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Conventions

Conventions

The following conventions are used throughout this manual and are described for your reference.

Bold text is typically used to highlight a specific button, keystroke, or menu option. It may also be used to highlight important text or terms.

Blue underlined text is typically used to highlight links and/or references to other sections of the manual. It may also be used to highlight references to other manuals or instructional material.

The gray box indicates general information that may be useful for improving assay performance. These notes may clarify other instructions or provide guidance to improve the efficiency of the assay workflow.



IMPORTANT: This symbol indicates important information that is critical to ensure a successful assay. Following these instructions may help improve the quality of your data.



WARNING: This symbol indicates the potential for bodily injury or damage to the instrument if the instructions are not followed correctly. Always carefully read and follow the instructions accompanied by this symbol to avoid potential hazards.

GeoMx DSP Workflow

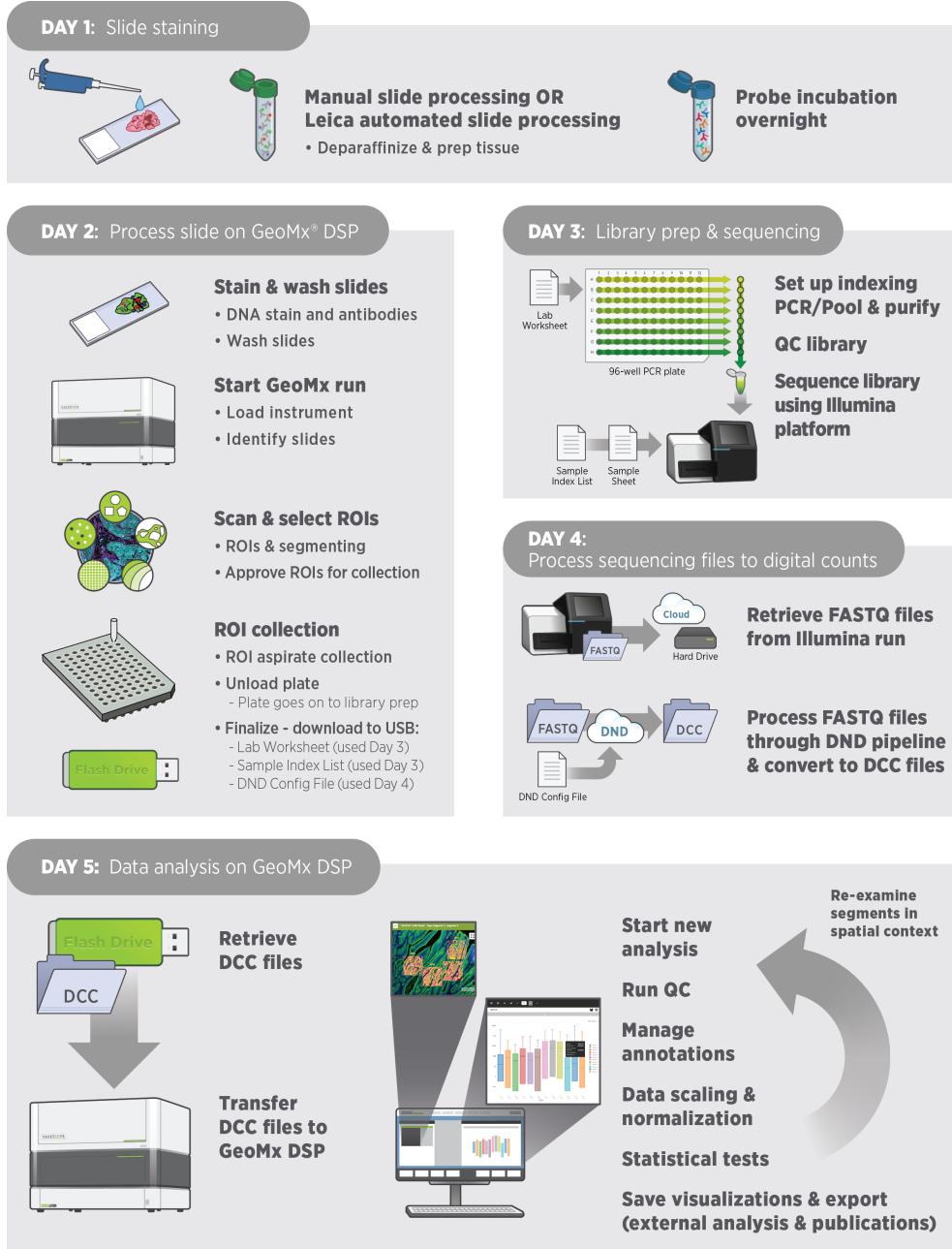


Figure 1: GeoMx DSP overall workflow

The GeoMx® Digital Spatial Profiling (DSP) technology is a novel platform developed by NanoString. This product relies upon antibody or RNA probes coupled to photocleavable oligonucleotide tags. After the hybridization of probes to slide-mounted tissue sections, the oligonucleotide tags are released from discrete regions of the tissue via UV exposure. Released

GeoMx DSP Workflow

tags are quantitated in an nCounter® or Illumina NGS assay, and counts are mapped back to tissue location, yielding a spatially-resolved digital profile of analyte abundance. The GeoMx DSP workflow describes the process of accomplishing these steps ([see Figure 1](#)).

- **Slide Staining** is mostly performed on Day 1. During this phase, you will prepare slides and hybridize biological targets with UV-cleavable biological probes.
- On Day 2, you will label cell types of interest with fluorescent morphology markers, then go into the next phase, **Process Slide on GeoMx DSP**. During this phase, you will load your prepared slides onto the GeoMx DSP instrument, enter identifying information for them, scan them to create fluorescent images, select regions of interest (ROIs), and then collect UV-cleaved oligos from these ROIs into the wells of a collection plate.
- If you are running your readout on a Next Generation Sequencing (**NGS**) platform, on Day 3, you will transfer the contents of the DSP collection to a PCR plate for **Library prep and Sequencing**. The products will be purified, pooled, then sequenced on an Illumina NGS instrument.
- On Day 4, you'll **process sequencing files to digital counts** (DCC files) using NanoString's GeoMx NGS Pipeline (DND) software.
- On Day 5, you'll transfer the DCCs to the GeoMx DSP **Data Analysis** Suite, where you will run platform and readout-specific quality control checks, perform data analysis, and generate analysis plots.

GEOFx DSP USER MANUALS AND OTHER USER DOCUMENTATION

- All of the GeoMx DSP user documentation exists in the **GeoMx DSP Online User Manual**, accessible from the help icon on the **GeoMx DSP Control Center** and online at <https://www.nanostring.com/geomx-online-user-manual>.
- PDF versions of GeoMx DSP documentation are also available for both nCounter and NGS readouts. The **Slide Prep**, **Instrument**, **Readout**, **GeoMx NGS Pipeline (DND)** (for NGS only), and **Data Analysis** user manuals are available for download from the **GeoMx DSP Online User Manual** (see above).
- Illumina platform documentation can be found in their respective manuals at <https://support.illumina.com/>.

Data Analysis Introduction

The Data Analysis module can be accessed from the **Data Analysis** button at the top of the GeoMx DSP Control Center. Choose to either create a **New study from queue** or **Open** an existing study. You may also launch this module from the **Records** section of the GeoMx DSP Control Center. See [Creating/Opening a Study on page 11.](#)

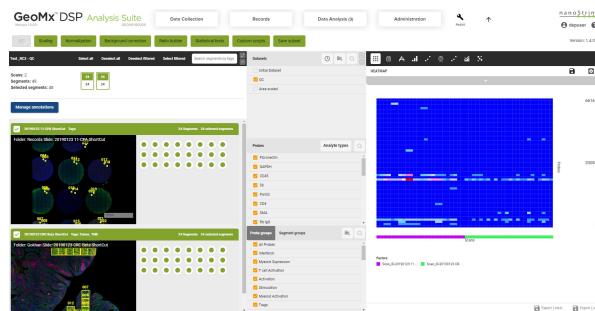


Figure 2: GeoMx DSP Data Analysis Suite

DATA ANALYSIS SCREEN ORIENTATION

The Data Analysis screen is divided into three panes and a toolbar. Here, we describe the functions of each pane and the interactions between all three.

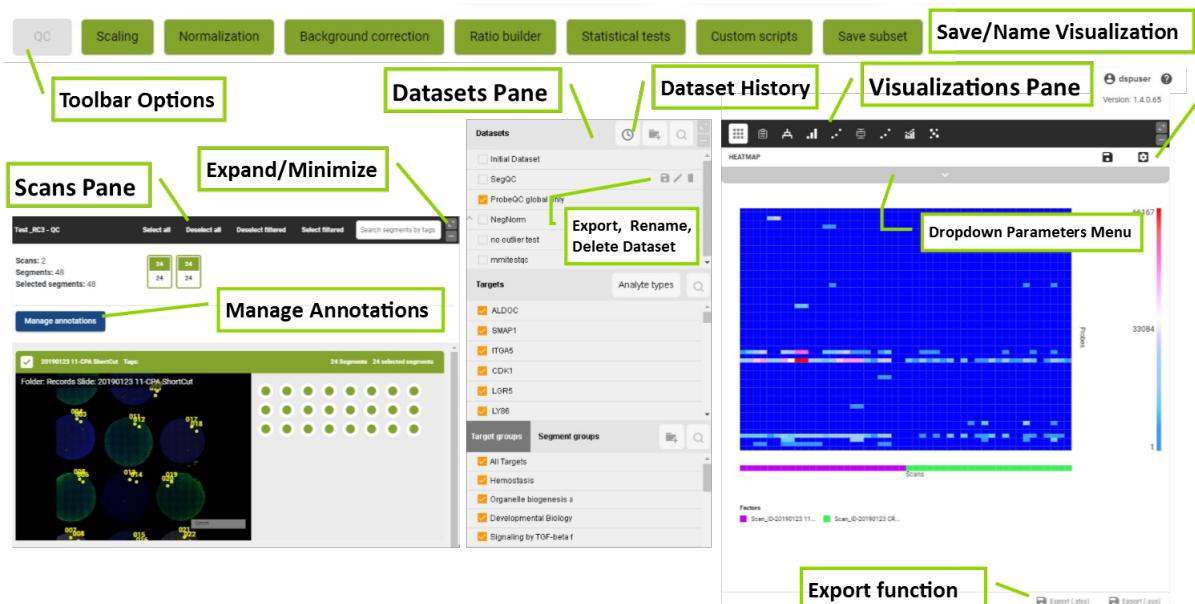


Figure 3: Data Analysis Suite diagram

The **Scans** pane is shown on the left side and contains individual image viewers of the scans. Scan icons representing each scan are located at the top of this pane and picker buttons representing each segment are located to the right of each image viewer. These icons and buttons are also toggle controls - a single click will switch the state of a scan or segment from selected to unselected and vice-versa. Hovering over an icon or picker button displays additional information such as name, tags, etc. See [Scans Pane on page 59.](#)

Data Analysis Introduction

Numerous **Toolbar Options** are available, however, only **QC** is active at first. Once QC has been run, other options become active. **Scaling**, **Background Correction**, and **Ratio Builder** are designed to be run once each (after they have been run, they will be inactive). Toolbar options are active or inactive based on the current selected dataset and the tasks allowed for that dataset. After each task, you will be prompted to save this new dataset; all datasets are available for selection in the central pane of the software. See [Toolbar Options on page 28](#).

The middle **Datasets** pane lists all datasets, targets, target groups, and segment groups associated with the current study. At a minimum, you will see the **Initial Dataset** (the calibrated set of imported data; this will appear at the top of the Dataset list) and the **All Targets** group. See [Datasets Pane on page 61](#).

The **Visualizations** pane is on the right. Visualizations are visual representations of the dataset selected, the targets selected, and the adjustments applied to the data from those targets.

Select an area of interest in any visualization to see the respective spatial location of the segments highlighted in the Scans pane. Right-click on your selection in the visualization to choose an action which may include:

- Creating a target group or segment group from the selection.
- Excluding the selected set of targets or segments from the current study.
- Creating a tag for the selected set of segments.

See [Visualizations Pane on page 67](#).

Data Analysis Introduction

DATA ANALYSIS QUICK GUIDE

Download the Data Analysis Quick Guide at <https://www.nanostring.com/geomx-online-user-manual>.

Creating/Opening a Study

A new analysis can be launched from the Data Analysis button on the GeoMx DSP Control Center ([see Figure 4](#)); the new analysis will be built using the scans in the Data Analysis Queue.

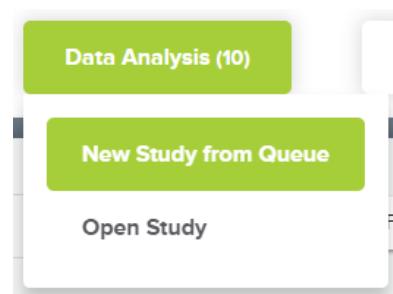


Figure 4: Data Analysis button

1 Data Analysis Queue

ASSEMBLING THE DATA ANALYSIS QUEUE

1. Select the **Records** button from the DSP Control Center.
2. Select the folder containing your scans ([see Figure 5](#)).
3. Review the status of each scan of interest. Only scans that are **Readout complete** can be added to the queue.
4. Select each scan of interest by clicking the check box in the upper-left corner. This will turn the header green. You may also view one slide at a time.

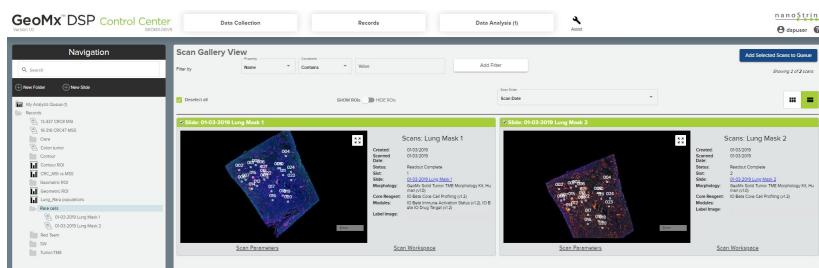


Figure 5: Scan Gallery View under Records; adding scans to Data Analysis queue

5. Click the **Add Selected Scans to Queue** button in the upper right corner of the window to queue the scan(s) together for an analysis. The number of scans currently in the Data Analysis Queue will appear in parentheses on the Data Analysis button; this number will change dynamically as scans are added to or removed from the queue.

Creating/Opening a Study

6. To view or edit the Queue, select the **My Analysis Queue** folder from the Navigation window (see [Figure 6](#)).

FILTERING

Use slide and scan metadata to filter scans. Select search parameters from the dropdowns in the upper left of the window.

- **Property:** Name, etc.
- **Constraint:** contains, equals, between, etc.
- **Value:** customizable field.

Select **Add Filter** to apply a filter rule. Applied filter rules will appear as gray boxes under the filter field. Use the **X** on each box to remove that filter, if desired.

Select **Clear Filters** to clear applied filter rules.

SELECT ALL, HIDE ROIs, & SCAN ORDER

- **Select all** scans using the check box. Alternatively, you may select one scan at a time using the check box in each scan's upper left corner.
- The **Scan Order** default is by scan date (from most to least recent); select another option from the drop-down (Scan Name or Slide Name), if desired.

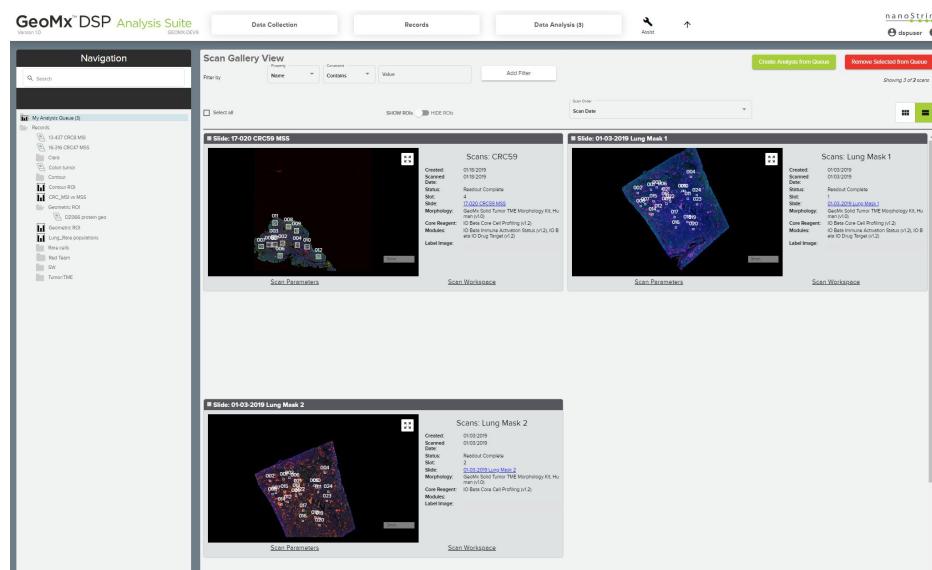


Figure 6: Scan Gallery View under Records; creating study from Data Analysis queue

2 Data Analysis Studies

CREATING A NEW STUDY

1. From the GeoMx DSP Control Center, hover over the **Data Analysis** button ([see Figure 7](#)).
2. **New Study from Queue** and **Open Study** buttons will appear. Select **New Study from Queue**. The number in parentheses on the Data Analysis button indicates the number of scans currently in the Queue. In the example here ([see Figure 7](#)), there are 10 scans presently in the queue.

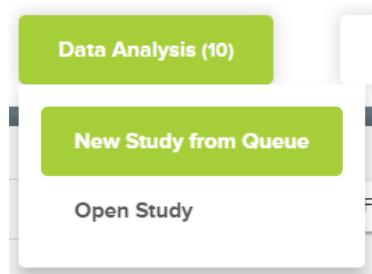


Figure 7: New Study from Queue button

You can also create a study by opening the **Data Analysis Queue** under **Navigation** and select **New Study from Queue**.

3. Enter a **Name**, **Description**, (optional) and the folder where you would like this study saved ([see Figure 8](#)).
4. Only the scans that you have added to your **Data Analysis Queue** (represented by the number in parentheses on the Data Analysis button) will be used to build your study.
5. Select **Create** to build an analysis from the scans in your Data Analysis Queue. Folder permissions can be set so that only defined users can view a study (see the **GeoMx DSP Instrument User Manual**).

Figure 8: Create New Study window

Creating/Opening a Study

OPENING A STUDY

1. From the GeoMx DSP Control Center, hover over the **Data Analysis** button ([see Figure 9](#)).
2. **New Study from Queue** and **Open Study** buttons will appear. Select **Open Study**.

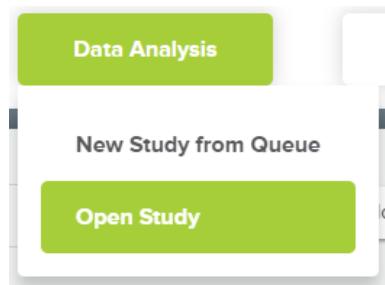


Figure 9: Open Study button

You can also find a previously-saved study in the **Navigation** window. Click on the **Records** button in the GeoMx DSP Control Center, locate your study in the Navigation window, and double click on it.

3. Browse previously created studies under the appropriate folder ([see Figure 10](#)). Note that a study can only be opened by one user at a time. A locked study indicates someone is working on it.
4. Select your study and select the **Open Study** button.

Once you have a study open or initiated, proceed to [Running QC](#).

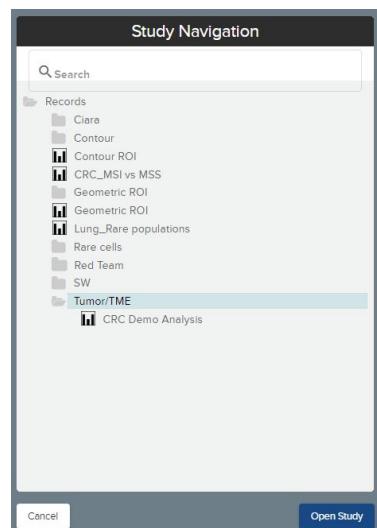


Figure 10: Open Study navigation

Creating/Opening a Study

Assess Data

A few tools are available in the Visualizations pane to assess your data initially and after Segment QC.

Line plot

The default live plot for NGS studies is the line plot ([see Figure 11](#)). This plots the **Raw reads**, **Trimmed reads**, **Stitched reads**, **Aligned reads**, and **Deduplicated reads** in \log_{10} space across all segments. For more information, see the **GeoMx-NGS Pipeline (DND) Software User Manual**.

- Examine this plot for differences between the line plots. Generally, the reads in all categories should be essentially the same. Deduplicated reads, however can be expected to plot lower than the rest; this indicates that many PCR duplicates were sequenced and that you have representative testing. Look for dips in the line plots, as this may indicate low reads and ineffective sequencing. In this example ([see Figure 11](#)), the dip on left may be due to small ROIs.
- Click the arrow to access the drop-down field. Choose **Linear**, **ratios**, or **Log₁₀** transformation. Select **tags** or **factors** to stratify by color.
- Click and drag to zoom in on any area of interest in the line plot.

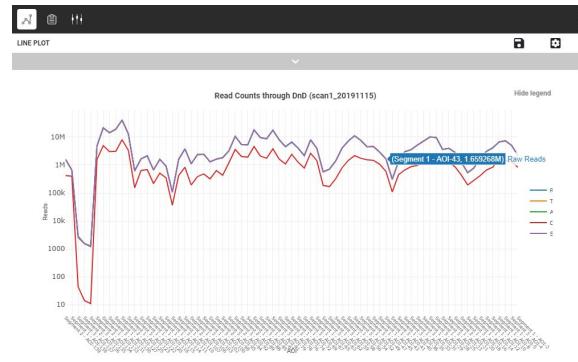


Figure 11: Line plot for NGS data

Study Summary

The study summary ([see Figure 11](#)) plots the total number of reads for each segment by each processing step outcome (trimmed, deduplicated, etc.). It also contains drop-down fields with **Segment Summary** ([see Figure 13](#)), **Probe Summary**, and **Target Summary**.

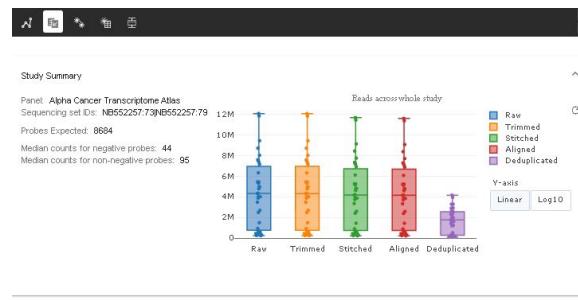


Figure 12: NGS Study Summary

Creating/Opening a Study

Segment summary includes Q30 values. Q30 refers to the percentage of bases that get a Q score of at least 30 (99.9% accuracy)

umiQ30 and rtsQ30 refer to the % of bases with Q>30 specifically in the UMI and RTS_ID portions of the reads, respectively.

Site name	Scan name	RCE name	Segment name	Tags
HTT3112.9	HTT3112.9	001	KMW_31-C01	Low Negative No
HTT3112.9	HTT3112.9	001	KMW_31-C02	Low Negative No
HTT3112.9	HTT3112.9	001	KMW_31-C03	Low Negative No
HTT3112.9	HTT3112.9	002	KMW_31-C04	Low Negative No
HTT3112.9	HTT3112.9	002	KMW_31-C05	Low Negative No
HTT3112.9	HTT3112.9	003	KMW_31-C06	Low Negative No
HTT3112.9	HTT3112.9	003	KMW_31-C07	Low Negative No
HTT3112.9	HTT3112.9	003	KMW_31-C08	Low Negative No
HTT3112.9	HTT3112.9	003	KMW_31-C09	Low Negative No
HTT3112.9	HTT3112.9	004	KMW_31-C10	Low Negative No
HTT3112.9	HTT3112.9	004	KMW_31-C11	Low Negative No
HTT3112.9	HTT3112.9	005	KMW_31-C12	Low Negative No
HTT3112.9	HTT3112.9	005	KMW_31-C13	Low Negative No
HTT3112.9	HTT3112.9	006	KMW_31-C14	Low Negative No
HTT3112.9	HTT3112.9	006	KMW_31-C15	Low Negative No
HTT3112.9	HTT3112.9	007	KMW_31-C16	Low Surface Area

Figure 13: Segment Summary

Pathway Map

The pathway map shows the coverage of pathways for entire dataset.

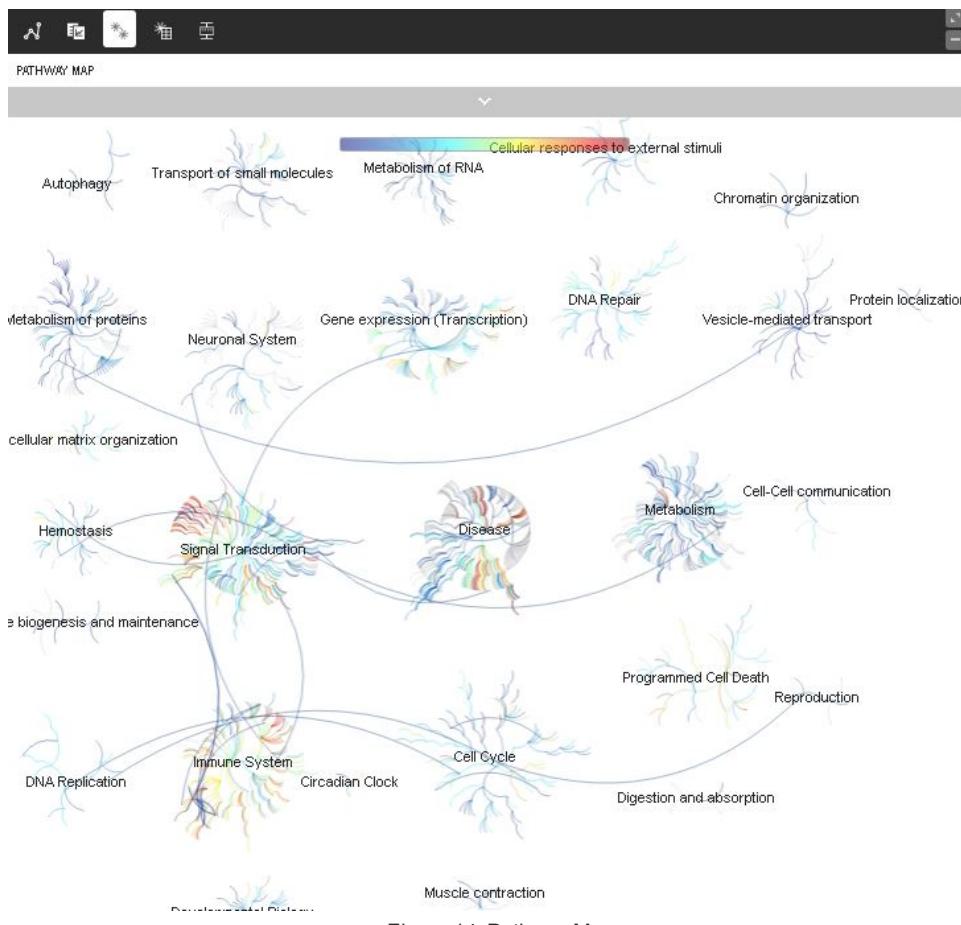


Figure 14: Pathway Map

Click the arrow to access the drop-down field. Here, you can change the color scheme of the Pathway Map.

This plot is most informative later in the analysis; return to this after running QC and filtering your data.

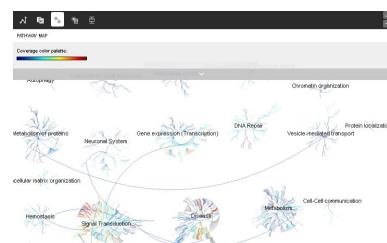
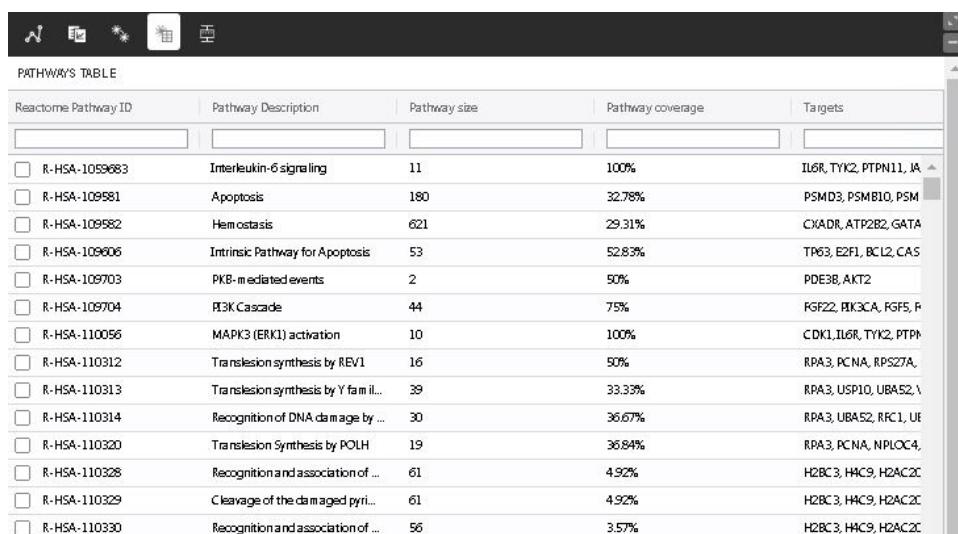


Figure 15: Pathway Map drop-down

Pathway Table

The Pathway Table summarizes the Pathway Map coverage. This value is calculated by dividing the **number of genes present in the dataset** by the **number of genes in present in the pathway**. The genes in the dataset are determined by the panel used for testing and the current selection in your dataset. The pathway information is obtained from reactome.org.



Reactome Pathway ID	Pathway Description	Pathway size	Pathway coverage	Targets
R-HSA-1059683	Interleukin-6 signalling	11	100%	IL6R, TYK2, PTPN11, JAK1, PSMD3, PSMB10, PSM
R-HSA-109581	Apoptosis	180	32.78%	CXADR, ATP2B2, GATA
R-HSA-109582	Hemostasis	621	29.31%	TP63, EZF1, BCL2, CAS
R-HSA-109606	Intrinsic Pathway for Apoptosis	53	52.83%	PDE3B, AKT2
R-HSA-109703	PKB-mediated events	2	50%	FGF22, PIK3CA, FGF5, PIK3CB
R-HSA-109704	R3K Cascade	44	75%	CDK1, IL6R, TYK2, PTPN11, RPA3, PCNA, RPS27A, RPA3, USP10, UBA52, H2BC3, HAC9, H2AC2C
R-HSA-110056	MAPK3 (ERK1) activation	10	100%	RPA3, PCNA, RPS27A, RPA3, UBA52, RFC1, U6, RPA3, PCNA, NPLOC4, H2BC3, HAC9, H2AC2C
R-HSA-110312	Translesion synthesis by REV1	16	50%	RPA3, PCNA, RPS27A, RPA3, UBA52, RFC1, U6, RPA3, PCNA, NPLOC4, H2BC3, HAC9, H2AC2C
R-HSA-110313	Translesion synthesis by Y family ...	39	33.33%	RPA3, PCNA, RPS27A, RPA3, UBA52, RFC1, U6, RPA3, PCNA, NPLOC4, H2BC3, HAC9, H2AC2C
R-HSA-110314	Recognition of DNA damage by ...	30	36.67%	RPA3, UBA52, RFC1, U6, RPA3, PCNA, NPLOC4, H2BC3, HAC9, H2AC2C
R-HSA-110320	Translesion Synthesis by POLH	19	36.84%	RPA3, PCNA, NPLOC4, H2BC3, HAC9, H2AC2C
R-HSA-110328	Recognition and association of ...	61	4.92%	RPA3, PCNA, NPLOC4, H2BC3, HAC9, H2AC2C
R-HSA-110329	Cleavage of the damaged pyrimidines by PCBP2	61	4.92%	RPA3, PCNA, NPLOC4, H2BC3, HAC9, H2AC2C
R-HSA-110330	Recognition and association of ...	56	3.57%	RPA3, PCNA, NPLOC4, H2BC3, HAC9, H2AC2C

Figure 16: Pathway Table

3 Running QC

The toolbar contains many options to adjust your data.

You must run **QC** first. After this, the other task buttons will become activated. QC allows you to specify parameters for evaluating the quality of your NGS data and to qualify segments by number of cells or by area. There are two steps in the QC process: **Segment QC** and **Biological probe QC**. Segments outside of desirable quality criteria are tagged with QC failure tags. Find more details on QC calculations in [Algorithm Details on page 100](#).

SEGMENT QC

Once you have imported data, the **QC** button will be active. In the Quality Control (QC) process, some basic metrics collected from the run are compared to an expected range of values to determine the overall quality of the data.

Select the **QC** button. Review the QC parameters and default values selected for your study ([see Figure 17](#)). You may change the selections and default values as appropriate based on the parameters of your study. After running QC, you are able to flag segments not meeting these QC parameters.

After each task option (QC, Normalization, etc.), the resulting data will be saved as a new dataset and appear in the dataset list (see **Datasets**). Enter a unique name for this dataset, a description (optional), and any tags (optional).

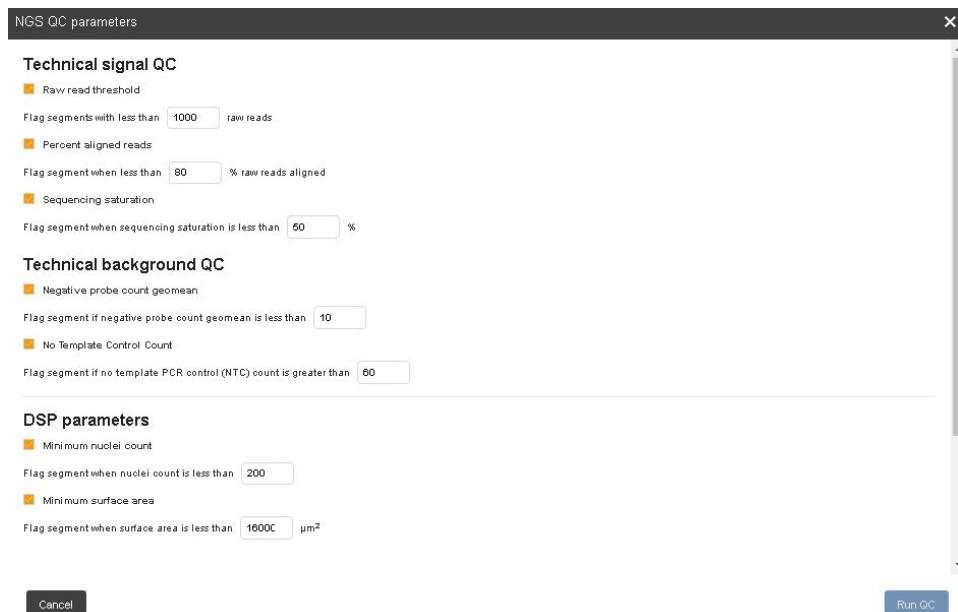


Figure 17: QC parameters window

Creating/Opening a Study

TECHNICAL SIGNAL QC

Technical signal QC assesses the quality of sequencing for each segment.

The **raw read threshold** is the lowest number of raw reads allowed.

The **percent aligned reads** establishes the minimum percent of raw reads aligned to target sequences allowed.

Sequencing saturation sets the minimum percent saturation allowed. The percent saturation is calculated as: $100\% - (\text{deduplicated reads}/\text{aligned reads}) \cdot 100\%$. 100% sequencing saturation indicates a representative sample, while 0% sequencing saturation indicates that all reads were unique. Values below 50% may need to be resequenced.

TECHNICAL BACKGROUND QC

The background QC is a GeoMx DSP run control.

The **Negative probe count geomean** establishes the level of technical noise expected and flags segments with signal below that level.

The **No Template Control Count** establishes the level at which counts in the NTC will be flagged. The No Template Control (NTC) is used to detect contamination in the library prep.

DSP PARAMETERS

Minimum Nuclei and Surface Area Count: The GeoMx chemistry is designed for an optimal range of nuclei and surface areas. The default value of each of these QC parameters will automatically fill with the minimum recommended value for the GeoMx product used in the present run. Note, however, that not all slides will have nuclei count, particularly if no DNA channel was selected. These may be safely deselected if ranges or consideration are not appropriate for the parameters of your study.

Select the **Run QC** button.

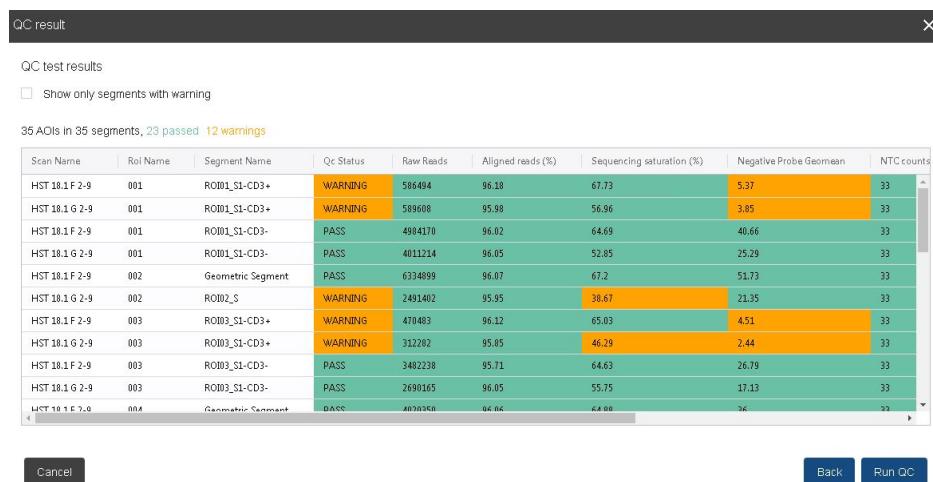
VIEWING SEGMENT QC RESULTS

When you click **Run QC**, a pop-up window will appear with preliminary QC results ([see Figure 18](#)). Scroll down the list.

Any samples not meeting the set QC criteria will appear in orange with a **WARNING** status. Check the **Show only segments with warning** box at the upper left to see this complete list.

Click the **Back** button to adjust QC parameters, if appropriate, or the **Run QC** button to accept these results. Wells designated with **Warning** will not be automatically removed from analysis; you must specifically deselect the well in the Scans pane prior to subsequent analyses.

Any QC parameter marked with a warning will materialize as a new tag. You can select or deselect segments associated with this tag from the search bar in the Scans pane. See [Tags on page 54](#).



Scan Name	ROI Name	Segment Name	QC Status	Raw Reads	Aligned reads (%)	Sequencing saturation (%)	Negative Probe Geomean	NTC counts
HST 18.1 F 2-9	001	ROI01_S1-CD3+	WARNING	586494	96.18	67.73	5.37	33
HST 18.1 G 2-9	001	ROI01_S1-CD3+	WARNING	599808	95.98	56.96	3.85	33
HST 18.1 F 2-9	001	ROI01_S1-CD3-	PASS	4984170	96.02	64.69	40.66	33
HST 18.1 G 2-9	001	ROI01_S1-CD3-	PASS	4011214	96.05	52.85	25.29	33
HST 18.1 F 2-9	002	Geometric Segment	PASS	6334899	96.07	67.2	51.73	33
HST 18.1 G 2-9	002	ROI02_S	WARNING	2491402	95.95	38.67	21.35	33
HST 18.1 F 2-9	003	ROI03_S1-CD3+	WARNING	470483	96.12	65.03	4.51	33
HST 18.1 G 2-9	003	ROI03_S1-CD3+	WARNING	312282	95.85	46.29	2.44	33
HST 18.1 F 2-9	003	ROI03_S1-CD3-	PASS	3482238	95.71	64.63	26.79	33
HST 18.1 G 2-9	003	ROI03_S1-CD3-	PASS	2690165	96.05	55.75	17.13	33
HCT 10.1 E 7-0	004	Geometric Segment	PASS	4070358	96.06	64.00	26	33

Figure 18: QC test results window

From the **Visualizations pane**, select the **Line plot**, **Summary**, or **Violin plot** buttons to assess segment QC (see [Assess Data on page 15](#)).

Creating/Opening a Study**BIOLOGICAL PROBE QC**

Once you have run QC on your segments, the **Biological probe QC** button will become active. Here, you determine the thresholds for excluding probes that appear to be outliers in the data.



Figure 19: Biological Probe QC window

Select the **Biological probe QC** button. Review the QC parameters and default values selected for your study ([see Figure 20](#)).



IMPORTANT: Checking the first box in this window will remove all segments that had a **warning** in the first QC step. The remaining parameters outlined here determine when to exclude outliers from the data. You may change the selections and default values as appropriate based on the parameters of your study.

GEOMEAN PROBE IN ALL SEGMENTS / GEOMEAN PROBES WITHIN TARGET

This is a filter for catching dropout probes (low outliers). It excludes probes for which the average counts across all segments are $\leq 10\%$ (or the value you enter) of the counts for all probes to that gene. This test catches probes that didn't actually make it into the final probe pool (which should be consistent across experiments) and removes probes that perform poorly relative to other probes to the same target.

Example: if four of five probes to GeneA yield 100 counts per segment on average and the fifth probe yields <10 counts on average, then the fifth probe will be called a global outlier (removed from ALL segments).

FAILS GRUBBS OUTLIER TEST

This is a filter for catching probes that are consistent outliers (always higher or lower than the other probes to the same gene). A Grubbs outlier test is performed on the probe counts to a

Creating/Opening a Study

given target in a given segment (5 values if there are 5 probes to the target). This is done for every target in every segment. If a particular probe is called an outlier in $\geq 20\%$ of segments, then it will be called a global outlier (removed from ALL segments). This test removes probes that are outliers in a user-defined proportion of segments from the entire dataset.

IF PROBE FAILS GRUBBS OUTLIER TEST IN A SEGMENT, EXCLUDE FROM TARGET COUNT CALCULATION IN THAT SEGMENT

This is a toggle that determines whether or not local outliers are removed from the data. A probe is a local outlier in a segment if it is called an outlier in that segment. If this box is checked, probes that are outliers in a given segment will be removed from that segment.

Note that this box is checked by default: **If probe fails Grubbs outlier test in a segment, exclude from target count calculation in that segment.**

Example: for all the Grubbs tests performed above, the outlier values will be removed from individual segments if this box is checked. If the box is not checked, the Grubbs tests are still performed to identify global outliers, but only global outlier probes are removed from the dataset.

CALCULATE LOQ

The limit of quantitation (LOQ) is set to be the negative probe geomean + some number of standard deviations of the negative probes. This is a confidence threshold rather than a detection threshold. A value below LOQ does not necessarily mean that a target is not expressed, but if the value is $>\text{LOQ}$ then we are confident that it is expressed. For CTA we recommend 2.5 as a stringent threshold and 2.0 for a slightly permissive threshold. LOQ is a threshold for high confidence detection.

It is only recommended to run the global outlier test on datasets with 24 or more ROIs/segments.

Click the **Run QC** button.

Creating/Opening a Study

VIEWING BIOLOGICAL QC RESULTS

When you click **Run QC**, a pop-up window will appear with preliminary biological probe QC results. Scroll down the list.

Any samples not meeting the set Biological QC criteria will appear in orange with a **WARNING** status. Check the **Show failed only** box at the upper left to see this complete list.

Click the **Back** button to adjust the Biological QC parameters, if appropriate, or the **Apply** button to accept these results. Wells designated with a **Warning** status will not be automatically removed from analysis; you must specifically deselect the well in the Scans pane prior to subsequent analyses.

This QC step distinguishes local outliers from global outliers. A **local outlier** is one appearing as an outlier in a few segments whereas a **global outlier** appears as an outlier in all segments.

Any QC parameter marked with a warning will materialize as a new tag. You can select or deselect segments associated with this tag from the search bar in the Scans pane. See [Tags on page 54](#).

QC result

QC tests results

Show failed only

8684 probes, 7878 passed, 732 local outliers, 74 global outliers

Target Name	Probe Name	Total Counts	Median Counts	Probe geometric / Probe set geometric mean	Outlier Status
PECAM1	PECAM1_01	12731	346	1.14	Passed
PECAM1	PECAM1_02	11011	246	0.94	Passed
PECAM1	PECAM1_04	7996	221	0.72	Passed
CREB3L4	CREB3L4_04	1839	61	0.98	Failed grubbs test in individual AOI, AOI proportion is: 0.04
CREB3L4	CREB3L4_03	1394	49	0.76	Failed grubbs test in individual AOI, AOI proportion is: 0.09
CREB3L4	CREB3L4_05	1815	61	0.97	Failed grubbs test in individual AOI, AOI proportion is: 0.04
CREB3L4	CREB3L4_02	2190	83	1.19	Passed
PRKCD	PRKCD_04	6257	162	1.08	Passed
PRKCD	PRKCD_05	6997	202	1.23	Passed
PRKCD	PRKCD_02	4574	145	0.8	Passed
PRKCD	PRKCD_01	5336	142	0.9	Passed

Cancel Back Apply

Figure 20: Biological QC results

Manage Annotations

4 Manage Annotations

The GeoMx DSP system incorporates a number of ways to group and filter data. For information on tagging in visualizations, see [Tags on page 54](#). If you do not have any annotations to create or import, proceed to [Toolbar Options on page 28](#).

MANAGE ANNOTATIONS

Segment annotations can be imported from a file (uploaded from a spreadsheet).

1. Click the **Manage Annotations** button in the Scans pane ([see Figure 21](#)).

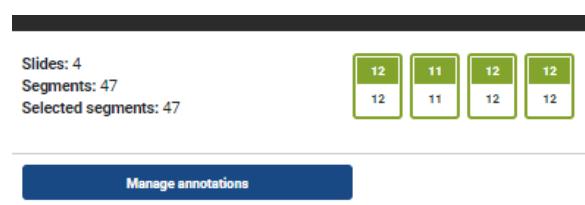


Figure 21: Manage Annotations window

2. Download the **Annotations template** file ([see Figure 22](#)).

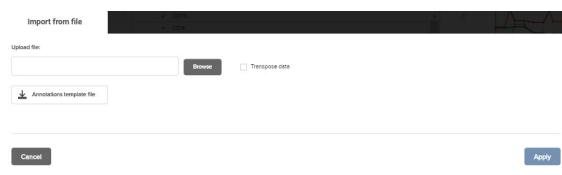


Figure 22: Manage Annotations window

3. Open the downloaded spreadsheet and review the pre-filled **Scan name**, **ROI (label)**, and **Segment (Name/Label)** columns. If you have any existing tags, they will be listed in the **Segment Tags** column. You may:

- Modify the annotations pre-loaded in the **Segment Tags** column and/or
- Create a new column(s) (choose any name for the header - this will become the first part of the tag) and add your tag names here. Tags in these columns can be imported as tags or as factors.

4. Delete any instruction rows at the top of the spreadsheet.

5. Save this spreadsheet as an **.xlsx** file on your computer and return to the **Manage Annotations** window.

	Scan name	ROI (label)	Segment (Name/Label)	Segment tags	Scan_ID	ROI_ID	Tags
5	D2074 85C	1	roi-001-segment-001		D2074 85C	1	
6	D2074 85C	2	roi-002-segment-001		D2074 85C	2	
7	D2074 85C	3	roi-003-segment-001		D2074 85C	3	
8	D2074 85C	4	roi-004-segment-001		D2074 85C	4	
9	D2074 85C	5	roi-005-segment-001		D2074 85C	5	
10	D2074 85C	6	roi-006-segment-001		D2074 85C	6	
11	D2074 85C	7	roi-007-segment-001		D2074 85C	7	
12	D2074 85C	8	roi-008-segment-001		D2074 85C	8	
13	D2074 85C	9	roi-009-segment-001		D2074 85C	9	
14	D2074 85C	10	roi-010-segment-001		D2074 85C	10	
15	D2074 85C	11	roi-011-segment-001		D2074 85C	11	
16	D2074 85C	12	roi-012-segment-001		D2074 85C	12	
17	roi-013-segment-001				roi-013 85C	13	

Figure 23: Adding a tags column

6. From the **Import from file** tab, **Browse** to your saved spreadsheet.

Manage Annotations

7. Choose the **Update type** from the drop-down:

- **Append** (default) adds new tags on to any existing tags in the dataset.
- **Merge** adds new tags to any untagged segments and replaces the existing tags of any tagged segments. Use this option for modifying existing tags with identical spelling but different type case (i.e., *tumor* to *Tumor*).
- **Replace** deletes any existing tags and replaces them with the new tags.

If importing factors only without modifying tags, check the **skip modifying tags** box.

8. Use the drop-downs to designate the columns that contain each piece of information requested and watch the grid populate with your selections ([see Figure 24](#)).

For **factors**, choose from **text**, **boolean** (yes/no), or **number** (numeric value) to describe the type of levels within the factor. Factors are tags that have multiple sub-categories which collectively span all segments in a dataset. For example, the factor *Tissue Type* would have the sub-categories *tumor*, *stroma*, and *invasive margin*.

Upload file:

[Annotations template file](#)

Update type for tags:

Update type for factors:

Set columns:

Scan:	Scan name
Segment:	Segment (Name/ Label)
ROI:	ROI (label)
Tags:	Segment tags

Figure 24: Importing annotations from file

9. Review the grid at the bottom of the window. Ensure that the desired columns of tags to be imported are selected; deselect any columns whose tags you do not want to be imported.
10. Select **Apply**.

Manage Annotations

Once you have selected the slides and segments for your study, consider which of the toolbar options to use in your study (see [Toolbar Options on page 28](#)).

Toolbar Options

5 Toolbar Options



The toolbar contains many options to adjust your data.

You must run **QC** first (see [Running QC](#)). After this, the other toolbar buttons will become activated. **Scaling**, **Background Correction**, and **Ratio Builder** are designed to be run once each (after they have been run, they will be inactive).

Scaling

In the Scaling window ([see Figure 25](#)), you can adjust counts to area or to the number of cells. Counts can be scaled to the geometric mean, median, or mean of the detected area or number of nuclei. Choose the desired **Average type** from the drop-down.

Find more details on scaling calculations in [Algorithm Details on page 100](#).

After each task option (QC, Normalization, etc.), the resulting data will be saved as a new dataset and appear in the dataset list (see **Datasets**). Enter a unique name for this dataset, a description (optional), and any tags (optional).

Figure 25: Scaling parameters window

Take care to avoid scaling to area or nuclei after normalizing to housekeepers or IgGs. Doing this effectively scales your data to area/nuclei twice.

Toolbar Options

SCALE TO AREA

This is a type of normalization correcting for variable surface areas. The Data Analysis software calculates the ratio of the geometric mean, median, or mean surface area to the measured surface area of each segment. This ratio is then used to adjust the counts detected in that segment.

SCALE TO NUCLEI

This is a type of normalization correcting for variable cell numbers, and may be preferable when studying expression per cell. The Data Analysis software calculates the ratio of the geometric mean, median, or mean number of nuclei to the measured number of nuclei of each segment. This ratio is then used to adjust the counts detected in that segment.

Select the **Run Scaling** button.

Toolbar Options

VIEWING SCALING RESULTS

From the **Visualizations** pane, select the **Summary** button and the **Scaling** tab.

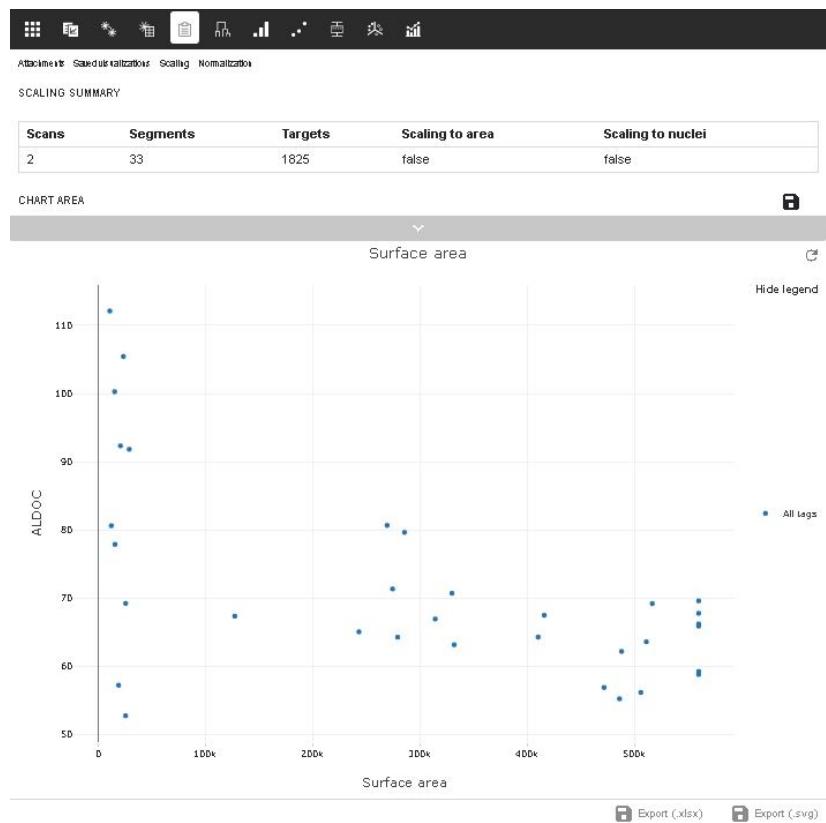


Figure 26: Scaling results plot

- The **Scaling Summary** section features a summary of the scans, segments, and probes that were included in the scaling, as well as any of the selections made.
- The **Chart area** ([see Figure 26](#)) provides visualizations plotting the scaling factors against adjusted counts for the selected probe; change this probe using the **Select probe** field.
- Click the arrow to access the drop down field.
 - Select **Area scaling factor**, **Surface area**, **Nuclei scaling factor**, or **Nuclei counts** to see the respective chart. The **Summary Table** lists the scaling factors for each segment. Ensure the scaling factors are all in a reasonable range.
 - Select the probe of interest.
 - **Select tags or factors** to filter and color the results based on tag.

Toolbar Options

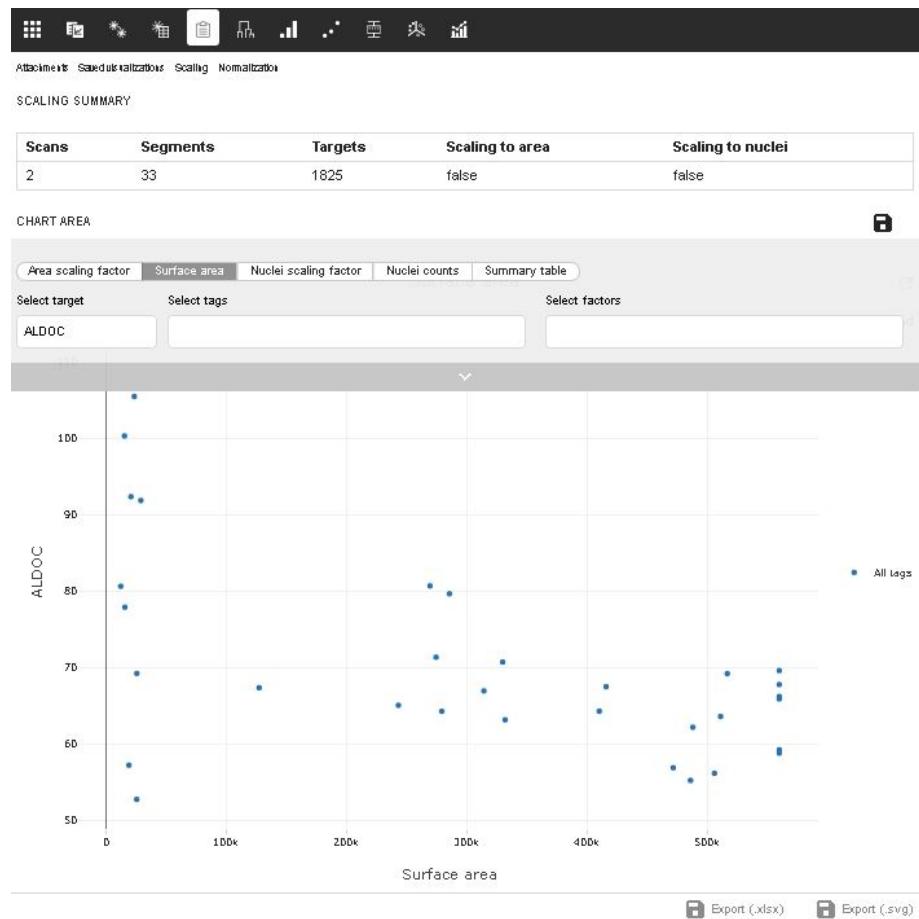


Figure 27: Scaling summary drop down menu

Toolbar Options

Normalization

Normalization is a data transformation that balances the results between segments within an analysis using the counts from a specific set of probes.

Find more details on normalization calculations in [Algorithm Details on page 100](#).



Figure 28: Normalization parameters window

A line plot displaying the expression of normalization probes will appear at the bottom of the Normalization window ([see Figure 28](#)). A set of good normalization genes can be expected to have correlated expression. You may choose to customize normalization using the following fields:

NORMALIZATION TYPE

Q3 (3rd quartile of all selected targets) is the recommended normalization method for NGS data for all targets that are above the limit of quantitation (LOQ). Q3 normalization uses the top 25% of expressers to normalize across ROIs/segments, so it is robust to changes in expression of individual genes and ideal for making comparisons across ROIs/segments.

To use this method, select **Normalization Type: Target Groups**, **All Targets**, then select **Average Type: Q3**.

After each task option (QC, Normalization, etc.), the resulting data will be saved as a new dataset and appear in the dataset list (see **Datasets**). Enter a unique name for this dataset, a description (optional), and any tags (optional).

Toolbar Options

HOUSEKEEPING

This option uses a CodeSet Content Normalization factor (also called Reference or Housekeeping Normalization factor), which is calculated using reference target endogenous to the biological sample to adjust for differences in analyte abundance and/or analyte quality across samples. This method assumes that a reference target is consistently expressed across all samples. If the assumptions are met this normalization can account for differences in the amount of cells analyzed.

Predefined target genes classified as controls will be automatically selected. Use the check boxes to make desired alterations. The default range of acceptable values for this is 0.1 - 10.

PROBESET

Probeset normalization allows you to normalize using the probe group of your choice. Use the check boxes in the list to select a probe group. This normalization has no set defaults.

CUSTOM

Custom normalization allows you to choose the probes to be applied as reference genes when predefined genes either don't exist or are not appropriate for the dataset. Ensure that the segments in your analysis are "like" segments. Use the check boxes to select probes for custom normalization.

NEGATIVE

Probes classified as Negative controls will be auto-selected. Negative normalization scales counts in a segment based on how the negative probe counts in that segment compare to those in other segments. If a segment has higher negative probes than average, its target counts will be reduced, and vice versa. This method is similar to calculating a signal-to-noise ratio, except counts are multiplied by a normalization factor rather than being divided by the negative probe geomean.

AVERAGE TYPE

Choose from Geometric mean, Median, Average, Sum, Q3, Minimum and Maximum. The drop-down menu defaults to use the geometric mean for calculations, since it weighs the low-concentration controls equally with the high-concentration controls. Q3 is the recommended option for NGS data for all targets that are above the limit of detection (LOQ).

Toolbar Options

Select the **Normalize** button.

Toolbar Options

VIEWING NORMALIZATION RESULTS

From the **Visualizations pane**, select the **Summary** button and the **Normalization** tab.

If scaling (area or nuclei) or background correction was performed, you may want to evaluate the visualization depicting normalization factor vs. scaling factor or normalization factor vs. background factor. High values for normalization factor, scaling factor, or mean background should be carefully evaluated.

- The **Normalization Summary** section features a summary of the scans, segments, and probes that were included in the normalization, as well as any of the selections made.
- The **Chart area** features visualizations which compare the normalization trend to the scaling or background adjustment trends (if those tasks have been run).
- Click the arrow to access the drop down field.
 - Select **Scaling** or **Background** from the slider to see the respective chart.
 - **Select tags or factors** to filter and color the results based on tag.

Toolbar Options

Background Correction

Background correction allows you to adjust for this non-target-specific adherence of antibody tags to the tissue. Understand that isotope protein controls act as surrogate markers for nonspecific signal but that every antibody will have a slightly different background.

In the Background Correction window ([see Figure 29](#)), you can customize background adjustment. Background is inherent non-target-specific antibody binding to the tissue. Calculations can be performed by the DSP Data Analysis software to minimize the impact of the background on the data counts arising from actual variants. Choose probes that have no ligand in your datasets' segments (for protein sets, this may be IgG controls, and for RNA sets, this may be all negative probes).

After each task option (QC, Normalization, etc.), the resulting data will be saved as a new dataset and appear in the dataset list (see **Datasets**).

Enter a unique name for this dataset, a description (optional), and any tags (optional).

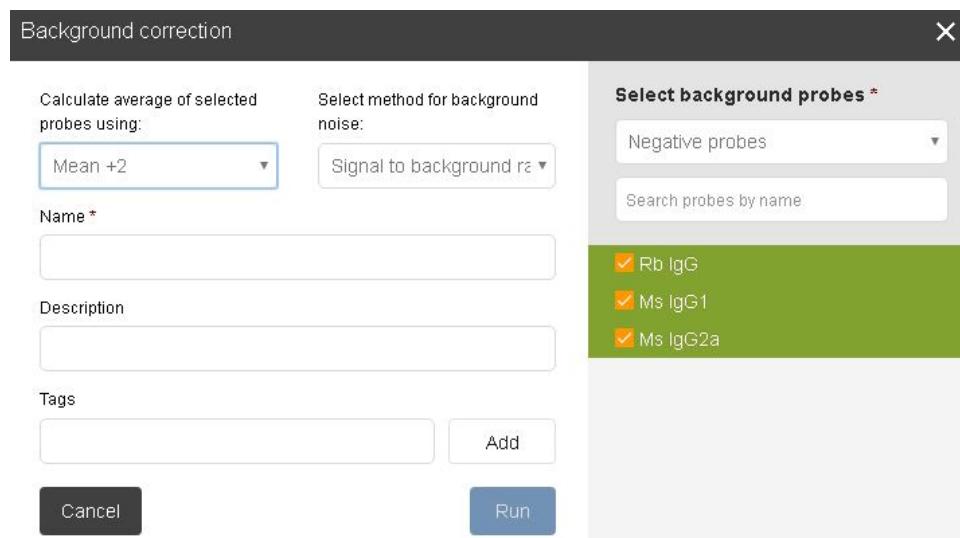


Figure 29: Background correction parameters window

AVERAGE METHODS

Choose from Maximum, Median, Mean, or Mean+n (n represents number of standard deviations).

Toolbar Options

BACKGROUND METHOD

Choose one of the following background correction methods.

SIGNAL TO BACKGROUND RATIO

For every probe in your segment, the Data Analysis software will calculate a ratio of the probe count to the average represented background, using the averaging method you selected and the counts from the probes you designated as negative.

THRESHOLDING

Choose the negative controls from the probes field for a negative control threshold. The system will take the average (using the method specified in the **Average method** field) of the negative control counts to determine the background level. Once the background level has been determined, all raw counts at or below this value will be floored to it.

SUBTRACTION

Though not recommended for most applications, Background Subtraction is available as an option. Choose the negative controls from the probes field for a negative control subtraction. The system will take the average (using the method specified in the **Average method** field) of the negative control counts to determine the background level. Once the background level has been determined, it will be subtracted from each of the raw counts.

BACKGROUND PROBES

Select groups of background probes (using **negative probes**, **probe sets**, or **tags**) using the drop-down menu and search bar. You can also select **manually** and select probes from the list.

Select the **Background correction** button.

Toolbar Options

VIEWING BACKGROUND RESULTS

From the **Visualizations** pane, select the **Summary** button and the **Background** tab.

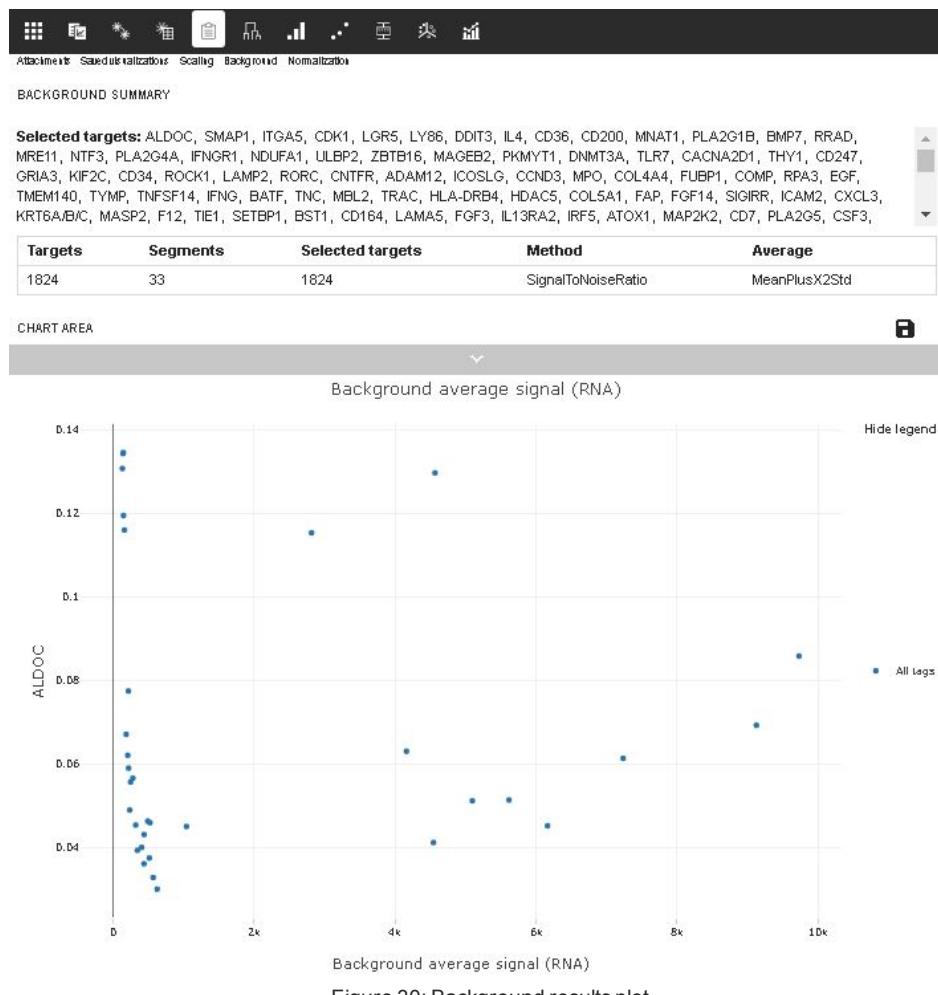


Figure 30: Background results plot

- The **Background Summary** section features a summary of the scans, segments, and probes that were included in the background correction, as well as any of the selections made.
- The **Chart area** ([see Figure 30](#)) provides visualizations plotting the background average signal against adjusted counts for the selected probe; change this probe using the **Select probe** field.
 - Click the arrow to access the drop down field.
 - Select **Protein** or **RNA** from the slider to see the respective chart.
 - Select tags or factors** to filter and color the results based on tag.

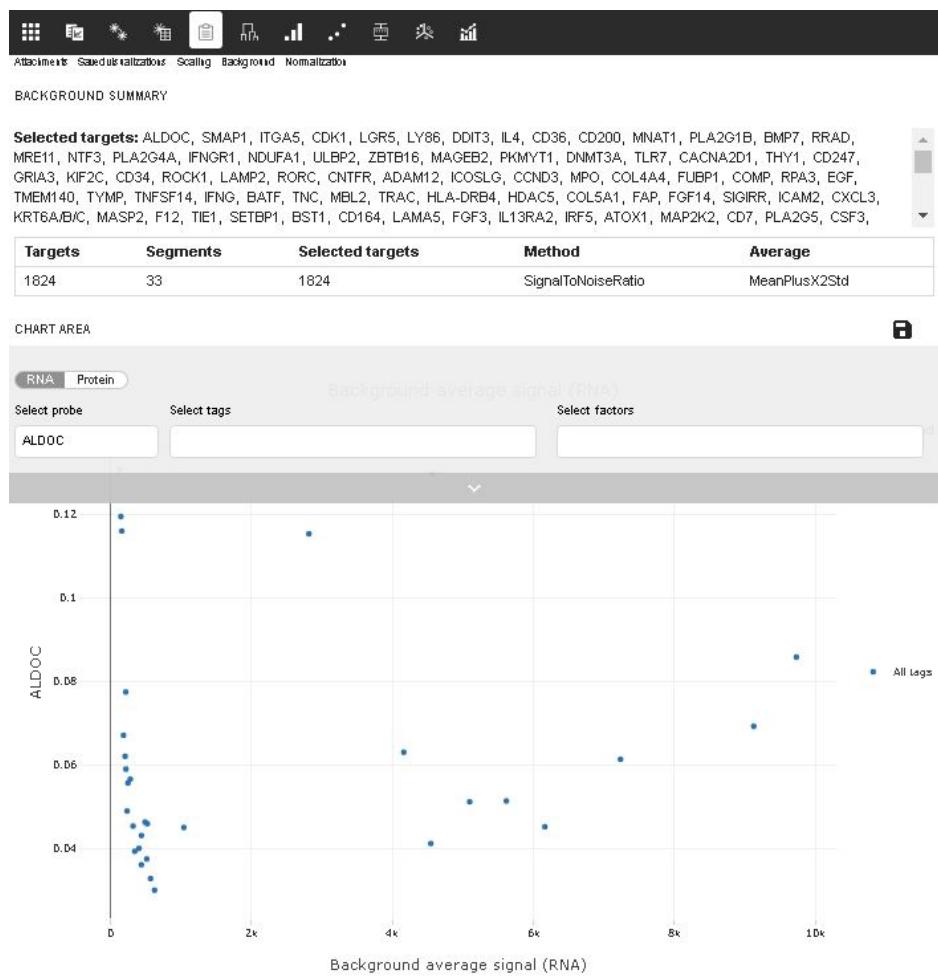
Toolbar Options

Figure 31: Background summary drop down field

Toolbar Options

Ratio Builder

Ratio Builder allows you to create ratios and estimate the relative expression of target probes. This can be used to compare expression of targets in a segment to the average of a group of segments delineated by tags, compare expression of target between segment pairs from within an ROI, or estimate relative expression of a target probe within a segment.

Ratios can be built only for the segments and probes that are actively selected in the Scans pane. Before opening Ratio Builder, select the segments you would like to include in the numerator of your ratio.

Choose one of the three tab options to build ratios ([see Figure 32](#)):



Figure 32: Ratio Builder tabs

- The **Ratio to a segment/group of segments** option calculates the ratio of each probe in each segment to the average of one or more segments selected as the baseline (denominator).
- The **Binary ratios** option calculates the relative expression of selected targets between a pair of segments belonging to the same ROI. For example, a binary ratio could compare a particular target probe's abundance between a tumor segment and a tumor microenvironment (TME) segment in a common ROI.
- The **Probe ratios** option calculates the relative expression of a probe pair within each segment. For example, one could use the probe ratio function to create a dataset comparing phospho-AKT counts to AKT counts for all selected segments in the dataset.

Toolbar Options**RATIO TO A SEGMENT/GROUP OF SEGMENTS**

Ratio to a segment/group of segments	Binary ratios	Probe ratios	HEATMAP
Average type Geometric mean	Select segments to ratio with (denominator) * Filtered segments: 12 Selected segments: 12	Tags for segments AND <input checked="" type="radio"/> OR Tumor <input type="button" value="X"/>	Ratios to create All segments: 48 Selected segments: 48
Name *	Exclusion tags for segments	<input type="button" value="Select filtered"/> <input type="button" value="Deselect all"/>	<input checked="" type="checkbox"/> 20190123 11-CPA ShortCut 001 roi-001-segment-001 <input checked="" type="checkbox"/> 20190123 11-CPA ShortCut 002 roi-002-segment-001 <input checked="" type="checkbox"/> 20190123 11-CPA ShortCut 003 roi-003-segment-001 <input checked="" type="checkbox"/> 20190123 11-CPA ShortCut 004 roi-004-segment-001 <input checked="" type="checkbox"/> 20190123 11-CPA ShortCut 005 roi-005-segment-001 <input checked="" type="checkbox"/> 20190123 11-CPA ShortCut 006 roi-006-segment-001 <input checked="" type="checkbox"/> 20190123 11-CPA ShortCut 007 roi-007-segment-001 <input checked="" type="checkbox"/> 20190123 11-CPA ShortCut 008 roi-008-segment-001 <input checked="" type="checkbox"/> 20190123 11-CPA ShortCut 009 roi-009-segment-001 <input checked="" type="checkbox"/> 20190123 11-CPA ShortCut 010 roi-010-segment-001
Description	Tags	<input type="button" value="Add"/>	
Tags			
<input type="button" value="Cancel"/>	<input type="button" value="Run"/>		

Figure 33: Ratio to average parameters window

1. Before you open Ratio Builder, select the scans and segments you would like to include in the numerator in the Scans pane (see [Scans Pane on page 59](#)). To use tags to filter segments, see [Tags on page 54](#).

It is important to select the appropriate segments and probes from the dataset prior to specifying parameters for ratio building; it is also important to choose the appropriate tags in selecting denominators.

2. Open **Ratio Builder** and confirm you are on the **Ratio to a segment/group of segments** tab ([see Figure 33](#)).
3. Choose the **average type** you'd like to use: Geometric mean (default and recommended), Median, Average, Sum, Minimum or Maximum.
4. In the central field, select the segment(s) you would like to include in the **denominator** of your ratio. Use the following steps to find segments of interest:
 - Use the search bar here to filter segments by tags. Select a tag from this drop-down.
 - The number of filtered segments will be reflected at the top of the central field and the names of the filtered segments will be listed down the central field. Review the list.

Toolbar Options

- Click **Select filtered** to include all the segments in this list in the denominator or individually select the ones you'd like included.
 - All segments that are selected for the denominator will be averaged without further stratifying the data.
5. In the right-most field, all segments chosen for the numerator will be listed, indicating that a ratio for each of these segments will be created. Click off any that you do not wish to create.
 6. In the left-most field, enter a unique name for the resulting dataset, a description (optional), and any tags (optional).
 7. Click the **Run** button. You will be returned to the main Data Analysis screen; your new dataset will be selected and the live visualization will reflect this, as well.

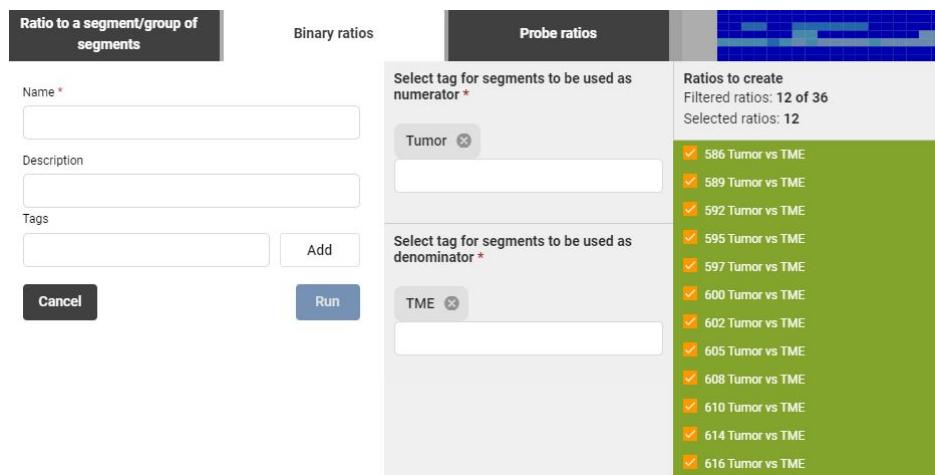
Toolbar Options**BINARY RATIOS**

Figure 34: Binary ratios parameters window

1. Before you open Ratio Builder, select the scans and segments you would like to include in the numerator in the Scans pane (see [Scans Pane on page 59](#)). To use tags to filter segments, see [Tags on page 54](#).

The binary ratios feature calculates the relative expression of a target between a pair of segments belonging to the same ROI. Ensure that the input data includes segments from a common ROI and that each segment type has a distinct tag.

2. Open **Ratio Builder** and click on the **Binary ratios** tab ([see Figure 34](#)).
3. In the central field, select the tag(s) you would like to choose for the **numerator**, then the **denominator** of your ratio. See note above. Use the search bars to select tags.
4. In the right-most field, review the ratios to be built. Click off any you do not wish to create.
5. In the left-most field, enter a unique name for the resulting dataset, a description (optional), and any tags (optional).
6. Click the **Run** button. You will be returned to the Data Analysis screen; your new dataset will be selected and the live visualization will reflect this, as well.

Toolbar Options**PROBE RATIOS**

Figure 35: Probe ratios parameters window

1. Before you open Ratio Builder, select the scans and segments you would like to include in the numerator in the Scans pane (see [Scans Pane on page 59](#)). To use tags to filter segments, see [Tags on page 54](#).

The Probe Ratios feature calculates the relative expression of a probe within a segment. Ensure you have selected probes from a common segment.

2. Open **Ratio Builder** and click on the **Probe ratios** tab ([see Figure 35](#)).
3. In the left-most field, click and drag probes from the list into the **numerator** and **denominator** boxes. Use the search bar to find probes, if necessary.
4. In the right-most field, review the ratios to be built.
5. In the left-most field, enter a unique name for the resulting dataset, a description (optional), and any tags (optional).
6. Click the **Run** button. You will be returned to the Data Analysis screen; your new dataset will be selected and the live visualization will reflect this, as well.

Once you run **Ratio Builder**, all task option buttons other than **Custom Scripts** and **Statistical Tests** will be inactive.

Toolbar Options

Statistical Tests

The statistical tests function can be used to define groups within a selected dataset and run hypotheses.

When up to two groups are established by selecting tag combinations, the following tests may be run:

- Unpaired T- test (useful for comparing two groups of independent samples)
- Paired T- test (useful for comparing two groups with a natural paired structure, e.g. pre- vs. post-treatment, or Tumor vs. Immune ROIs from the same collection of samples.)
- Mann-Whitney U-test (useful for data that is extremely skewed or heavy-tailed)
- Linear mixed models (useful for data with repeated measurements from each sampling unit, e.g., multiple ROIs from each sample)

After each task option (QC, Normalization, etc.), the resulting data will be saved as a new dataset and appear in the dataset list (see **Datasets**). Enter a unique name for this dataset, a description (optional), and any tags (optional).

When performing statistical analysis, if background subtraction has already been run, beware of statistical anomalies that can occur when values are thresholded to background or subtracted to 1.

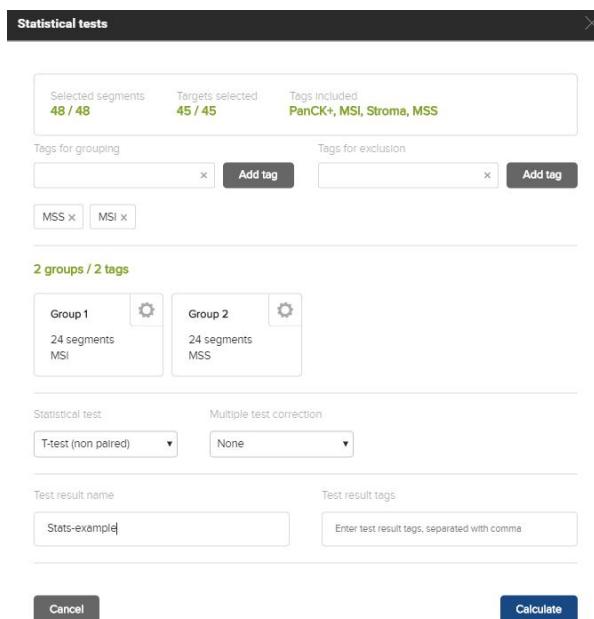


Figure 36: Statistical tests parameters window

Toolbar Options

1. In the box at the top of the Statistical tests window ([see Figure 36](#)), review the selected segments, targets, and tags to be included in this analysis. If these are not correct, exit this window and select or create another dataset.
2. Select the tag you'd like to use in your first group in the **Tags for grouping** field. Select **Add tag** to the right of this field.
3. Select tags you'd like to exclude from your groups in the **Tags for exclusion** field. Select **Add tag** to the right of this field.
4. Review the groups created. For each group, hover over the gear icon to review the segments included in that group; click off any you'd like to exclude from analysis. Select **Update** if you made any changes.
5. Select the Statistical Test desired.
 - **T-test (unpaired)**: The unpaired t-test should be selected when comparing the means of two groups from independent observations. For example, an unpaired t-test would be used to test whether the expression of a probe in the TME is significantly different between tissue specimens originating from tumors which responded to treatment vs. tissue specimens originating from tumors which did not respond to treatment. T-tests assume a normal distribution.

Under the null hypothesis, there is not a significant difference in the mean target probe expression between the two groups.

- **T-test (paired)**: The paired t-test should be selected to compare means between paired observations, such as between segments originating from the same ROI. For example, a paired t-test would be used in comparing the expression of a probe target in the TME before and after treatment for matched tissue samples. T-tests assume a normal distribution.

Under the null hypothesis, there is not a significant difference in the mean target probe expression between the two groups.

- **Mann-Whitney test**: The Mann-Whitney U-test is a non-parametric test of the null hypothesis. It does not require the assumption of normal distribution.
 - Under the null hypothesis, the distributions of both populations are equal.
 - Under the alternative hypothesis, the distributions of the populations are not equal; a randomly selected measurement from one group will be greater or smaller than the randomly selected observation from the second group. The test returns the probability that the median expression of a target probe in one group is greater than the other.

Toolbar Options

- **Linear Mixed Model:** Linear Mixed Models (LMMs) are designed to handle data with repeated measurements from the same sampling unit. For example, in a study where 6 ROIs are collected from each sample, the ROIs from any one sample will all be influenced by the idiosyncratic biology of that sample, sharing a tendency for higher expression of some genes/proteins and lower expression of others. LMMs explicitly model this tendency.

In contrast to LMM, the t-test is often dramatically wrong in data with repeated measurements. By treating multiple ROIs from the same sample as independent observations, the t-test inappropriately inflates the sample size of the study. For example, given 100 ROIs from one treated and one control sample, the t-test will act as if there are 100 independent samples from the treated and control groups, while a LMM will correctly acknowledge that there is just one independent sample from each group, with many repeated measurements.

6. Select the Multiple test correction desired. The method chosen will be used to adjust the p-values of individual genes. A gene's FDR is the proportion of significant genes that are expected to be false discoveries. FDR can be used as a more conservative and informative alternative to p-values.
 - **None:** this selection is an option to forgo calculating FDR.
 - **Permutation q-Value:** this method requires a large number of tests and may not be the most effective method for GeoMx DSP analysis.
 - **BH:** the Benjamini-Hochberg procedure is a standard method in controlling FDR and is the **recommended method to be used for DSP Protein analysis.** It assumes that the tests are positively correlated for a subset of genes/proteins, which usually holds for gene/protein expression data.
 - **BY:** the Benjamini-Yekutieli procedure is an adjustment to BH. It controls FDR in a more general setting, but poses a more strict threshold that leads to fewer significant calls. With the BY method, it's very unlikely to have any significant gene in DSP analysis.
7. Enter a unique Test result name and list the test result tags.
8. Select **Calculate.**

VIEWING STATISTICAL TEST RESULTS

The results from the statistical tests task can be accessed by clicking the arrow next to the dataset on which the test was run in the second pane. Select the test of interest from the drop-down list and the summary of results will appear in the 3rd Data Analysis pane.

Toolbar Options

Custom Scripts

Custom Scripts allows you to run custom R scripts on your data.

ADDING A CUSTOM SCRIPT

1. Select a dataset that has been through **QC**.
2. Select the **Custom Scripts** button from the **Toolbar Options**.
3. On the **Manage** tab, click the **Add** button ([see Figure 37](#)).
4. Enter a name, description (optional) and any tags associated (optional).
5. In the **Main Scripts** field, click the **+** button.
6. Browse for the desired R file, select it, and click **Open**.
7. Click the **Save** button. The script will appear in the **Main Script** box. You can manually make changes here
8. Click **Save** in the **Main Script** box to save any changes made, or **Revert** to go back to the originally uploaded version. This script will now be available to run.

RUNNING A CUSTOM SCRIPT

1. Select the **Custom Scripts** button from the **Toolbar Options**.

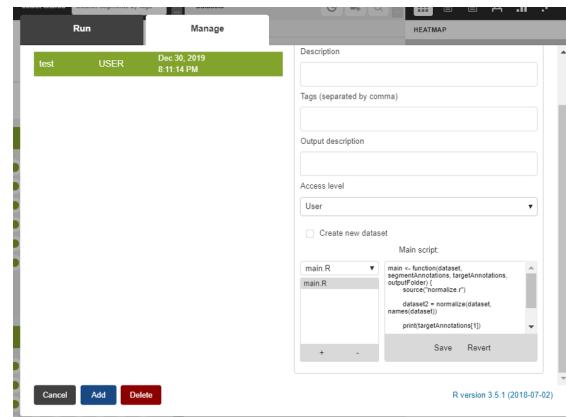


Figure 37: Adding a custom script

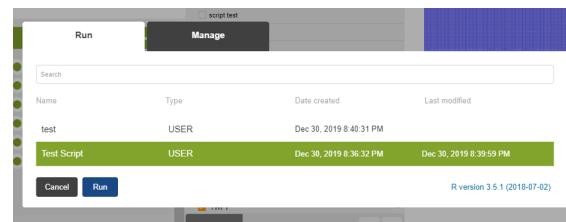


Figure 38: Running a custom script

2. On the **Run** tab, select a saved script from the list and select **Run** ([see Figure 38](#)).
3. Select the **Manage** tab to Add, Edit, or Delete a script ([see Figure 39](#)).
4. Check the **Create new dataset** box to save as a distinct dataset.

Toolbar Options

5. A **Successful!** message will appear, notifying you that the custom R script successfully executed. Select **OK**.
6. Navigate to the Visualizations pane of the Data Analysis Suite. Click on the **Dataset Summary** icon and select the **Attachments** tab. The Custom Scripts you just ran should appear.
7. Select the **Save** icon to download this visualization or the **Trash** icon to discard it.

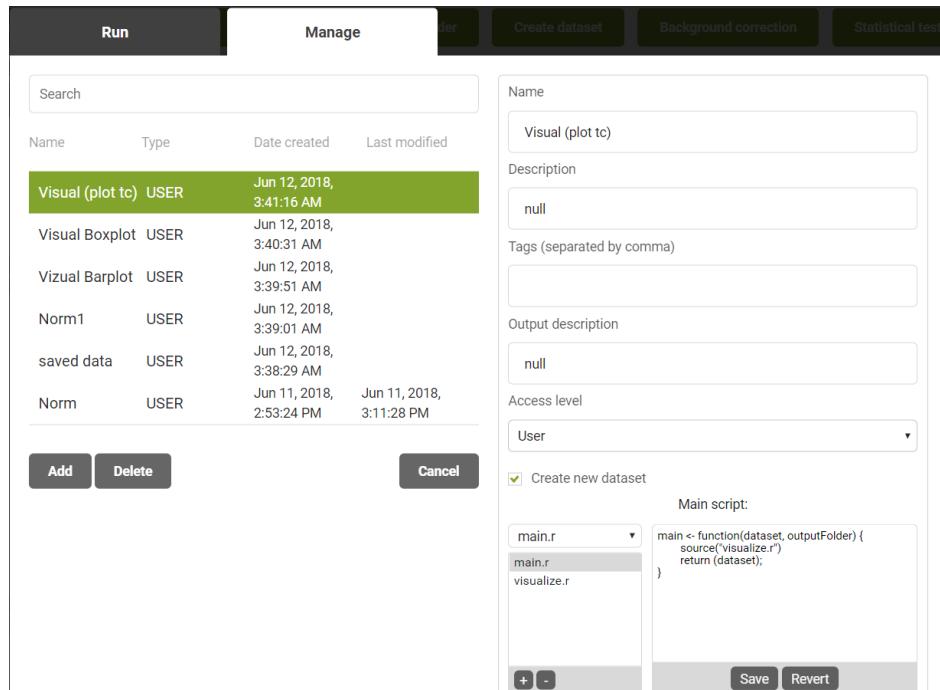


Figure 39: Custom Scripts window

Toolbar Options

Save Subset

The **Save Subset** function is available to save your current dataset settings and selections as a distinct dataset. For example, you may use the search bar to select and remove a subset of undesirable segments from a dataset (such as flagged QC) or to separate segments of interest to analyze separately (such as only tumor segments). Each of these selections can be saved as a subset.

To save your current dataset settings and selections, select the **Save subset** button.

Define: **Name**, **Description** (optional), and **Tags** (optional).

Select **Save**.

Toolbar Options

Pathway Analysis

The Pathway Analysis button is only active in datasets after a t-test or linear mixed model has been run and in datasets with 1000 or more targets ([see Figure 40](#)).

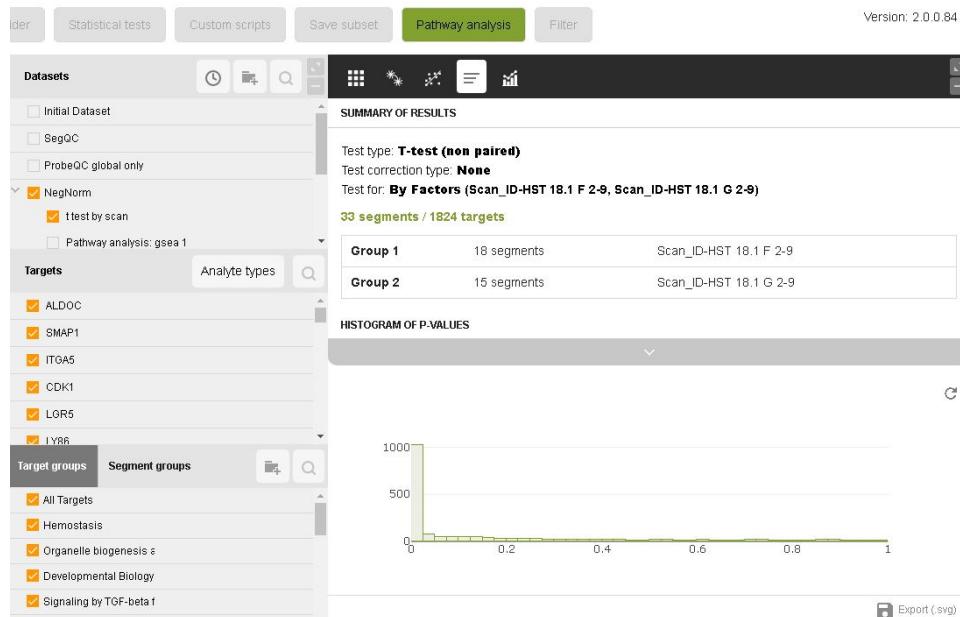


Figure 40: Active Pathway Analysis button

Select a dataset that fits the above criteria and click the **Pathway analysis** button. In the Pathway analysis window ([see Figure 41](#)), set thresholds and click the **Run** button.

This is a configuration window titled "Pathway analysis". It contains two main sections: "Set thresholds for coverage of a pathway present in the dataset" and "Set thresholds for size of pathways to be included in GSEA".

- Set thresholds for coverage of a pathway present in the dataset:**
 - Minimum coverage of genes in the pathway*: 20 %
 - Minimum number of genes in the pathway*: 5
- Set thresholds for size of pathways to be included in GSEA:**
 - Minimum pathway size*: 15 genes
 - Maximum pathway size*: 500 genes
 - Number of permutations*: 10000

Below these sections are input fields for "Analysis name*" and "Pathway analysis tags". The "Analysis name*" field contains "Enter analysis name" and the "Pathway analysis tags" field contains "Enter test result tags, separated with a comma". At the bottom are "Cancel" and "Run" buttons.

Figure 41: Pathway Analysis window

Toolbar Options

Resulting visualization options include the **Pathway Analysis Table**, **Bargraph**, **Volcano plot**, and **Pathways map**.

Select pathways of interest in the table ([see Figure 42](#)) to activate the **Create Target Group** button.

Click on a point (pathway) of interest on the Volcano plot to reveal that pathway's heatmap below ([see Figure 43](#)).

Reactome Pathway ID	Pathway Description	Pathway size	Pathway coverage
R-HSA-38551	Apoptosis	381	32.78%
R-HSA-38552	Hemostasis	621	28.87%
R-HSA-38666	Intrinsic Pathway for Apoptosis	53	52.87%
R-HSA-39764	PDK Cascade	44	75%
<input checked="" type="checkbox"/> R-HSA-12185	Opioid Signaling	91	28.57%
<input checked="" type="checkbox"/> R-HSA-12240	G-protein mediated events	55	29.89%
R-HSA-12243	PLC beta mediated events	54	26.67%
R-HSA-12259	IGF-mediated signaling	41	37.83%
R-HSA-12260	RAF-independent MAPK2/3 act...	23	32.63%
R-HSA-12442	Activation of BH1-only protein	38	53.33%
R-HSA-12464	GPI0-mediated activation casc...	35	48%
R-HSA-12468	Phosphatase degradation	23	27.83%
R-HSA-128572	Downstream signaling events	93	43.27%
R-HSA-128601	Activation of NF-kappaB in B c...	67	33.21%
R-HSA-129408	EDG5 activated mechanism	73	26.67%

Figure 42: Pathway Analysis Table

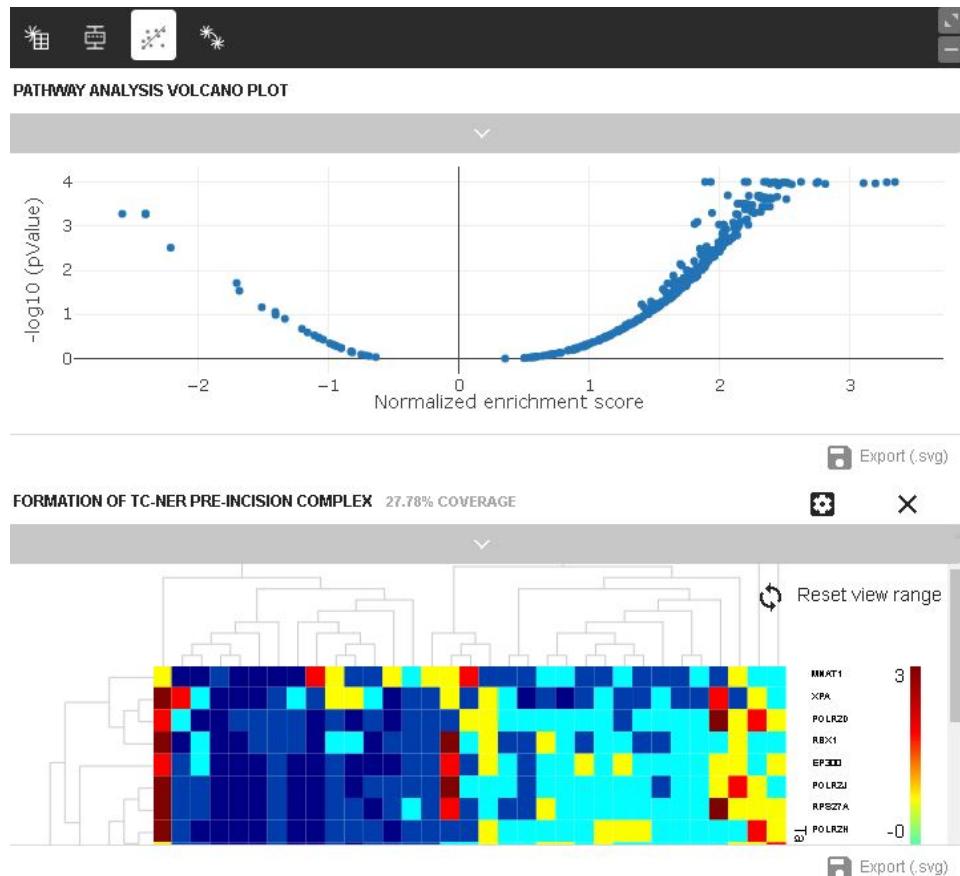


Figure 43: Heatmap from Volcano plot

Toolbar Options

Filter

The **Filter Parameters** ([see Figure 44](#))

allow you to establish an expression threshold and a frequency at which the targets/segments can be below that threshold.

Select the **expression threshold - LOQ (limit of quantitation, see below)**, **user defined value**, or the **higher** of the two. This directs the system to deselect targets/segments at or below this value in your present dataset.

Note that a value below LOQ does not necessarily mean that a target is not expressed; if the value is > LOQ then we are confident that it is expressed.

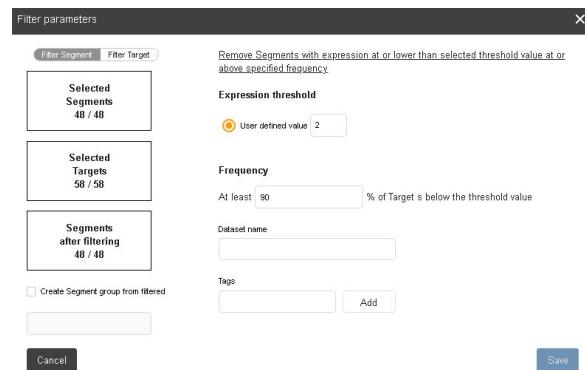


Figure 44: Filter parameters

Set the **frequency** to the percentage of targets/segments that should be filtered out.

Enter a **Dataset name** and any **Tags** you would like associated with the new dataset (optional). You may also check the **Create Segment group from filtered** box.

Click **Save**.

After completing Toolbar Options, your resulting datasets will be available in the Dataset list in the second pane. In this pane, you can also select or create new probe groups for analysis. [Datasets Pane on page 61](#).

6 Analyze in Spatial Context

Use the inter-connectivity of the three panes ([see Figure 45](#)) to re-examine segments in spatial context. See below for information on tagging. See [Scans Pane on page 59](#), [Datasets Pane on page 61](#), and [Visualizations Pane on page 67](#) for more information on what they each feature and how they interact. On most visualizations, you can select a set of data points representing segments and add tags to them. In the heatmap and cluster heatmap, you can click-and-drag a box across one or more columns, selecting those segments of interest. You can then right-click and add the tags you'd like to be associated with the selected segments. Turning to the Scans pane, you'll see that the selected segments in the plots are indicated by pink picker buttons. When you zoom into the scan image, you will see that the selected segments are highlighted with a pink border to easily identify them and to evaluate their spatial context.

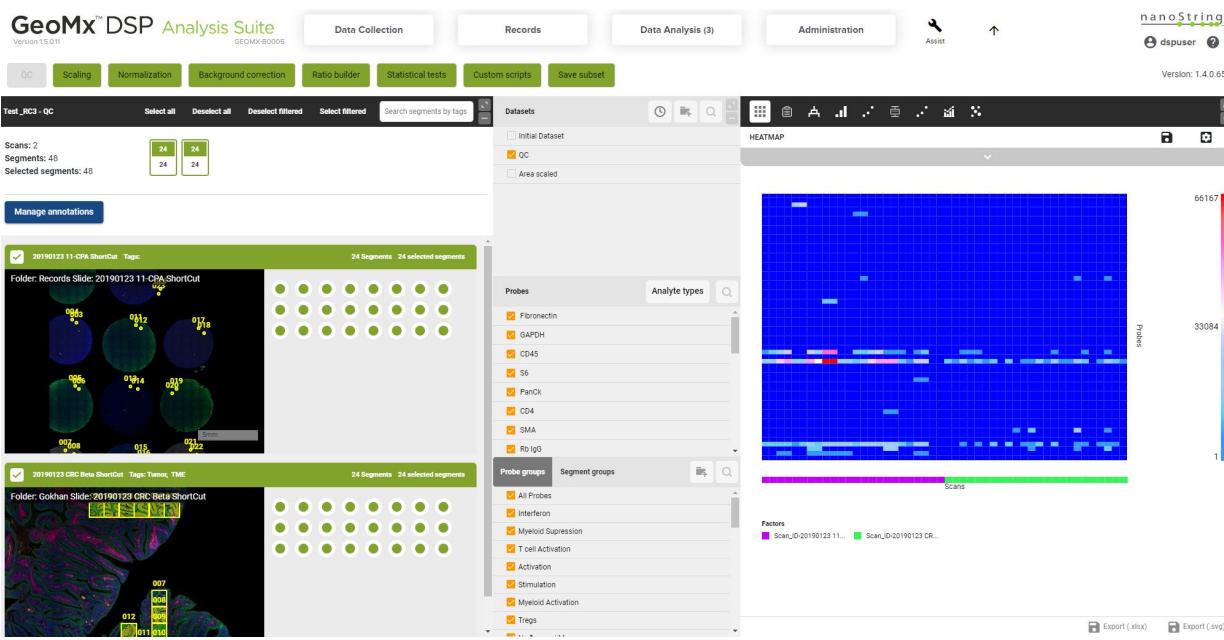


Figure 45: Data Analysis Suite

TAGS

Tags allow you to group segments by type and can then be used to categorize and filter data for analysis.

Analyze in Spatial Context**CREATING A NEW TAG FOR A SPECIFIC GROUP OF SEGMENTS**

1. Select only those segments of interest in the first Data Analysis pane ([see Figure 46](#)).

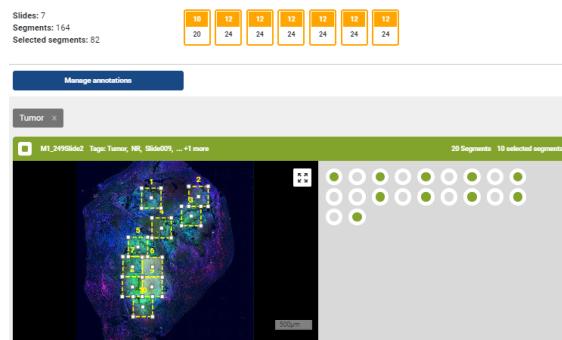


Figure 46: Selecting segments using segment picker buttons

2. Click and drag a box across the whole resulting heatmap in the third (visualizations) pane, such that the box includes all segments (it does not matter what probes are included in this box).
3. Right-click on this selection box. From the resulting menu ([see Figure 47](#)), select **Add tags to segments**.

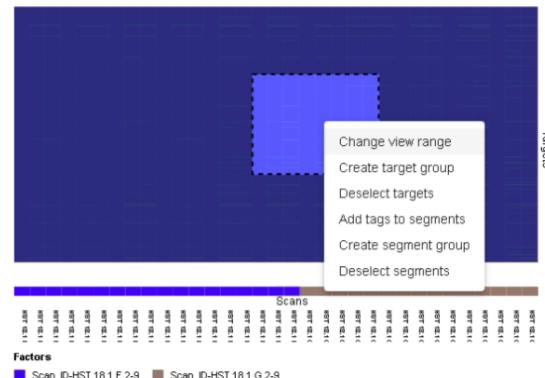


Figure 47: Adding tags to selected segments

4. In the **Add tags to segments** window (see [Figure 48](#)), review the list of **Selected segments**, then type the name of the first tag you would like to add to these segments and select the gray **Add tag** button.
5. Type the name of any additional tags you would like to add to these segments and select the gray **Add tag** button for each.
6. Select the blue **Add tags** button when you are done tagging this group of segments.

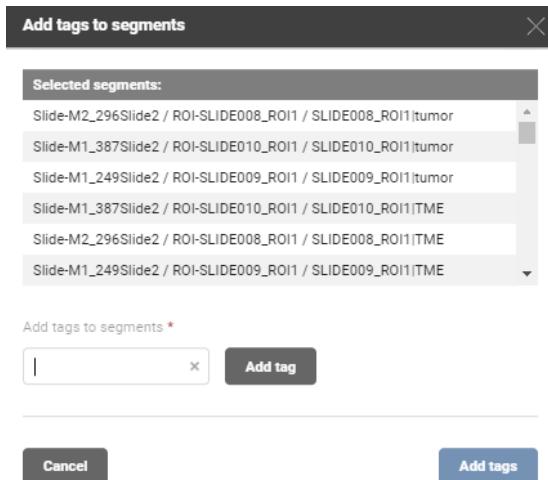


Figure 48: Segments selected for tag

Analyze in Spatial Context**FILTERING DATA BASED ON TAGS**

1. In most cases, you'll want to start with all scans and segments selected; each slide icon should be green ([see Figure 49](#)); if a slide icon is orange or gray, click on it until it toggles to the green status (meaning all segments are selected).
2. Click in the search bar at the top of the Data Analysis window to select an available tag(s). To create a new tag, see [Tags on page 54](#).
3. Any available tags will appear in the drop-down. Select the first tag of interest. Typing into the search box will limit the list of tags displayed to those that have at least a partial match to the typed term.
4. The chosen tag will appear as a gray button and the scan icons will show a preview of the segments included in this selection ([see Figure 50](#)). Note that the filter *has not yet been applied*; the segment picker buttons next to each image viewer indicate the current selection.
5. To apply this filter to the analysis, click **Select Filtered**. Alternatively, you may click **Deselect Filtered** to remove the segments associated with this tag from analysis. The selected scans and segments should change dynamically to reflect this tag choice ([see Figure 51](#)). In addition, the plot in the visualization pane will be redrawn to reflect the current selection.

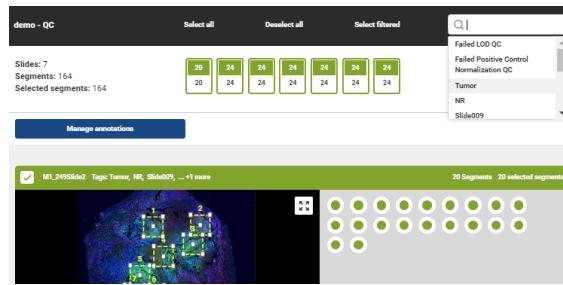


Figure 49: All scans selected

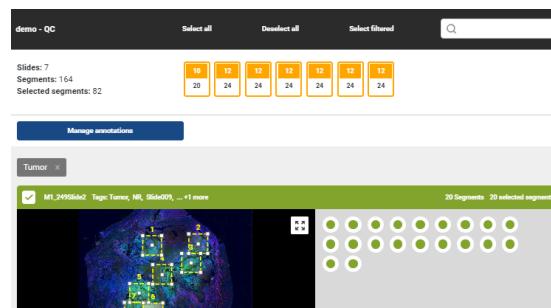


Figure 50: Tag selected for filtering, but not yet applied

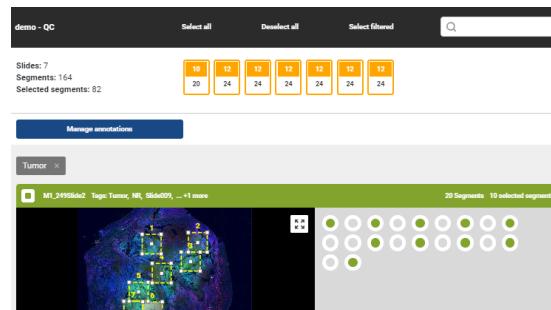


Figure 51: Segment selection, filtered by tag

Analyze in Spatial Context

6. Repeat this process to filter by additional tags, if desired. Select the **x** on a button to stop filtering using that tag.

Selections can be changed in a number of ways including by clicking off a gray tag box (see step 4), by clicking Select all/Deselect all, or by adding additional filters. To save your current selection, select the **Save Subset** button from the Toolbar (see [Toolbar Options on page 28](#)).

Scans Pane

Scans Pane

In the first pane of the DSP Data Analysis interface ([see Figure 52](#)), you may select the scans and segments to include in your study. All scans in the study will be represented as scan icons at the top of this pane and as scan images listed downward. By default, all scans and segments will be initially selected for analysis.

Use the Maximize button to make this pane fill the screen or the Minimize button to hide it as a tab.

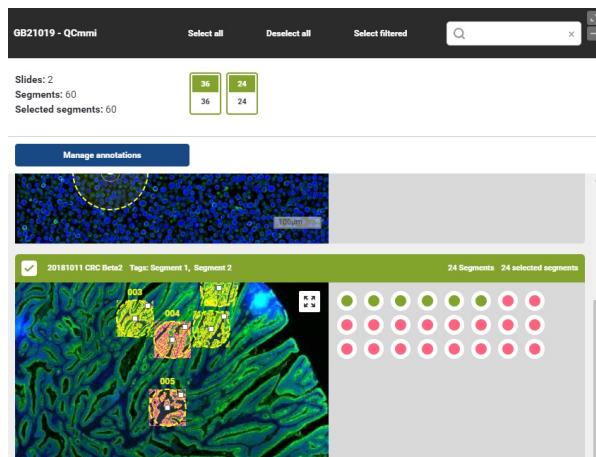


Figure 52: Scans pane

SELECT ALL/DESELECT ALL

Select all and **Deselect all** buttons at the top of the window select and deselect (respectively) all scans and all segments within them.

SCAN ICONS

Scan icons provide a visual preview of:

- The number of segments selected for analysis (top half)
- The total number of segments on the scan (bottom half)
- The general proportion of segments selected for analysis:



All segments selected for analysis is indicated by a green outline and top half.

Some segments selected for analysis is indicated by an orange outline and top half.

No segments selected for analysis is indicated by a gray outline and top half.

Click on a slide icon to toggle between selecting (green or orange) or deselecting (gray) for analysis. Specify which segments will be selected using the slide images (see [Image Viewer on page 60](#)).

MANAGE ANNOTATIONS

Segment annotations can be uploaded to a Data Analysis study from a spreadsheet using the Manage Annotations button in the Scans Pane. See [Manage Annotations on page 25](#).

IMAGE VIEWER

Each image viewer ([see Figure 53](#)) portrays the scan and the spatial placement of the ROIs and segments. The check box in the upper left corner indicates whether that scan is selected for analysis.

- Selected scans have a green header
- Deselected scans have a white header.

You may adjust the scan image to assist in viewing, selecting, and deselecting segments.

- **Zoom in and out** using the roll button on your mouse or zoom feature on your touch screen or touch pad.
- Click the **full-screen button** to expand the image to full-screen.

Each ROI in the image corresponds with one or more segment picker buttons to the right of the image. Hovering over an ROI on the image viewer will initiate a pop-up providing more information.

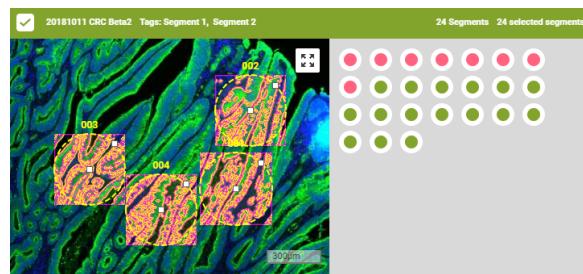


Figure 53: Image viewer and segment picker buttons

Datasets Pane

The central pane of the DSP Data Analysis screen lists all datasets and target groups associated with the current study. At a minimum, you will see the **Initial Dataset** (the raw set of imported data; this will appear at the top of the Dataset list) in the Data sets field. The **All Targets** group, as well as any additional target groups defined in your core and module kit configuration files (see the **GeoMx DSP Instrument User Manual** for details regarding Scan Parameters) will populate the Target groups field.

Use the Maximize button to make this pane fill the screen or the Minimize button to hide it as a tab.



Datasets

Anytime you run a task (QC, Normalization, etc.), you will be prompted to save the updated dataset. This new dataset will be listed in the dataset list ([see Figure 54](#)).

Select the **new folder icon** on the **Datasets** header to create a new folder to organize your datasets. Click and drag datasets to place them in the folder of your choice.

Select the search button on the **Datasets** header to search by tag or text.

To save a selected subset of scans and/or segments as a distinct dataset, use the **Save Subset** button on the Toolbar (see [Toolbar Options on page 28](#)).

Datasets Pane**MANAGE DATASETS**

- At any time, you may select the **History** icon at the top of the datasets pane ([see Figure 54](#)) to see the adjustments that have been made to the selected dataset.



Figure 54: History bar in Datasets pane

- An **arrow** next to a dataset indicates that statistical tests have been run on that dataset; click on this **arrow** and select a test result box ([see Figure 55](#)) to see visualizations with those results.

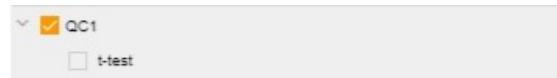


Figure 55: Statistical tests datasets

- Hovering over a dataset name reveals **export**, **edit**, and **trash** icons ([see Figure 56](#)).
- Export the whole dataset in .xlsx format by selecting the **export** icon that appears when you hover over it. The downloaded file will be a multi-tab spreadsheet.
- Rename the dataset by selecting the **edit** icon that appears when you hover over it.
- Delete the dataset by selecting the **trash** icon that appears when you hover over it.



Figure 56: Export, edit, and trash icons in the datasets pane

Datasets Pane

MERGE DATASETS

You may merge two datasets into one if the two datasets have either:

- **Different segments** from each other but the *same set of probes* or
- **Different probes** from each other but the *same set of segments*.

To merge:

1. Select the first dataset you'd like to use in the merge.
2. Hold down the Control (**Ctrl**) button and select any other datasets you would like to merge with the first.
3. Right-click and select **Merge datasets**.
4. Confirm or edit your selections using the check boxes. If a message appears, prohibiting this merge, revisit the restrictions listed above.
5. Define the **Name**, **Description** (optional), and **Tags**.
6. Select **Merge**.

Datasets Pane

Targets

All targets detected in the configuration file (see the **GeoMx DSP Instrument User Manual** for details regarding Scan Parameters) of the dataset will be listed in the probe list in the Datasets pane. Each target with a checked box will be included in the study ([see Figure 57](#)).

Targets	Analyte types
<input checked="" type="checkbox"/> ALDOC	
<input checked="" type="checkbox"/> SMAP1	
<input checked="" type="checkbox"/> ITGA5	
<input checked="" type="checkbox"/> CDK1	
<input checked="" type="checkbox"/> LGR5	
<input checked="" type="checkbox"/> LY86	

Figure 57: Probes list section of datasets pane

SELECTING/DESELECTING TARGETS

Uncheck boxes to remove targets from analysis.

Filter targets using one of the following buttons:

- Analyte Type
- Search: use the **Search** button to search for probes by **text** and/or by **tag**.

Target Groups & Segment Groups

Target groups and segment groups are listed in the Datasets pane ([see Figure 58](#)).

Target groups are sets of targets organized together in a designated group. The first target group (and only one that cannot be deleted) is the group **All Targets**. Other pre-defined target groups may autopopulate in this field as they are defined in the core or module kit configuration file (see the **GeoMx DSP Instrument User Manual** for details regarding Scan Parameters).

Select the **new folder icon** on the **Target/Segment groups** header to create a new folder to organize your target groups or segment groups. Click and drag target/segment groups to place them in the folder of your choice.

Select the **search button** on the **Target/Segment groups** header to search by tag or text.

Target groups	Segment groups
<input checked="" type="checkbox"/> All Targets	
<input checked="" type="checkbox"/> Hemostasis	
<input checked="" type="checkbox"/> Organelle biogenesis	
<input checked="" type="checkbox"/> Developmental Biology	
<input checked="" type="checkbox"/> Signaling by TGF-beta	

Figure 58: Probe and segments group section of Datasets pane

Datasets Pane**CREATE NEW TARGET GROUP**

1. Start with an existing target group (such as **All Targets**).
2. Alter this group by deselecting targets you'd like to remove and/or clicking on targets you'd like to add. You can also "highlight" targets by selecting an area on a visualization in the third pane.
3. Right-click in the targets list and select **Create Group (selected)** (if you clicked on/off probes in the Targets list) or **Create Group (highlighted)** (if you highlighted targets using a visualization) to save this as a new target group ([see Figure 59](#)).
4. Enter the Target Group name and add any tags desired (optional).
5. Click the **Create Target Group** button.

You may also select a region of the live visualization in the third pane and right-click on it to create a new target group from those targets in the selected region or to deselect them ([see Figure 60](#)).

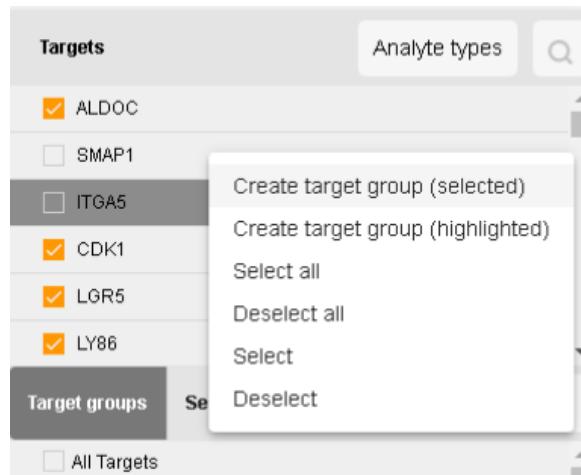


Figure 59: Creating a probe group from the probes section of the Datasets pane

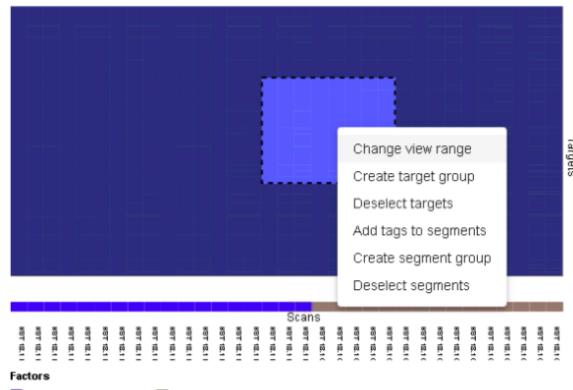


Figure 60: Creating a probe group from a visualization

Datasets Pane**CREATE NEW SEGMENT GROUP**

Select a region of the live visualization in the third pane, right-click, and select **Create segment group** from the segments in the selected region ([see Figure 61](#)). You can also deselect them from this menu.

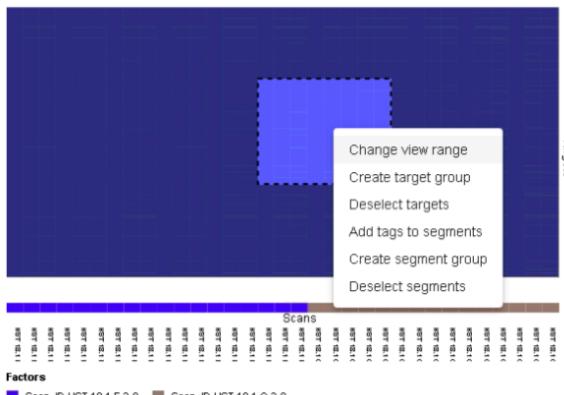


Figure 61: Creating a segment group from a visualization

MANAGE TARGET GROUPS & SEGMENT GROUPS

Hovering over a target group or segment group name reveals **edit** and **trash** icons.

- Rename the group by selecting the **edit** icon that appears when you hover over it.
- Delete the group by selecting the **trash** icon that appears when you hover over it. You cannot delete the **All Targets** group.

Visualizations Pane

Visualizations Pane

The third pane of the DSP Data Analysis window is the **Visualizations pane** ([see Figure 62](#)). Visualizations are visual representations of the selected dataset and probes. They will change dynamically in response to the dataset, segments, and/or probes selected for analysis. Find more details on some visualizations in [Algorithm Details on page 100](#).

Use the Maximize button to make this pane fill the screen or the Minimize button to hide it as a tab.



Visualization option icons line the header of this pane. The icon in white is presently visible; the icon in gray is not available for the present dataset. Upon starting an NGS study and through the first QC step, the live plot in the visualization pane will be a line plot. **Summary** and **Violin** plot options will also be available. See [Viewing Segment QC Results](#).

The **Save** and **Gear** icons are available to save and name visualizations, respectively. See [Accessing Saved Visualizations on page 98](#) for information on viewing saved plots.

Click and drag to select any area of interest on a visualization. Any segments in this area of interest will be shown as highlighted in the **Scans pane**. Right-click on your selection to view options such as:

- Creating a probe group or segment group for the selection.
- Excluding the selected set of probes or segments from the present study.
- Creating a tag for the selected segments.

Export the present visualization in .svg format using the **Export(.svg)** button. Export the data upon which this visualization is based in .xlsx format using the **Export(.xlsx)** button.

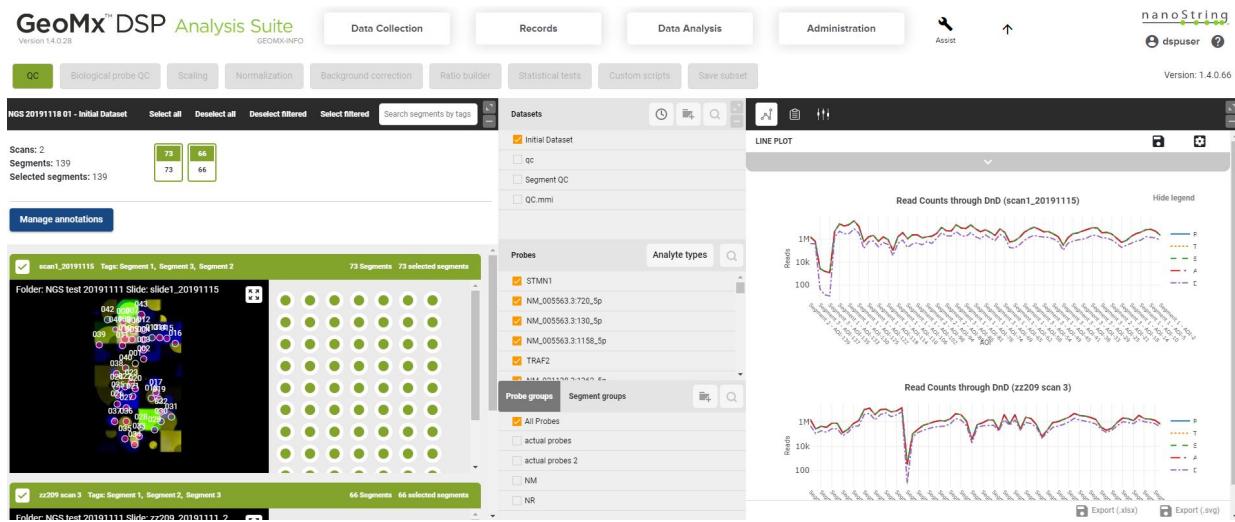


Figure 62: Live line plot for NGS data

Visualizations Pane

Heatmap

The heatmap ([see Figure 63](#)) is an image that depicts counts by color. Segments are aligned along the x-axis and targets on the y-axis.

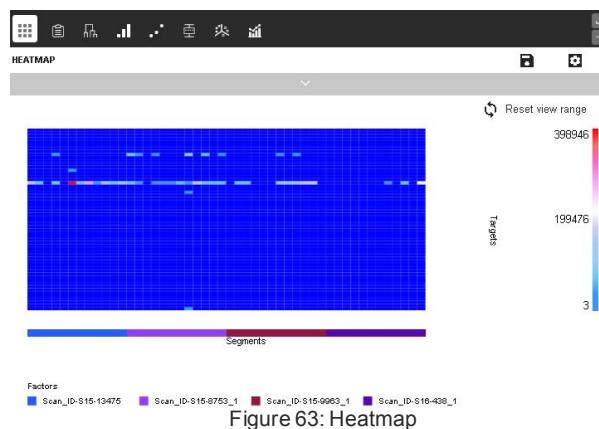


Figure 63: Heatmap

- Hover over an area of the heatmap to see a pop-up displaying the segment, target, count, and any tags.
- To name the visualization, select the **gear icon** ([see Figure 64](#)), enter the desired name, and select **Save**. Use the check boxes to **Export Columns** and **Display Legend** here, as well.
- To save the visualization, select the **Save** icon. Access the saved image later under the **Summary** tab. For more information on accessing saved visualizations, exporting visualizations, or exporting data from visualizations, see [Saving & Exporting on page 97](#).



Figure 64: Save and gear icons

Click and drag to select part or all of the heatmap. Right-clicking within this selected area summons a menu ([see Figure 65](#)) with which you can:

- Create a probe group comprised of the selected probes.
- Deselect the selected probes from the current analysis.
- Add tags to the selected segments.

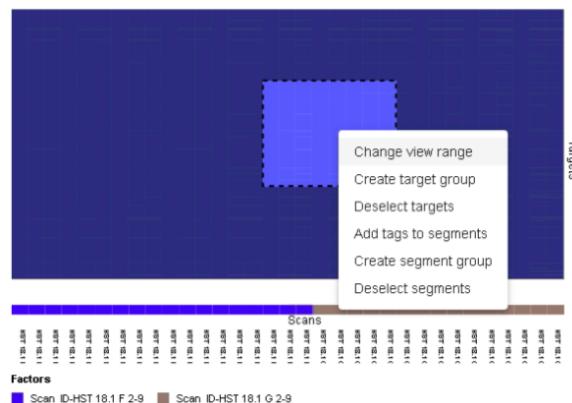


Figure 65: Right-click visualizations menu

Visualizations Pane

- Create a segment group comprised of the selected segments.
- Deselect the selected segments from the current analysis.

Click the arrow to access the drop-down field ([see Figure 66](#)).

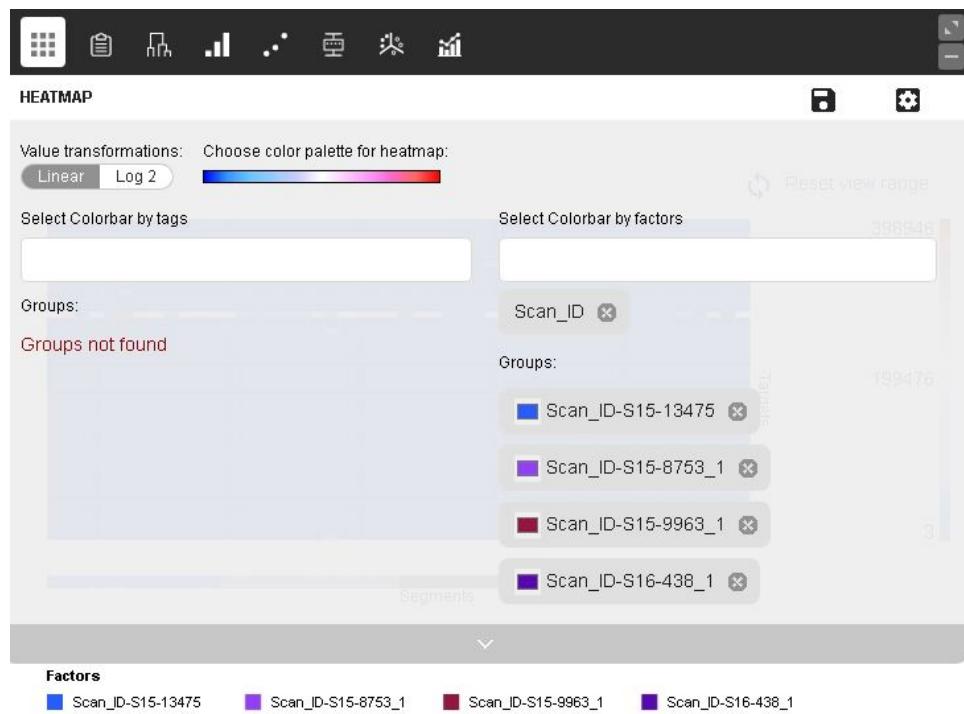


Figure 66: Heatmap drop down field

Here, you may customize the graph by deciding whether to:

- Use the slider to choose between **Linear** and **Log₂** data. You may also select **Ratio**, if available (only available if the selected dataset contains ratio data).
- Click on the color bar legend to change the color scheme.
- Establish colorbar by tags or factors.

Visualizations Pane

Study Summary

The study summary ([see Figure 67](#)) plots the total number of reads for each segment by each processing step outcome (trimmed, deduplicated, etc.). It also contains drop-down fields with **Segment Summary** ([see Figure 63](#)), **Probe Summary**, and **Target Summary**.

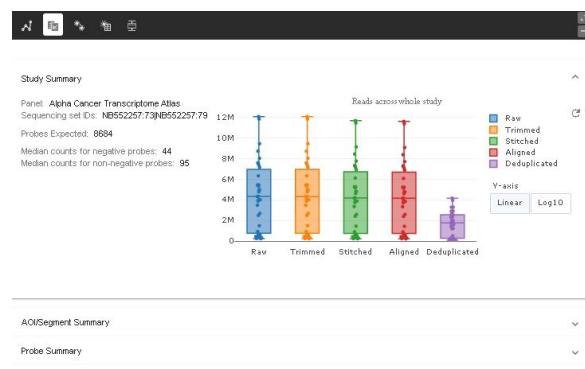


Figure 67: NGS Study Summary

Segment summary includes Q30 values.

Q30 refers to the percentage of bases that get a Q score of at least 30 (99.9% accuracy)

umiQ30 and rtsQ30 refer to the % of bases with Q>30 specifically in the UMI and RTS_ID portions of the reads, respectively.

AOI/Segment Summary					
Site name	Barcode	ROI name	Segment name	Tags	
HT36112.9	HGT18112.9	004	KM0L21<204	Low Negative Pro	
HT36112.9	HGT18102.9	004	KM0L21<204	Low Negative Pro	
HT36112.9	HGT18112.9	004	KM0L21<204	Low Negative Pro	
HT36112.9	HGT18112.9	004	KM0L21<204	Low Negative Pro	
HT36112.9	HGT18112.9	002	KM0L21<204	Geometric Segment	
HT36112.9	HGT18102.9	002	KM0L21<204	Low Sequencing?	
HT36112.9	HGT18112.9	003	KM0L21<204	Low Negative Pro	
HT36112.9	HGT18102.9	003	KM0L21<204	Low Sequencing?	
HT36112.9	HGT18112.9	003	KM0L21<204	Low Negative Pro	
HT36112.9	HGT18112.9	003	KM0L21<204	Low Sequencing?	
HT36112.9	HGT18112.9	004	KM0L21<204	Geometric Segment	
HT36112.9	HGT18112.9	004	KM0L21<204	Low Negative Pro	
HT36112.9	HGT18112.9	004	KM0L21<204	Low Negative Pro	
HT36112.9	HGT18112.9	006	KM0L21<204	Low Surface Area	
HT36112.9	HGT18102.9	006	KM0L21<204	Low Surface Area	

Figure 68: Segment Summary

Pathway Map

The pathway map shows the coverage of pathways for entire dataset.

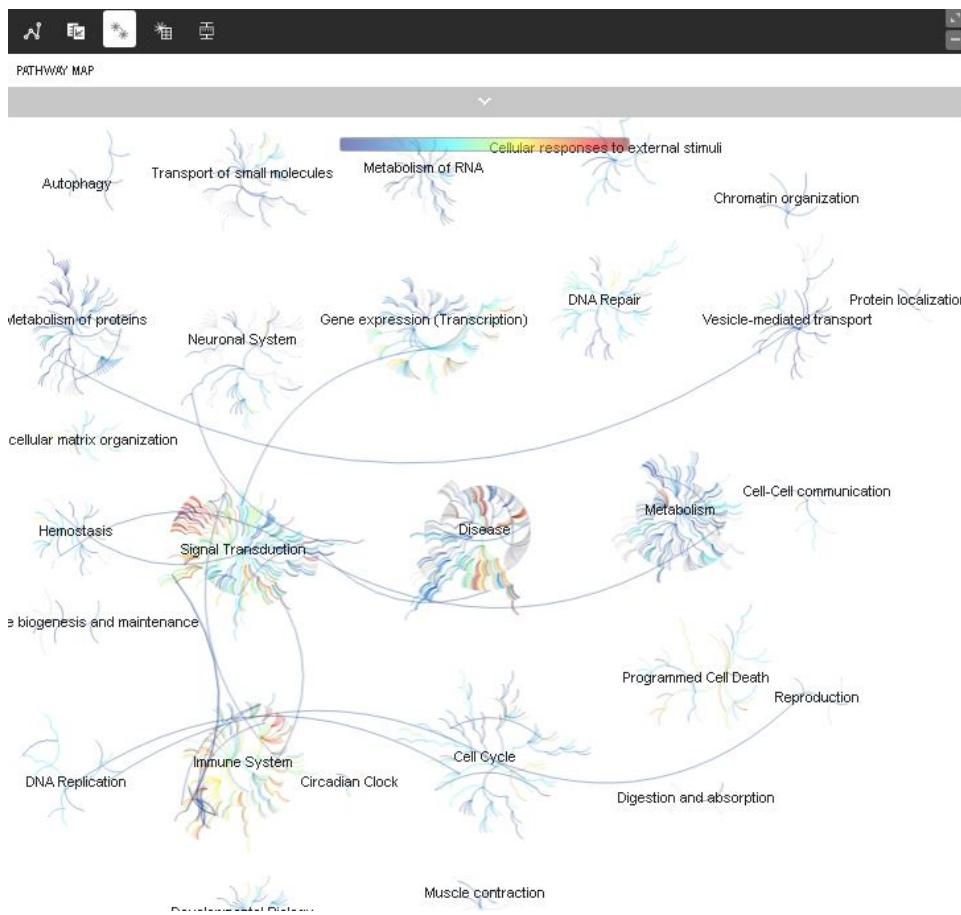


Figure 69: Pathway Map

Click the arrow to access the drop-down field. Here, you can change the color scheme of the Pathway Map.

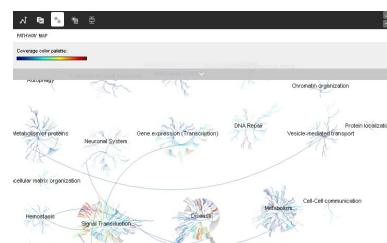


Figure 70: Pathway Map drop-down

Visualizations Pane**Pathway Table**

The Pathway Table summarizes the Pathway Map coverage. This value is calculated by dividing the **number of genes present in the dataset** by the **number of genes in present in the pathway**. The genes in the dataset are determined by the panel used for testing and the current selection in your dataset. The pathway information is obtained from reactome.org.

Reactome Pathway ID	Pathway Description	Pathway size	Pathway coverage	Targets
R-HSA-1059683	Interleukin-6 signalling	11	100%	IL6R, TYK2, PTPN11, JAK1, PSMD3, PSMB10, PSM
R-HSA-109581	Apoptosis	180	32.78%	CXADR, ATP2B2, GATA
R-HSA-109582	Hemostasis	621	29.31%	TP63, EZF1, BCL2, CAS
R-HSA-109606	Intrinsic Pathway for Apoptosis	53	52.83%	PDE3B, AKT2
R-HSA-109703	PKB-mediated events	2	50%	FGF22, PIK3CA, FGF5, PIK3CB
R-HSA-109704	R3K Cascade	44	75%	CDK1, IL6R, TYK2, PTPN11, RPA3, PCNA, RPS27A, RPA3, USP10, UBA52, H2BC3, HAC9, H2AC2C
R-HSA-110056	MAPK3 (ERK1) activation	10	100%	RPA3, PCNA, RPS27A, RPA3, UBA52, RFC1, U6, RPA3, PCNA, NPLOC4, H2BC3, HAC9, H2AC2C
R-HSA-110312	Translesion synthesis by REV1	16	50%	RPA3, PCNA, RPS27A, RPA3, UBA52, RFC1, U6, RPA3, PCNA, NPLOC4, H2BC3, HAC9, H2AC2C
R-HSA-110313	Translesion synthesis by Y family ...	39	33.33%	RPA3, PCNA, RPS27A, RPA3, UBA52, RFC1, U6, RPA3, PCNA, NPLOC4, H2BC3, HAC9, H2AC2C
R-HSA-110314	Recognition of DNA damage by ...	30	36.67%	RPA3, UBA52, RFC1, U6, RPA3, PCNA, NPLOC4, H2BC3, HAC9, H2AC2C
R-HSA-110320	Translesion Synthesis by POLH	19	36.84%	RPA3, PCNA, NPLOC4, H2BC3, HAC9, H2AC2C
R-HSA-110328	Recognition and association of ...	61	4.92%	RPA3, PCNA, NPLOC4, H2BC3, HAC9, H2AC2C
R-HSA-110329	Cleavage of the damaged pyrimidines by ...	61	4.92%	RPA3, PCNA, NPLOC4, H2BC3, HAC9, H2AC2C
R-HSA-110330	Recognition and association of ...	56	3.57%	RPA3, PCNA, NPLOC4, H2BC3, HAC9, H2AC2C

Figure 71: Pathway Table

Visualizations Pane

Dataset Summary

Selecting the summary button in the Visualizations pane of the Data Analysis window may reveal multiple tabs, depending on what tasks have been run: **Segment Summary**, **Attachments**, **Saved visualizations**, **Background**, **Normalization**, **Scaling**, and **Statistics**.

Scan Name	ROI Name	Segment Name	Binding Density	Imaging	Pos Control R2
S15-13475	001	Geometric Segment	1.19	1	NA
S15-13475	002	Geometric Segment	0.92	1	NA
S15-13475	003	Geometric Segment	0.73	1	NA
S15-13475	004	Geometric Segment	1.1	1	NA
S15-13475	005	Geometric Segment	0.53	1	NA
S15-13475	006	Geometric Segment	1.1	1	NA
S15-13475	007	Geometric Segment	0.81	0.95	NA
S15-13475	008	Geometric Segment	1.31	1	NA
S15-13475	009	Geometric Segment	0.95	0.99	NA
S15-13475	010	Geometric Segment	1.21	1	NA
S15-13475	011	Geometric Segment	0.94	1	NA

Figure 72: Summary in visualizations pane

SEGMENT SUMMARY

This table lists the scan name, ROI name, segment name, surface area, nuclei count, and any tags associated with each segment. Select or deselect listed segments to add or remove them from the current analysis.

Depending on the analysis performed, additional data may appear here to summarize information for each segment. This includes QC status, binding density, FoV registration QC, positive normalization factor, QC flags, and average background.

ATTACHMENTS

This tab contains any files generated from custom scripts for the current dataset. See [Toolbar Options on page 28](#).

SAVED VISUALIZATIONS

This tab contains any saved visualizations for the current dataset. See [Saving a Visualization on page 97](#) and [Accessing Saved Visualizations on page 98](#).

Background Summary - see [Viewing Background Results on page 38](#).

Normalization Summary - see [Viewing Normalization Results on page 35](#).

Scaling Summary - see [Viewing Scaling Results on page 30](#).

Statistics Summary - see [Viewing Statistical Test Results on page 47](#).

Visualizations Pane

Cluster

A dendrogram ([see Figure 73](#)) is an interactive tree which makes inferences about relationships among data points.

Data points belonging to the same branch of a cluster are similar to each other at some level; data points in separate branches are less similar.

Segments are aligned along the x-axis and targets on the y-axis.

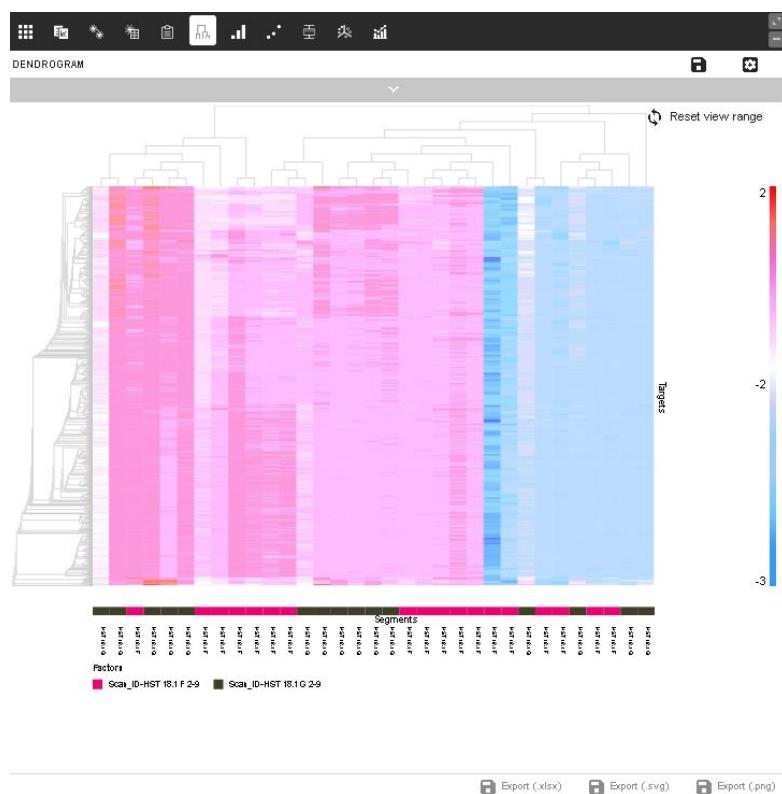


Figure 73: Cluster

- Hover over an area of the dendrogram to see a pop-up displaying the segment, target, count, and any tags.
- Select a branch of the dendrogram to zoom.
- To name the visualization, select the **gear icon** ([see Figure 74](#)), enter the desired name, and select **Save**. Use the check boxes to **Export Columns** and **Display Legend** here, as well.
- To save the visualization, select the **Save icon**. Access



Figure 74: Save and gear icons

Visualizations Pane

the saved image later under the **Summary** tab. For more information on accessing saved visualizations, exporting visualizations, or exporting data from visualizations, see [Saving & Exporting on page 97](#).

Click and drag to select part or all of the dendrogram. Right clicking within this selected area summons a menu ([see Figure 75](#)) with which you can:

- Create a probe group comprised of the selected probes.
- Deselect the selected probes from the current analysis.
- Add tags to the selected segments.
- Create a segment group comprised of the selected segments.
- Deselect the selected segments from the current analysis.

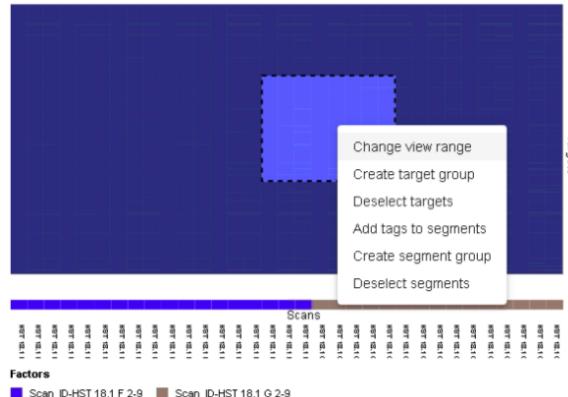


Figure 75: Right-click visualizations menu

Click the arrow to access the drop-down field ([see Figure 76](#)).

Visualizations Pane

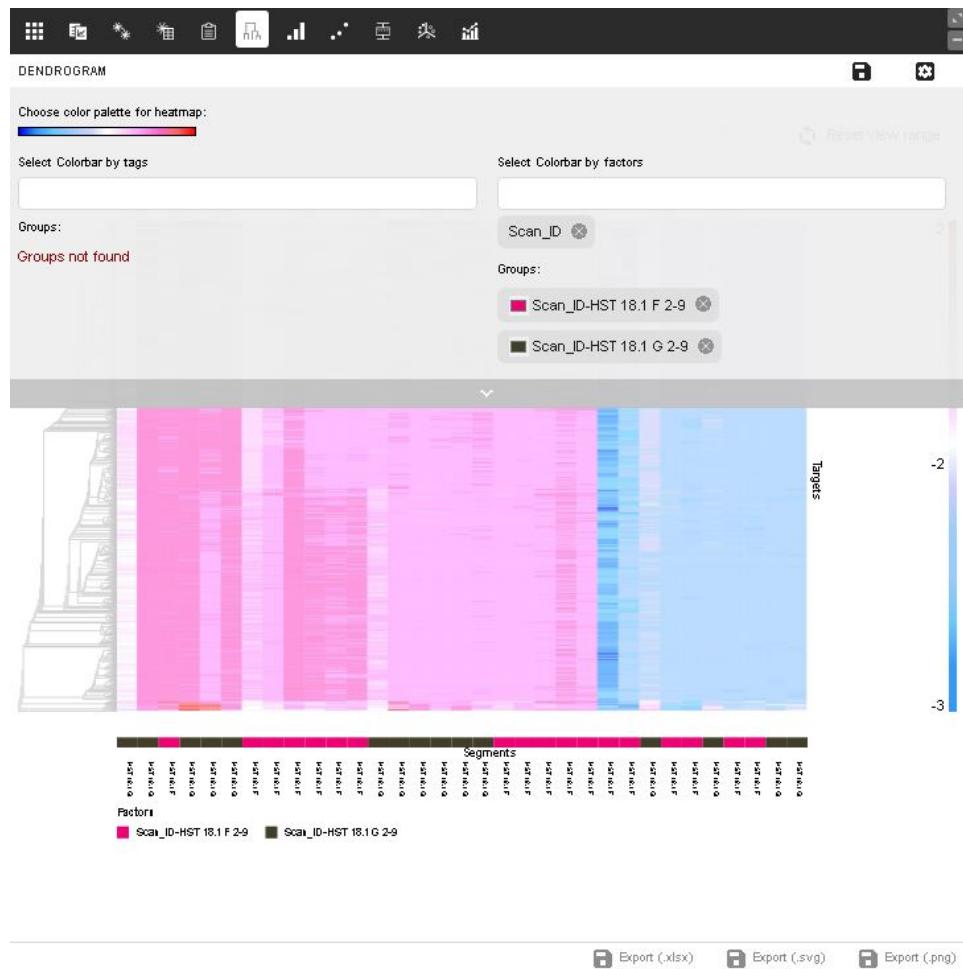


Figure 76: cluster drop down field

Here, you may customize the graph by deciding whether to:

- Use the slider to choose between **Linear** and **Log₂** data. You may also select **Ratio**, if available (only available if the selected dataset contains ratio data).
- Click on the color bar legend to change the color scheme of the diagram.
- Establish colorbar by tags or factors.

Visualizations Pane**Bar chart**

The bar graph ([see Figure 77](#)) represents the count values of all probes across all segments included in the study. Segments are listed along the x-axis and counts along the y-axis. The height of each bar represents the frequency of each count defined by the bins.



Figure 77: Bar graph

- Zoom in or out using the + and - buttons and scroll to view all results.
- Below the plots, select whether bars should be colored by **Counts**, **Probe**, **Segments**, or **Tags**. The resulting legend will be listed to the right of the plots.
- To name the visualization and/or the y-axis, select the **gear icon** ([see Figure 78](#)), enter the desired name, and select **Save**.



Figure 78: Save and gear icons

Visualizations Pane

- To save the visualization, select the **Save** icon. Access the saved image later under the **Summary** tab. For more information on accessing saved visualizations, exporting visualizations, or exporting data from visualizations, see [Saving & Exporting on page 97](#).

Click the arrow to access the drop-down field ([see Figure 79](#)).

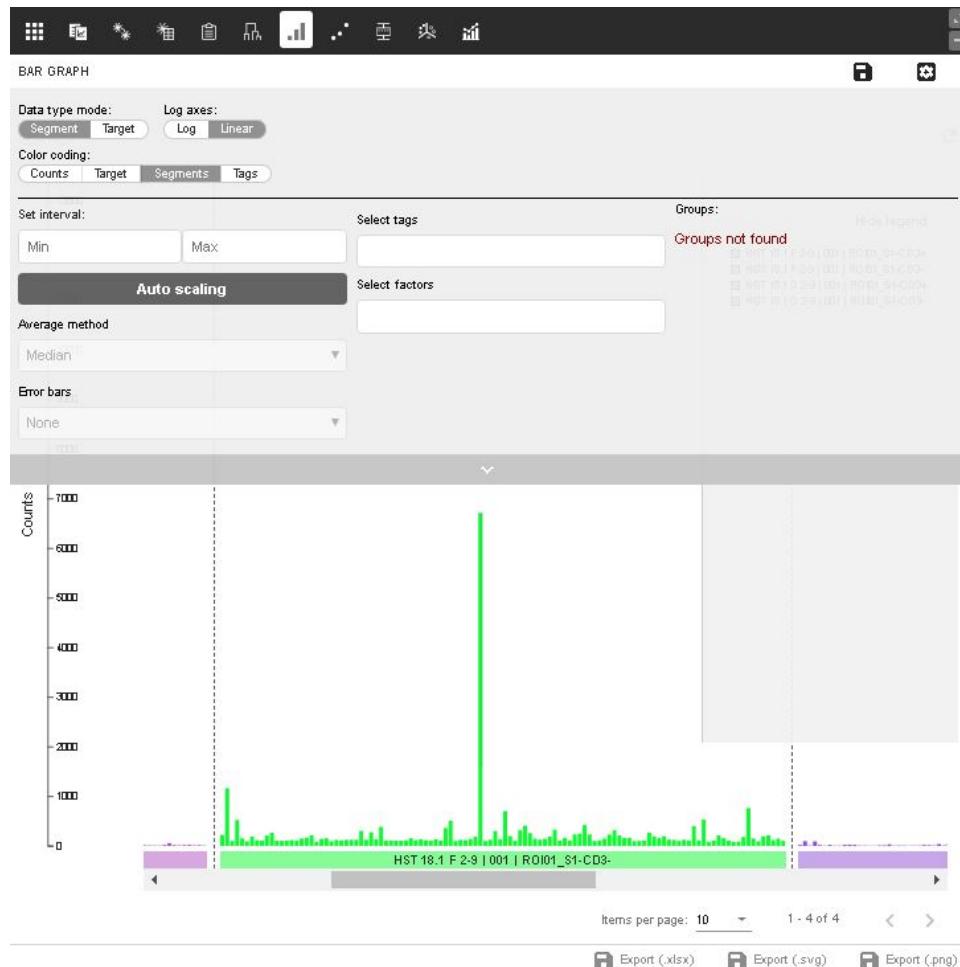


Figure 79: Bar graph drop-down field

Here, you may customize the graph by deciding whether to:

- View segments or probes on the x-axis using the **Segment/Probe** slider.
- View intensity data in linear or log space using the **Linear/Log** slider.
- View ratio data (if applicable) as **Ratios**, **Fold Changes**, or **Log₂** ratio.
- Apply auto-scaling by entering a **Min Count Value** and/or a **Max Count Value**(only available when viewing linear intensity data, not in log scale).

Visualizations Pane

- Apply grouping by selecting **Tags**, **Factors**, **Average method** (median, geomean, average), and **Error bars** (SE, SD, none).

Visualizations Pane**Correlation plot**

The correlation plot ([see Figure 80](#)) is a visualization that plots one segments' results on the x-axis and a different segments' results on the y-axis. Alternatively, you may choose to plot one targets' results vs. another probes' results (see below).

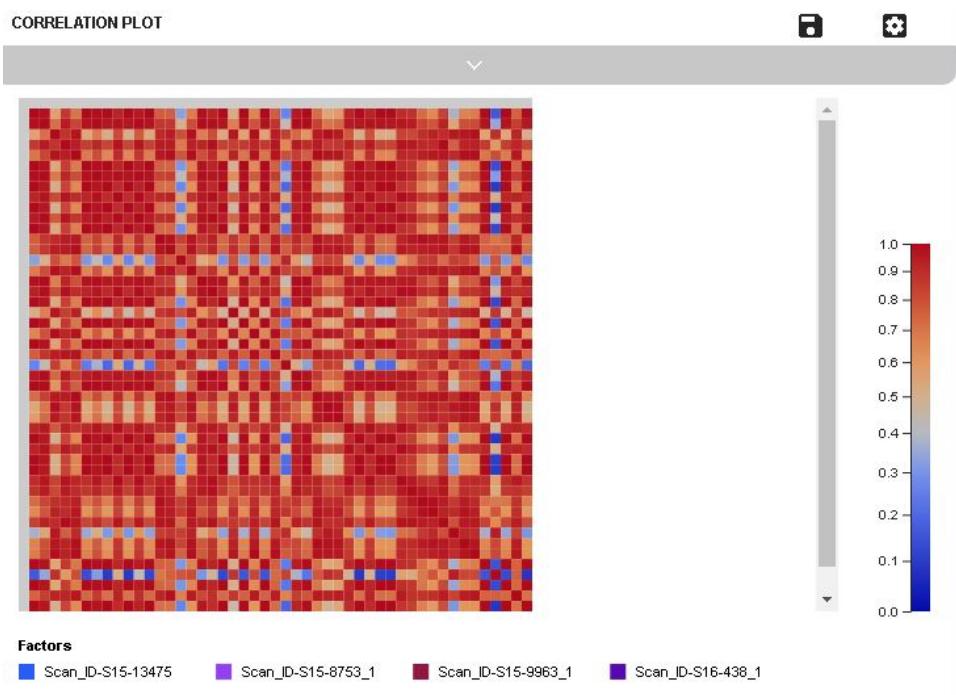


Figure 80: Correlation plot

- Hover over a cell on the correlation plot to see a pop-up displaying the represented segments and correlation coefficient.
- To name the visualization, x-axis, or y-axis, select the **gear icon** ([see Figure 81](#)), enter the desired name(s), and select **Save**. Use the check box to **Display trendline** or show or hide the **x-** or **y-axis** labels here, as well.
- To save the visualization, select the **Save** icon. Access the saved image later under the **Summary** tab. For more information on accessing saved visualizations, exporting visualizations, or exporting data from visualizations, see [Saving & Exporting on page 97](#).



Figure 81: Save and gear icons

Click the arrow to access the drop-down field ([see Figure 82](#)).

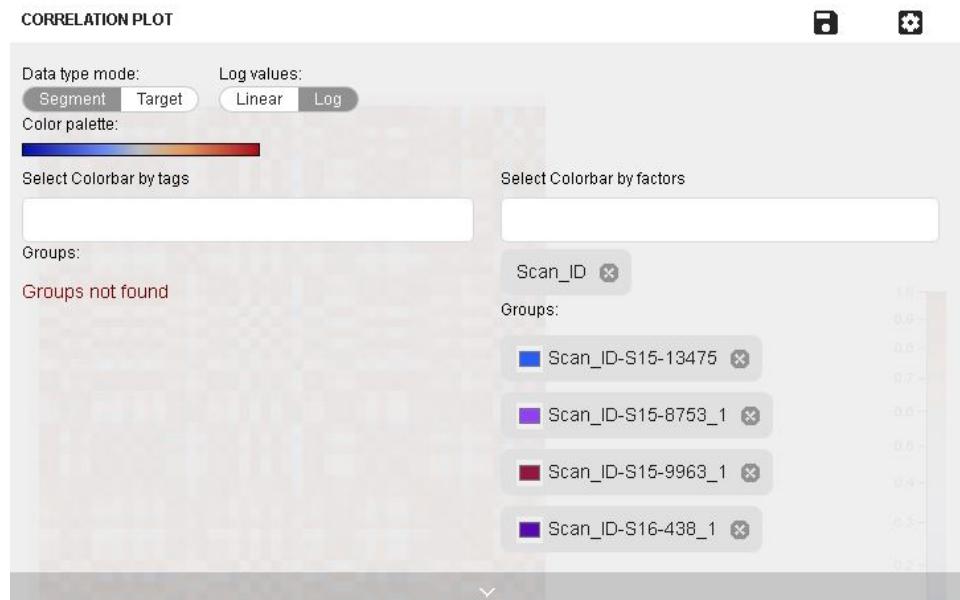
Visualizations Pane

Figure 82: Correlation plot drop-down field

Here, you may customize the graph by deciding whether to:

- View segments or probes on the x-axis using the **Segment/Target** slider.
- View intensity data in linear or log space using the **Linear/Log** slider.
- Change the color scheme

Click and drag to select part or all of the correlation plot. Right-clicking within this selected area summons a menu ([see Figure 83](#)) with which you can:

- **Zoom in.**
- **Add tags** to segments.
- **Create a target/segment group** from the selection. See [Create New Target Group on page 65](#).
- **Deselect targets/segments** in the selection.

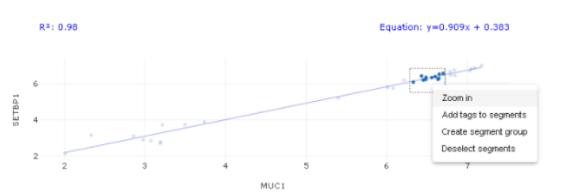


Figure 83: Right-click scatter plot menu

Visualizations Pane**Box plot**

Box plots ([see Figure 84](#)) are a convenient way of depicting subsets of your experiment through their quartiles. Box plots have lines extending vertically from the boxes (whiskers) that indicate variability outside the upper and lower quartiles. Outliers may be plotted as individual points. These visualizations display differences between subsets of an experiment without making any assumptions about the underlying statistical distribution; they are non-parametric.

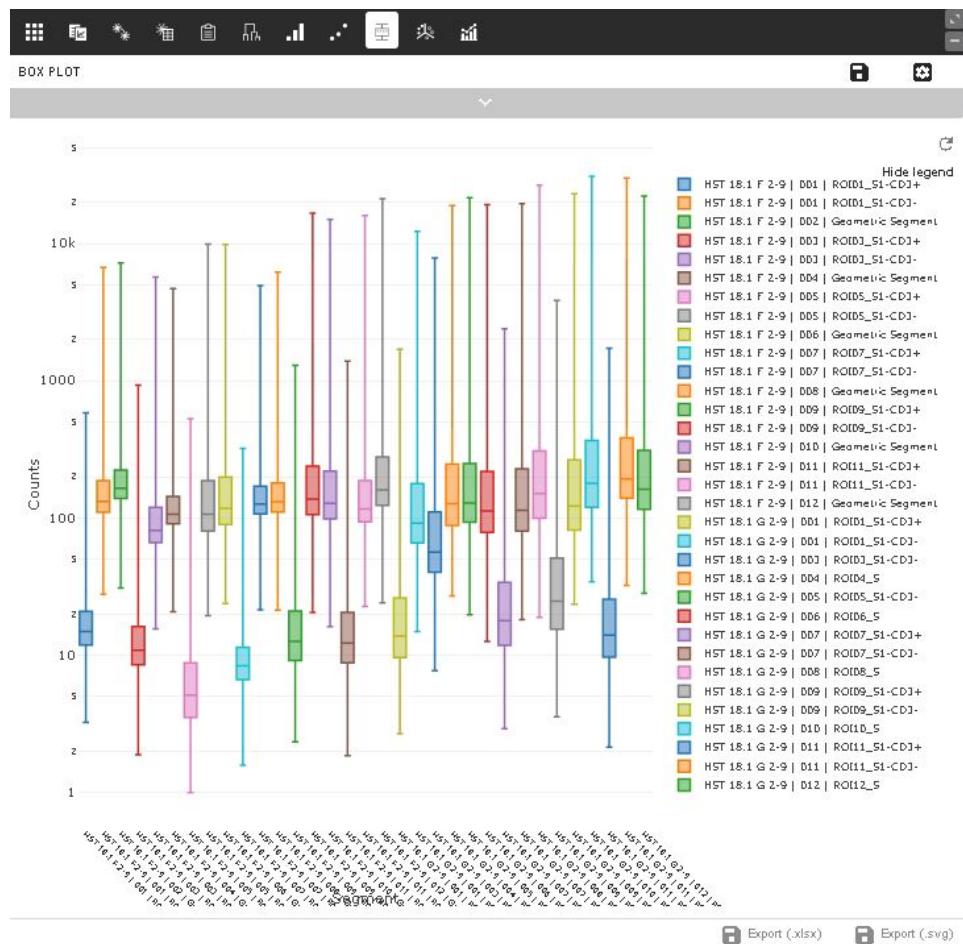


Figure 84: Box plot

- Hover over a box on the plot to see a pop-up displaying the segment, tags, and the values for the median, maximum, and first and third quartiles.
- Click and drag over an area of interest on the box plot to zoom in.
- The legend shows the color assigned to each plot and its corresponding label.
- Click a color box in the legend to either display or hide the plot.

Visualizations Pane

- To name the visualization, x-axis, or y-axis, select the **gear icon** (see [Figure 85](#)), enter the desired name(s), and select **Save**.
- To save the visualization, select the **Save** icon. Access the saved image later under the **Summary** tab. For more information on accessing saved visualizations, exporting visualizations, or exporting data from visualizations, see [Saving & Exporting on page 97](#).



Figure 85: Save and gear icons

Click the arrow to access the drop-down field (see [Figure 86](#)).

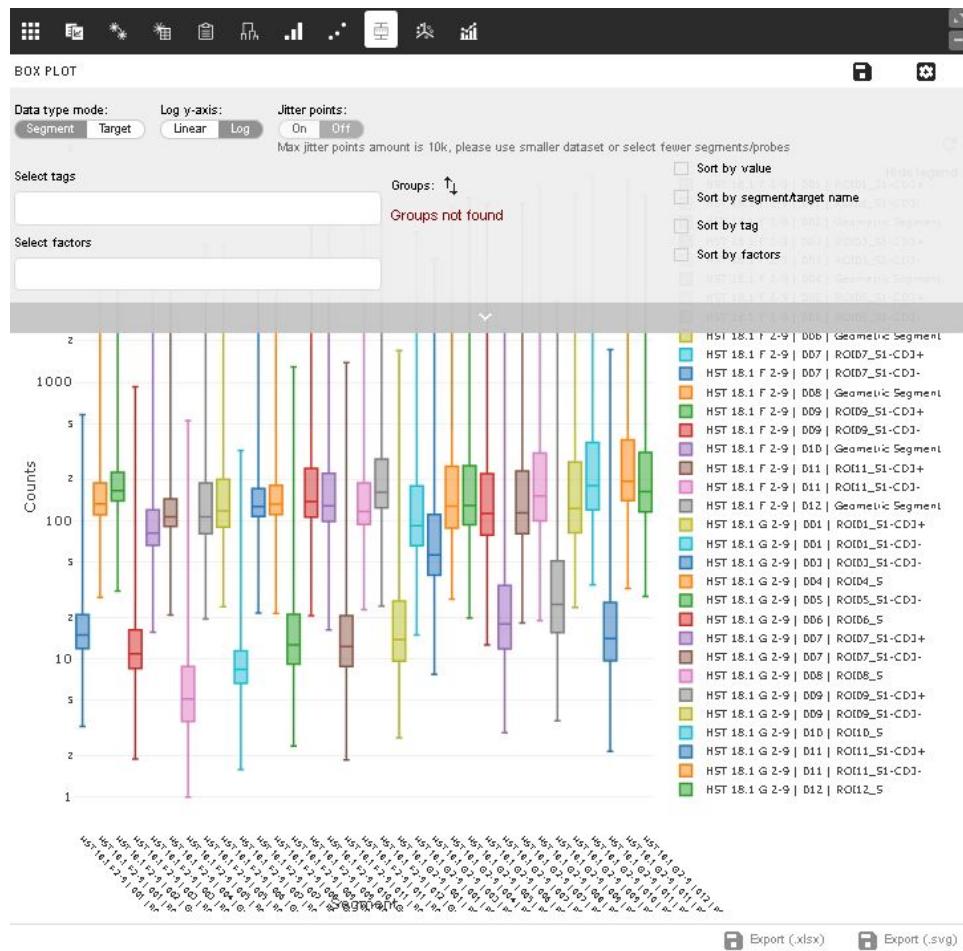


Figure 86: Box plot drop-down field

Here, you may customize the graph by deciding whether to:

- View segments or probes on the x-axis using the **Segment/Target** slider.
- View intensity data in linear or log space using the **Linear/Log** slider.

Visualizations Pane

- **Jitter points** can be turned on **On/Off** using the slider
- **Group** by tags, if desired.
- Use the check boxes to **Sort by value, name, tag, or factor**.

Visualizations Pane**Forest Plot**

The forest plot ([see Figure 87](#)) shows the distribution of ratio values for individual probes across all segments or groups of segments. Forest plots can only be generated from a ratio data dataset. Fold changes are depicted as box and whisker plots along the horizontal axis against each probe name (listed vertically). A vertical axis is shown at ratio value equal to 1 (0 on a Log₂ scale).



Figure 87: Forest plot

- Hover over a box to see a tooltip with the statistics for the distribution, depicted by the plot for each probe.
- The boxes span the first and third quartile of the distribution with a line indicating the median. Whiskers extend between the 95% confidence limits for the data.
- The legend shows the color assigned to each plot and its corresponding label.
- Click a color box in the legend to either display or hide the plot.
- Click and drag over an area of interest on the forest plot to zoom in.

Visualizations Pane

- To name the visualization or x-axis, select the **gear icon** ([see Figure 88](#)), enter the desired name(s), and select **Save**.
- To save the visualization, select the **Save** icon. Access the saved image later under the **Summary** tab. For more information on accessing saved visualizations, exporting visualizations, or exporting data from visualizations, see [Saving & Exporting on page 97](#).



Figure 88: Save and gear icons

Click the arrow to access the drop-down field ([see Figure 89](#)).

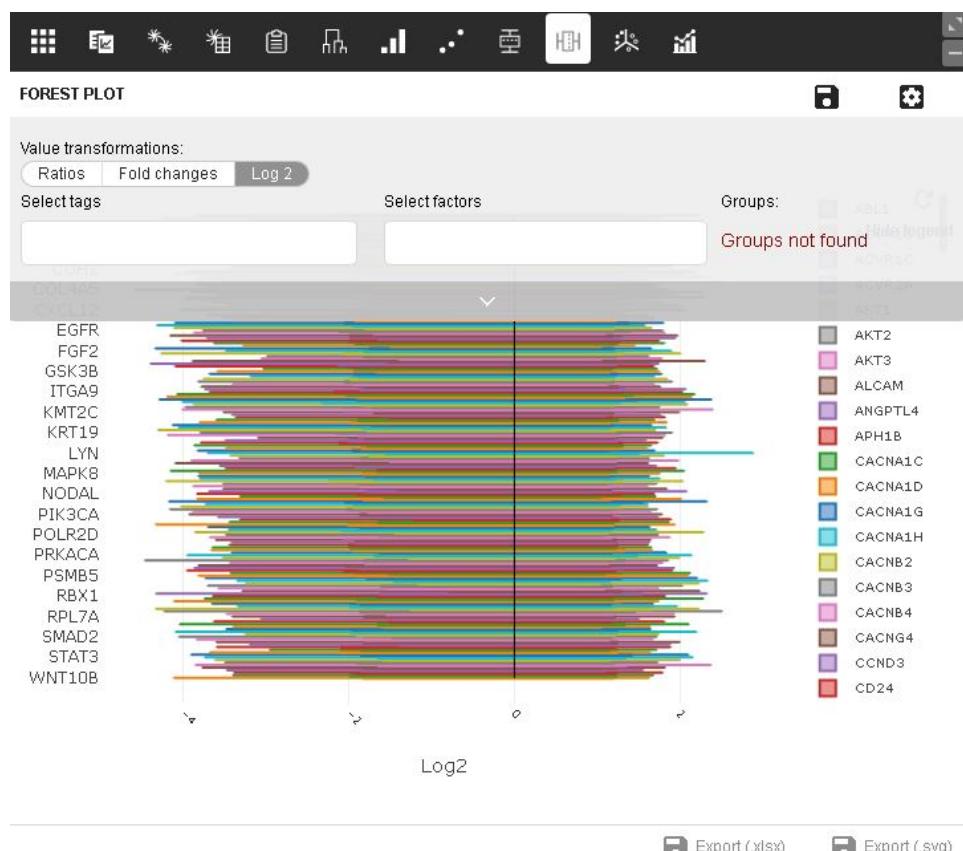


Figure 89: Forest plot drop-down field

Here, you may customize the graph by deciding whether to:

- View intensity data as **Ratios**, **Fold changes**, or in **Log₂**.
- Stratify and color data by **grouping by tags** and tag combinations.
- Color data points by tags:

Visualizations Pane

- Select one or more segment tag for coloring (combination groups will be created, as well).
- Boxes not included in tag groups will be shown as gray.
- To change the color of the grouping, click on the group's color box in the drop-down field; a color selection window will appear.

Visualizations Pane

Principal Component Analysis (PCA) Plot

The PCA plot ([see Figure 90](#)) depicts the first three principal components for the selected dataset along the x-axis, y-axis, and z-axis of a three-dimensional plot.

PCA is a dimensionality reduction technique for visualizing variation within a dataset. Users should view PCA when they want to get a sense of how similar or different a segment is to the others. This technique can be used to identify clusters of similar segments. Each principal component (PC1-3 are plotted as x,y,z axes by default) explains a certain percentage of the variation in the data (PC1 explains the most variation, PC2 second, etc). It's also possible using PCA data to determine which targets contribute to each PC, that is, which are responsible for variation between segments.

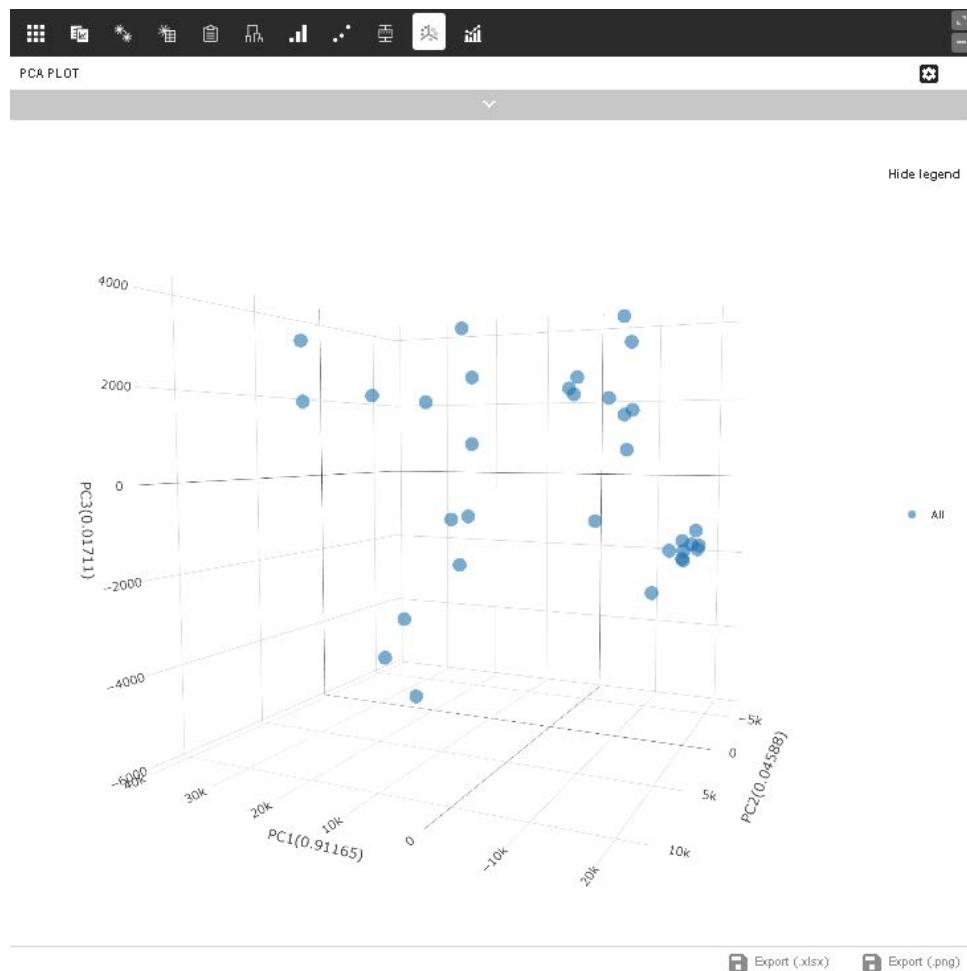


Figure 90: PCA plot

- Click on the plot and drag to rotate along the x, y, or z axis to view the plot in different axes' perspectives.

Visualizations Pane

- Click on a data point to automatically highlight the segment in the segments pane and the scan image viewer.
- Hover over a datapoint on the plot to see a pop-up ([see Figure 91](#)) displaying the segment name it represents, associated tags, and each of its coordinates and to see its three dimensions defined.

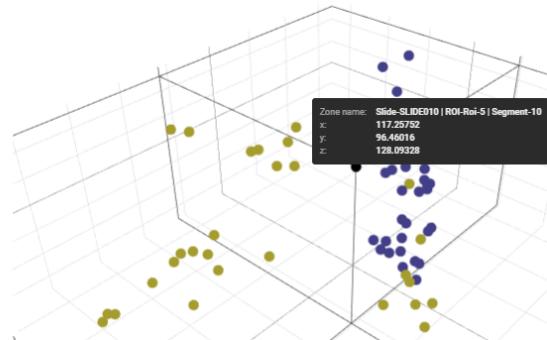


Figure 91: PCA plot hover-over pop-up

- To name the visualization, select the **gear icon** ([see Figure 92](#)), enter the desired name, and select **Save**. Use the check boxes to **Export Columns** and **Display Legend** here, as well.
- To save the visualization, select the **Save** icon. Access the saved image later under the **Summary** tab. For more information on accessing saved visualizations, exporting visualizations, or exporting data from visualizations, see [Saving & Exporting on page 97](#).



Figure 92: Save and gear icons

Click the arrow to access the drop-down field ([see Figure 93](#)).

Visualizations Pane

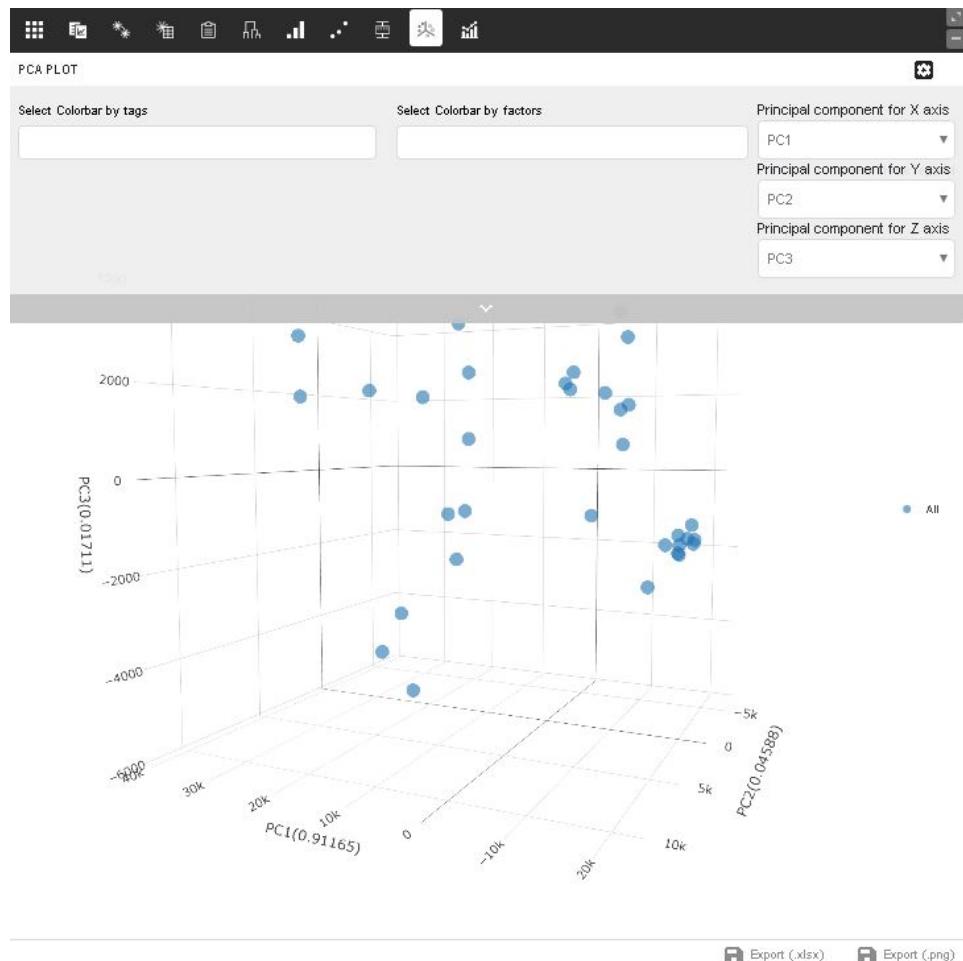


Figure 93: PCA drop-down field. Here, you may customize the graph to:

Here, you may customize the graph to:

- Color segments by tags/factors
- Change the Principal Component axis names

Volcano Plot

A volcano plot ([see Figure 94](#)) is a scatter plot showing the measure of significance (-log₁₀ of p-values) on the y-axis vs the difference in geometric means of probe expression between two groups of segments compared in a statistical test. This visualization is available for datasets which contain t-tests (p-values).

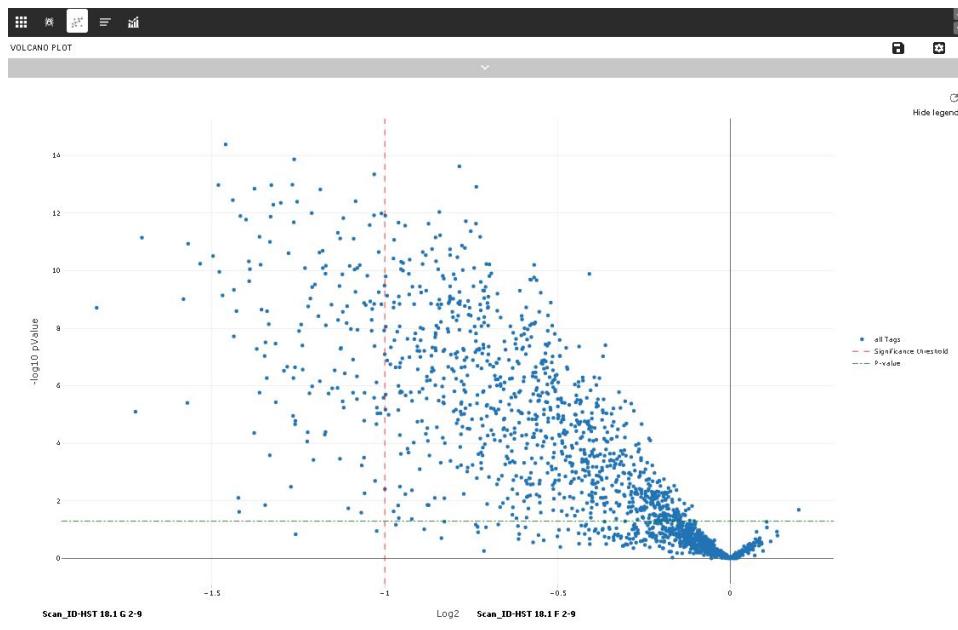


Figure 94: Volcano plot

- If there is more than one statistical test associated with a dataset, select the test of interest at the top of the window.
- Hover over a data point on the plot to see a pop-up displaying the target, p-value, and ratio value associated with that point.
- To name the visualization, x-axis, or y-axis, select the **gear icon** ([see Figure 95](#)), enter the desired name(s), and select **Save**.
- To save the visualization, select the **Save** icon. Access the saved image later under the **Summary** tab. For more information on accessing saved visualizations, exporting visualizations, or exporting data from visualizations, see [Saving & Exporting on page 97](#).



Figure 95: Save and gear icons

Click the arrow to access the drop-down field ([see Figure 96](#)).

Visualizations Pane

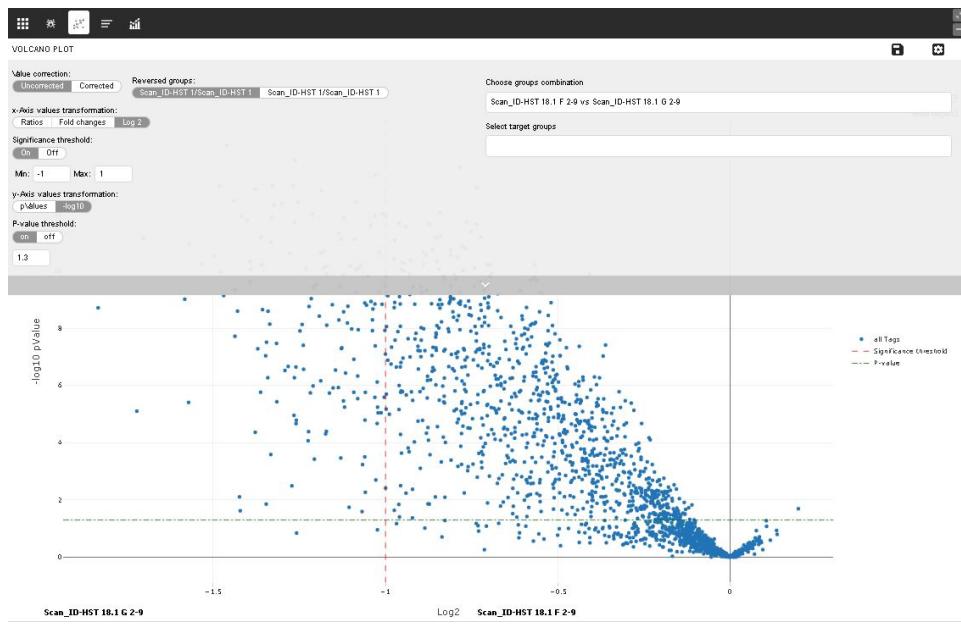


Figure 96: Volcano plot drop-down field

Here, you may customize the graph by deciding whether to:

- Display corrected or uncorrected data using the **Uncorrected/Corrected** slider.
- Select the **ratio** (e.g., Tumor/Immune or Immune/Tumor) to use in the plot by toggling the **Reversed groups**.
- View ratio data as **Ratio**, **Fold changes**, or **Log2 ratio** on the x- and y-axes
- Establish **Significance** or **P-value** threshold settings.

Click on any datapoint in the volcano plot to see detailed box and scatter plot of the data behind it ([see Figure 97](#)).

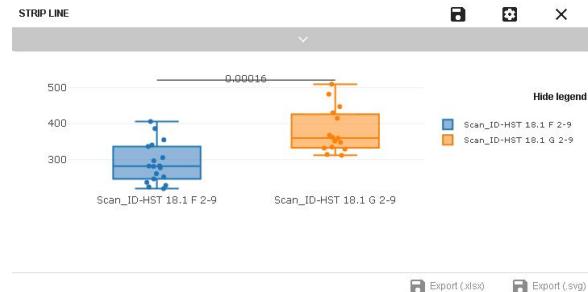


Figure 97: Box and scatter plot

Visualizations Pane**Trend Plot**

Trend plots ([see Figure 98](#)) show line graphs for all selected probes in the dataset. Segments are ordered along the x-axis, while probe counts are along the y-axis.

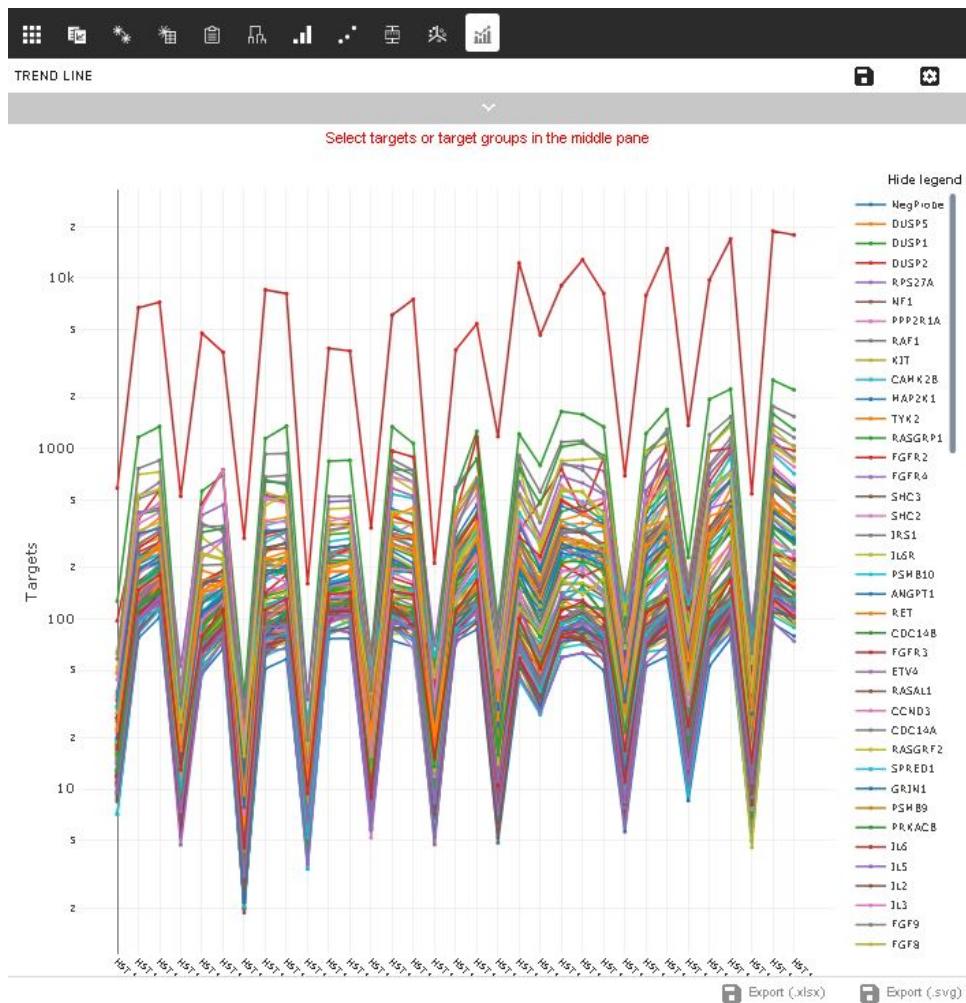


Figure 98: Trend plot

- In NGS data, by default, no probes are selected. Select the probes of interest to see the trend plot materialize.
- Hover over lines to see a pop-up with probe names and p-values.
- To name the visualization, select the gear icon ([see Figure 99](#)), enter the desired name, and select Save. Use the check boxes to Export Columns and Display Legend here, as well.



Figure 99: Save and gear icons

Visualizations Pane

- To save the visualization, select the **Save** icon. Access the saved image later under the **Summary** tab. For more information on accessing saved visualizations, exporting visualizations, or exporting data from visualizations, see [Saving & Exporting on page 97](#).

Click the arrow to access the drop-down field ([see Figure 100](#)).

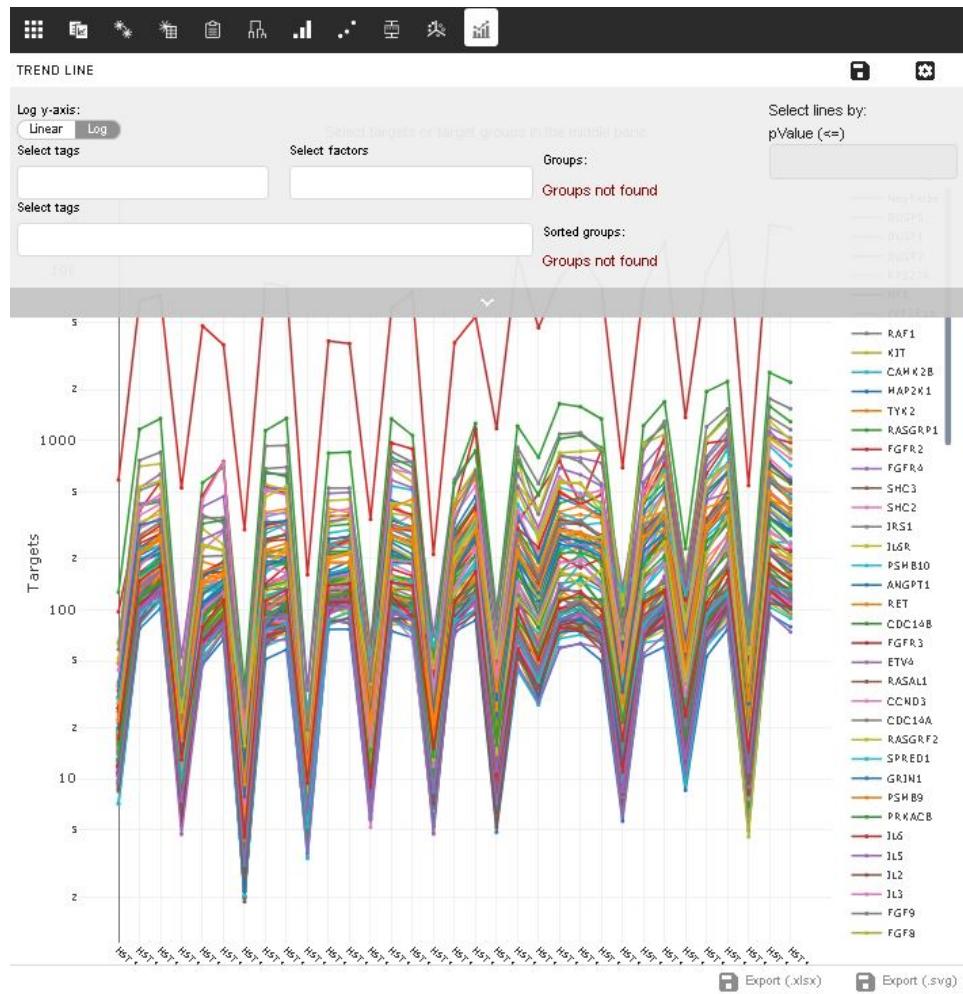


Figure 100: Trend line drop-down field

Here, you may customize the graph by deciding whether to:

- Use the slider to switch between **linear** and **log** values for the y-axis.
- Choose **Segment grouping by tags** or **factors** or to **sort segments by tags**.
- You can select lines by **establishing the maximum p-value**. Any lines that are representing results with that p-value or better will be selected.

Visualizations Pane

For information on saving & exporting images, see [Saving & Exporting on page 97](#).



Figure 101: Save and gear icons

Saving & Exporting

7 Saving & Exporting

Save and Export functions are primarily featured in the **Visualizations pane**.

Save the present visualization using the **Save** icon ([see Figure 102](#)) and access it later under the **Summary** tab.

Establish a name for the present visualization using the **gear** icon ([see Figure 102](#)).

Export the present visualization in **.svg** format using the **Export(.svg)** button ([see Figure 103](#)).



Figure 102: Save and gear icons



Figure 103: Export data from visualizations (.xlsx) or visualization images (.svg)

Heatmap, Cluster, and Bar graphs also have the option to be exported as scalable **.png** files.

Export the data upon which this visualization is based in **.xlsx** format using the **Export(.xlsx)** button ([see Figure 103](#)).

Export the whole dataset in **.xlsx** format from the Datasets pane ([see Manage Datasets on page 62](#)).

SAVING A VISUALIZATION

1. Select the **Save** icon to save the present visualization.
2. Give this visualization a name, description, and tags (if desired) ([see Figure 104](#)).
3. Select **Save**.

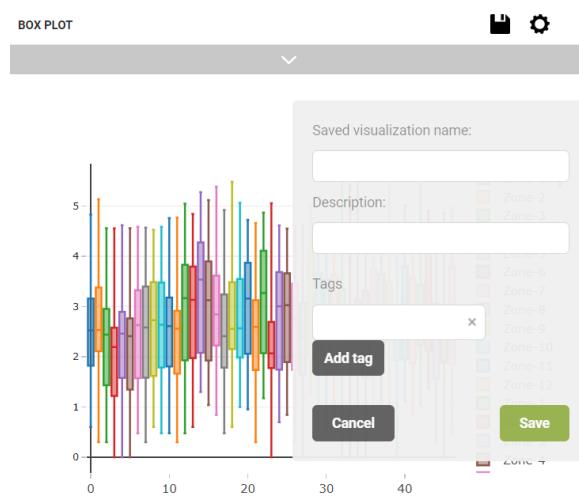


Figure 104: Saving a visualization

ACCESSING SAVED VISUALIZATIONS

1. Select the **Summary** tab in the **Visualizations pane**.
2. Select **Saved Visualizations** ([see **Figure 105**](#)).
3. Select the visualization of interest from the list.

4. You may select another visualization (if available) to compare to the present image by selecting it at the bottom of the window ([see **Figure 106**](#)).
5. You may review the segments and probes that make up this image by selecting the arrow at the top of the window.



Figure 105: Saved visualizations tab under Summary on Visualizations pane

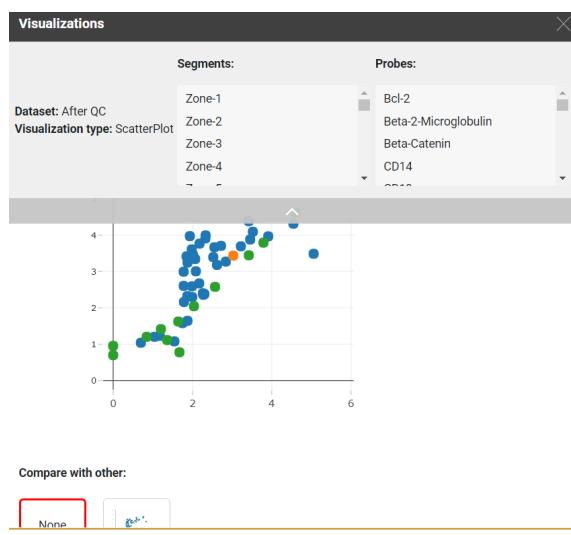


Figure 106: Comparing saved visualizations

Saving & Exporting

EXPORTING VISUALIZATIONS

1. Select the **Export(.svg)** or **Export(.png)** (not available for all visualizations) button at the bottom of the **Visualizations pane** to export the present visualization.
2. The visualization will be exported from the DSP system in respective format. Exports in *.png* are scalable.

EXPORTING DATA ON WHICH A VISUALIZATION IS BASED

1. Select the **Export(.xlsx)** button at the bottom of the **Visualizations pane** to export the data upon which the present visualization is based.
2. The spreadsheet will be exported from the DSP system in *.xlsx* format.

Algorithm Details

Algorithm Details

QC

See below for details on calculations performed during QC.

MINIMUM NUCLEI COUNT

Segments will be flagged when the minimum nuclei counts is less than the provided value.

MINIMUM SURFACE AREA

Segments will be flagged when the minimum surface area is less than the value provided (in μm^2).

BIOLOGICAL PROBE QC

Grubbs test looks for outlier probes either low or high within a target and ROI. Probes that are outliers in a proportion of ROIs above the user-set threshold are removed from all ROIs and considered "global" outliers. Probes that are outliers in a proportion of ROIs below the user-set threshold are removed only from those ROIs in which they are an outlier and are considered "local" outliers.

The limit of quantitation (LOQ) for each AOI is calculated as {user-defined value} geometric standard deviations above the geometric mean of the negative probes. This is calculated after the exclusion of outlier probes.

NORMALIZATION

Normalization factor for each segment shall be calculated as the ratio of the average of all geometric mean or arithmetic mean of selected normalization probes across all segments to the geometric mean or arithmetic mean of selected normalization probes for that segment.

SCALING

When scaling to surface area is selected, probe count values for a particular segment shall be multiplied by a surface area scaling factor. Scaling factor for each segment shall be calculated as the ratio of the calculated geometric mean, average, median, maximum or minimum surface area across all actively selected segments in the dataset to the surface area of that segment.

Algorithm Details

When scaling to nuclei count is selected, probe count values for a particular segment shall be multiplied by a nuclei scaling factor. The scaling factor is calculated as the ratio of the calculated geometric mean, average, median, maximum or minimum nuclei count across all actively selected segments in the dataset to the nuclei count of that segment.

HEATMAP

Heatmaps are displayed as linear counts or \log_2 transformed values.

CLUSTER

The cluster displays an unsupervised hierarchical clustering where the selected data represents log-transformed and z-scored values. Clustering is performed based on correlation to determine the position in the clustering dendrogram. The cluster heatmap will plot each segment-probe cell according to determined position in the color representing the z scores. When exporting data from visualization, you will be exporting only the values you see, which are z-scores.

SCATTER PLOT

R^2 value is calculated as the Pearson correlation coefficient; **RSQ** formula in Excel. Equation is also per slope and the intercept formula in Excel.

BAR GRAPH

Bar graphs are displayed as linear or \log_2 transformed values.

When grouping: Median.

Error bars are either the Standard deviation of values in a group, or standard error.

BOX PLOTS

Box plots are a convenient way of depicting subsets of your experiment through their quartiles. Box plots have lines extending vertically from the boxes (whiskers) that indicate variability outside the upper and lower quartiles. Outliers may be plotted as individual points. These visualizations display differences between subsets of an experiment without making any assumptions about the underlying statistical distribution; they are non-parametric. The box extends between 25th and 75th quartile. The whiskers extend to minimum and maximum.

Algorithm Details

STRIP PLOTS

Strip plots depict one probe per visualization. Dots on the strip plot represent each value. The line shows the median for the group. Min, max and +_ 1.5IQR are also displayed.

FOREST PLOTS

The forest plot shows the distribution of ratio values for individual probes across all segments or groups of segments. Forest plots can only be generated from a ratio data dataset. Fold changes are depicted as box and whisker plots along the horizontal axis against each probe name (listed vertically). A vertical axis is shown at ratio value equal to 1 (0 on a log₂ scale). Also displayed are: median, q1, q3, and 95% confidence limits.

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