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Making Cancer History®

SOP For Data Management Using the GeoMx DSP Analysis Software Suite

Data visualization, quality control, normalization, data analysis and exporting data

1. Technical platform

The GeoMx® Digital Spatial Profiling (DSP) technology is a novel platform developed by NanoString. This product relies upon antibodies coupled to photocleavable oligonucleotide tags. After the incubation of antibodies to slide-mounted tissue sections, cell types of interest with fluorescent morphology markers to create fluorescent images for selecting region of interest (ROIs). The oligonucleotide tags are released from discrete regions of the tissue via UV exposure. Released tags are quantitated in an nCounter® and counts are mapped back to tissue location, yielding a spatially resolved digital profile of analyte abundance.

This SOP describes the workflow and process to manage data outputs generated from the DSP assay. The DSP analysis software is used to obtain initial data set data mapped to the image slide, and to perform quality control, normalization and basic statistical analysis.

2. Generation of Initial Dataset from a study

Description: Generate the initial data set that will be used for experiment quality control and normalization.

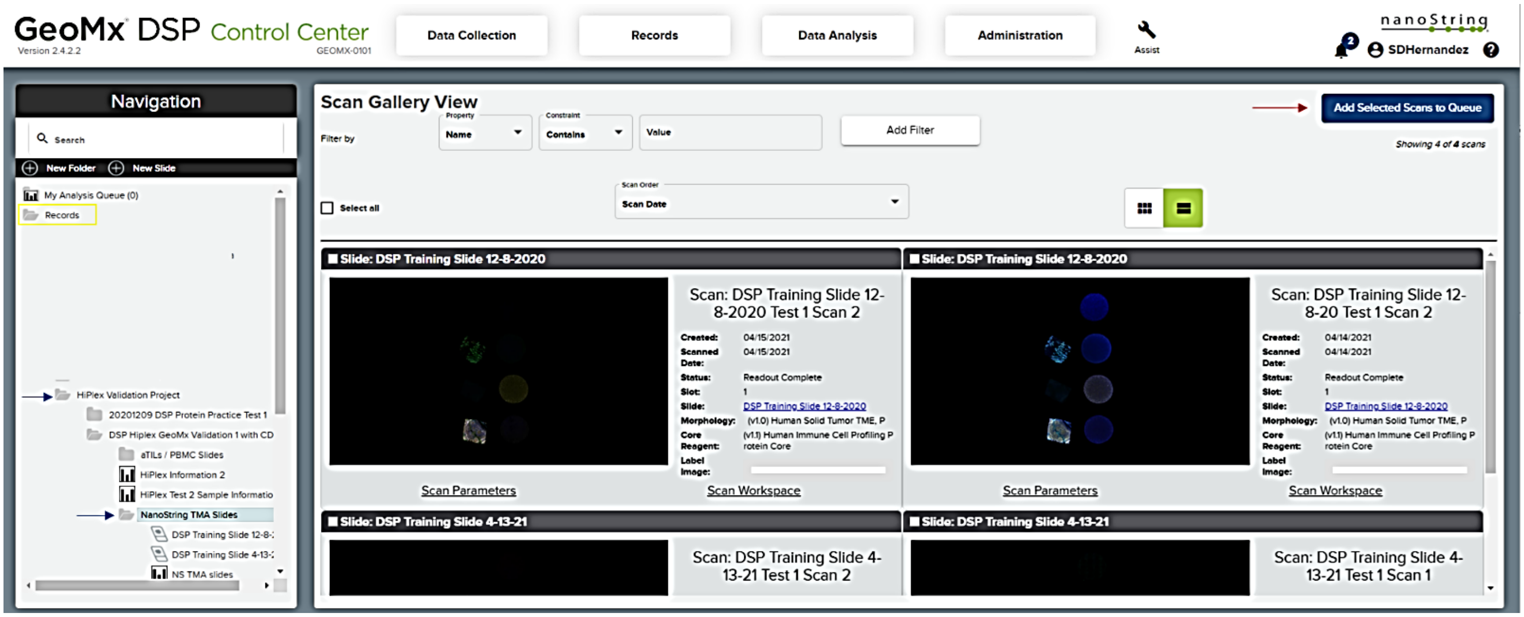
-In the GeoMx DSP analysis suite click in “Records” (Yellow rectangle in screenshot 1)

-Select the folder and sub-folder with the project (e.g. blue arrows in screenshot 1).

-From the “Scan Gallery view”, select all the slides that belong to the specific study by checking on “select all” or checking each square next to the Slide ID (on the top of each image)

-click “Add Selected Scans to Queue” (red arrow in screenshot 1).

Screenshot 1:



Property: Name

Constraint: Contains

Value

Scan Order: Scan Date

Slide: DSP Training Slide 12-8-2020

Scan: DSP Training Slide 12-8-2020 Test 1 Scan 2

Created: 04/15/2021
Scanned Date: 04/15/2021
Status: Readout Complete
Slot: 1
Slide: DSP Training Slide 12-8-2020
Morphology: (v1.0) Human Solid Tumor TME, P
Core Reagent: (v1.1) Human Immune Cell Profiling Protein Core
Label Image:

Slide: DSP Training Slide 12-8-2020

Scan: DSP Training Slide 12-8-20 Test 1 Scan 2

Created: 04/14/2021
Scanned Date: 04/14/2021
Status: Readout Complete
Slot: 1
Slide: DSP Training Slide 12-8-2020
Morphology: (v1.0) Human Solid Tumor TME, P
Core Reagent: (v1.1) Human Immune Cell Profiling Protein Core
Label Image:

Slide: DSP Training Slide 4-13-21

Scan: DSP Training Slide 4-13-21 Test 1 Scan 2

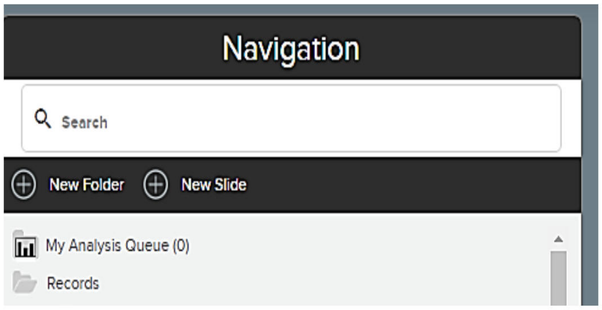
Slide: DSP Training Slide 4-13-21

Scan: DSP Training Slide 4-13-21 Test 1 Scan 1

- Click “Data Analysis”
  - Click “New Study from Queue”
  - Label the Study with Project Name.

An Initial Dataset will be generated and can be accessed from the list of project in “Records” in the “Navigation pane” (the following label recognizes Analysis files 📊, see screenshot 2)

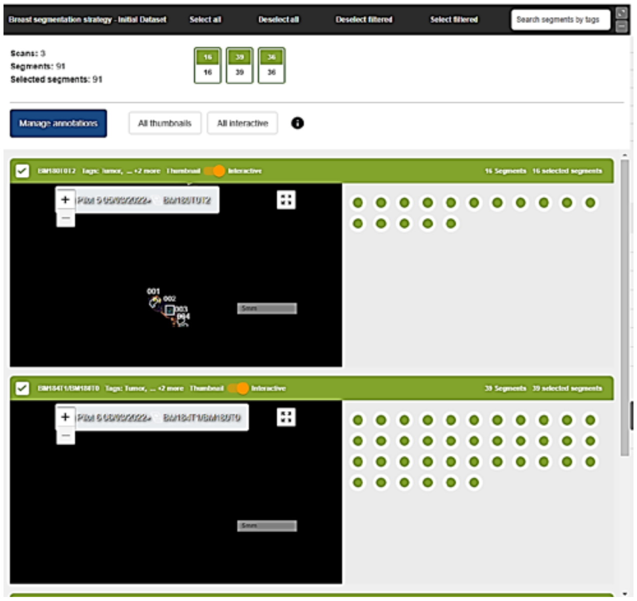
Screenshot 2:



The following notes are provided as a guide to locate specific tools in the DSP analysis suite window:

-Image slides with ROI and segmentation are located in the left pane (screenshot 3).

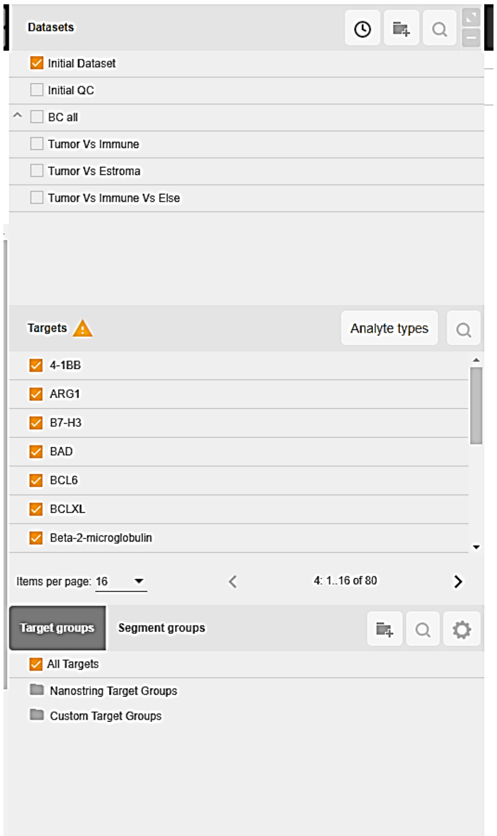
<u>Screenshot 3</u>



Scans: 3
Segments: 91
Selected segments: 91

-All datasets are displayed in the middle of the window including Initial Dataset, and any other analysis done to each set. This area will also display Targets and targets groups (screenshot 4).

<u>Screenshot 4:</u>



☑ Initial Dataset
☐ Initial QC
☐ BC all
☐ Tumor Vs Immune
☐ Tumor Vs Estroma
☐ Tumor Vs Immune Vs Else

☑ 4-1BB
☑ ARG1
☑ B7-H3
☑ BAD
☑ BCL6
☑ BCLXL
☑ Beta-2-microglobulin

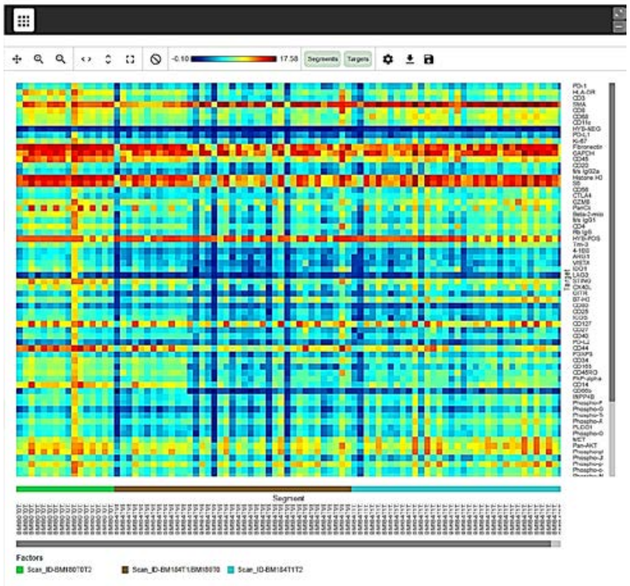
Items per page: 16

4: 1..16 of 80

☑ All Targets
Nanostring Target Groups
Custom Target Groups

-The initial dataset heatmap, statistical test results and other visualization tools are found in the right pane of the window (screenshot 5).

Screenshot 5:



- A heatmap of the initial dataset is automatically generated when a study is created.

-Perform a visual observation of the heatmap obtained from the Initial Dataset.

-Identify if there is any visual pattern that may indicate an experiment issue such as negative DSP counts in all markers (Blue in the heatmap) including positive controls, or columns with consistently high DSP counts for all markers, including negative controls.

-If the regions of interest have any area with known biological biomarker expression (e.g. CK+ in epithelial AOI vs CK- in Immune AOI) review if the expected pattern is observed.

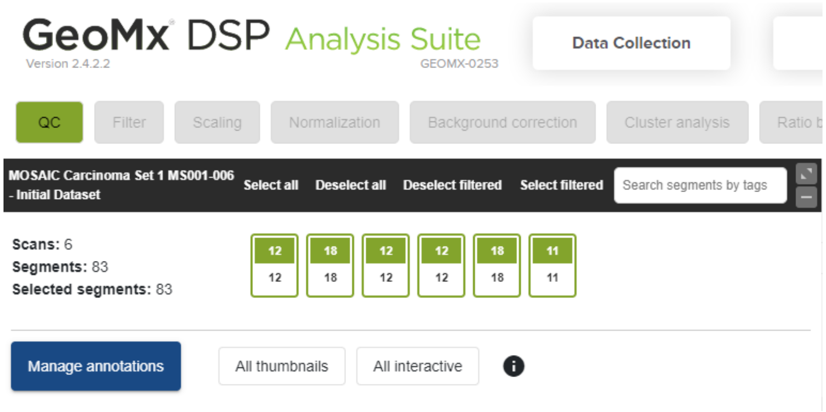
-After initial visualization of the Initial Dataset, we will proceed to the experiment Quality Control.

3. Experiment Quality Control (QC).

Description: Computes and returns nanoString quality control metrics and flags.

-Click the “QC” icon to select parameters for quality control. This is the only icon available when a study is recently created and only an initial dataset visualized (screenshot 6)

Screenshot 6:



Scans: 6
Segments: 83
Selected segments: 83

-Select Quality control (QC) parameters: In most cases, there is no need to change the default QC settings. They are customizable; however, altering these parameters may be appropriate to prevent flagging of useful data.

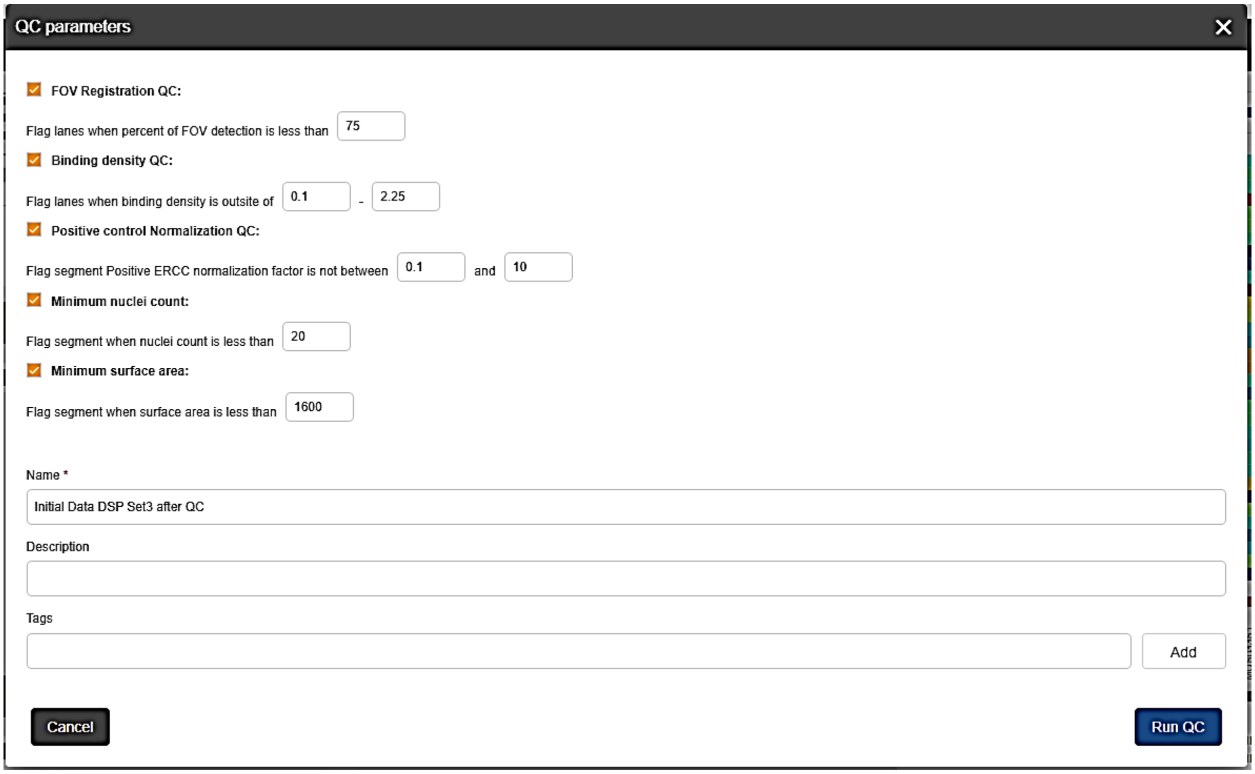
The default QC settings are the following (screenshot 7):

- FOV Registration QC.
  - Flag lanes when percent of FOB detection is less than **75**
- The Binding Density QC:
  - Flag lanes when binding density is outside of **0.1-2.25**
- The Positive Control Normalization QC:
  - Flag segment Positive ERCC normalization factor is not between: **0.1 and 10** (This parameter is the only one that TMP modified from the default settings 0.3 and 3 to 0.1 and 10)
- Minimal nuclei count:
  - Flag segments when nuclei count is less than **20**
- Minimal surface area
  - Flag segments when surface area is less than **1600**.

-After selecting QC parameters write “Name” (e.g. DSP_set_1_QC) and any additional description as needed.

-Click Run QC.

Screenshot 7:



☑ **FOV Registration QC:**

Flag lanes when percent of FOV detection is less than 75

☑ **Binding density QC:**

Flag lanes when binding density is outside of 0.1 - 2.25

☑ **Positive control Normalization QC:**

Flag segment Positive ERCC normalization factor is not between 0.1 and 10

☑ **Minimum nuclei count:**

Flag segment when nuclei count is less than 20

☑ **Minimum surface area:**

Flag segment when surface area is less than 1600

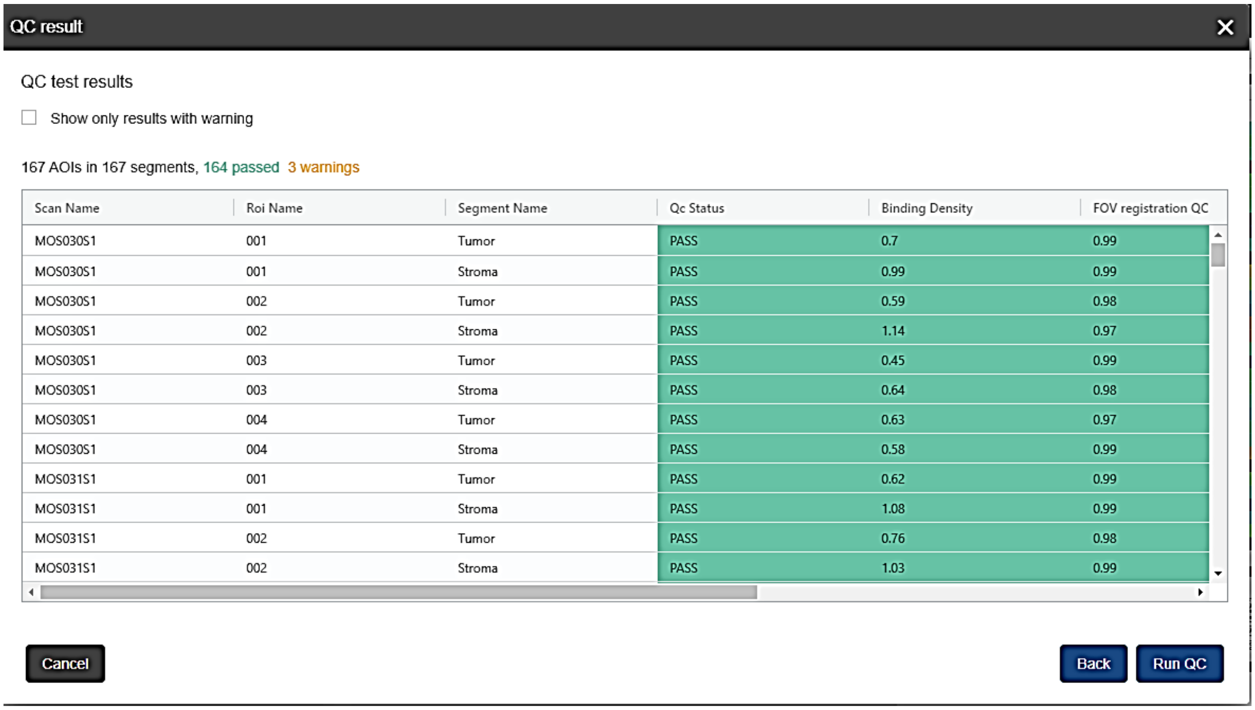
Name * Initial Data DSP Set3 after QC

Description

Tags

-A window will be displayed showing the results of the QC including warnings (screenshot 8).

Screenshot 8:



QC test results

☐ Show only results with warning

167 AOIs in 167 segments, 164 passed 3 warnings

Scan Name	Roi Name	Segment Name	Qc Status	Binding Density	FOV registration QC
MOS030S1	001	Tumor	PASS	0.7	0.99
MOS030S1	001	Stroma	PASS	0.99	0.99
MOS030S1	002	Tumor	PASS	0.59	0.98
MOS030S1	002	Stroma	PASS	1.14	0.97
MOS030S1	003	Tumor	PASS	0.45	0.99
MOS030S1	003	Stroma	PASS	0.64	0.98
MOS030S1	004	Tumor	PASS	0.63	0.97
MOS030S1	004	Stroma	PASS	0.58	0.99
MOS031S1	001	Tumor	PASS	0.62	0.99
MOS031S1	001	Stroma	PASS	1.08	0.99
MOS031S1	002	Tumor	PASS	0.76	0.98
MOS031S1	002	Stroma	PASS	1.03	0.99

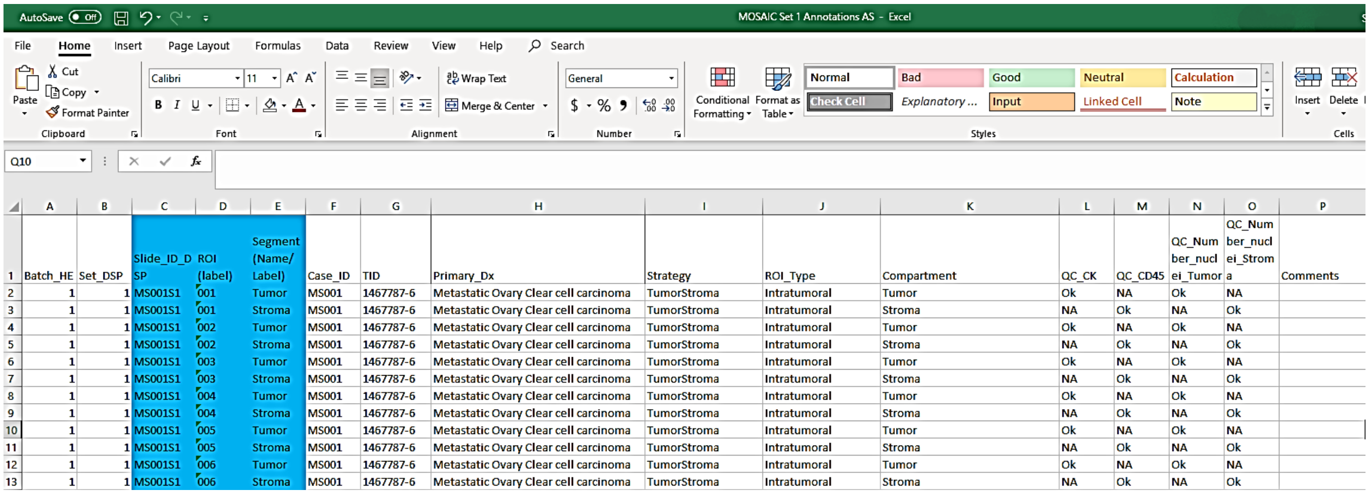
-Click “Run QC”.
-Check for QC Flags: Samples that fail to pass one of the QC parameters will display a flag in QC status. QC flags requires a closer examination of the affected lane prior to proceed with analysis. Oftentimes, flags are raised due to intended attributes of a CodeSet or sample. QC flags do NOT necessarily mean that data is unreliable. To determine whether a QC flag is indicating a critical problem, examine the raw and normalized data.

<u>What to do if a QC flag is displayed</u>
We recommend reviewing carefully the AOI flagged:
-Low binding density or high positive control normalization usually indicates issue related to the assay.
-Low nuclei count, indicates that the number of nuclei using the DSP analysis suite is lower than the threshold (20), review image and confirm accuracy of this calculations (The number of nuclei DSP outputs may be overestimated or underestimated, this requires confirmation by a pathologist).
-Low surface area refers to the threshold of area UV lighted for AOI. Review thresholds agreed for specific study, and number of nuclei in the area.

In case of Flag, discuss with Data analysis team and pathologist to consider them included or excluded from the analysis.

4. Pathology Annotations and metadata

Description: Pathology or relevant metadata to define sample groups should be assigned for experiments in which fold-change estimates and their statistical significance will be studied. These annotations can also be used in Advanced Analysis.
The following annotations are recorded as standard at TMP-IL: diagnosis; strategy for tumor segmentation, spatially defined ROI type (, e.g. intratumoral, versus invasive margin), biological compartment obtained after segmentation (e.g. Tumor, vs. Immune) and a pathology quality control of immunofluorescence biomarkers by each ROI.
The file used to annotate this data should have similar identifiers to easily merge with DSP annotation template file (Slide ID, ROI, Segment).
Additional metadata such as clinical information of recurrence and time points can be also added in this file. Example of this annotations are illustrated in screenshot 9.
<u>Screenshot 9:</u>



Batch_HE	Set_DSP	Slide_ID_DSP	ROI (label)	Segment (Name/ Label)	Case_ID	TID	Primary_Dx	Strategy	ROI_Type	Compartment	QC_CK	QC_CD45	QC_Number_nuclei_Tumor	QC_Number_nuclei_Stroma	Comments
1	1	MS001S1	001	Tumor	MS001	1467787-6	Metastatic Ovary Clear cell carcinoma	TumorStroma	Intratumoral	Tumor	Ok	NA	Ok	NA	
1	1	MS001S1	001	Stroma	MS001	1467787-6	Metastatic Ovary Clear cell carcinoma	TumorStroma	Intratumoral	Stroma	NA	Ok	NA	Ok	
1	1	MS001S1	002	Tumor	MS001	1467787-6	Metastatic Ovary Clear cell carcinoma	TumorStroma	Intratumoral	Tumor	Ok	NA	Ok	NA	
1	1	MS001S1	002	Stroma	MS001	1467787-6	Metastatic Ovary Clear cell carcinoma	TumorStroma	Intratumoral	Stroma	NA	Ok	NA	Ok	
1	1	MS001S1	003	Tumor	MS001	1467787-6	Metastatic Ovary Clear cell carcinoma	TumorStroma	Intratumoral	Tumor	Ok	NA	Ok	NA	
1	1	MS001S1	003	Stroma	MS001	1467787-6	Metastatic Ovary Clear cell carcinoma	TumorStroma	Intratumoral	Stroma	NA	Ok	NA	Ok	
1	1	MS001S1	004	Tumor	MS001	1467787-6	Metastatic Ovary Clear cell carcinoma	TumorStroma	Intratumoral	Tumor	Ok	NA	Ok	NA	
1	1	MS001S1	004	Stroma	MS001	1467787-6	Metastatic Ovary Clear cell carcinoma	TumorStroma	Intratumoral	Stroma	NA	Ok	NA	Ok	
1	1	MS001S1	005	Tumor	MS001	1467787-6	Metastatic Ovary Clear cell carcinoma	TumorStroma	Intratumoral	Tumor	Ok	NA	Ok	NA	
1	1	MS001S1	005	Stroma	MS001	1467787-6	Metastatic Ovary Clear cell carcinoma	TumorStroma	Intratumoral	Stroma	NA	Ok	NA	Ok	
1	1	MS001S1	006	Tumor	MS001	1467787-6	Metastatic Ovary Clear cell carcinoma	TumorStroma	Intratumoral	Tumor	Ok	NA	Ok	NA	
1	1	MS001S1	006	Stroma	MS001	1467787-6	Metastatic Ovary Clear cell carcinoma	TumorStroma	Intratumoral	Stroma	NA	Ok	NA	Ok	

-After merging and uploading the data to DSP analysis suite software, perform a QC to make sure the file has not be shifted. This is accomplished by reviewing random cases or searching Specific Tags.

5. Manage Annotations

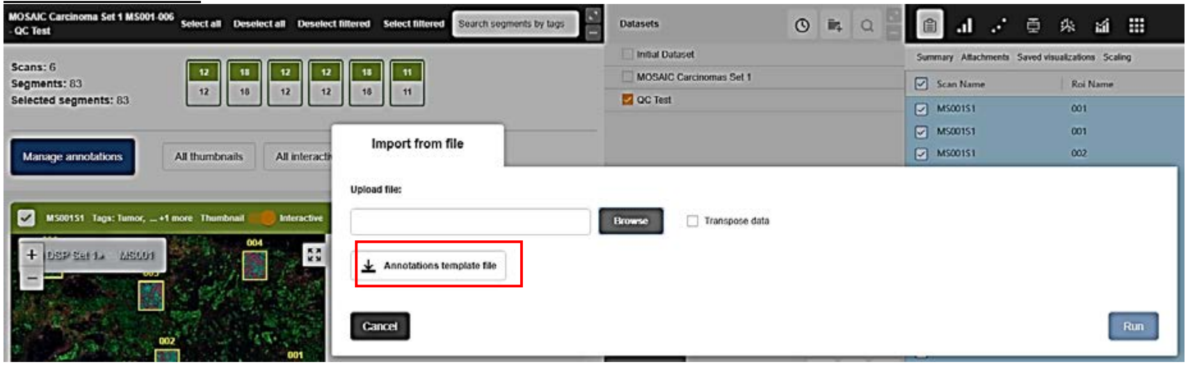
Description: The GeoMx DSP analysis suite allows to incorporate annotations for further analysis.

-First, download annotation template file
-Add metadata in the template file, and finally
-Upload merged file to DSP analysis suite.

To accomplish this, follow these steps in the DSP analysis suite:

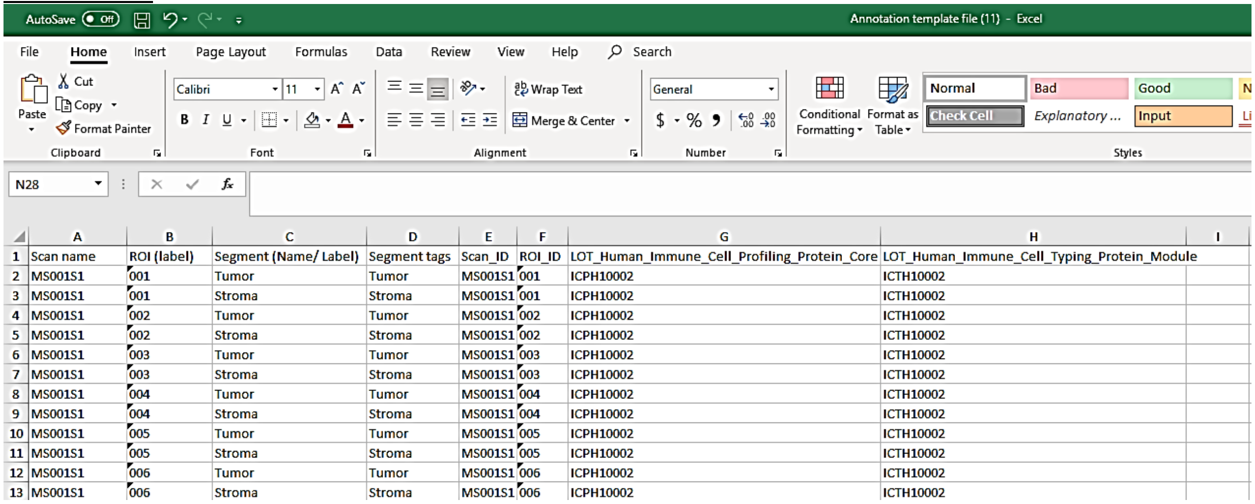
-Click manage annotations (Blue box in the left pane, screenshot 10) then Click “🡇Annotation template file”

Screenshot 10:



The file will provide data of Scan name, ROI, Segments, and reagent lot number used from each core and modules (example showed in screenshot 11).

Screenshot 11:



	A	B	C	D	E	F	G	H	I
1	Scan name	ROI (label)	Segment (Name/ Label)	Segment tags	Scan_ID	ROI_ID	LOT_Human_Immune_Cell_Profiling_Protein_Core	LOT_Human_Immune_Cell_Typing_Protein_Module	
2	MS001S1	001	Tumor	Tumor	MS001S1	001	ICPH10002	ICTH10002	
3	MS001S1	001	Stroma	Stroma	MS001S1	001	ICPH10002	ICTH10002	
4	MS001S1	002	Tumor	Tumor	MS001S1	002	ICPH10002	ICTH10002	
5	MS001S1	002	Stroma	Stroma	MS001S1	002	ICPH10002	ICTH10002	
6	MS001S1	003	Tumor	Tumor	MS001S1	003	ICPH10002	ICTH10002	
7	MS001S1	003	Stroma	Stroma	MS001S1	003	ICPH10002	ICTH10002	
8	MS001S1	004	Tumor	Tumor	MS001S1	004	ICPH10002	ICTH10002	
9	MS001S1	004	Stroma	Stroma	MS001S1	004	ICPH10002	ICTH10002	
10	MS001S1	005	Tumor	Tumor	MS001S1	005	ICPH10002	ICTH10002	
11	MS001S1	005	Stroma	Stroma	MS001S1	005	ICPH10002	ICTH10002	
12	MS001S1	006	Tumor	Tumor	MS001S1	006	ICPH10002	ICTH10002	
13	MS001S1	006	Stroma	Stroma	MS001S1	006	ICPH10002	ICTH10002	

-Follow instructions in the first raw of the Annotation template file to add metadata.
-Save file using a name that can be identified later.
-Click “Manage annotations”
-Click “Browse”
-Select the file with annotation template file containing pathology annotation and metadata.
-Click “Run”

6. Normalization of Initial Dataset after QC.

Description: To select the best normalization approach we use the nanoString normalization R- scripts, the results allows to:

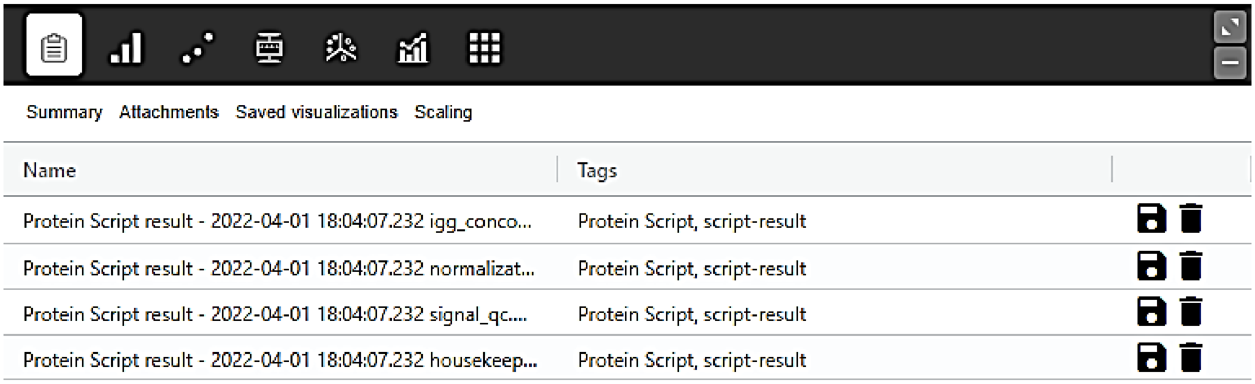
1. Review quality control of ROI/AOI and DSP counts of Probe Targets,
2. Review QC plots for normalization factors,

3. Assess concordance among the different factors, and finally
4. Choose the appropriate normalization method.

The R-scripts can be found in the following webpage https://nanostring.com/products/geomx-digital-spatial-profiler/geoscript-hub/) . In TMP-IL, they are already uploaded and available in the GeoMx DSP analysis suite.

To run the R-scripts, perform the following steps:
-Click "Custom script" (green box located in the upper pane of screen).
-Select proper QC R-scripts depending of assay: Protein, 96-plex RNA, CTA and WTA.
-Click Run

To review files obtained after running scripts:
Use the right pane to Click" Data set summary" and then "Attachments" (Screenshot 12)
Screenshot 12:



Name	Tags
Protein Script result - 2022-04-01 18:04:07.232 igg_conco...	Protein Script, script-result
Protein Script result - 2022-04-01 18:04:07.232 normalizat...	Protein Script, script-result
Protein Script result - 2022-04-01 18:04:07.232 signal_qc....	Protein Script, script-result
Protein Script result - 2022-04-01 18:04:07.232 housekeep...	Protein Script, script-result

For protein analysis, review the following documents:

-Normalization Factor concordance: This script plots HK geomean, negative geomean, area and nuclei data to review concordance and review distribution of the samples by slides and segments. This plot also can be used to detect any cluster of ROI/AOI with low signal strength. A quality control of ROI/AOI to exclude ROI/AOI with poor data (low signal strength) can also be performed by evaluating the Housekeeper Geomean and IgG Geomean by tissue type or other metadata.

-Signal QC: Review the computed "signal-to background" ratio per target (each AOI's data divided by its IgG geomean).Target probes with lower DSP counts than negative control probes and with limited expression range should be considered carefully and potentially excluded .

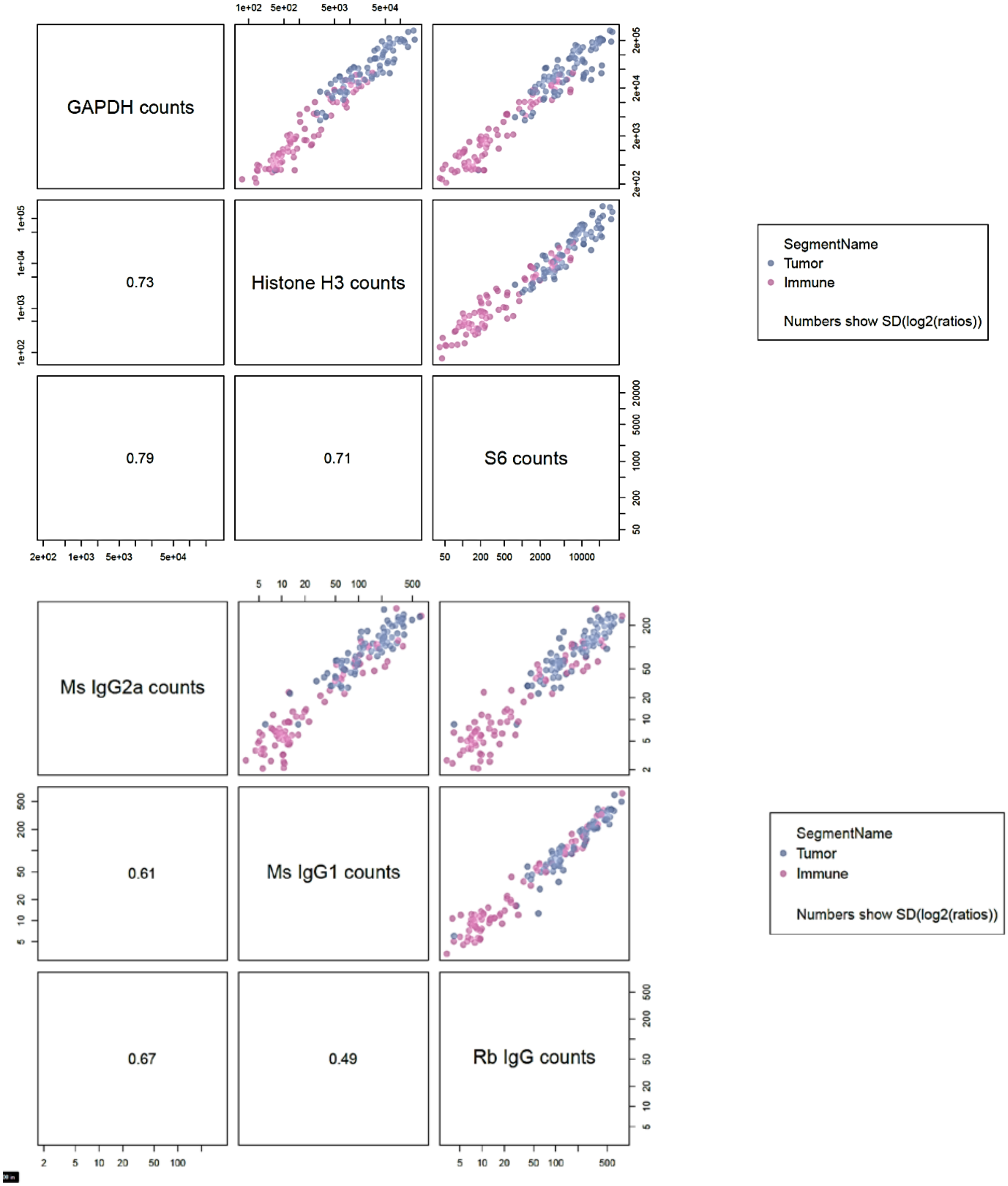
-Housekeeper and IgG concordance: Review correlations among housekeeper proteins and determine the biomarkers with higher concordance for further normalization by housekeeperproteins. Similarly review correlations among negative controls (IgGs) and determine the biomarkers with higher concordance for further normalization by background correction.

After selection of the normalization factors **perform two normalization strategies,** Normalization by background correction and by housekeeper

Follow these steps:
-Click "Normalization" (located in the upper green boxes)
-Select "Normalization Type: Housekeeper or Negative Control
-Average type: Geometric mean.

-Select targets used for normalization based in housekeeper or negative controls with the highest concordance among them. Of note, concordance between Housekeepers and concordance between IgGs can be visualized in the scripts such as the following plots (Screenshot 13):

<u>Screenshot 13:</u>



-Write “Name”: normalization used (e.g. “BC all IgGs”)
-Add any description as needed.
-Click “Run”
-Review data

Cluster Analysis
-Perform cluster analysis with each normalization strategy to evaluate if the data cluster follows any particular trend. To perform this follow these steps:

-Click Cluster Analysis (located in the upper green boxes)

-Write a name and click ”run”. An arrowhead down will appear next to the normalized data file used for the cluster, to visualize it press the arrowhead and then click on “cluster”.

Advanced analysis:
Advanced analysis can be done following “Statistical test” option (located in the upper green boxes), and should be done with guidance of statistician or data analyst.

7. Export Data

Description: All datasets and statistical analysis can be exported to keep documentation of every step of the assay.

As standard, the following data from each project is exported :

a) Initial Data set
b) Initial Data set after QC
c) Normalization by Housekeeper (indicating HK used for normalization)
d) Normalization by Negative control (background correction)

The above files (a-d) can be exported by clicking in the disk image displayed next to each Data Set.

e) Summary of Quality control.

To export this data follow these steps:

   i. Select “Initial Data set after QC”
   ii. In the right pane a summary of the quality control will be displayed, click “Segment Summary” located in the lower bar from the right pane.

f) DSP Scripts

To export this data follow these steps:

   i. Select the “Initial data set after QC”, in the right pane, Use the right pane to Click” Data set summary” and then “Attachments”, then click in the disk next to each script.

Finally, generate a word document with the information of exported data sets, annotations uploaded to them, and parameters used for QC and normalization (See **Annex 1**).

References

1. Van TM, Blank CU. A user's perspective on GeoMxTM digital spatial profiling. Immuno-Oncology Technology. 2019;1:11-8.

2. Hernandez S, Lazcano R, Serrano A, Powell S, Kostousov L, Mehta J, et al. Challenges and Opportunities for Immunoprofiling Using a Spatial High-Plex Technology: The NanoString GeoMx(®) Digital Spatial Profiler. Front Oncol. 2022;12:890410.

3. NanoString I. GeoMx® Protein Assays for Immuno-Oncology 2021 [cited 2022 09/05/2022]. Available from: chrome-extension://efaidnbmnnnibpcajpcglclefindmkaj/https://nanostring.com/wp-content/uploads/PB_MK3350_DSP_IO_Protein_PB_R28.pdf.

4. NanoString I. MAN-10109-01 GeoMxDSPExampleDataAnalysis 2019 [cited 2022 09/05/2022]. Available from: chrome-extension://efaidnbmnnnibpcajpcglclefindmkaj/https://nanostring.com/wp-content/uploads/MAN-10109-01_GeoMx_DSP_Example_Data_Analysis.pdf.

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Signature (Luisa M. Solis Soto) **Date: 09/06/2022**

Annex 1

PARAMETERS USED FOR QC AND NORMALIZATION IN THE EXPORTED DATA SETS *(template)*

DSP project #:

Name of the project:

Project PI:

1. Exported data sets

The files obtained correspond to the following Datasets:

1. Initial Data set
2. Initial Data set after QC
3. Normalization by Housekeeping
4. Normalization by Negative control (background correction)
5. Summary of Quality control
6. DSP Scripts

2. Annotations:

The following pathology annotations and metada were uploaded in the data sets using the GeoMx DSP analysis suite: *(list data uploaded to DSP for data set generation, if none, just indicate none)*

3. Quality control

Quality control was performed in the initial data set with the following parameters (*check if the below parameters were used, if not, change as needed*):

- FOV Registration QC.
    - Flag lanes when percent of FOB detection is less than **75**
- The Binding Density QC:
    - Flag lanes when binding density is outside of **0.1-2.25**
- The Positive Control Normalization QC:
    - Flag segment Positive ERCC normalization factor is not between: **0.1 and 10**
- Minimal nuclei count:
    - Flag segments when nuclei count is less than **20**
- Minimal surface area
    - Flag segments when surface area is less than **1600**.

4. Normalization

Two independent normalization strategies were performed: by background correction and by housekeeping proteins.

The normalization was performed in the “initial data set after DSP quality control”

Normalization strategies were evaluated based in pairwise correlation of the housekeeping proteins (Histone H3, S6 and GAPDH) or negative control normalization (background correction) (Ms IgG1, Rb IgG nd IgG2a).

Based on the above results,

-Normalization by background correction was performed with the following parameters:

-Normalization type: Negative Control

-Average: Type: Geometric mean

-Targets for normalization: e.g. *Ms IgG1 and Rb IgG (IgG2 was not used for normalization based in the low concordance with Ms IgG1 and Rb IgG)*

-Housekeeping normalization was performed with the following parameters:

-Normalization type: Housekeeper

-Average: Type: Geometric mean

-Targets for normalization: e.g. *Histone H3 and S6 (GAPDH was not used for normalization based in the low concordance with Histone H3 and S6).*
