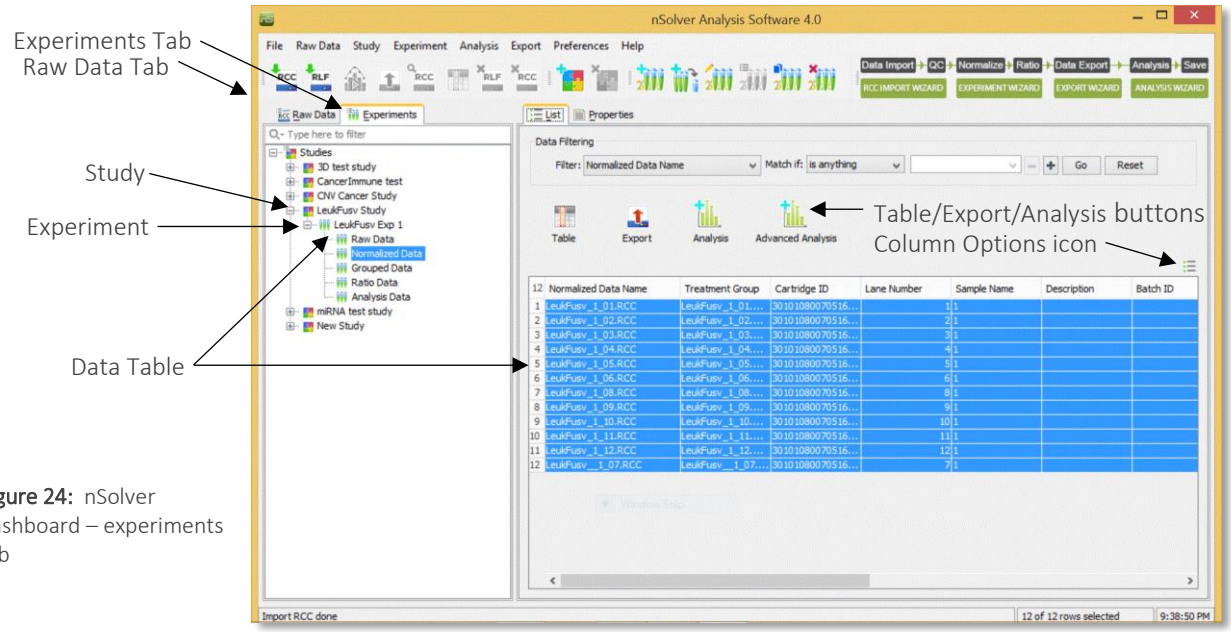


Figure 24: nSolver dashboard – experiments tab

Creating Experiments with PlexSet Data

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

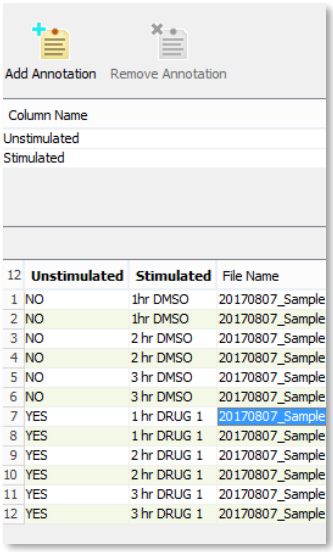
A **Study** is an organizational folder used to store experiments; select the **New Study** button to create a study.

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.



	Unstimulated	Stimulated	File Name
1	NO	1hr DMSO	20170807_Sample
2	NO	1hr DMSO	20170807_Sample
3	NO	2 hr DMSO	20170807_Sample
4	NO	2 hr DMSO	20170807_Sample
5	NO	3 hr DMSO	20170807_Sample
6	NO	3 hr DMSO	20170807_Sample
7	YES	1 hr DRUG 1	20170807_Sample
8	YES	1 hr DRUG 1	20170807_Sample
9	YES	2 hr DRUG 1	20170807_Sample
10	YES	2 hr DRUG 1	20170807_Sample
11	YES	3 hr DRUG 1	20170807_Sample
12	YES	3 hr DRUG 1	20170807_Sample

Figure 25: Annotation window

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**.

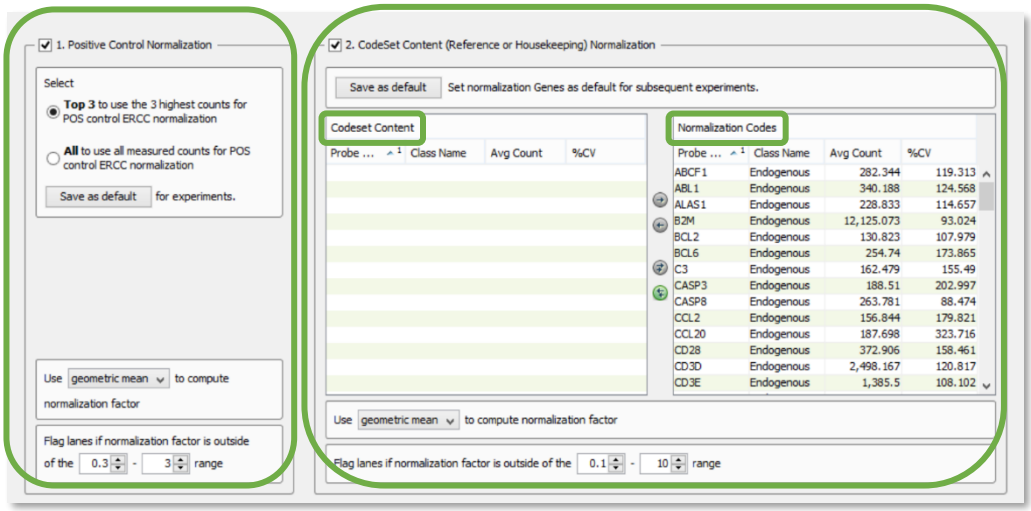


Figure 26: Normalization window

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.***

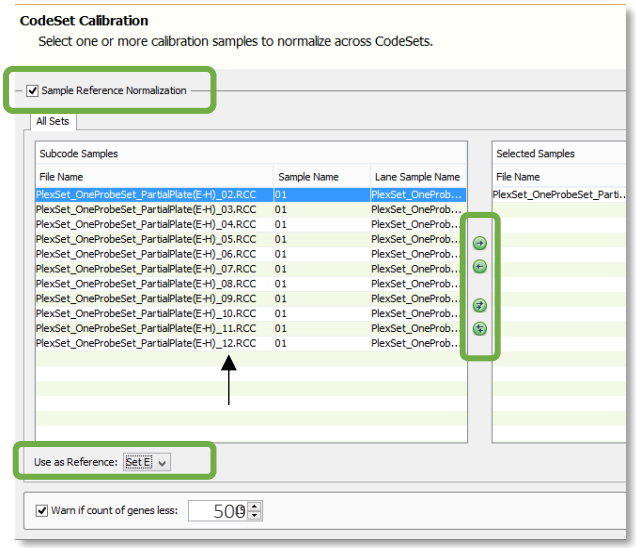


Figure 27: Reference Calibration window for sample data

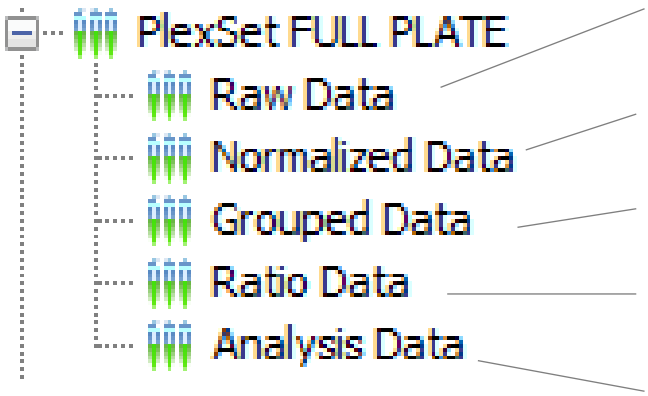
Ensure that the box **Warn if count of genes is less: …** is checked and selecting the value **500** from the dropdown will ellicit a popup, warning you of the genes whose counts fall below that value. Readings from empty wells in partial plates may trigger this warning. Select **Next**.

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.

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.

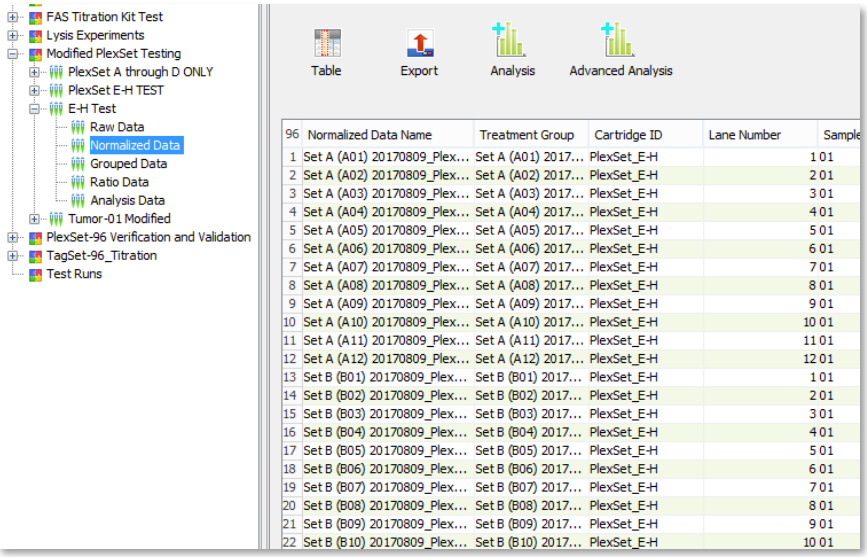
Figure 28: Experiment data hierarchy

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).



96	Normalized Data Name	Treatment Group	Cartridge ID	Lane Number	Sample
1	Set A (A01) 20170809_Plex...	Set A (A01) 2017...	PlexSet_E-H	1	01
2	Set A (A02) 20170809_Plex...	Set A (A02) 2017...	PlexSet_E-H	2	01
3	Set A (A03) 20170809_Plex...	Set A (A03) 2017...	PlexSet_E-H	3	01
4	Set A (A04) 20170809_Plex...	Set A (A04) 2017...	PlexSet_E-H	4	01
5	Set A (A05) 20170809_Plex...	Set A (A05) 2017...	PlexSet_E-H	5	01
6	Set A (A06) 20170809_Plex...	Set A (A06) 2017...	PlexSet_E-H	6	01
7	Set A (A07) 20170809_Plex...	Set A (A07) 2017...	PlexSet_E-H	7	01
8	Set A (A08) 20170809_Plex...	Set A (A08) 2017...	PlexSet_E-H	8	01
9	Set A (A09) 20170809_Plex...	Set A (A09) 2017...	PlexSet_E-H	9	01
10	Set A (A10) 20170809_Plex...	Set A (A10) 2017...	PlexSet_E-H	10	01
11	Set A (A11) 20170809_Plex...	Set A (A11) 2017...	PlexSet_E-H	11	01
12	Set A (A12) 20170809_Plex...	Set A (A12) 2017...	PlexSet_E-H	12	01
13	Set B (B01) 20170809_Plex...	Set B (B01) 2017...	PlexSet_E-H	1	01
14	Set B (B02) 20170809_Plex...	Set B (B02) 2017...	PlexSet_E-H	2	01
15	Set B (B03) 20170809_Plex...	Set B (B03) 2017...	PlexSet_E-H	3	01
16	Set B (B04) 20170809_Plex...	Set B (B04) 2017...	PlexSet_E-H	4	01
17	Set B (B05) 20170809_Plex...	Set B (B05) 2017...	PlexSet_E-H	5	01
18	Set B (B06) 20170809_Plex...	Set B (B06) 2017...	PlexSet_E-H	6	01
19	Set B (B07) 20170809_Plex...	Set B (B07) 2017...	PlexSet_E-H	7	01
20	Set B (B08) 20170809_Plex...	Set B (B08) 2017...	PlexSet_E-H	8	01
21	Set B (B09) 20170809_Plex...	Set B (B09) 2017...	PlexSet_E-H	9	01
22	Set B (B10) 20170809_Plex...	Set B (B10) 2017...	PlexSet_E-H	10	01

Figure 29: Partial plate data in the central table

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.

		PlexSet A												PlexSet B							
		Lane 1	Lane 2	Lane 3	Lane 4	Lane 5	Lane 6	Lane 7	Lane 8	Lane 9	Lane 10	Lane 11	Lane 12	Lane 1	Lane 2	Lane 3	Lane 4	Lane 5	Lane 6	Lane 7	Lane
		Well A01	Well A02	Well A03	Well A04	Well A05	Well A06	Well A07	Well A08	Well A09	Well A10	Well A11	Well A12	Well B01	Well B02	Well B03	Well B04	Well B05	Well B06	Well B07	Well B
PlexSet A	NEG_1	2	12	3	9	2	5	2	7	2	1	9	1	2	12	3	9	12	10	11	
PlexSet B	NEG_2	1	4	1	2	2	6	7	2	4	1	2	5	1	4	4	2	2	6	7	
PlexSet C	NEG_3	2	6	1	6	9	2	8	2	8	7	3	4	2	6	1	6	9	19	8	
PlexSet D	NEG_4	1	11	3	5	7	2	8	8	4	5	6	2	1	11	3	5	7	4	8	
PlexSet E	NEG_5	2	3	1	5	1	2	5	6	7	0	3	5	2	3	1	5	1	2	5	
PlexSet F	NEG_6	1	7	3	1	4	0	4	3	5	8	5	2	1	7	3	1	4	2	4	
PlexSet G	NEG_7	1	5	2	1	4	6	2	4	7	1	4	2	1	5	2	1	4	6	2	
PlexSet H	NEG_8	1	7	1	1	5	8	2	4	3	7	4	5	1	7	1	1	5	8	2	
PlexSet A	POS_1	3	5	2	7	4	4	1	4	1	1	1	5	3	10	15	14	9	14	1	
PlexSet B	POS_2	3	4	2	6	5	4	6	1	4	5	2	7	3	15	15	6	16	16	6	
PlexSet C	POS_3	2	2	1	6	2	1	8	4	1	1	4	7	2	15	1	6	2	17	16	
PlexSet D	POS_4	7	7	2	3	8	1	5	2	6	6	5	3	7	7	14	3	8	1	11	
PlexSet E	POS_5	4279	4471	3815	3709	3938	4169	2867	3921	4607	4785	4706	3089	4279	4471	3815	3709	3938	4169	2867	39
PlexSet F	POS_6	4958	4035	3269	3043	4588	2636	4110	2850	4635	4374	4445	4003	4958	4035	3269	3043	4588	2636	4110	28
PlexSet G	POS_7	3159	3628	4581	3278	4640	2711	3531	3618	2586	2820	2545	2600	3159	3628	4581	3278	4640	2711	3531	36
PlexSet H	POS_8	4003	3599	4520	2956	4019	3451	2565	2968	3415	4930	2524	2842	4003	3599	4520	2956	4019	3451	2565	29

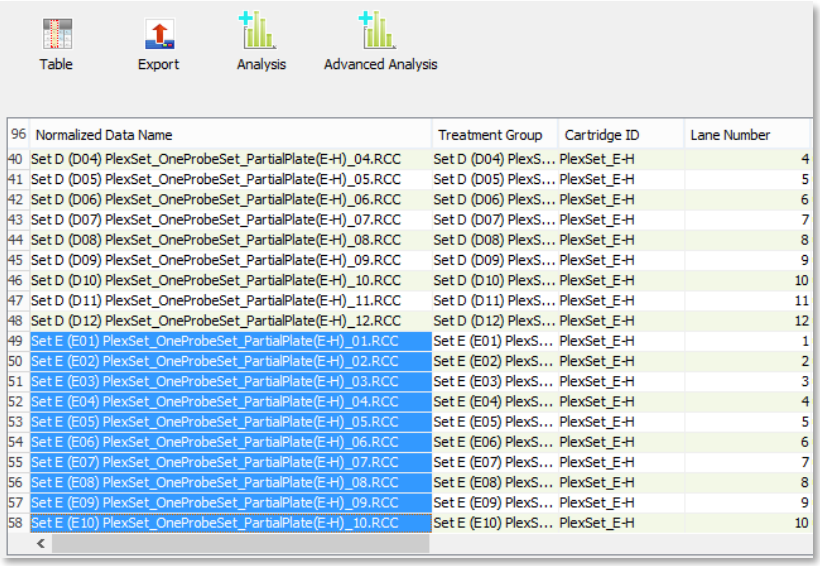
Figure 30: POS and NEG controls in exported data from partial plate

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.



96	Normalized Data Name	Treatment Group	Cartridge ID	Lane Number
40	Set D (D04) PlexSet_OneProbeSet_PartialPlate(E-H)_04.RCC	Set D (D04) PlexS...	PlexSet_E-H	4
41	Set D (D05) PlexSet_OneProbeSet_PartialPlate(E-H)_05.RCC	Set D (D05) PlexS...	PlexSet_E-H	5
42	Set D (D06) PlexSet_OneProbeSet_PartialPlate(E-H)_06.RCC	Set D (D06) PlexS...	PlexSet_E-H	6
43	Set D (D07) PlexSet_OneProbeSet_PartialPlate(E-H)_07.RCC	Set D (D07) PlexS...	PlexSet_E-H	7
44	Set D (D08) PlexSet_OneProbeSet_PartialPlate(E-H)_08.RCC	Set D (D08) PlexS...	PlexSet_E-H	8
45	Set D (D09) PlexSet_OneProbeSet_PartialPlate(E-H)_09.RCC	Set D (D09) PlexS...	PlexSet_E-H	9
46	Set D (D10) PlexSet_OneProbeSet_PartialPlate(E-H)_10.RCC	Set D (D10) PlexS...	PlexSet_E-H	10
47	Set D (D11) PlexSet_OneProbeSet_PartialPlate(E-H)_11.RCC	Set D (D11) PlexS...	PlexSet_E-H	11
48	Set D (D12) PlexSet_OneProbeSet_PartialPlate(E-H)_12.RCC	Set D (D12) PlexS...	PlexSet_E-H	12
49	Set E (E01) PlexSet_OneProbeSet_PartialPlate(E-H)_01.RCC	Set E (E01) PlexS...	PlexSet_E-H	1
50	Set E (E02) PlexSet_OneProbeSet_PartialPlate(E-H)_02.RCC	Set E (E02) PlexS...	PlexSet_E-H	2
51	Set E (E03) PlexSet_OneProbeSet_PartialPlate(E-H)_03.RCC	Set E (E03) PlexS...	PlexSet_E-H	3
52	Set E (E04) PlexSet_OneProbeSet_PartialPlate(E-H)_04.RCC	Set E (E04) PlexS...	PlexSet_E-H	4
53	Set E (E05) PlexSet_OneProbeSet_PartialPlate(E-H)_05.RCC	Set E (E05) PlexS...	PlexSet_E-H	5
54	Set E (E06) PlexSet_OneProbeSet_PartialPlate(E-H)_06.RCC	Set E (E06) PlexS...	PlexSet_E-H	6
55	Set E (E07) PlexSet_OneProbeSet_PartialPlate(E-H)_07.RCC	Set E (E07) PlexS...	PlexSet_E-H	7
56	Set E (E08) PlexSet_OneProbeSet_PartialPlate(E-H)_08.RCC	Set E (E08) PlexS...	PlexSet_E-H	8
57	Set E (E09) PlexSet_OneProbeSet_PartialPlate(E-H)_09.RCC	Set E (E09) PlexS...	PlexSet_E-H	9
58	Set E (E10) PlexSet_OneProbeSet_PartialPlate(E-H)_10.RCC	Set E (E10) PlexS...	PlexSet_E-H	10

Figure 31: Highlighting relevant cells for export

	A	B	C	D	E	F	G	H	I
1	Probe Name	Accession #	NS Probe ID	Class Name	Analyte Type	% Samples above Threshold	Annotation	Set A (A01) PlexSet_OneProbeSet_FullPlate_01.RCC	Set A (A02
2								Set A (A01) PlexSet_OneProbeSet_FullPlate_01.RCC	Set A (A02
3									
4	ABCF1	NM_001090.	NM_001090.2:	Endogenous	mRNA	100		573.04	206.81
5	ABL1	NM_005157.	NM_005157.3:	Endogenous	mRNA	100		607.88	206.81
6	ALAS1	NM_000688.	NM_000688.4:	Endogenous	mRNA	100		319.51	206.81

scroll down

100	NEG_1	ERCC_00019.	NEG_1	Negative	SYSTEM	91.67		12	2
101	NEG_2	ERCC_00019.	NEG_2	Negative	SYSTEM	91.67		4	1
102	NEG_3	ERCC_00019.	NEG_3	Negative	SYSTEM	91.67		6	2
103	NEG_4	ERCC_00019.	NEG_4	Negative	SYSTEM	91.67		11	1
104	NEG_5	ERCC_00019.	NEG_5	Negative	SYSTEM	83.33		3	2
105	NEG_6	ERCC_00019.	NEG_6	Negative	SYSTEM	83.33		7	1
106	NEG_7	ERCC_00019.	NEG_7	Negative	SYSTEM	83.33		5	1
107	NEG_8	ERCC_00019.	NEG_8	Negative	SYSTEM	75		7	1
108	POS_1	ERCC_00002.	POS_1	Positive	SYSTEM	100		3586	7473
109	POS_2	ERCC_00002.	POS_2	Positive	SYSTEM	100		2302	4871
110	POS_3	ERCC_00002.	POS_3	Positive	SYSTEM	100		2186	4668
111	POS_4	ERCC_00002.	POS_4	Positive	SYSTEM	100		2900	6245
112	POS_5	ERCC_00002.	POS_5	Positive	SYSTEM	100		2705	6000
113	POS_6	ERCC_00002.	POS_6	Positive	SYSTEM	100		2607	5829
114	POS_7	ERCC_00002.	POS_7	Positive	SYSTEM	100		2793	5817
115	POS_8	ERCC_00002.	POS_8	Positive	SYSTEM	100		1973	4236

Figure 32: Analyzing PlexSet data

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**.

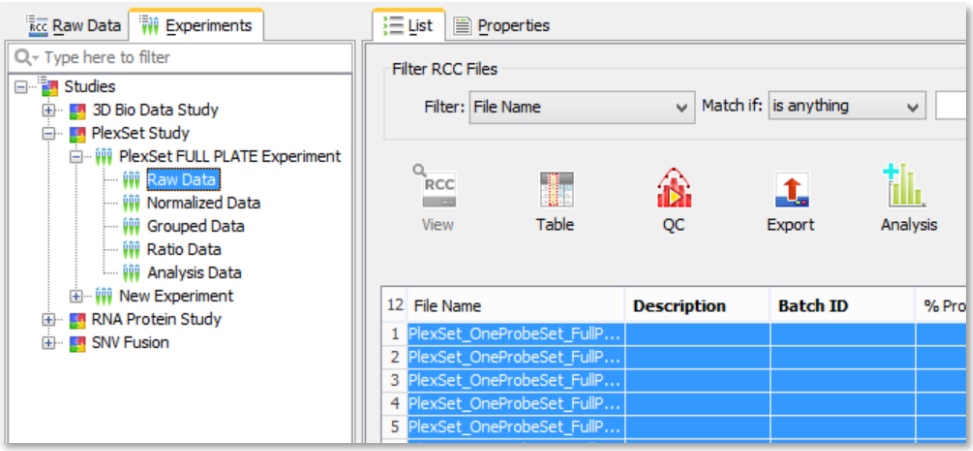


Figure 33: Creating an Analysis

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.

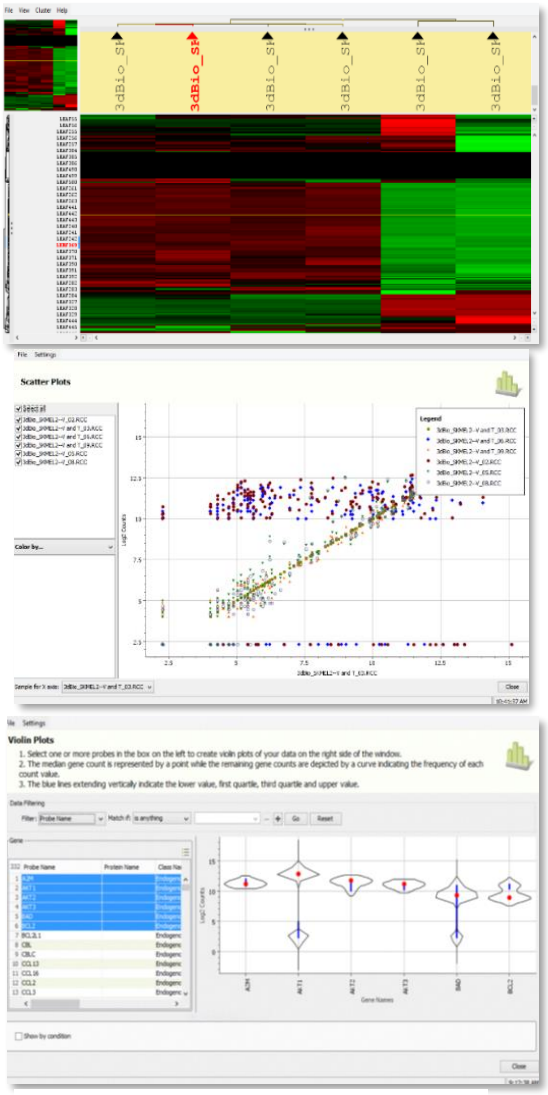


Figure 34: Sample visualizations in nSolver