

Feb 06, 2024

Sinai SCENT TMC - Olink NPX manager for Olink Data Analysis

DOI

dx.doi.org/10.17504/protocols.io.bp2l6x5xzlqe/v1

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Protocol Citation: Hui Xie 2024. Sinai SCENT TMC - Olink NPX manager for Olink Data Analysis. **protocols.io**
<https://dx.doi.org/10.17504/protocols.io.bp2l6x5xzlqe/v1>

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Protocol status: Working

We use this protocol and it's working

Created: February 02, 2024



Last Modified: February 06, 2024

Protocol Integer ID: 94624

Keywords: npx manager for olink data analysis, time pcr analysis software, olink data analysis, npx manager software, ct value of the pcr reaction, time pcr analysis, npx manager, time pcr, pcr reaction, assay data, normalized relative protein expression value, using fluidigm, pcr, protein expression, npx, fluidigm

Abstract

To describe the procedures for using Fluidigm “Real-Time PCR Analysis” software and “Olink NPX manager” software to analyze Olink Assay data and generate final result reports. Fluidigm Real-Time PCR Analysis software generates the Ct value of the PCR reaction and Olink NPX manager software calculates the Normalized relative Protein Expression value (NPX) from the Ct value.

Materials

- Computer
- Fluidigm Real-Time PCR Analysis
- Olink NPX manager

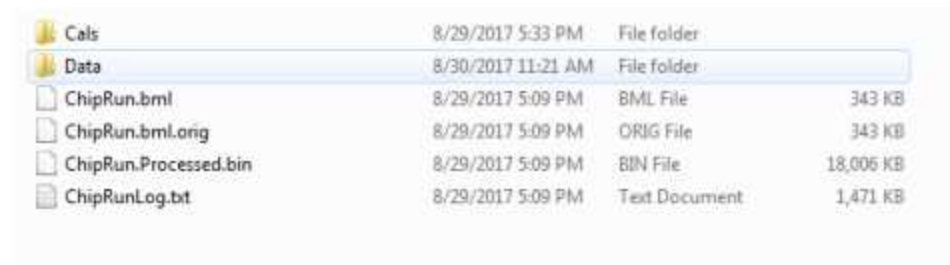
Troubleshooting

Safety warnings

 Not Applicable

Raw data Ct value analysis

- 1 Copy the Biomark File (entire Chip-run folder corresponding to the Olink assay) from the Biomark Computer to USB drive, the size (~1.3 GB). There should be always 2 sub-folders and 4 files in the Chip run folder.



Cals	8/29/2017 5:33 PM	File folder	
Data	8/30/2017 11:21 AM	File folder	
ChipRun.bml	8/29/2017 5:09 PM	BML File	343 KB
ChipRun.bml.orig	8/29/2017 5:09 PM	ORIG File	343 KB
ChipRun.Processed.bin	8/29/2017 5:09 PM	BIN File	18,006 KB
ChipRunLog.txt	8/29/2017 5:09 PM	Text Document	1,471 KB

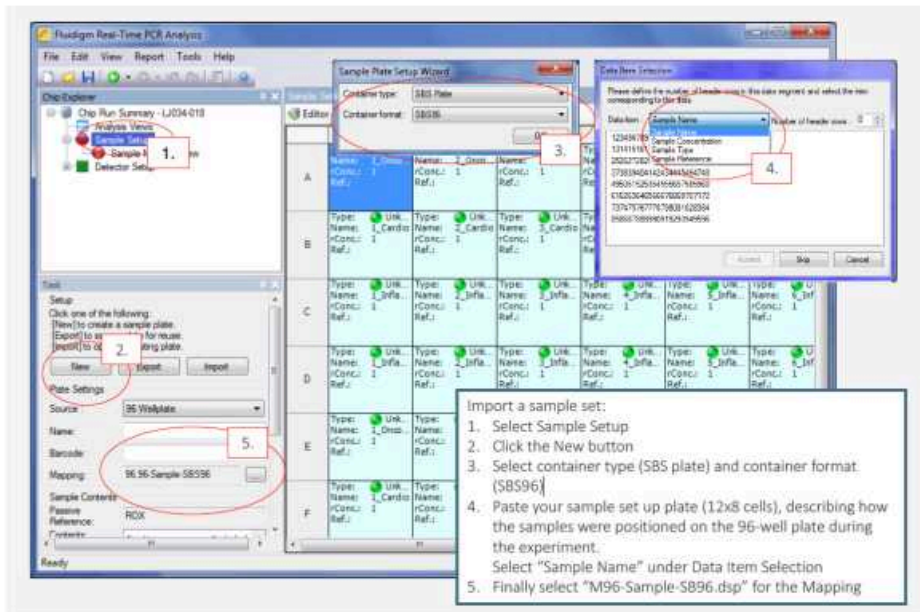
- 2 Connect USB drive from above step with HIMC computer with installed Fluidigm Olink PCR analysis software
- 3 Start the Fluidigm "Real-Time PCR analysis software (Version 4.5.2)"
- 4 Open the Chip run under "Quick Tasks". This will redirect to a new window. Locate and double click the Chip run file (.bml-format) in the copied folder in Step 1.

Notes: Only one Chip run-file can be open at a time. Before opening a new run-file, the first chip run file must be closed.



5 Import a sample set.

- 5.1 Prepare a 96-well-plate sample layout for the Olink Assay in excel according to HIMC format and save in HIMC shared drive or Google drive.
- 5.2 Double click Sample Setup in the Fluidigm Olink PCR analysis software. Select container type (SBS plate) and container format (e.g. SBS96) in the Sample Plate Set-up Wizard, press OK.
- 5.3 Copy the Olink assay sample layout from the plate layout excel file saved in Step 5.1, right click well A1 in the Sample Set-up "Editor" panel.
- 5.4 Select "Sample Name" under Data Item Selection.
- 5.5 Write Run name in the "Name" section under plate settings. Select mapping will open a new window. Different mapping files will be seen in that window.
- 5.6 Select "M96-Sample-SB96.dsp" for the Mapping.



6 Import a Detector setup:

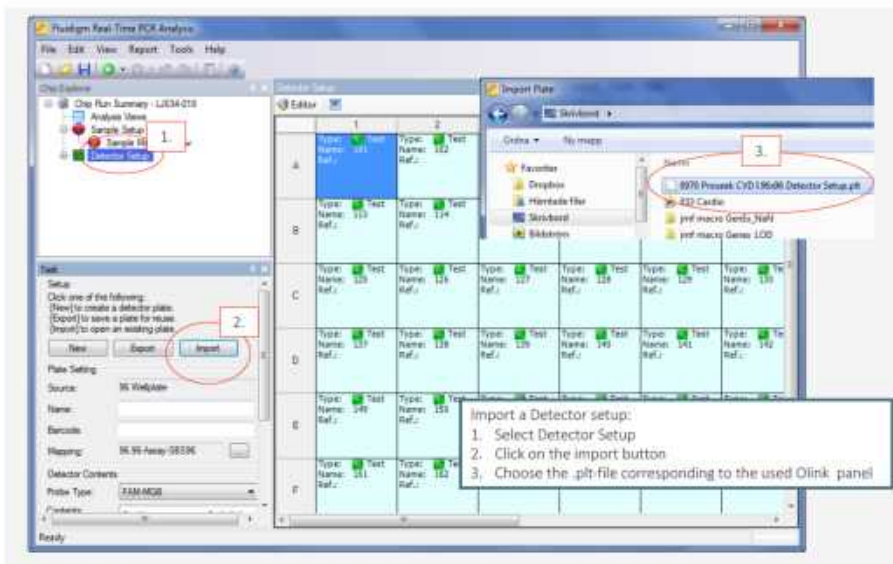
6.1 Select Detector Setup and Click the New button.

6.2 Click on the import button. Choose the .plt-file corresponding to the Olink panel kit used for the assay (i.e. for Inflammation panel use "Mapped Inflammation I 96x96 Detector Setup.plt").

Notes: Check the Olink kit contents Paper insert for Lot # and the proper .plt file information. New version of .plt file may need to be downloaded before analysis based on kit lot#. All the assay .plt files from Olink are downloaded from Olink website and saved on:

J:\gtherapy\HumanImmuneMonitoringCenter\Olink_proteomic2016\NPX_analysisSoftware\mt Sinai

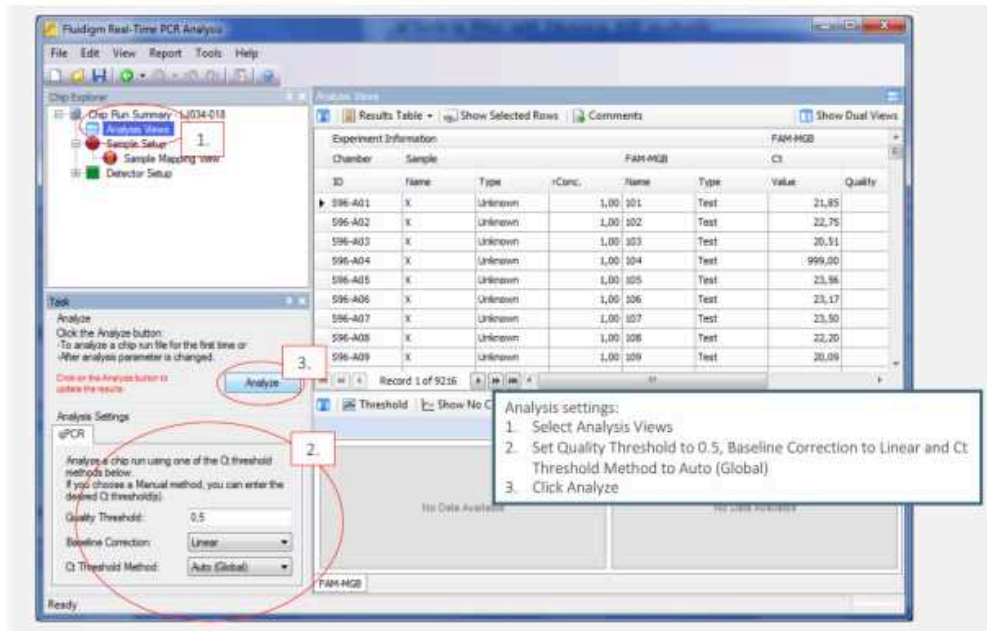
6.3 In the case of a project with different lot # kits, one will need to have the same reference samples in plates with biological samples for normalization purposes. However, if the "Data File No: XXXX" which comes in the paper with the kit is the same, then the data from the different kit lot numbers can be analyzed as a project.



7 Analysis settings:

7.1 Select Analysis Views/ Details Views.

7.2 Set Quality Threshold to "0.5", Baseline Correction to "Linear" and Ct Threshold Method to "Auto (Global)", (by changing the default settings) and Click Analyze button. These settings are common for all the Olink panels.

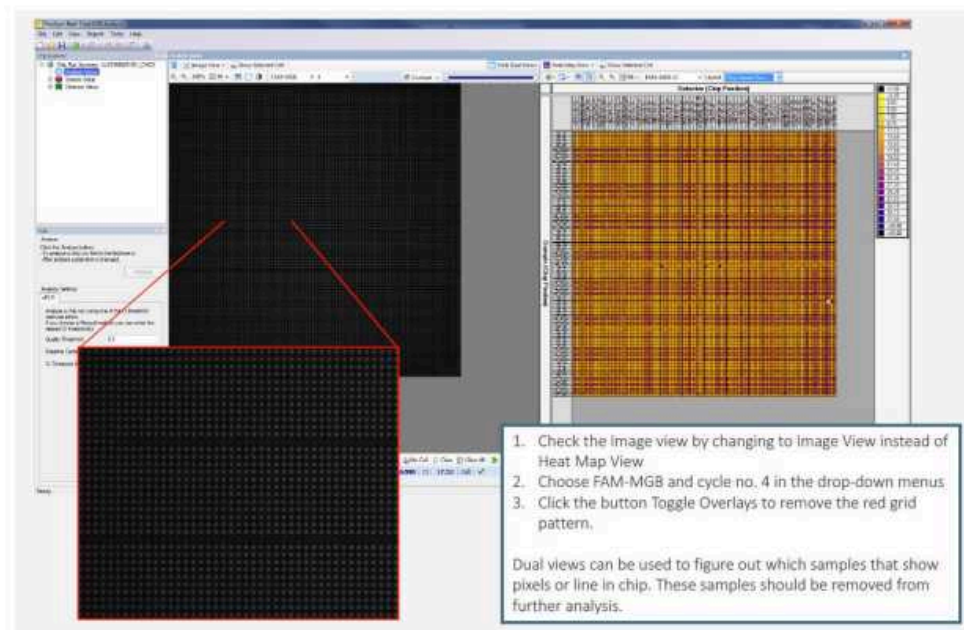


8 Data preview:

8.1 Select "Results Table" and drop down. Select Heat Map View.

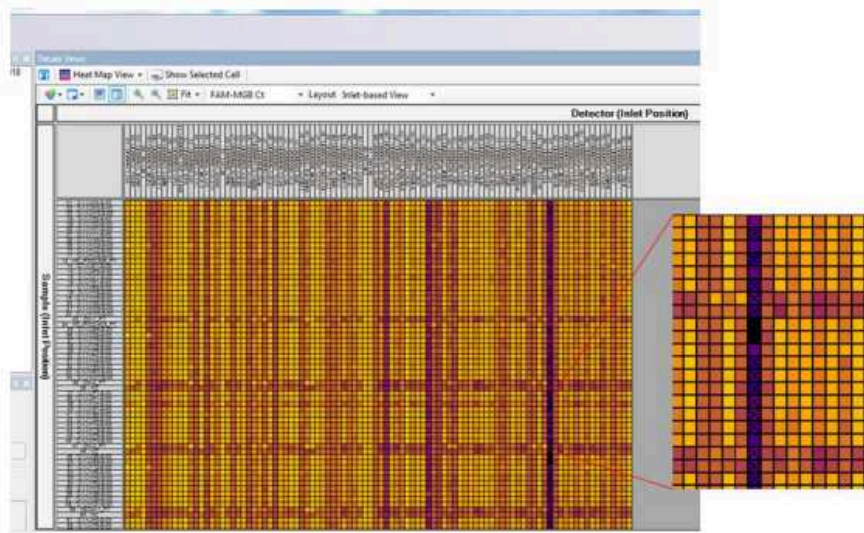
8.2 Review the Heat Map. The Negative Ctrl samples should give a darker "line" from left to right and IPC samples should give a brighter "line" from left to right (Bright color corresponds to more fluorescence, i.e. a lower Ct value and therefore a higher protein concentration). Check if any line of samples/assays has a Black square point that means the assay failed in that Chip well.

8.3 Check the Image view by changing to Image View instead of Heat Map View. Choose FAM-MGB and cycle no. 4 in the drop-down menus. Then Click the button Toggle Overlays (square) to remove the red grid pattern. Dual views can be used to figure out which samples that show pixels or line in chip. These samples should be removed from further analysis.



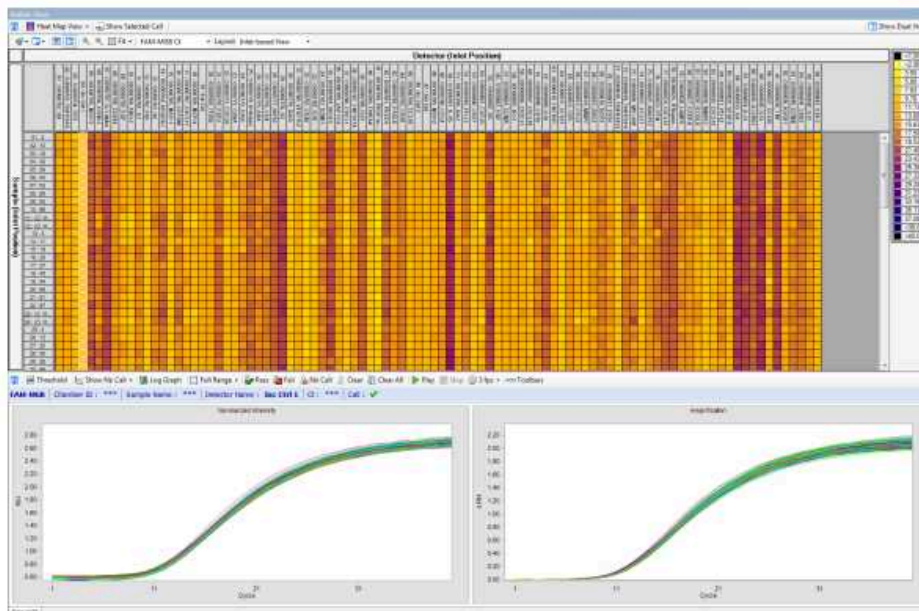
8.4

Check the Heat Map View by selecting the Heat map view tab on the top of detail view. If there is any analyte or sample has dark purple line with black crossed out square, then remove the data of this analyte/sample in the Olink NPX manager for further analysis. Follow the Step 28 to remove the data from NPX manager.

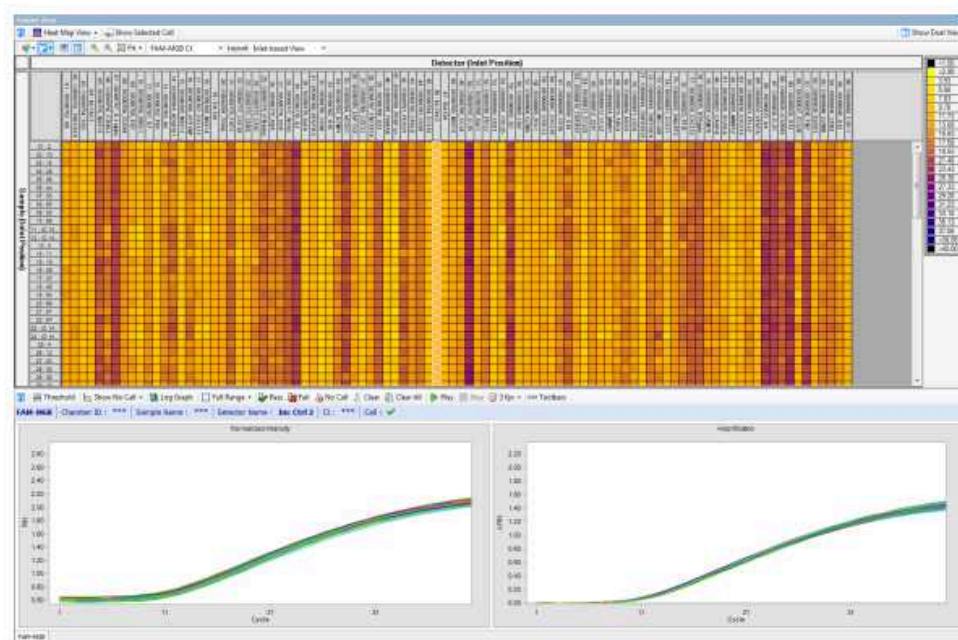


Note: The following figures are examples of different Ctrls/sample/analyte conditions and can be used for reference view only, while analyzing other plate data. These examples are taken from Olga_IO_10162017.

- 8.5 Select Incubation Ctrl-1 in the Detector on heat map and it will appear as the following figure with the amplification curves.



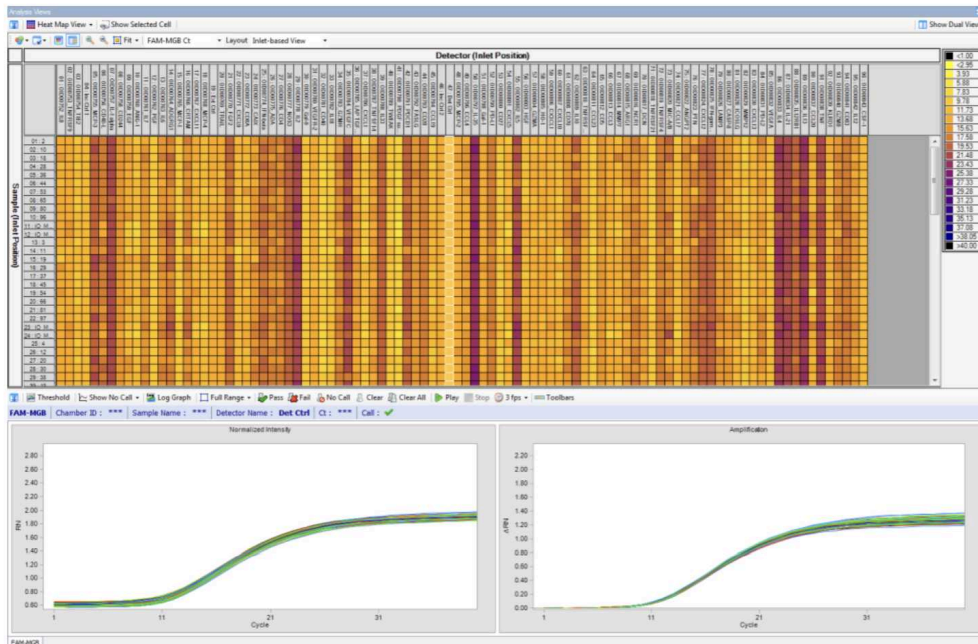
- 8.6 Select Incubation Ctrl-2 in the Detector on heat map and it will appear as the following figure with the amplification curves.



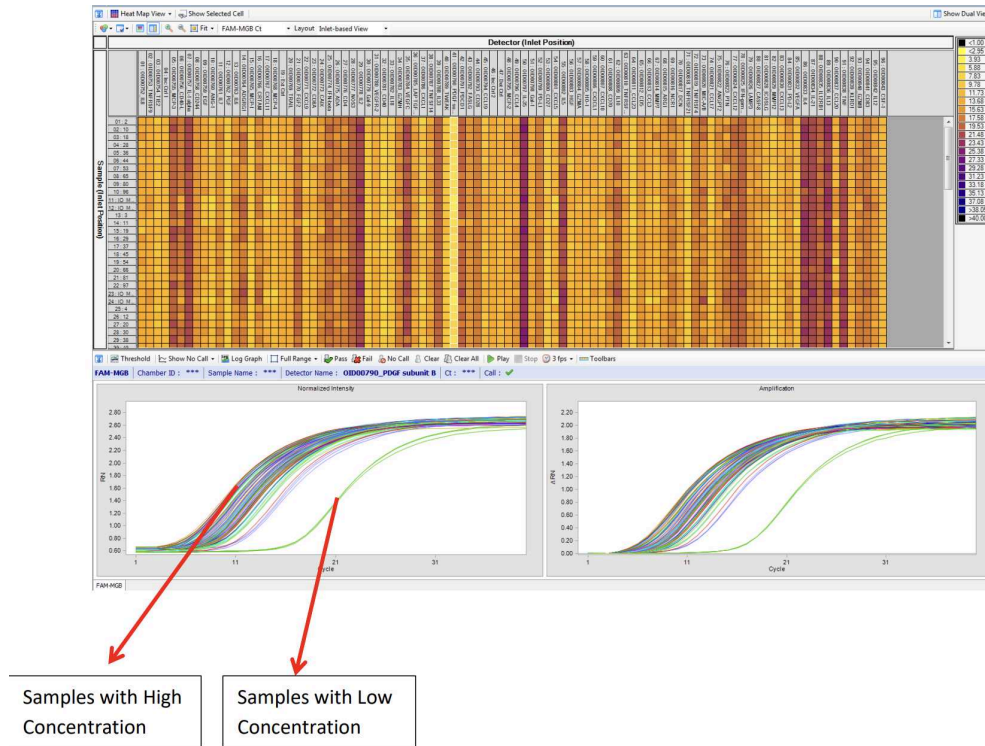
- 8.7 Select Extension Ctrl in the Detector on heat map and it will appear as the following figure with the amplification curves.



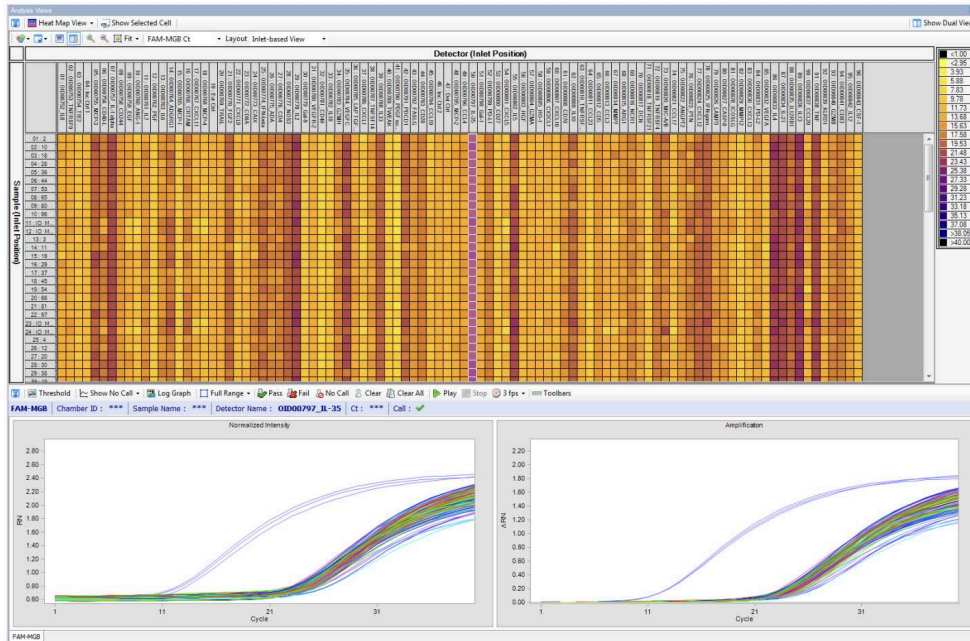
8.8 Select Detection Ctrl in the Detector on heat map and it will appear as the following figure with the amplification curves.



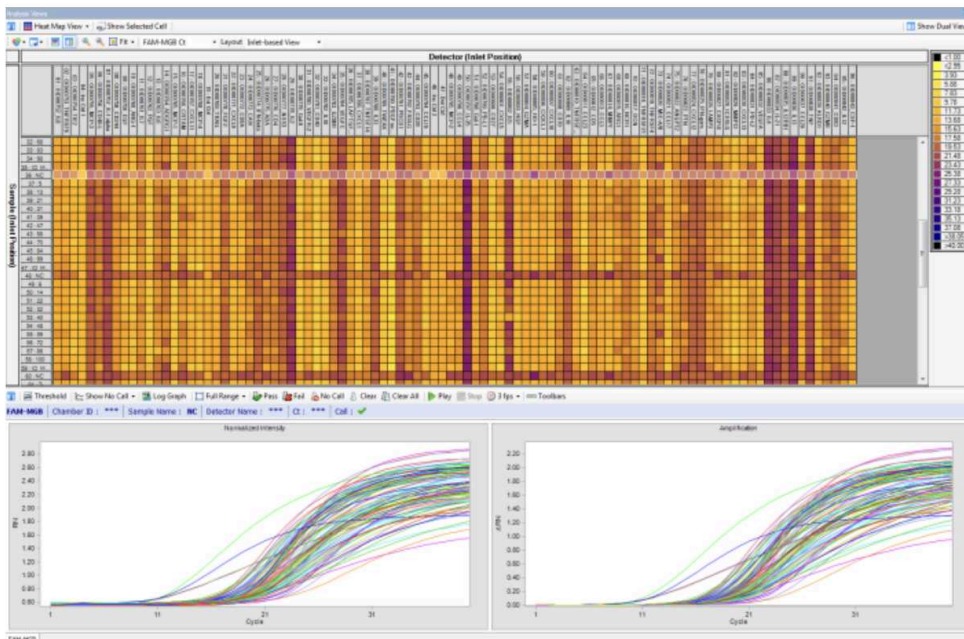
- 8.9 Select an assay with bright yellow color in whole column in the Detector on heat map and it will appear as the following figure with the amplification curves. This is the highly expressed/more concentration of protein.



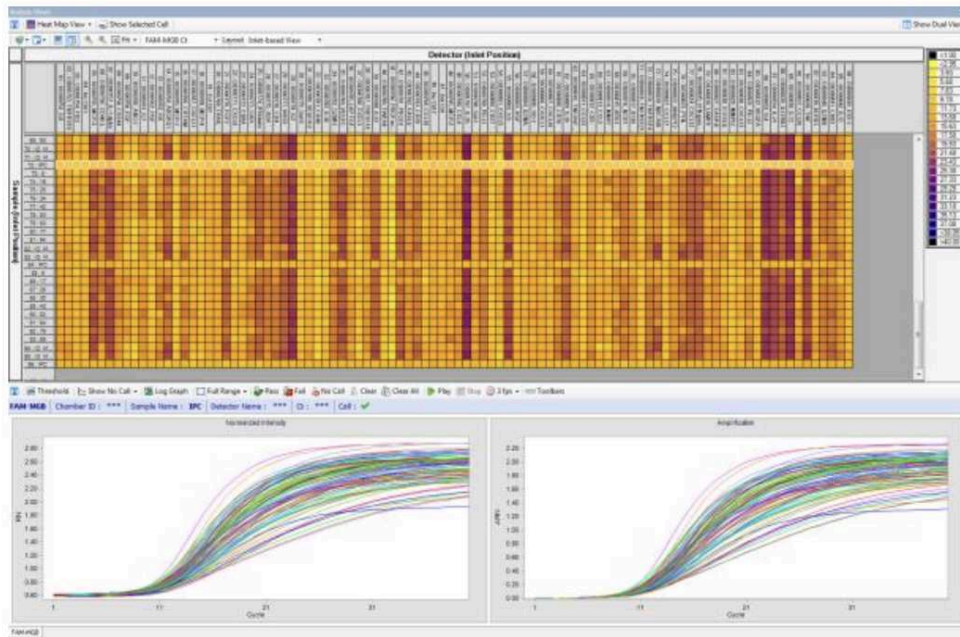
- 8.10 Select an assay with Dark purple color in whole column in the Detector on heat map and it will appear as the following figure with the amplification curves. This is the lowest expressed/low concentration of protein.



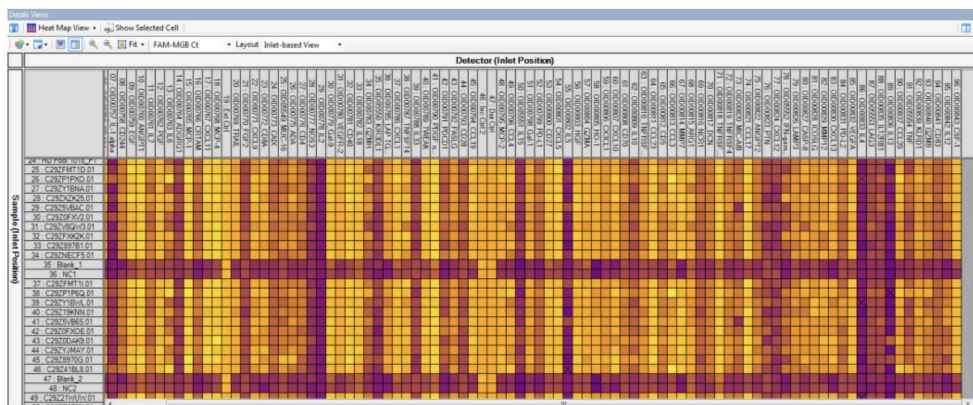
8.11 Select NC (Negative Ctrl) in Samples on heat map and it will appear as the following figure with the amplification curves. This should always be purple (dark line) color because it is negative Ctrl.



- 8.12 Select IPC (Positive Ctrl) in Samples on heat map and it will appear as the following figure with the amplification curves. This should always be yellow (bright line) color as it is positive ctrl.



The X marked wells/squares shown in below heat map picture in Analyte IL-4 indicates there is no Data.



Data Export

- 9 Select File>Export. Make sure to choose Heat Map Results (*.csv) for File format from "Save as Type" drop down. Name the excel file according to HIMC format "HIMC_PL_Date_Ct.csv"
- 10 Open the exported CSV file in excel software to ensure of the header, Olink ID, sample names and Fluidigm QC result.

The output should be in heatmap format

Olink ID	Sample names	Fluidigm QC result
1	Sample 1	Pass
2	Sample 2	Pass
3	Sample 3	Pass
4	Sample 4	Pass
5	Sample 5	Pass
6	Sample 6	Pass
7	Sample 7	Pass
8	Sample 8	Pass
9	Sample 9	Pass
10	Sample 10	Pass
11	Sample 11	Pass
12	Sample 12	Pass
13	Sample 13	Pass
14	Sample 14	Pass
15	Sample 15	Pass
16	Sample 16	Pass
17	Sample 17	Pass
18	Sample 18	Pass
19	Sample 19	Pass
20	Sample 20	Pass
21	Sample 21	Pass
22	Sample 22	Pass
23	Sample 23	Pass
24	Sample 24	Pass
25	Sample 25	Pass
26	Sample 26	Pass
27	Sample 27	Pass
28	Sample 28	Pass
29	Sample 29	Pass
30	Sample 30	Pass
31	Sample 31	Pass
32	Sample 32	Pass
33	Sample 33	Pass
34	Sample 34	Pass
35	Sample 35	Pass
36	Sample 36	Pass
37	Sample 37	Pass
38	Sample 38	Pass
39	Sample 39	Pass
40	Sample 40	Pass
41	Sample 41	Pass
42	Sample 42	Pass
43	Sample 43	Pass
44	Sample 44	Pass
45	Sample 45	Pass
46	Sample 46	Pass
47	Sample 47	Pass
48	Sample 48	Pass
49	Sample 49	Pass
50	Sample 50	Pass
51	Sample 51	Pass
52	Sample 52	Pass
53	Sample 53	Pass
54	Sample 54	Pass
55	Sample 55	Pass
56	Sample 56	Pass
57	Sample 57	Pass
58	Sample 58	Pass
59	Sample 59	Pass
60	Sample 60	Pass
61	Sample 61	Pass
62	Sample 62	Pass
63	Sample 63	Pass
64	Sample 64	Pass
65	Sample 65	Pass
66	Sample 66	Pass
67	Sample 67	Pass
68	Sample 68	Pass
69	Sample 69	Pass
70	Sample 70	Pass
71	Sample 71	Pass
72	Sample 72	Pass
73	Sample 73	Pass
74	Sample 74	Pass
75	Sample 75	Pass
76	Sample 76	Pass
77	Sample 77	Pass
78	Sample 78	Pass
79	Sample 79	Pass
80	Sample 80	Pass
81	Sample 81	Pass
82	Sample 82	Pass
83	Sample 83	Pass
84	Sample 84	Pass
85	Sample 85	Pass
86	Sample 86	Pass
87	Sample 87	Pass
88	Sample 88	Pass
89	Sample 89	Pass
90	Sample 90	Pass
91	Sample 91	Pass
92	Sample 92	Pass
93	Sample 93	Pass
94	Sample 94	Pass
95	Sample 95	Pass
96	Sample 96	Pass
97	Sample 97	Pass
98	Sample 98	Pass
99	Sample 99	Pass
100	Sample 100	Pass

Transform Ct values to NPX using "Olink NPX manager"

- 11 Open NPX manager software version 2.1.0.224 on the same computer.
- 12 Default mode/setting for the Project Options are shown below:

Project Options

☐ Samples Randomized

☐ Ignore QC

Display NPX Values < LOD As

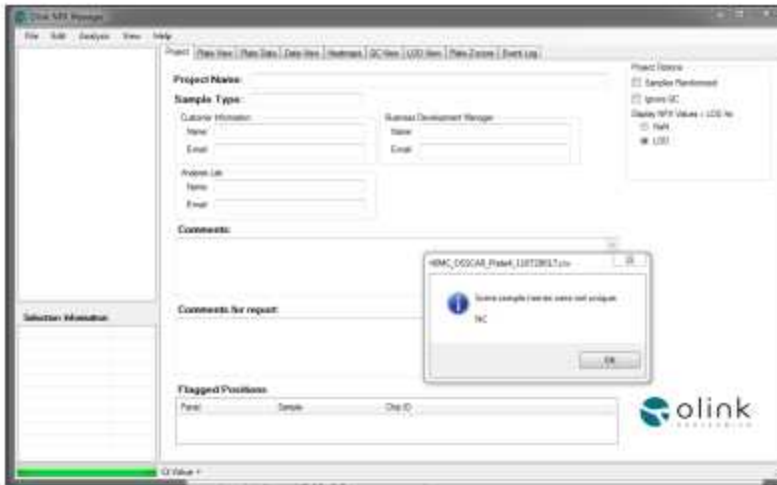
☐ NaN

☐ LOD

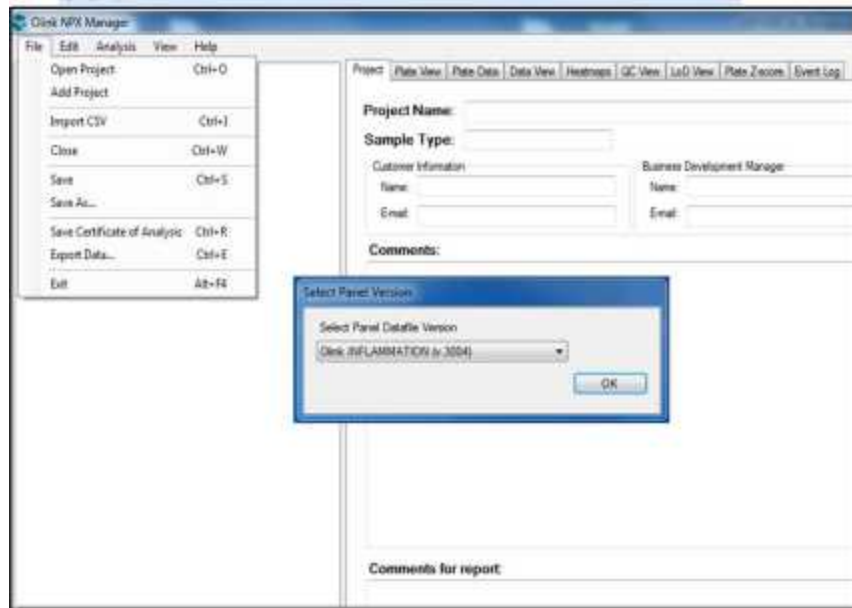
☒ Actual Value

- 13 Import Ct Value/.csv file data: Select "File" > "Import CSV" to import the xxxx.csv file. e.g. "HIMC_PI_Date_Ct.csv"
- 13.1 During import, Olink NPX Manager Software will check for duplicate sample names. If duplicate sample names are found, a message will be shown with the names of the duplicate samples. Press OK.

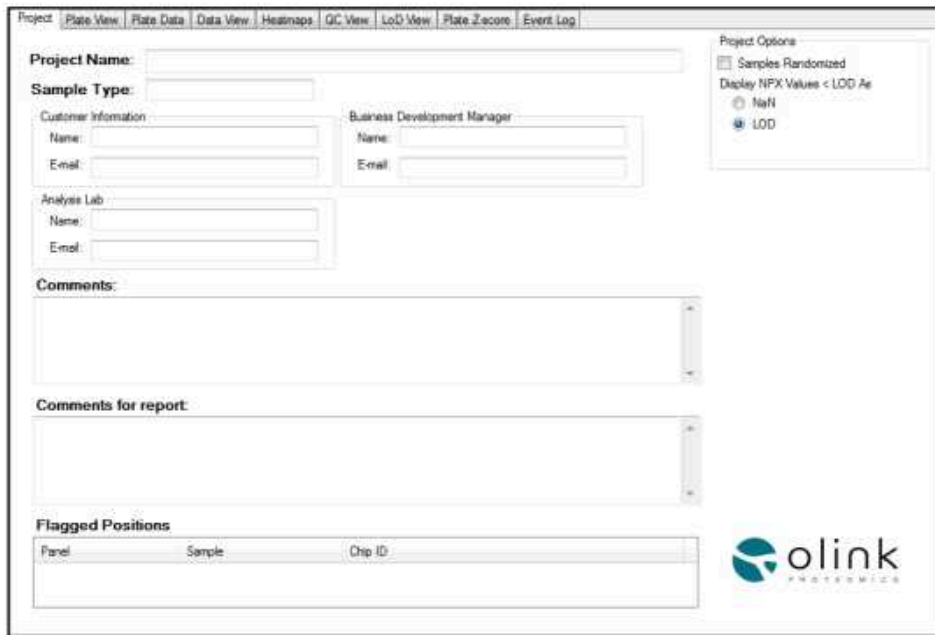
Note: Multiple files within the same project can be selected for import at the same time.



- 13.2 Next screen is for the "Select Panel Version"



- 13.3 Each imported run is automatically annotated with the correct Olink Panel. Click OK
- 14 Enter project information:
- 14.1 Go to the "Project". Project Name will be displayed in the data export file and the Certificate of Analysis (CoA). Information about Sample type, customer, business development manager and analysis lab will be displayed in the CoA. Comments will be saved in the Olink NPX Manager Project (oaf) file. Comments for report and Flagged Positions will be displayed in the Certificate of Analysis (CoA).
- 14.2 Fill out required Project information.



15 Go to "Plate View" Verify sample and data annotation.

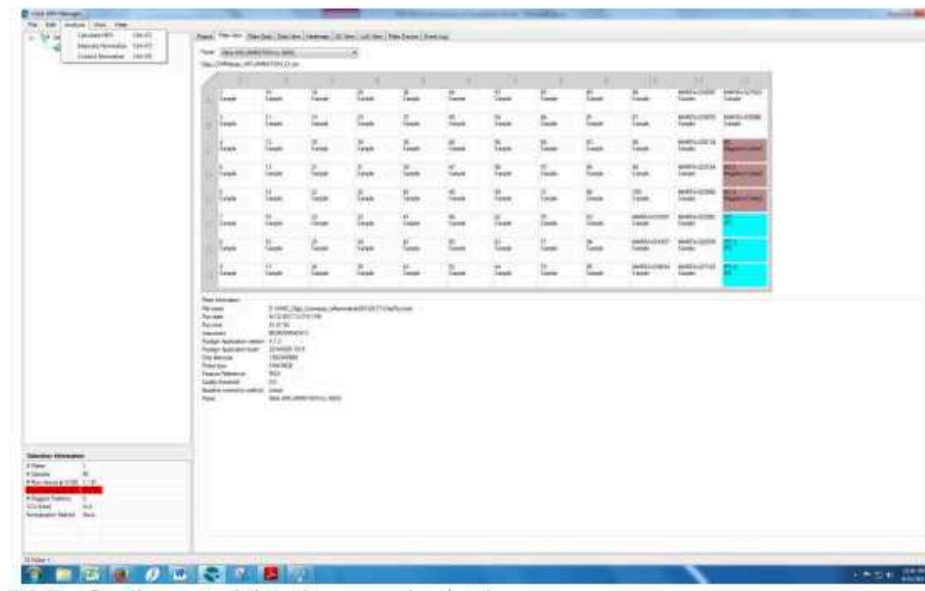
15.1 Verify the plate layout. The layout can be changed by right-clicking on a selected well, or a selection of wells, and changing the well type to any of the available selections: Sample, Control, Negative Control, IPC and Not Used.

15.2 Select all 3 NC wells (NC, NC-2, NC-3) and mark them as Negative Control. Repeat Steps 15.1 and 15.2 for all imported plates.

16 Calculate NPX

16.1 Highlight an Olink Panel in the left pane tree node. Click on "menu Analysis" > "Calculate NPX". Randomized studies with more than one sample plate will be intensity normalized if we select the "Samples Randomized" check box under the project tab before normalization or by clicking on Analysis" > Intensity Normalize v.2. Other study set ups will be IPC normalized by default. The "Intensity Normalization" will calculate the median of all the plates and individual plate NPX values will be normalized based on the median value of the all plates.

16.2 Default view is Ct Value. To view NPX values, select NPX Value in the status bar in the top left corner of the main window and go to Data view tap.



17 Quality control data (per sample plate)

17.1 Select a plate in the left pane tree node and go to QC View

17.2 Check acceptance criteria Run QC:

- Number of flagged samples less than 1/6th of the total number of samples (16 samples in a full plate)
- Standard deviation < 0.2 NPX for each of Incubation control 1, Incubation control 2 and Detection control

17.3 Acceptance criteria Sample QC

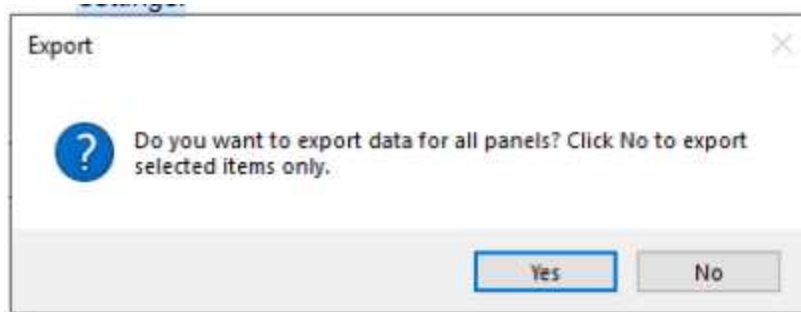
- Incubation Control 2 deviations from median of plate: < +/- 0.3 NPX
- Detection Control deviation from median of plate: < +/- 0.3
- NPX Samples that deviate more than +/- 0.3 NPX will be marked as flagged

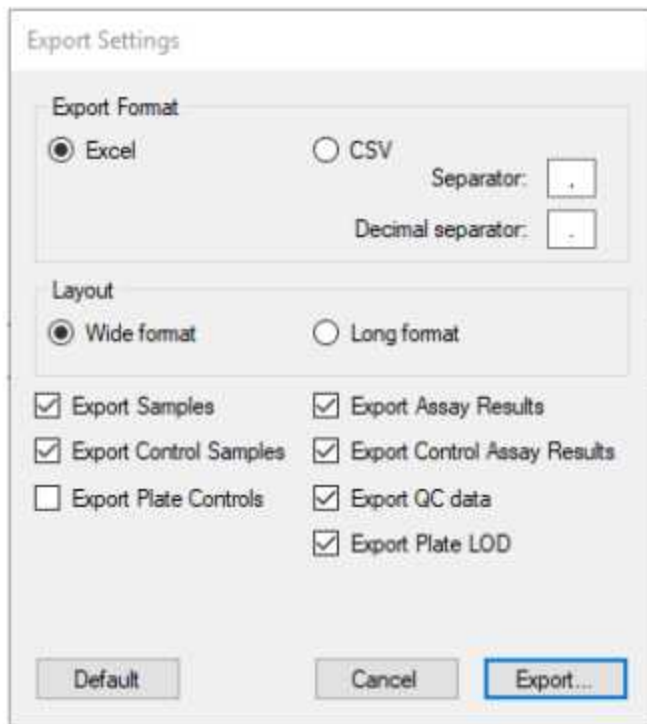
18 Evaluate results in selection information (based on NPX values)

18.1 Plate ANOVA (if more than one sample plate per panel) < 10

18.2 Row/Column ANOVA: < 30 OK, 40-60 Investigate further

- 18.3 If more than 5 LODs highlighted yellow under LoD view tab, deviating from the values observed (+/- 2.5 NPX: which is Olink recommended value), check the following steps:
- If One of the negative Control samples is flagged in the NPX software, this control has to be marked "not used sample" before calculating the NPX
 - Check the Heat map view to show only negative controls to see if any one negative ctrl is different from other two that it has many dark "BLUE" values (= low Ct = high NPX which affects the LOD value) by going through the "assays".
 - In such a case remove that negative ctrl by marking it "Not Used" and reanalyze the NPX data.
- 19 Export data
- 19.1 Select NPX Value in the status bar in the topleft corner of the main window
- 19.2 Under Project Options in the Project tab, select whether NPX values below LOD should be exported as NaN or replaced with the value for LOD or Actual Value (below LOD). Select menu File > Export Data.
- 19.3 Click "Yes" if exporting data for all plates together and "No" for exporting data on selected plate only. Select desired export settings.





The 'Export Settings' dialog box contains the following options:

- Export Format:** Radio buttons for 'Excel' (selected) and 'CSV'.
 - Separator:** A text box containing a comma (,).
 - Decimal separator:** A text box containing a period (.)
- Layout:** Radio buttons for 'Wide format' (selected) and 'Long format'.
- Checkboxes:**
 - ☒ Export Samples
 - ☒ Export Assay Results
 - ☒ Export Control Samples
 - ☒ Export Control Assay Results
 - ☐ Export Plate Controls
 - ☒ Export QC data
 - ☒ Export Plate LOD
- Buttons:** 'Default', 'Cancel', and 'Export...' (highlighted with a blue border).

- 19.4 Enter filename and export format (Excel files *.xlsx or CSV-files*.csv) in the Save-dialog and click the Save button.
- 20 Export certificate of analysis: Select menu File > Report > Save Certificate of Analysis.

Analysis for Multi-plates Project Per Olink Panel

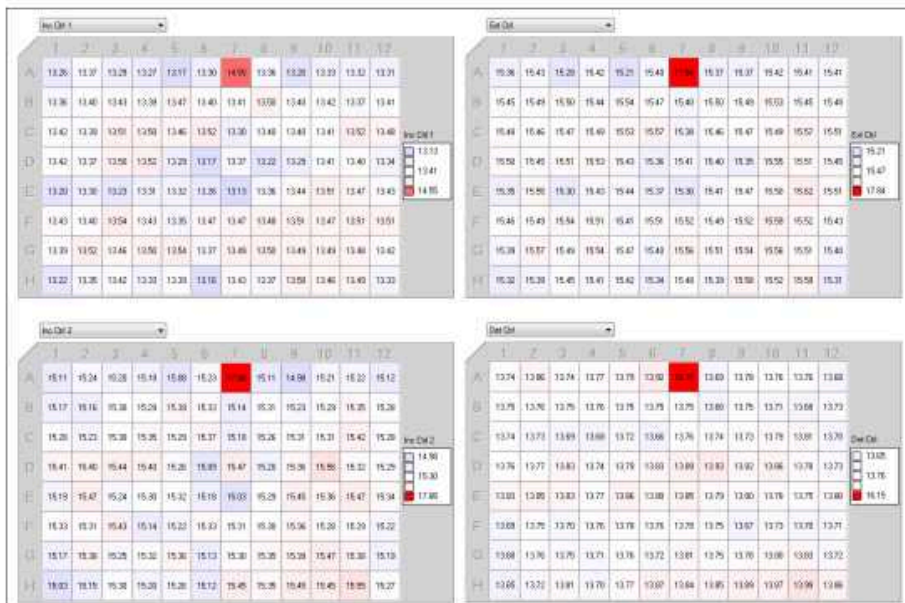
- 21 For randomized projects with more than one plate per Olink panel, follow Steps 11 through 15.2 for all plates one after another.
- 22 Imported plates are grouped by Olink panel and displayed in the left pane tree view. Plates can be reorganized in the tree view by drag-and-drop.
- 23 Then check the Samples Randomized box under Project Options to analyze data using Intensity Normalization. Intensity Normalization can also be applied by Clicking intensity Normalize v.2 under Analysis tab. Follow the Steps from 16 and further analysis.
- 24 Click the + sign next to an Olink Panel name to expand the tree node and reveal all imported runs for the Olink panel. Highlight the first run and go to the Plate View tab

Verify the data file version

- 25 For using the NPX software do the multi-plate analysis, check the plate Z-score for each analyte. If one/more sample spots are far from the 0- line, then check the Ct value, NPX value and also check the Chip image in RT-PCR analysis software if there is any Chip line has issues- meaning dark line mentioned in Step 8.3. The sample/s should be removed from analysis.

Troubleshooting

- 26 EXAMPLE OF data set without any flagged samples by NPX manager but needed to be corrected manually
- When 1 or more samples have Ct value VERY different (darker red or darker blue color) from the rest of the wells on the plate but that sample is not flagged by NPX manager
 - The sample is not flagged because the NPX-values for Incubation control 2 and Detection control were within accepted limits.
 - All four controls are affected for that sample and when using the extension control to get the NPX value, the NPX values for Incubation control 2 and Detection control happen to be within accepted limits.
 - When the extension control is affected in the same way as the other controls, meaning that all four controls have lower or higher Ct values than the rest of the plate, this difference can disappear in the normalization step.
 - In this case, the sample should be marked as "failed" in the plate view tab and the data for that sample will disappear and will show "No Data" in data view tab. For example in DEP Culture samples inflammation Olink panel, see below:



- 26.1 If sample/s is flagged in a row/column and all internal controls and analytes have high/low Ct values compared to that of the whole plate, it could be an error while adding detection mixture in those wells.
- 26.2 If same samples of the study or healthy donor plasma/serum samples were on all plates they will be used as control for analysis, Olink uses Plasma pool of 20 HD samples. The intra assay %CV for control sample is recommended <10% and that of inter assay is <15%.

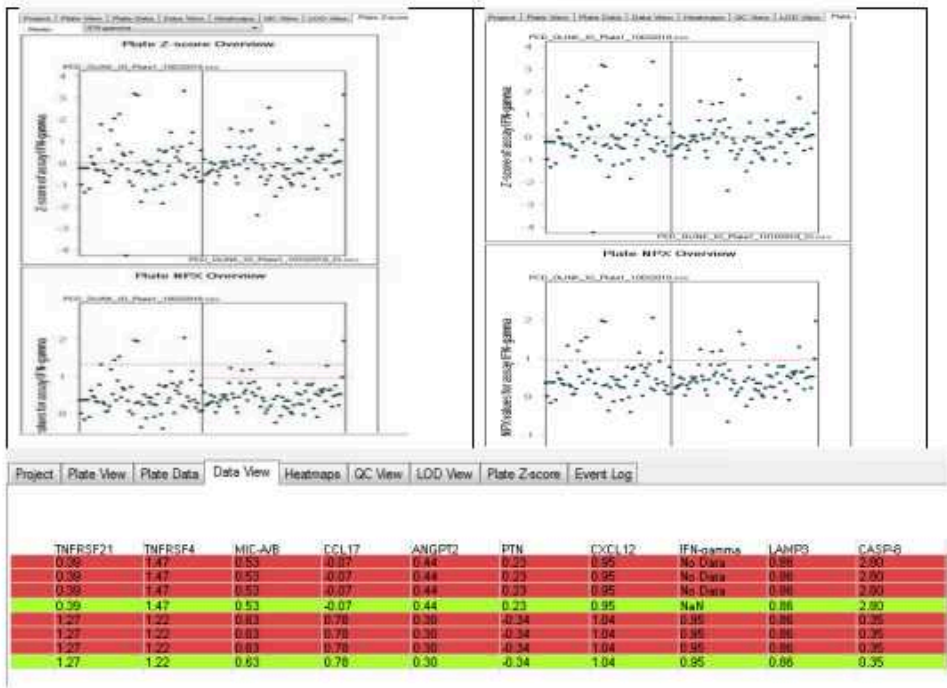
NPX manager uses highest LoD value out of all plates LoD values.

- 27 Check if the chosen (highest) LoD affects more than 50% of the samples for that analyte/protein detected in all the plates meaning this high LoD value is used for more than 50% samples on all plate.
- 28 In this case, mark that assay failed for that Highest LoD value analyte on that one specific plate.
1. by selecting that plate on the left pane tree view, go to Data View
 2. Select analyte by RIGHT click on the analyte and mark as failed.
 3. A comment box will appear where associated comment can be made and all values for that analyte will be shown as "No Data".
 4. Now select Olink panel (all plates) on left pane tree view to recalculate.
 5. Confirm % of protein detected for the analyte in question with LOD.

Project	Plate View	Plate Data	Data View	Heatmaps	GC View	LOD View	Plate Z-score	Event Log
C00L1	C00L10	C070	JL10	TNFR5P126	C03L2	C05	C03	
12.91	11.13	16.86	13.71	No Data	8.91	15.89	12.28	
15.12	16.39	16.94	21.53	No Data	10.29	15.70	13.10	
12.94	13.27	17.95	20.44	No Data	9.85	16.10	12.86	
15.04	14.41	16.34	20.30	No Data	9.79	15.87	12.21	
13.99	13.03	14.95	19.59	No Data	9.72	16.39	13.68	
13.69	15.07	15.45	20.47	No Data	10.25	16.94	14.08	
13.85	12.96	15.90	18.86	No Data	9.19	15.85	13.64	
14.91	13.13	16.36	20.44	No Data	10.44	16.20	14.10	
19.50	21.46	19.32	21.00	No Data	19.08	20.12	18.36	
13.63	11.88	16.02	19.91	No Data	10.75	15.99	13.46	
12.91	13.52	15.14	19.92	No Data	9.91	15.40	12.79	
14.16	14.01	16.44	19.50	No Data	9.39	15.59	13.75	
13.70	11.14	14.35	18.93	No Data	9.61	15.93	13.51	
19.92	21.47	19.45	22.02	No Data	18.90	20.07	18.31	
14.91	14.48	17.00	21.18	No Data	10.32	15.01	13.33	
12.34	12.96	14.71	19.27	No Data	10.05	15.79	12.99	
11.77	12.07	15.88	19.42	No Data	9.49	16.37	12.49	
10.96	12.74	12.36	18.96	No Data	9.86	16.59	12.20	
19.75	21.96	19.51	21.79	No Data	18.88	20.11	18.92	
12.57	12.40	16.99	19.73	No Data	9.49	15.38	12.51	
12.42	11.66	14.87	19.19	No Data	9.30	15.58	12.30	
11.85	12.70	15.91	21.16	No Data	9.76	16.68	13.07	
14.00	12.78	15.72	19.60	No Data	9.40	15.19	9.45	
11.26	12.89	12.26	13.34	No Data	11.94	13.60	16.71	
14.30	13.45	15.07	19.93	No Data	10.40	16.75	13.52	
10.95	11.12	15.17	19.20	No Data	9.09	16.34	12.21	
14.18	13.67	16.65	18.55	No Data	10.06	15.60	12.57	
11.43	13.08	12.43	13.50	No Data	12.12	13.82	12.86	
16.70	21.07	16.95	21.45	No Data	15.23	20.13	8.11	
11.16	12.88	12.10	12.26	No Data	11.87	13.79	12.50	

HOWEVER: optionally,

Note: When we have too very different LODs between the two plates for some analyses and, therefore, lose the data points of one plate (with low LOD) due to the other plate, which has the higher LODs for that specific analytes, one can remove the "NEGATIVE CONTROL". AT THE Data View- Display only "Neg Ctrl", right-click the NC, NC2, NC3 for the specific analyte (depends on the variation- remove all or the only one w HIGH LOD) and select the " Mark as Failed". This will remove the high- LOD for that analyte.



Protocol references

REFERENCE- Fluidigm Real-Time PCR Analysis and Olink NPX manager user Guide
<http://www.olink.com/products/document-download-center/>