Olink® NPX Manager

User Manual

1078, v6.1, 2022-04-1

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1. Introduction

Olink high-multiplex immunoassay panels provide an efficient and innovative tool for targeted human protein biomarker discovery, development and validation.

1.1 About this manual

The Olink® NPX Manager User Manual provides you with the instructions needed for data processing when running Olink® Target 48, Olink® Target 96 panels and Olink® Explore panels with a locally installed set up of software. Specific instructions for Olink® Target 48 and Olink® Explore are highlighted in the manual:

Olink® Target 48

Olink® Explore

NOTE: The information in the Olink® NPX Manager User Manual is believed to be accurate. However, the displayed screenshots may differ from actual user intreface and should be considered as examples.

1.1.1 Intended use

Olink® NPX Manager is a data analysis software that is designed for the Olink® Target analysis platform, including Olink® Target 48, Olink® Target 96, and Olink® Explore platform. It allows for importing data, validating data quality, and normalizing Olink data for subsequent statistical analysis.

Olink®NPX Manager is intended for research use only. Not for use in diagnostic procedures. All trademarks and copyrights contained in this material are the property of Olink® Proteomics AB unless otherwise stated. For questions, guidance, and support, contact Olink Support at support@olink.com.

1.1.2 Intended target group

Olink® NPX Manager is intended to be used by trained users of Olink® Target analysis platform and staff certified to run the Olink® Explore platform. For the Olink® Explore platform, Quality control should be performed by trained users that determines whether data from a run can be approved for further analysis.

1.2 Requirements for analysis

System Requirements:

- Windows® 10 or later
- NET™ Framework 4.7.1 or later
- Recommended system: Intel® Core™ i5 or higher, 8 GB RAM

Files and information needed for analysis (qPCR readout):

- Run data (bml-file)
- Plate layout with sample names
- Detector setup file (.plt) from https://www.olink.com/members-site/ (which PLT file to use is stated on the lot configuration sheet)
- Data file number (provided on the insert delivered with the kit)

Files and information needed for analysis (NGS readout):

- Run data in csv format and run metadata in json format (NGS raw data in bcl format pre-processed using bcl2counts)
- Plate layout with sample names
- Panel Lot number of reagents used (provided on the Lot configuration insert delivered with the kit)

1.3 Process

1.3.1 qPCR Readout (Olink® Target)

The *Fluidigm® Biomark™ Data Collection* software captures images of the processed runs throughout the real-time PCR cycling, and those images are imported into the *Fluidigm® Real-Time PCR Analysis* software. The *Real-Time PCR Analysis* software is a tool for analyzing real-time PCR data and exporting heatmaps of Ct values for downstream pre-processing using Olink® NPX Manager.

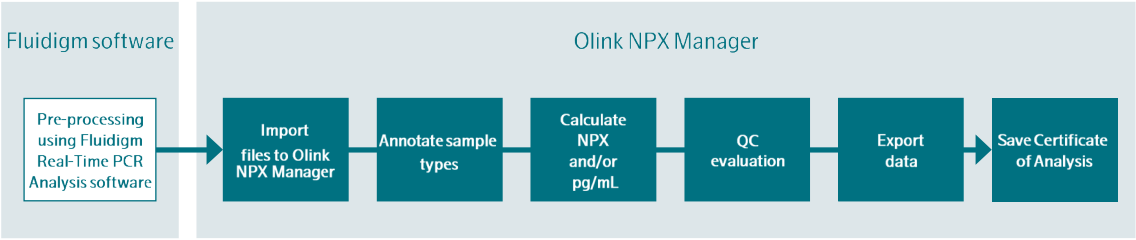


Figure 1. Flowchart describing steps involved in the analysis of Olink data.

1.3.2 NGS Readout (Olink® Explore)

Olink® Explore

The NGS raw data output must be pre-processed using bcl2counts to generate counts csv files and run metadata json files. The files are imported into Olink® NPX Manager for generating NPX data, data QC and normalization.

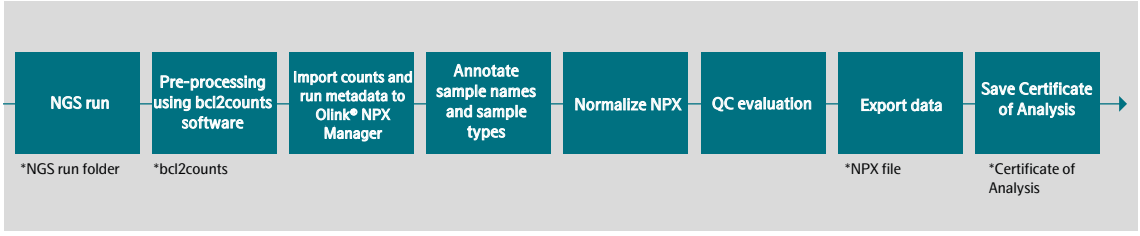


Figure 2. Flowchart describing steps involved in the analysis of Olink data.

1.4 Limitations

This manual contains step-by-step instructions for exporting heatmaps of Ct values in CSV format for analysis in Olink® NPX Manager and some *Real-Time PCR Analysis* software troubleshooting. For additional information and troubleshooting, please see the documentation for *Fluidigm® Real-Time PCR Analysis* software, refer to *1.6.2 Other documents.*

The manual also contains step-by-step instructions for generating counts files from NGS runs using bcl2counts for analysis in Olink® NPX Manager.

1.5 List of abbreviations

%CV	Coefficient of Variance
CoA	Certificate of Analysis
Ct	Cycle threshold
IFC	Integrated Fluidic Circuits
IPC	Inter-Plate Control
LOD	Limit of Detection
MAD	Median Absolute Deviation
NaN	Not a Number
NGS	Next Generation Sequencing
NPX	Normalized Protein eXpression
PCR	Polymerase Chain Reaction
QC	Quality Control

1.6 Associated documentation

1.6.1 Olink documentation

All relevant Olink documentation is available from the Olink website. www.olink.com/downloads.

1.6.2 Other documents

For information on the Fluidigm IFC Controller HX, IFC Controller MX, Juno System and Fluidigm BioMark System, read the following User Guides that can be found on www.fluidigm.com:

- Fluidigm® IFC Controller User Guide - PN 68000112
- Fluidigm® Juno System User Guide - PN 100-7070
- Fluidigm® Real-Time PCR Analysis User Guide - PN 68000088
- Fluidigm® Data Collection Software User Guide - PN 68000127

1.7 Technical support

For questions, guidance and support, contact Olink® Proetomics at support@olink.com

2. Olink® NPX Manager user interface

The Olink NPX Manager user interface consists of several views on separate tabs where information and results are displayed. The views are mostly the same for Olink Target 48 and 96 panels and for Olink Explore panels. Specific instructions for Olink Target 48 and Olink Explore are highlighted. The views are described in this chapter. Specific instructions for Olink Target 48 and Olink Explore are highlighted in the manual:

Olink® Target 48

Olink® Explore

2.1 General

2.1.1 Search

Use Ctrl + F to open a search field. The search function works on most views for either plain text or sample names, assay names or Uniprot IDs. The first match found will be highlighted. The search function is also available from **Edit** > **File**.

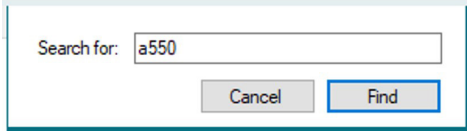


Figure 3. Search field.

2.1.2 Help

Press F1 to open the Olink NPX Manager User Manual as a PDF file.

2.1.3 Data display setting

Click the text in the colored panel in the upper left corner of the screen to change display type in the drop-down menu. Switch between **Ct Value**, **NPX Value** and **Quantified Value**.

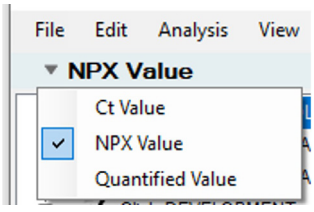


Figure 4. Drop-down menu for Target 48 and Target 96.

To better identify which data display setting is currently being used, note the color of the data display bar. If Ct Value has been selected, the data display bar will be yellow, and if **NPX Value** or **Quantified Value** has been selected, the data display bar will be blue.

Olink® Target 48

Quantified Value is only available when analyzing data from Olink® Target 48 panels. This setting will make all views display the quantified values calculated with the calibrator normalization. Refer to *4.4 Calibrator normalization*.

Olink® Explore

Ct Value displays counts values.

2.2 Tree view

The Tree View displays the imported runs, grouped by Olink panel.

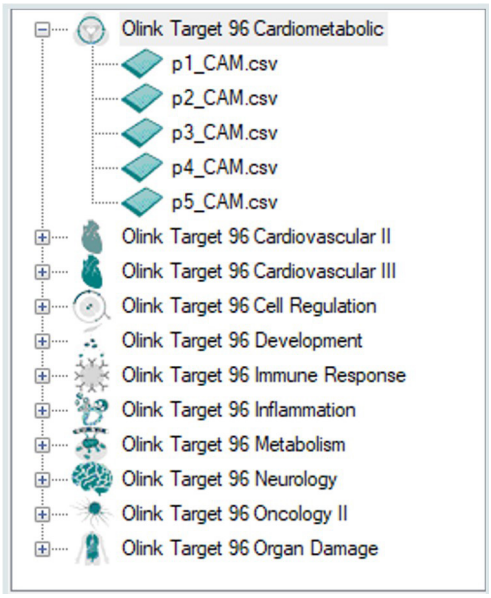


Figure 5. Olink Panels Tree View.

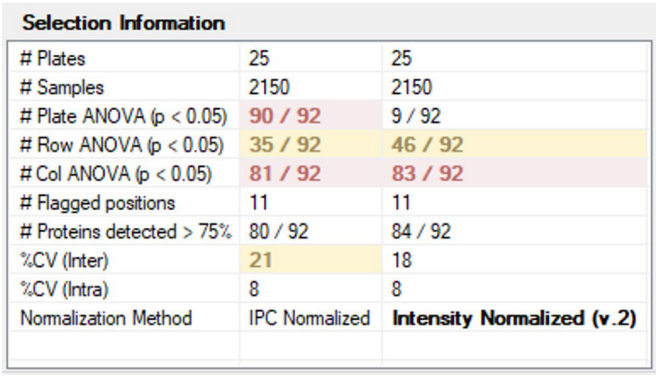
- Click on the **+** next to the Olink panel name to display the imported runs for each Olink panel.
- Highlight an Olink panel to display information about all runs in that panel. If an Olink panel is highlighted in the Tree View, information for the first run is displayed on the run specific tabs. On the **LOD View** and **Plate Z-score** tabs, data from all runs in the selected Olink panel are shown.
- Highlight an individual run to see information about that specific run.
- Change the order of the runs in the Tree View by dragging and dropping a run to its desired location.
- Drag-and-drop Fluidigm CSV files on the Tree View to import new runs. Drag-and-drop a folder to import multiple CSV files at once.
- Remove a run or Olink panel from a project by highlighting the item in the Tree View and clicking **Edit > Delete**. A pop-up audit window asks for the reason for removing runs or Olink panels. Enter the reason and click **OK**.
- Use drag-and-drop, or click **File > Add project**, to combine two projects.

NOTE: Only projects with the same panel version can be combined.

NOTE: A project cannot include different panel types at the same time. It can include either runs from Olink® Explore, runs from Olink® Target 48 panels, or runs from Olink® Target 96 panels.

2.3 Selection information

The **Selection Information** view in the lower left corner displays information about the currently selected item in the Tree View: all sample plates for an Olink panel or an individual sample plate. This information gives you an indication of the overall data quality.



Selection Information

# Plates	25	25
# Samples	2150	2150
# Plate ANOVA (p < 0.05)	90 / 92	9 / 92
# Row ANOVA (p < 0.05)	35 / 92	46 / 92
# Col ANOVA (p < 0.05)	81 / 92	83 / 92
# Flagged positions	11	11
# Proteins detected > 75%	80 / 92	84 / 92
%CV (Inter)	21	18
%CV (Intra)	8	8
Normalization Method	IPC Normalized	**Intensity Normalized (v.2)**

Figure 6. Selection Information view for a Olink® Target 96 panel with the NPX setting.

Refer to *Table 1* for a description of all content in the **Selection Information** pane. The values displayed in the table depend on the selection in the upper left corner of the screen.

- If **Ct** is selected: Number of plates, number of samples and ANOVA based on Ct values will be listed.
- If **NPX** or **Quantified Value** is selected: Number of plates, number of samples and ANOVA based on NPX values will be listed as well as the number of flagged samples, detection, %CV and the normalization method used.

Table 1. Description of Selection Information.

Item	Description
# Plates	Number of runs in selected Olink panel.
# Samples	Number of samples, excluding control samples, in selected Olink panel or run.
# Plate ANOVA (p < 0.05) # Row ANOVA (p < 0.05) # Column ANOVA (p < 0.05)	Number of assays where the variation between plates/rows/columns is larger than the variation within the plates/rows/columns. A high number indicates measurable systematic variation. For more information, Refer to *8.5 Inconsistent results detected in Olink® Target using Olink® NPX Manager*. The p-value is calculated using the selected display unit ("Ct Value" or "NPX Value"). For specific limits, Refer to *7.5 Perform quality controls*. Olink® Explore Not displayed for Olink® Explore
# Flagged Positions	Number of flagged positions (samples that did not pass the quality control, including control samples) in the selected Olink panel or plate.
# Proteins detected > 75%	Number of proteins detected above LOD in more than 75% of the samples. Olink® Target 48 When quantified view is used, the number of proteins detected within LOQ in more than 50% of the samples are displayed instead. Olink® Explore Not displayed for Olink® Explore.
%CV (Inter)	Mean inter-plate CV for all assays based on linear NPX values. %CV will only be calculated if control samples are included on the sample plate and annotated as "Control" in the **Plate View**. Refer to *3.2 Run QC* for more information. Values >20% are highlighted in yellow, values > 25% are highlighted in red. Olink® Target 48 Mean inter-plate %CV for all assays is calculated using values in pg/mL within LOQ for non-flagged Sample Controls. Olink® Explore Not displayed for Olink® Explore.
%CV (Intra)	Mean intra-plate CV for all assays based on linear NPX values. %CV will only be calculated if control samples are included on the sample plate and annotated as "Control" in the **Plate View**. Refer to *3.2 Run QC* for more information. Values >10% are highlighted in yellow, values > 15% are highlighted in red. Olink® Target 48 Mean intra-plate %CV for all assays is calculated using values in pg/mL within LOQ for non-flagged Sample Controls. Olink® Explore Not displayed for Olink® Explore.

Item	Description
Normalization method	Displays which normalization method has been used. A column is added for each type of normalization that has been performed. The most recent, and currently applied, normalization will be highlighted in bold text. IPC normalization is the default for single plate runs. Sample intensity normalization is the default for randomized studies. Olink® Target 48 Calibration normalization is always used for these panels. Olink® Explore Olink Explore Plate control normalization and Intensity normalization can be used. Plate control normalization is the default for single plate runs. Intensity normalization is the default for randomized studies.

2.4 Project

The **Project** tab contains fields for entering and saving project information and some of this information is displayed on the Certificate of Analysis (CoA), as described in this section.

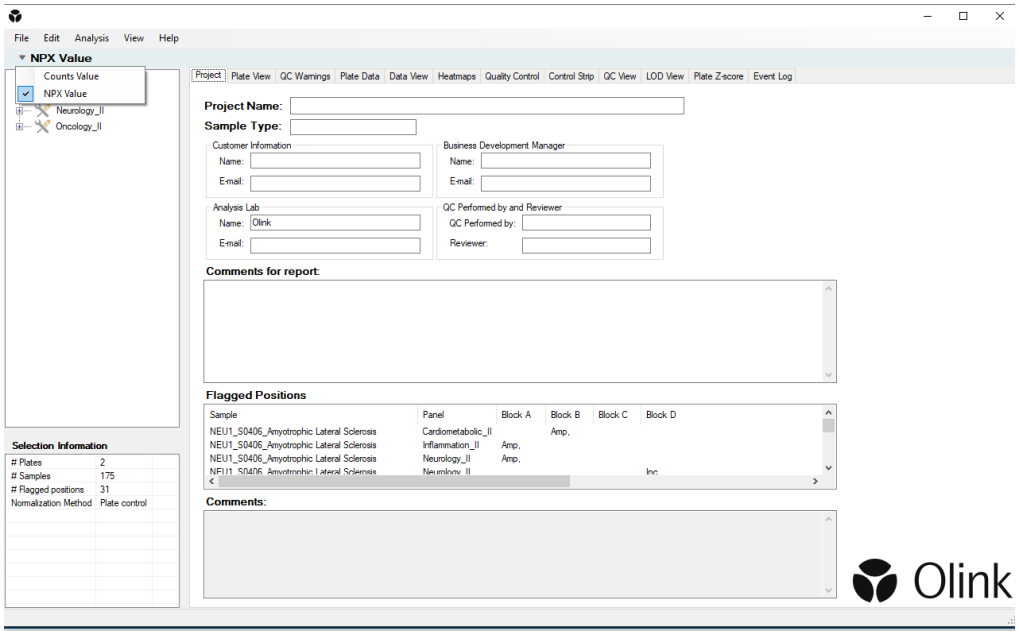


Figure 7. The Project tab.

2.4.1 Project Name

The project name is displayed in the NPX data output file and the Certificate of Analysis.

2.4.2 Sample Type

This field is used to describe the sample matrix, e.g. “Plasma” or “Serum”. The information is displayed on the Certificate of Analysis.

2.4.3 Customer Information, Analysis Lab and Business Development Manager

Information entered in these fields is displayed on the first page of the Certificate of Analysis.

2.4.4 QC Performed by and Reviewer

These fields can be used to add names of the persons responsible for QC and review. Names entered in these fields are displayed on the Certificate of Analysis cover page with a signature placeholder.

2.4.5 Comments for report

Information entered in this field is displayed on the Certificate of Analysis. This field can be used for project information that is not covered by the default Certificate of Analysis. Click on the **Comments for report** header to open the comments field in a separate window for easy access from other views.

2.4.6 Flagged Positions

This field shows the Olink panel, sample names and plate name for flagged samples. The list is automatically populated when the NPX values have been calculated. If a sample is flagged in the QC, every assay in that Olink panel is flagged for that sample.

2.4.7 Comments

This field can be used for any comments and is not included in Certificate of Analysis or data export. Click on the **Comments** header to open the comments field in a separate window for easy access from other views.

2.4.8 Project Options

2.4.8.1 Samples Randomized

Check this box if samples are randomized across plates and intensity normalization is used. For more information, refer to *4. Normalization methods*.

2.4.8.2 Display NPX values < LOD As

Select how Olink NPX Manager should display NPX values below the LOD (Limit of Detection):

- **NaN** (Not a Number): NaN is displayed for values below LOD.
- **LOD**: Values below LOD are replaced with the calculated LOD for that assay.
- **Actual Value**: The actual NPX value is displayed. This is the default.

2.5 Plate View

The **Plate View** tab shows the sample plate setup for the selected run, and general information about the Fluidigm run.

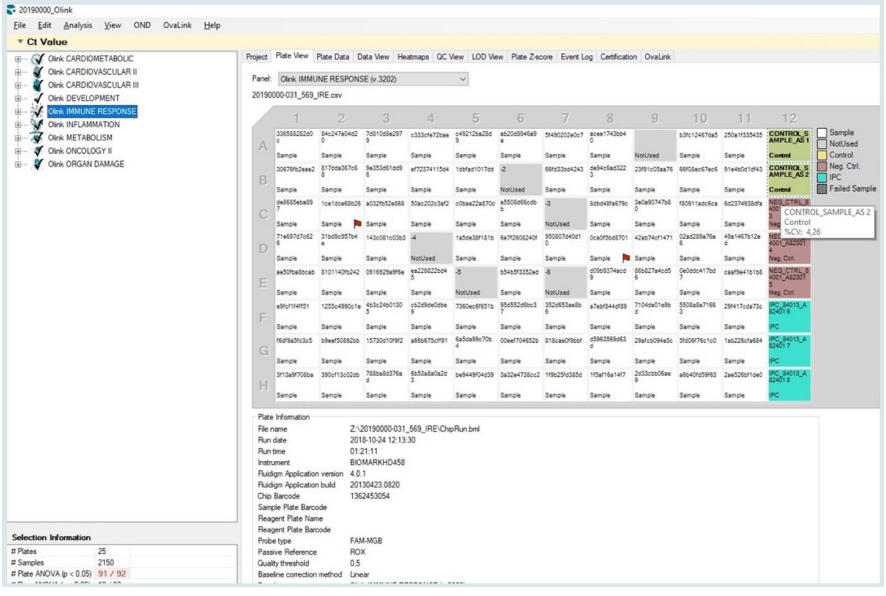


Figure 8. The Plate View tab for a project with Olink® Target 96 panels.

On the **Panel** drop-down menu, it is possible to select which data file version to use, with the data file selected during import set as default. The data file version is linked to the lot number of the reagent kit for the Olink panel that was used. The lot number can be found in the *Article and lot configuration* sheet delivered together with the analysis kit. The same data file version must be used for all runs/sample plates analyzed with the same Olink panel within a project. Each plate position is automatically annotated to one of the following content types, based on the sample naming:

- Sample
- Control
- Negative Control
- IPC (Olink Target 96)
- Plate Control (Olink Explore)
- Not Used

Olink® Target 48

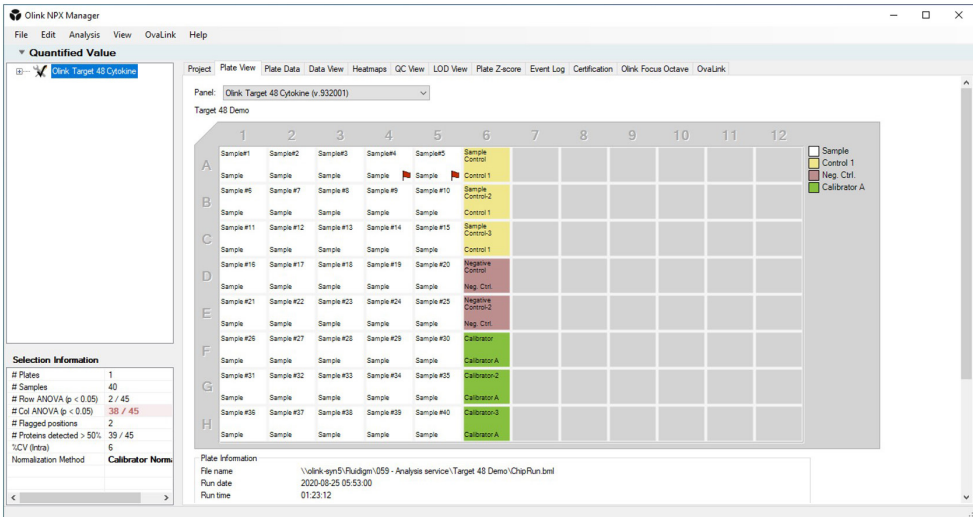


Figure 9. The Plate View tab for a project with Olink® Target 48 panels.

Additional content types are Control 1-4 and Calibrator A. For Olink Target 48 Cytokine, Calibrator A, Negative Controls and Control 1 types must be annotated for calibrator normalization and QC to be performed. Up to four different control sample types (Control 1-4) can be selected, but only Control 1 should be used for Olink Target 48 Cytokine.

Olink® Explore

Control, Plate Control, and Negative Control have different coloring than Olink Target panels.

2.5.1 Incorrect annotation

If a position has been annotated incorrectly:

1. Olink® Target 48 and Olink® Target 96

   Click on the cell or click and drag over several cells to select adjacent cells. Click and hold the ctrl or shift key to select additional cells.

2. Right-click on the selection and select the correct content type.

2.5.2 Tooltip for %CV

Selecting more than one well will show a tooltip with the %CV for the selection.

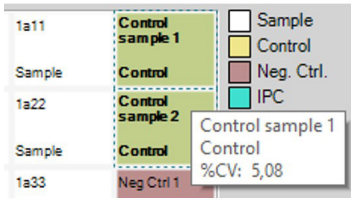


Figure 10. The tooltip showing the CV% for the selection.

2.5.3 Flagged samples

Wells with QC warnings are marked with flags; red for automatic and yellow for manual set QC warnings. Refer to *2.6 QC Warnings (Olink® Explore projects).*

To remove wells from the calculation, the well(s) can be set as **Not Used**. Right-click on a sample and select **Not Used**. The flag will disappear and the well is marked dark grey.

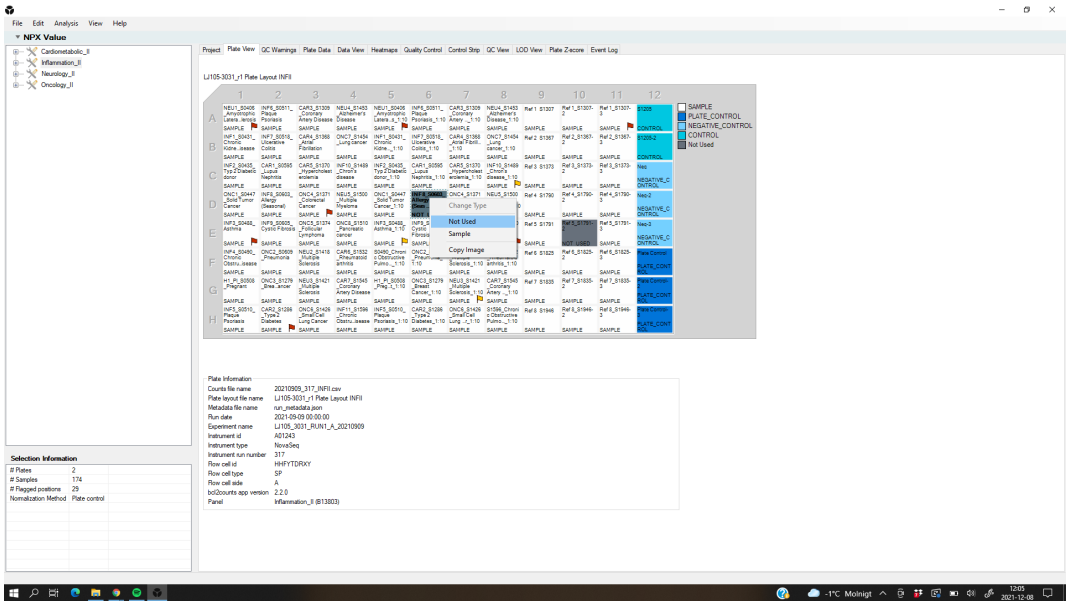


*Figure 11. Set **Not Used** status.*

The software automatically calculates new QC values without the Not Used samples and the plate QC data can be reviewed again. For samples marked as not used, no data is shown in the NPX file. Refer to *7.5 Perform quality controls* for more information.

NOTE: NPX calculation and normalization will automatically be recalculated for all plates from the same Olink panel if a sample, assay or datapoint has been set as not used.

Olink® Target 48

Data for flagged samples is exported to the results file in standard concentration units, pg/mL but the results will be written in red and the sampe will be noted with Warning in the QC warning column. For samples, assays, or data points marked as not used, **No Data** is shown in the results file. The calculation of concentration in pg/mL and normalization will be automatically recalculated for all plates from the same Olink panel if a sample, assay or datapoint has been set as not used.

2.6 QC Warnings (Olink® Explore projects)

The **QC Warnings** pane displays the plate layout with automatic QC warnings (red flag) and manual QC warnings (yellow flag).

All manuals are listed below the plate layout.

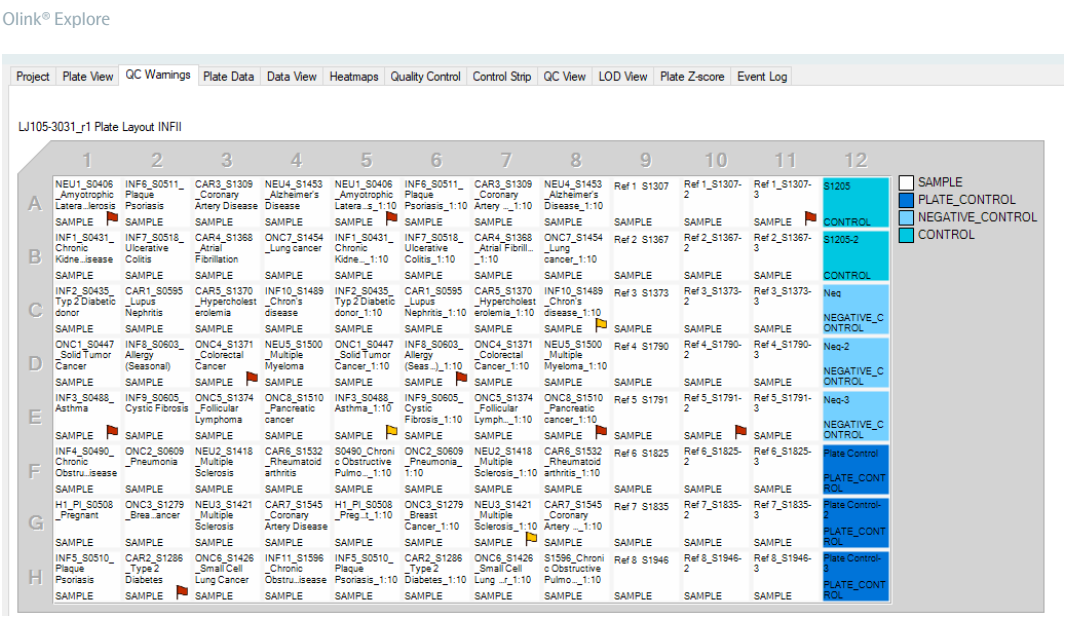


Figure 12. QC overview dislaying automatic (red flag) and manual (yellow flag) QC warnings.

1. To add or edit a manual QC warning:
2. Click on preferred sample well. To mark a cluster of wells, click and drag over preferred wells.
3. In the pop-up menu, select preferred block(s) by cklicking the checkbox(es).
4. Fill in reasons for creating a QC warning.

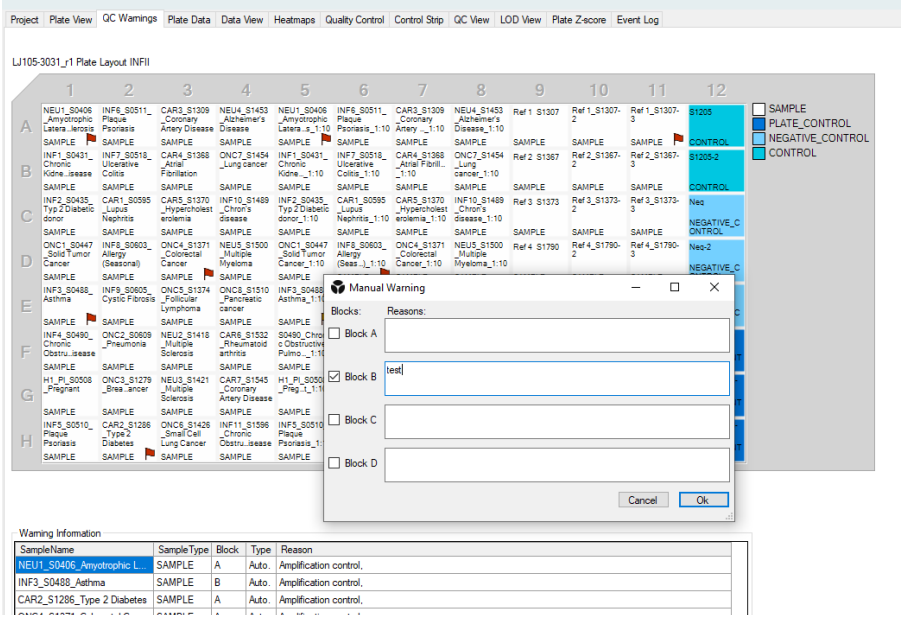


Figure 13. Edit manual QC warning menu

5. Click **Ok**. A yellow flag will appear on the sample well(s).
6. Repeat step 1-4 until all affected wells are marked.
7. Click **Save**.

2.7 Plate Data

The **Plate Data** tab shows by default heatmaps of the four internal control assays, Incubation Control 1, Incubation Control 2, Extension Control and Detection Control. This view is useful for identifying position effects within a plate (e.g. if one column of samples deviates from the rest, this could indicate a pipetting error).

Olink® Target 48

In Olink® Target 48 panels only one Incubation Control is included. The default heatmaps show the three internal control assays, Incubation Control (shown in two maps), Extension Control and Detection Control.

Olink® Explore

In Olink® Explore panels, the heatmaps show assays available in each abundance block.

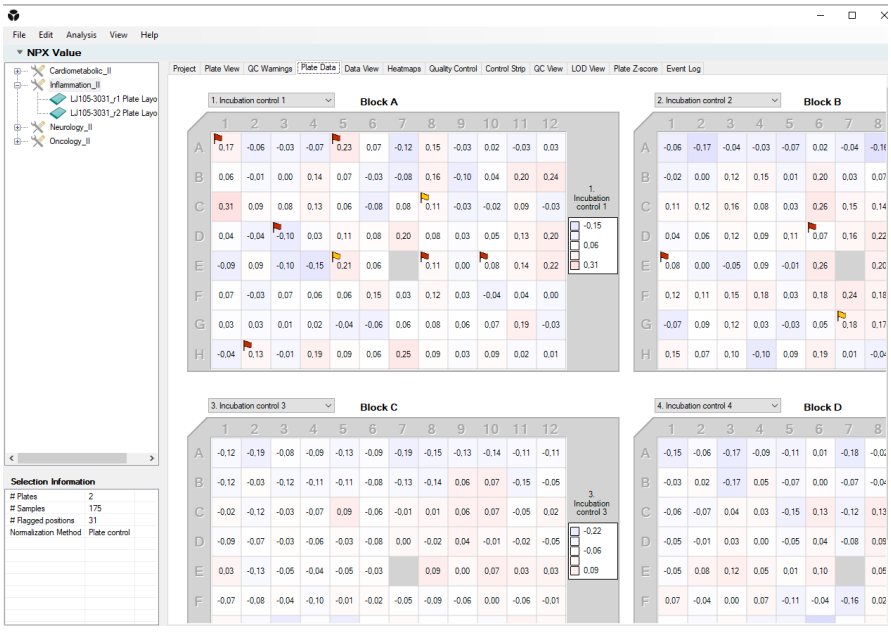


Figure 14. The Plate Data tab.

The heatmap displays deviations from the plate median for the selected assay using the colors blue and red. Blue values are below median and red values are above median. The values displayed in the view are either Ct-values, NPX-values or quantified values in pg/mL depending on the selection in the upper left corner of the screen.

Selecting more than one well will show %CV for the selection as a tooltip in the same way as in Plate View.

Use the assay drop-down menu to change which assay is displayed in the heatmap.

2.8 Data View

The **Data View** tab displays data (Ct or NPX) in a grid view, depending on the selection in the upper left corner of the screen. If NPX values are displayed, the plate LOD is also displayed after each run in the list.

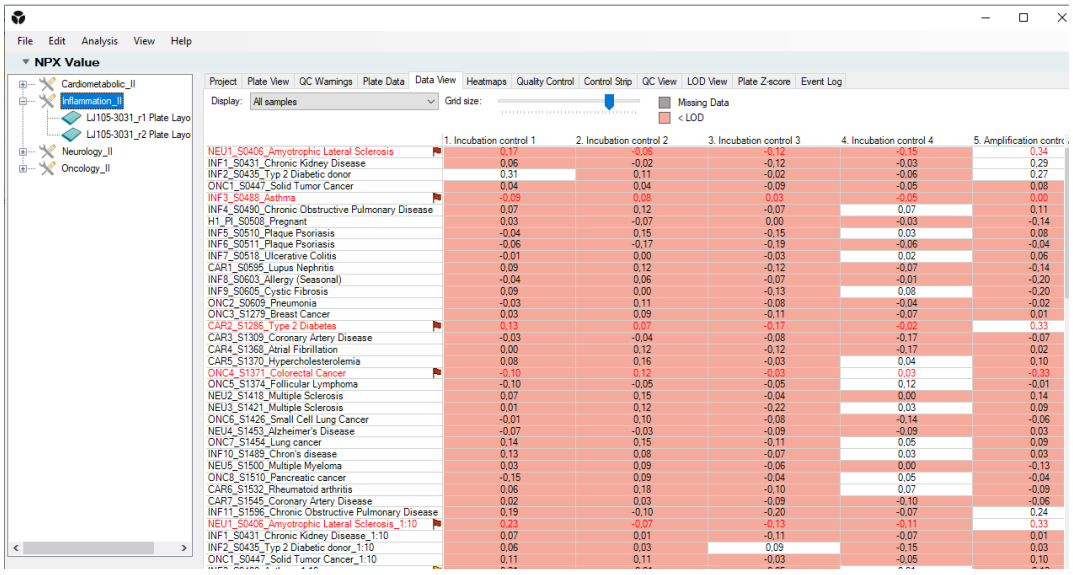


Figure 15. The Data View tab displaying data in NPX.

- Use the **Display** drop-down menu to select if you want to display data for all sample types or for samples, controls, Negative Control or IPC only.
- Use the **Grid size** slider to decrease or increase font and cell size of the grid to make it easier to read or to see patterns between runs.

Olink® Target 48

- To mark samples, assays or data points as Not Used, right-click on the failed entity and select “Mark as Failed” from the popup menu. A pop-up audit window asks for the reason for the change. Enter the reason and click **OK**. This information is displayed on the Certificate of Analysis. The exported data output file displays empty cells for data points or samples marked as “Failed”. To undo this operation, select “Mark as Valid” from the popup menu.

Olink® Target 48

For Olink® Target 48 panels, the Data View can also be displayed in pg/mL using the **Quantified Value** setting.

- For Extension, Incubation and Detection Controls it is not possible to calculate quantified values, therefore the cells remain blank.
- Failed data points are indicated by **No data** in grey cells.
- Data higher than ULOQ is indicated as >ULOQ with a red background.
- Cells that contain data values below LLOQ (default) or plate LOD (when plate LOD> LLOQ) are indicated by a red background.
- Data below the lowest fitting parameter in the 4PL curve fit model cannot be calculated and is therefore indicated as **NaN** in red cells. Refer to *4.4 Calibrator normalization*.
- For samples or assays with a QC warning, results are shown as described above but highlighted in red text.
- For each plate and assay, values for LLOQ, ULOQ, LQL and LOD are presented on separate rows below the data for the samples of each plate. Lowest Quantifiable Level (LQL) is defined as the value used as lower limit, LLOQ (default) or plate LOD (when plate LOD > LLOQ).

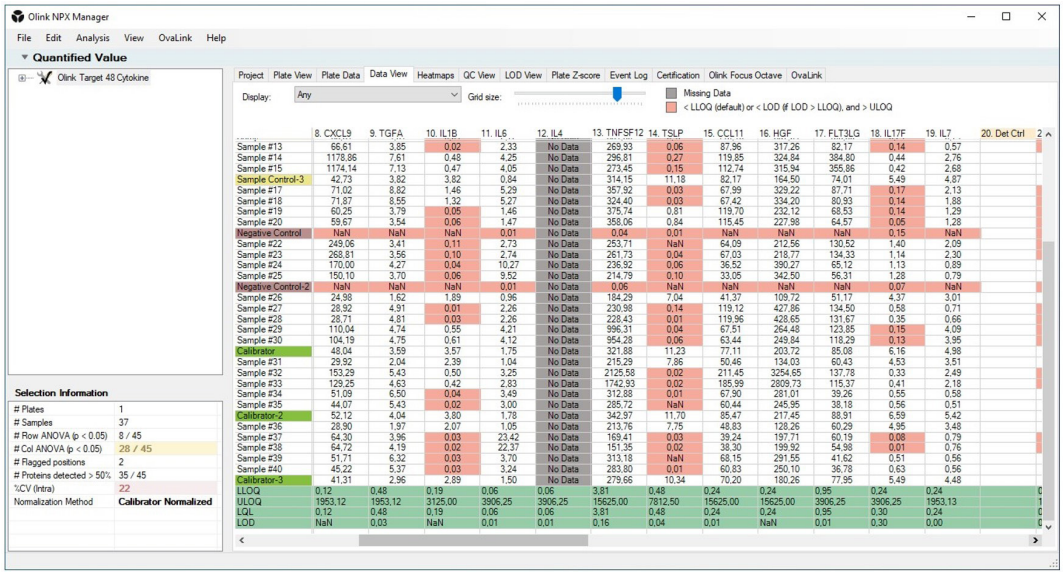


*Figure 16. The Data View tab with the **Quantified Value** setting, displaying data in pg/mL.*

2.9 Heatmaps

The **Heatmaps** tab contains a color-coded view for all assays and samples in the selection. The heatmap uses a color scheme of red and blue to display any deviations from the plate median for each assay.

Displayed values are either Ct (Counts for Olink® Explore) or NPX values, depending on the selection in the upper left corner of the screen. For NPX, blue values are below the median and red values are above the median. For Ct values, red values are below the median and blue values are above the median. This view is useful for finding patterns, such as samples or plates where the levels of many proteins are higher or lower than the plate median, or outlier samples for specific assays.

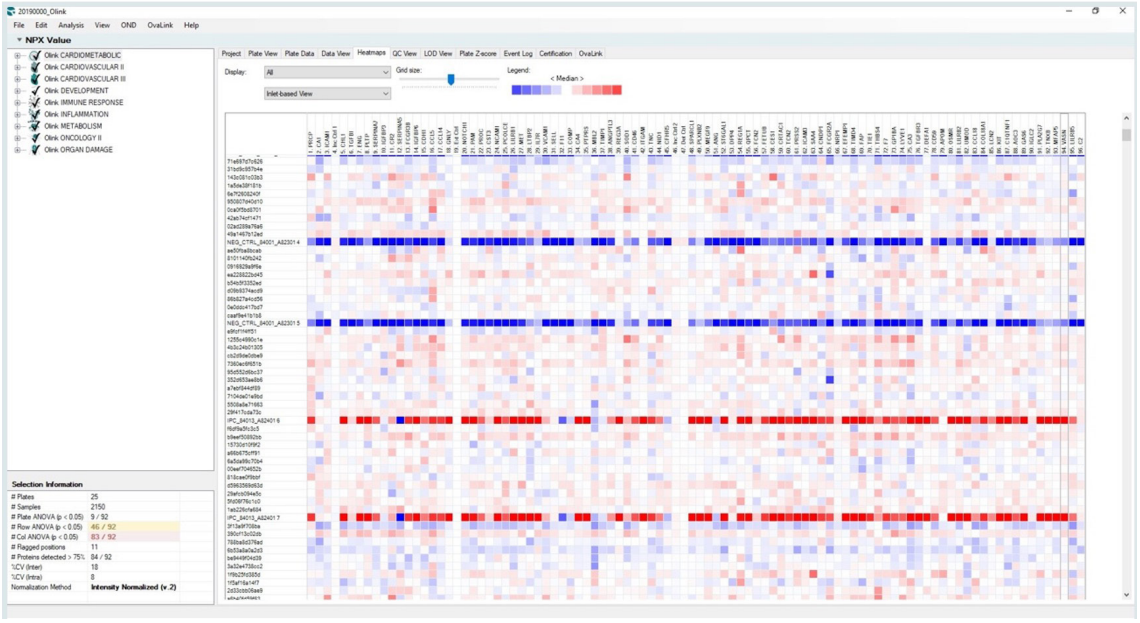


Figure 17. Heatmaps view showing samples' deviation from mean per assay.

- Use the **Display** drop-down list to select the sample type to display (Sample, IPC, NegCtrl, Control).
- Use the **Grid size** slider to increase or decrease the size of the heatmap to make it easier to read or to see patterns between plates.
- Gridlines are displayed to highlight the selected sample and assay.
- Display samples column- or row-wise according to their position on the 96-well plate, or chip, or as inlet-based view according to the IFC layout from Fluidigm.

Olink® Target 48

For Olink® Target 48 panels, Calibrators are available as sample type from the **Display** drop-down menu. When the Quantified Values setting is used, the heatmap shows the quantified values in pg/mL where blue values are above the plate median and red values are below the plate median. For failed assays, samples, data points and values with **NaN**, the cells are marked in black.

2.10 Control strip (Olink® Explore projects)

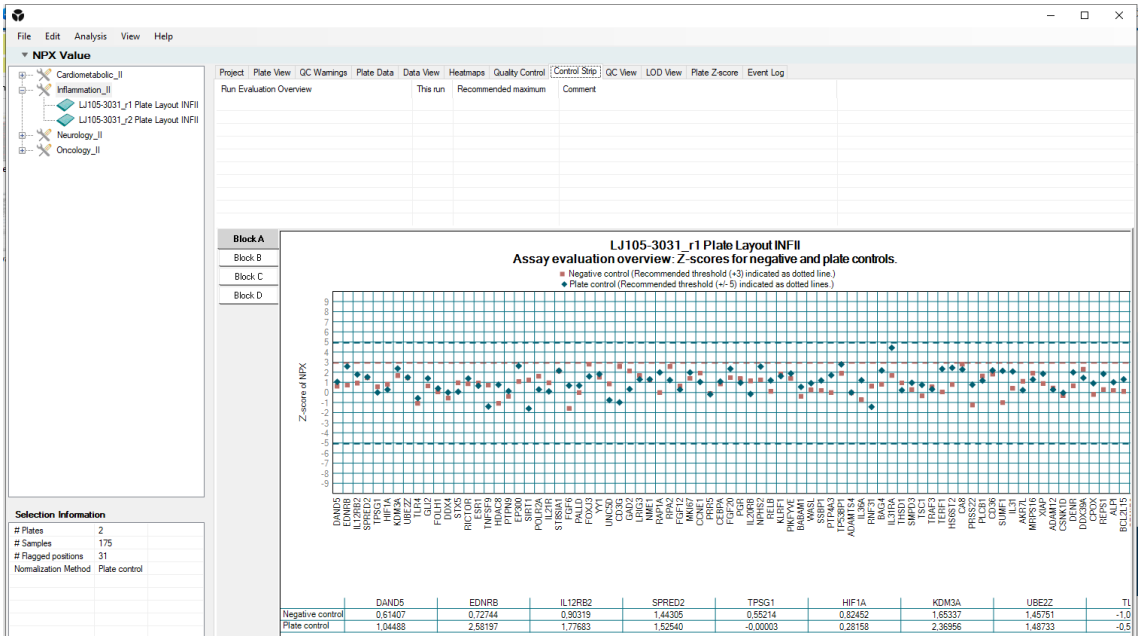
The **Control Strip** tab is only displayed for Olink® Explore projects.

The Plate Controls have specific QC criteria for precision, measured as median absolute deviation (MAD) of the Z-score, and accuracy, measured as median of the Z-score. The Z-scores are computed using predefined values set for each assay.

The Control strip view displays z-scores for Negative Controls (red squares) and Plate Controls (blue diamond) in a scatterplot per block. Yellow color denotes deviations that are out of bounds of the graph. The median absolute deviation (MAD) of the Z-score of Plate controls must not exceed 1.5, the absolute value of the median Z-score of Plate controls must not exceed 5.

In the tree view, select the plate to view.

Hovering over a data point in the plot will display the sample name and the deviation from the median.

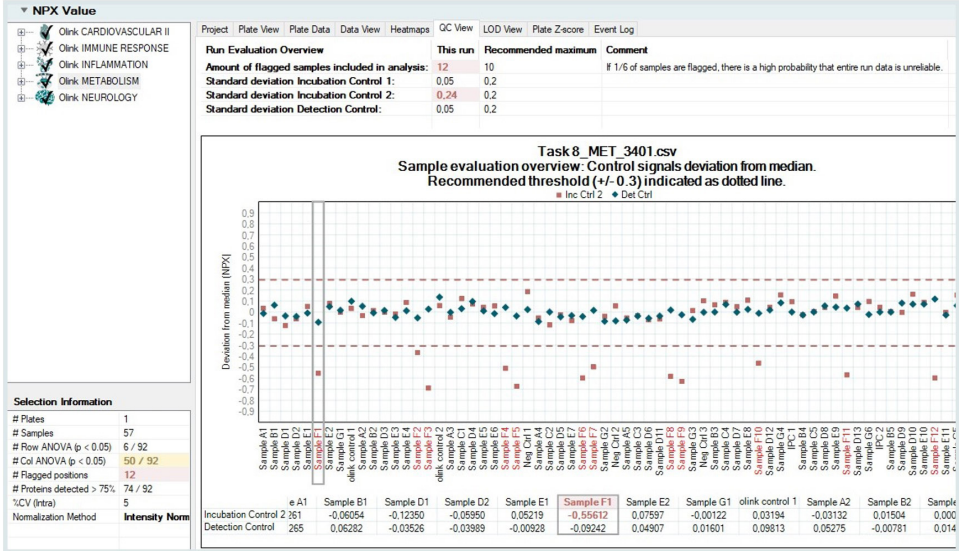


	DAND5	EDNRB	IL12RB2	SPRED2	TPSG1	HIF1A	KDM3A	UBE2Z	TL
Negative control	0,61407	0,72744	0,90319	1,44305	0,55214	0,82452	1,65337	1,45751	-1,0
Plate control	1,04488	2,58197	1,77683	1,52540	-0,00003	0,28158	2,36956	1,48733	-0,5

Figure 18. The Control Strip View tab for Olink® Explore.

2.11 QC view

The **QC View** tab displays quality control data for the plate selected in the Tree View. The quality control is performed individually for each plate during the NPX calculation. All values are set to zero until the NPX has been calculated.



	e A1	Sample B1	Sample D1	Sample D2	Sample E1	Sample F1	Sample E2	Sample G1	olink control 1	Sample A2	Sample B2	Sample
Incubation Control 2	261	-0,06054	-0,12350	-0,05950	0,05219	-0,55612	0,07597	-0,00122	0,03194	-0,03132	0,01504	0,000
Detection Control	265	0,06282	-0,03526	-0,03989	-0,00928	-0,09242	0,04907	0,01601	0,09813	0,05275	-0,00781	0,014

Figure 19. The QC View tab for Olink® Target 96.

Olink® Target 48 and Olink® Target 96

- The top table displays the number of flagged samples on the selected sample plate and the standard deviation for the internal control assays: Incubation Control 1 and 2 and Detection Control. Values that do not meet the QC specifications are highlighted in red.
- For Target panels, the scatter plot displays the NPX signal deviation from median for Incubation Control 2 and Detection Control for all samples. If either of these values exceed the ±0.3 NPX threshold (the red dotted lines), the sample is marked as flagged. Flagged samples can be excluded from the QC analysis by right-clicking on the flagged

sample and selecting **Exclude Flagged Sample From QC Analysis**. The QC data is then recalculated without the excluded samples. To undo this operation, select **Include Flagged Sample In QC Analysis**. The sample name of a sample that did not pass the QC will be highlighted in red.

- Click the control name in the scatter plot legend above the plot to toggle the display of the controls on and off.
- Click a sample on the grid to automatically display its values in the table below, or click on a value in the table to highlight the sample in the scatter plot.

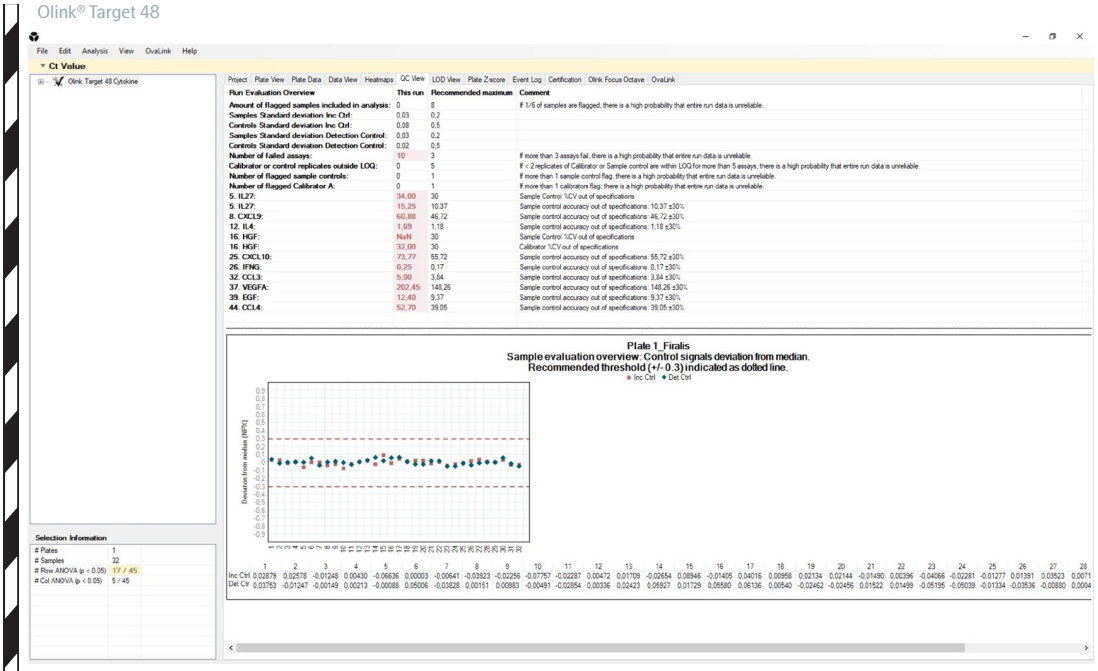


Figure 20. The QC View tab for Olink® Target 48.

- For Olink® Target 48 panels, the top table shows the standard deviation for the internal control assays: Incubation Control and Detection Control. The standard deviation of the NPX values is presented separately for samples and controls and should be below 0.2 for samples and below 0.5 for controls.
- Olink® Target 48 also has additional QC criteria compared to Olink® Target 96, which are listed in the top table as seen in the figure above. These include Number of failed assays, Calibrator or control replicates outside LOQ, Number of flagged Sample Controls and Number of flagged Calibrators. For descriptions of the criteria Refer to *3.2 Run QC*.
- Assays where the Calibrator CV%, or the Sample Control CV% or accuracy are outside specifications are listed in the top table. Under **This run**, the calculated CV for the assay will be highlighted in red, and under **Recommended maximum**, the recommended maximum CV% will be displayed. For accuracy of the Sample Control, the quantified value in pg/mL highlighted in red is listed under **This run**, and the expected value in pg/mL is listed under Recommended maximum.
- If a Calibrator is flagged, this one calibrator can and should be excluded from the analysis. If a Sample Control is flagged, this one Sample control can and should be excluded from the analysis. If more than one Sample Control or Calibrator are flagged, the entire run is considered unreliable and the plate should be re-run. If Sample Control CV% and/or accuracy are outside specification for several assays (Refer to *8.3 Deviating controls for Olink® Target in Olink® NPX Manager*), a maximum of one sample control can be excluded from the analysis. If Calibrator CV% is outside specification for several assays (Refer to *8.3 Deviating controls for Olink® Target in Olink® NPX Manager*), a maximum of one Calibrator can be excluded from the analysis.
- The scatterplot for Olink® Target 48 panels displays the NPX signal deviation from the median for Incubation Control 1 and Detection Control for all samples. If either of these values exceed the ±0.3 NPX threshold, the sample is marked as flagged.

Olink® Explore

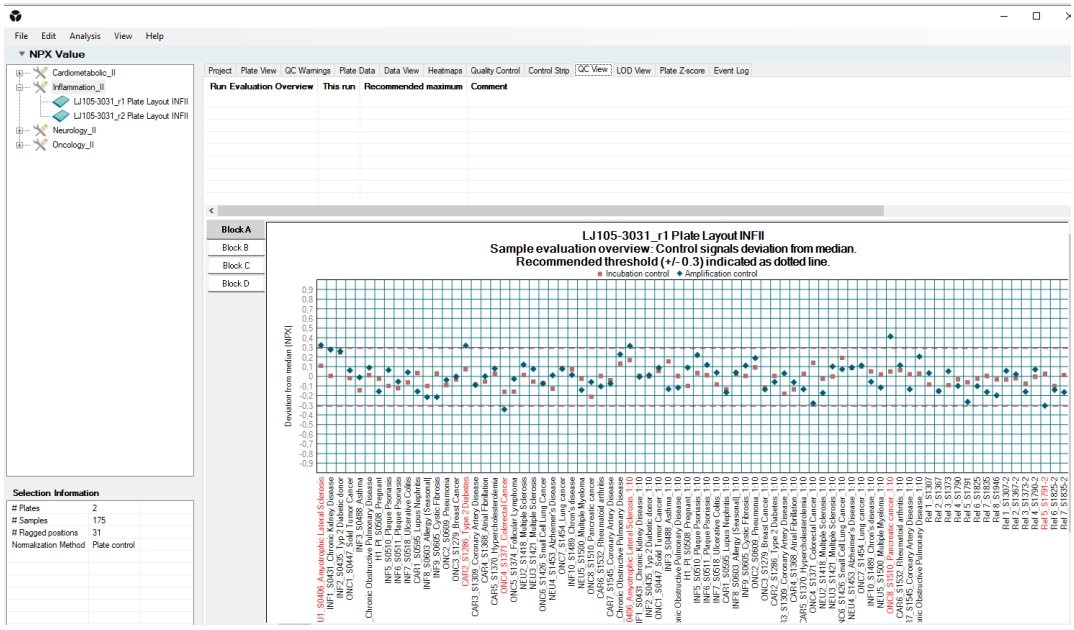


Figure 21. The QC View tab for Olink® Explore.

The **QC** View tab displays the deviation from the median (zero) in all samples for Incubation Control (red squares) and Amplification Control (blue diamonds). Red dotted lines depict the QC criteria cutoff of 0.3 NPX deviations from the median. Choose which block is displayed by clicking the corresponding block button. The x-axis is ordered by row, so the sample at A1 is far left, followed by the sample at A2.

For more information about the QC process, refer to *3. Quality control*.

Apart from the **QC View**, Olink® NPX manager offers several tools for troubleshooting and issue detection. Guidelines for what is considered an issue are available for the **LOD View** and the **Selection Information** view and can serve as a good starting point. For more information about the **Selection Information** pane, see *Table 1*. Known issues are described in the Troubleshooting section. The purpose of these guidelines is to help the Olink user and operator to produce high quality data.

2.12 Quality Control (Olink® Explore projects)

Olink® Explore

The **Quality Control** tab is only displayed for Olink® Explore projects.

The Quality control tab displays an overall quality assessment for the selected plate, and a green checkmark or a red x indicates if the run passes QC or not. Refer to *3.1 Sample QC*, section *3.2 Run QC* and section *3.3 Assay QC* for description of the QC criteria.

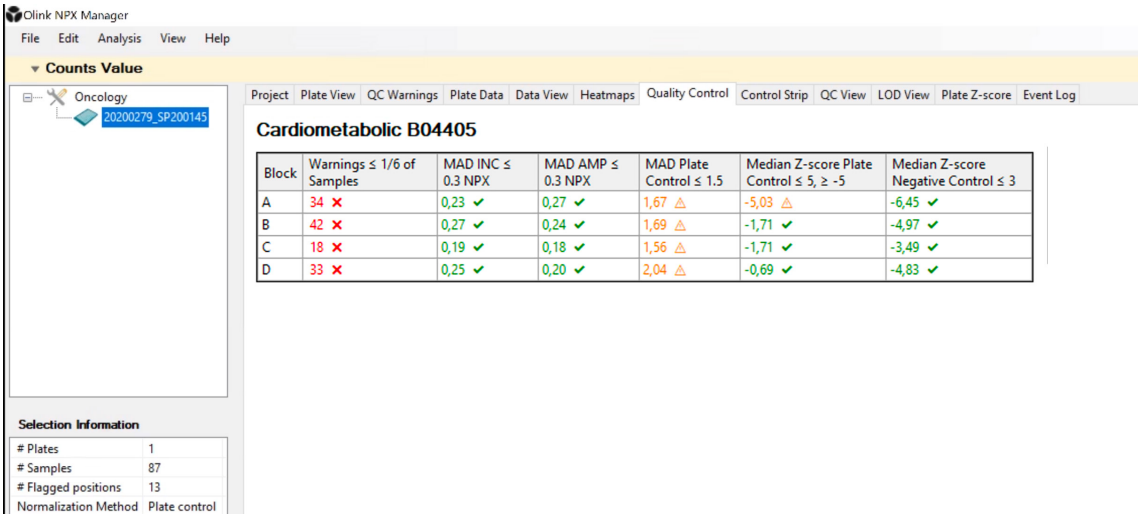


Figure 22. Quality Control tab for Olink® Explore.

2.13 LOD View

The **LOD View** contains a bar chart showing frequency of missing data for all plates in the selected Olink panel or for a specific plate only. When multiple plates are analyzed, the maximum LOD is used as the data cut-off.

The table below the bar chart shows the Limit of Detection in NPX for all plates in the selected Olink panel or for an individual plate.

LOD is estimated using NPX values and the following formula:

$$LOD_{Assay\ X} = Average\ (Negative\ control_{Assay\ X}) + 3 * Fix\ SD_{Assay\ X}$$

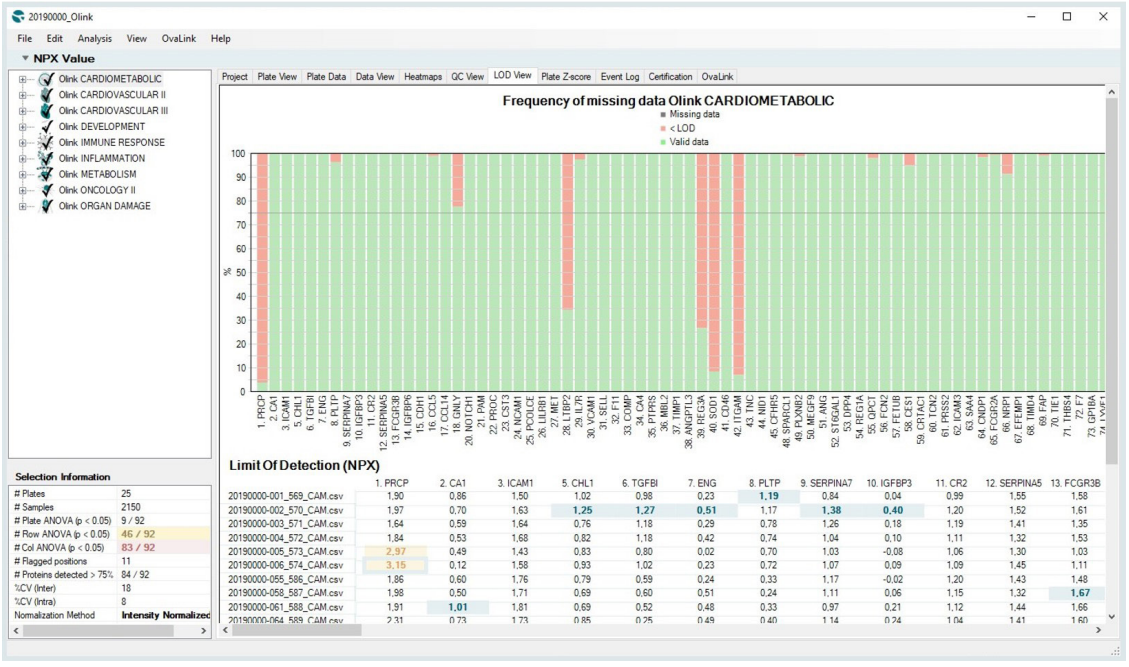


Figure 23. The LOD View tab for Olink® Target 96 panels.

- The maximum LOD value for each assay is highlighted in blue when a panel with more than one plate is selected. For assays highlighted in yellow, only the frame is highlighted in blue.
- An assay value highlighted in yellow signifies an assay with LOD higher than +2.5 or lower than -2.5. A high LOD means that the signal for the Negative Controls is higher than what was measured during validation of the panel. If a high LOD results in low detectability, you might want to investigate whether the LOD affects the data, Refer to *8.3 Deviating controls for Olink® Target in Olink® NPX Manager*.
- Click on an assay in the bar chart to highlight the assay in the table below.

- When the **Quantified Value** display setting is used, assays that are outside the LOQ range will be indicated in red in the bar chart. The lower limit of quantification is LLOQ (default) or plate LOD (when plate LOD >LLOQ). The upper limit is ULOQ. The approximate limits for LLOQ and ULOQ are defined during the development of the panel and specifications for LLOQ and ULOQ are established for each lot.
- There is no highlighting of assays with high LOD for Olink® Target 48 panels. LOD for some assays in Olink® Target 48 panels can have values above 2.5 without having a high LOD issue. If the high LOD results in a low number of samples that can be quantified above LOD, it is recommended to investigate whether or not the LOD affects the data (Refer to *8.3 Deviating controls for Olink® Target in Olink® NPX Manager*).

Figure 24. The LOD View tab for Olink® Target 48 panels.

Olink® Explore

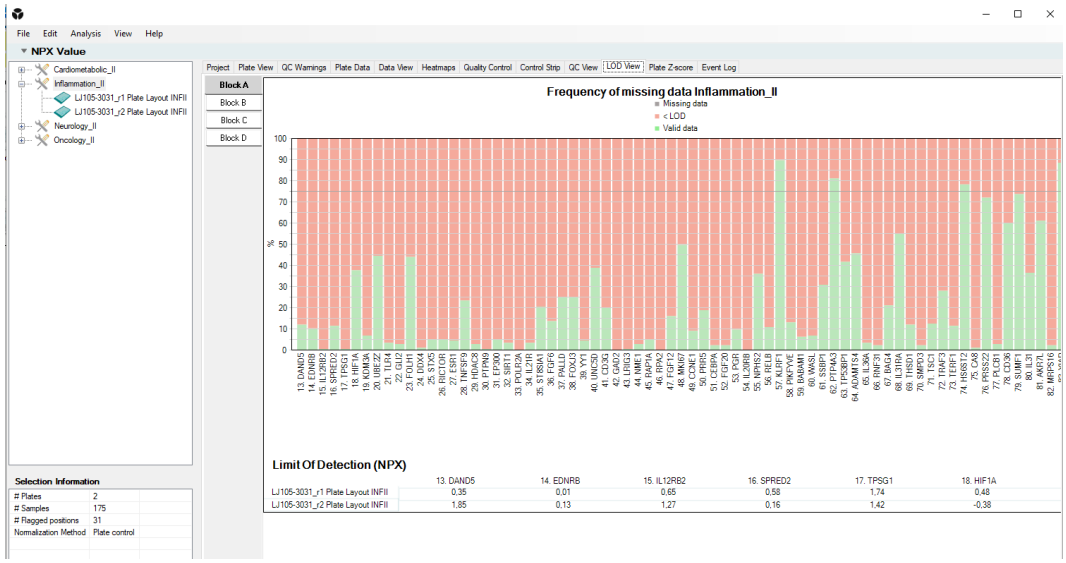


Figure 25. The LOD View tab for Olink® Explore panels

- LOD graph is displayed per block. Use the buttons to the upper left next to the graph to switch between blocks.
- Assays with deviating Negative Controls +3 SD in Z-score are highlighted in yellow In the list below the graph. Assays with Z-scores > 5 SD that receive an assay warning in the results file are highlighted in orange in this list.

2.14 Plate Z-score

The **Plate Z-score** tab displays a box plot which represents the deviation of total intensity of each sample from the plate median. Use this plot to detect systematic variance in total intensity between plates or to identify samples with deviating total intensities.

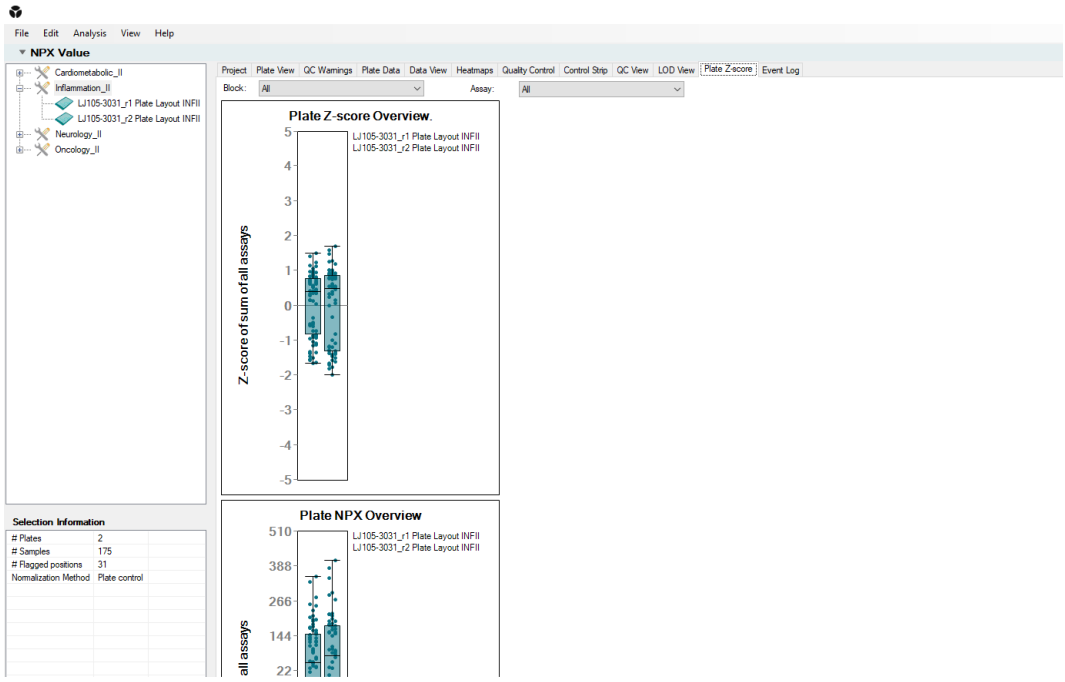
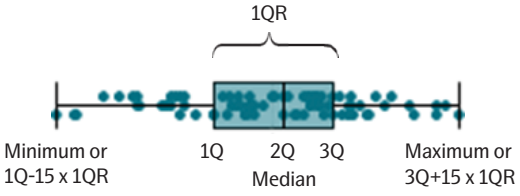


Figure 26. The Z-score tab displaying z-score box plot for the sum of all assays per plate.

A box plot is a graph that indicates how the values in the data are spread out. It shows outliers, symmetry, how tightly the data is grouped, and if and how the data is skewed. The box plot splits the data set into quartiles. The first quartile, (1Q), is the 25th percentile and the third quartile (3Q) is the 75th percentile. A vertical line is drawn at the median, the second quartile (2Q). The interquartile range (IQR) is a measure of where the bulk of the values in the data set lie, and how they cluster around the mean.



- The sum of NPX for each sample is calculated and converted to Z-scores. This means that a sample at +1 on the plot has one standard deviation higher than the average NPX value for all the samples.
- Use the **Assay** drop-down menu to determine whether the plot should visualize the Z-score distribution for all assays or for an individual assay in the panel.
- An outlier sample will be highlighted in bright red.
- Use the search function to search for a specific sample name. The matching sample will then be highlighted in dark red in the boxplot.

2.15 Event log

The **Event Log** tab displays all events for the current project.

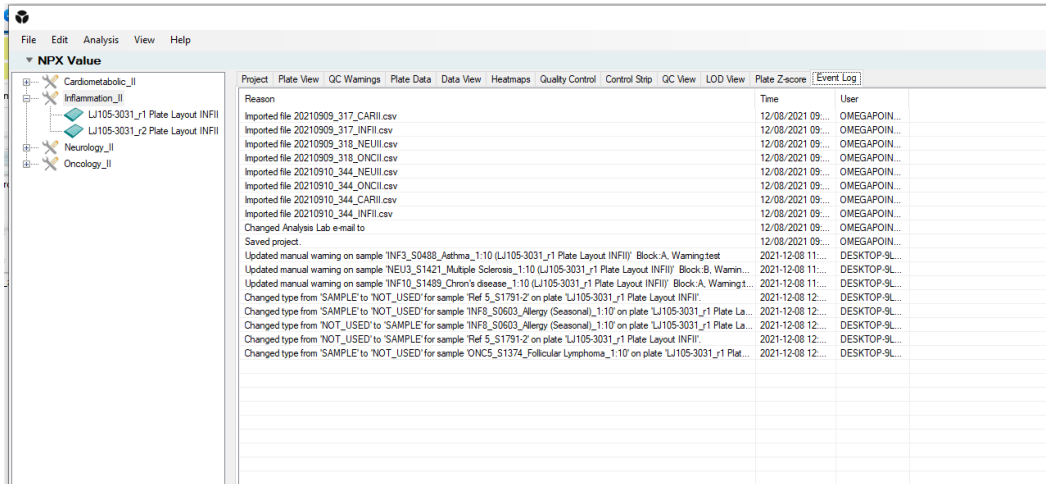


Figure 27. The Event Log tab displaying all changes performed on the project.

3. Quality control

Olink has built-in quality controls in all multiplex panels. Each 92-plex panel contains 96 assays. Four of these are internal controls that allow for an in-process quality control designed to monitor different steps of the protocol: immuno reaction, extension and amplification/detection. The controls included are illustrated in *Figure 28*.

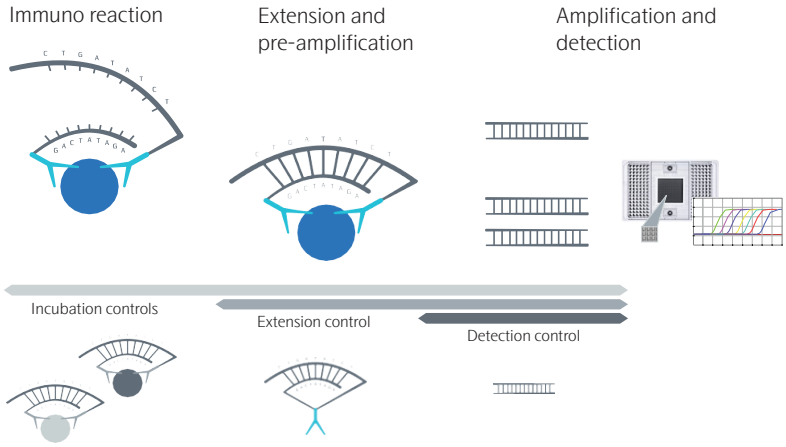


Figure 28. Outline of Olink internal controls for Olink® Target 96 panels.

- The two Incubation Controls consist of two different non-human antigens measured with PEA assays: Incubation Control 1 and 2. These controls monitor potential variation in all three steps of the reaction.
- The Extension Control is an antibody coupled to both DNA-tags (hence always in proximity). This control monitors the extension/pre-amplification and amplification/detection step and is used for normalization of the data.
- The Detection Control is a complete double stranded DNA amplicon which does not require any proximity binding or extension step. This control monitors the amplification/detection step.

The internal controls are used for both sample and run QC as described below. The quality control of data is performed separately for each sample plate.

Olink® Target 48

Each Olink Target 48 panel contains 48 assays, three of which are internal controls. These are: Incubation Control, Extension Control and Detection Control. Only one Incubation Control is used in Olink Target 48 panels, Incubation Control 1. The controls are used for sample and run QC as described below.

Olink® Explore

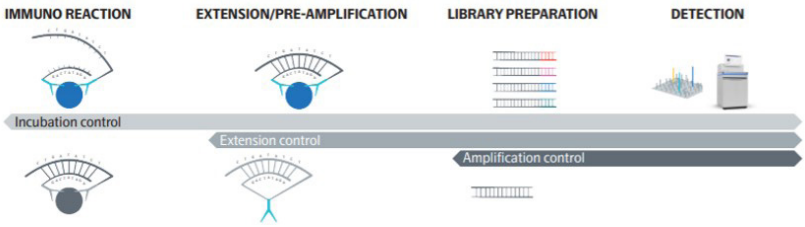


Figure 29. Outline of Olink internal controls for Olink® Explore panels.

Each Olink Explore 384 panel contains three internal controls. These are: Incubation Control, Extension Control and Amplification Control. The controls are used for sample and run QC as described below.

3.1 Sample QC

Each of the internal controls is spiked into all samples at a set concentration. The signals for these are therefore expected to be the same over the entire plate. Sample QC is performed using the Detection Control and Incubation Control 2. Within each run, the levels of these controls are monitored for each sample and compared against the plate median of all samples. If either of the controls deviate more than the acceptance criteria allow (see below), the sample is flagged. The Extension Control is used in the normalization step and in generation of NPX, and hence is not included in the quality control of data.

Acceptance criteria for passing a sample:

- Incubation Control 2 and Detection Control deviates <±0.3 NPX from the plate median.

Deviating values for the internal controls can be caused by, for example, errors in pipetting or pre-analytical factors in the samples that affect the performance of the controls. For more information on troubleshooting flagged samples, Refer to *8.3 Deviating controls for Olink® Target in Olink® NPX Manager*.

Olink® Target 48

Incubation Control and Detection Control are used for the sample QC of the Olink® Target 48 panels. The acceptance criteria for passing a sample are that Incubation Control and Detection Control deviate no more than ±0.3 NPX from the plate median.

Olink® Explore

Incubation Control deviation < ±0.3 NPX from plate median

Amplification Control deviation < ±0.3 NPX from plate median

Average counts per sample > 500 counts

3.2 Run QC

The internal controls are also used in the run QC. This QC assesses the variation over the plate for each of the Incubation Controls 1 and 2 and the Detection Control. If the variation for one of the controls is too large (see below) the entire run is considered unreliable.

Acceptance criteria for passing a run:

- Standard deviation of Incubation Control 1 and 2 and Detection Control: < 0.2 NPX
- Number of flagged samples: ≤ 1/6 of the total number of samples (i.e. ≤ 15 in a full plate)

Olink® Target 48

- Standard deviation of Incubation Control and Detection Control in samples: < 0.2 NPX
- Standard deviation of Incubation Control and Detection Control in controls: < 0.5 NPX
- Number of flagged samples: ≤ 1/6 of the total number of samples (i.e. ≤ 8 in a full plate)
- Number of failed assays: ≤ 3 out of 45 (Refer to *3.3 Assay QC*)
- ≤ 1/3 of the Sample Control or Calibrator replicates are outside of LOQ for < 5 assays
- Number of flagged Sample Controls: ≤ 1
- Number of flagged Calibrators: ≤ 1

Olink® Explore

- Number of flagged samples ≤ 1/6 of total number of samples on plate (15/88 samples on a full plate)
- MAD of Incubation Control ≤ 0.3 NPX
- MAD of Amplification Control ≤ 0.3 NPX
- MAD of Plate Control ≤ 1.5 NPX
- Median of Z-score Plate Control ≤ 5, ≥ -5
- Median of Z-score Negative Control ≤ 3

If a too large variation is observed for either of the controls, go to the **Plate View** tab to evaluate the data. For example, if individual samples show extreme values or if a certain sample column is affected, these samples can be marked as failed, and the QC redone and re-evaluated. For more information on troubleshooting of this step, refer to *8.3 Deviating controls for Olink® Target in Olink® NPX Manager*.

In addition to passing or failing individual plates, ensure that no systematic bias is present in the data. The **Selection Information** view in the bottom left corner alerts you to such issues.

%CV is calculated using control samples if present in duplicates on each sample plate. The reported %CV is the mean %CV over all assays, and this is only calculated using data over LOD. A high %CV does not fail a run automatically but should be a cause for further investigation.

- Reference value for Inter %CV: < 25%
- Reference value for Intra %CV: < 15%

Olink® Target 48

%CV is calculated using the Sample Control present in triplicates on each sample plate. The reported %CV is the mean %CV over all assays, and this is only calculated using data in pg/mL from non-flagged replicates within LOQ. A high %CV does not fail a run automatically but should be a cause for further investigation.

- Reference value for Inter %CV: < 25
- Reference value for Intra %CV: < 15%

3.3 Assay QC

Olink® Target 48

Quality control of assays is performed for Olink® Target 48. An expected concentration value for the Sample Control for each assay is defined during the validation of the panels. The accuracy and precision for the calculated concentration values are evaluated for each assay.

Acceptance criteria for passing an assay:

- The accuracy of the calculated concentration mean for the Sample Control and Calibrator for each assay must fall within ±30% of the expected concentration.If this criterion is not met, the assay will be marked with assay warning and results are presented as pg/mL in a grey cell in the result file.
- The precision of the calculated concentration for the Sample Control must be < 30% for intra-CV. If this criterion is not met, the assay will be marked with assay warning and results are presented as pg/mL in a grey cell in the result file.
- A maximum of one of the Calibrator replicates and/or the Sample Control replicates can fall **outside** of the limits of quantification (LOQ). If the criterion is not met, the assay is noted as "No Data" in the results file.
- The precision of the calculated concentration for the Calibrator is evaluated and should be <30% for Intra-CV. If this criterion is not met, the assay will be marked with assay warning and results are presented as pg/mL in a grey cell in the result file.

3.4 Additional quality assessment

Olink® Explore

Click the following views in the left menu to assess the quality of the data in each view and further determine if any reruns are required (panes are described in section Quality control view):

QC View: View deviation from the median in all samples per plate and panel. Each block is displayed. Assess samples deviating > 0.3 NPX from the median.

Plate Data View: View the values of internal controls and assays (change assay in the drop-down menu above the plate layout) for each sample plate and panel. Each block is displayed. Assess if there are patterns in the plate (i.e., row or column) representing a technical error. Change the plate and panel using the dropdown menus at the top of the page.

Plate Z-score: Display differences in sample distributions within and between plates.

NOTE: For intensity normalized projects, the assessment of this view should be performed after normalization, section 4. Normalization methods.

Limit of detection: Evaluate detectability for each plate, panel, and block. Assays with a high limit of detection are listed below the graph. Assess if there are deviations between sample plates within the same block.

Heat map: Search for extreme outlier samples.

4. Normalization methods

Olink NPX Manager supports three methods for between-plate normalization. Between-plate normalization adjusts median NPX levels based either on customer samples, IPC controls or Calibrators. Calibrator normalization is used for Olink Target 48, refer to *4.4 Calibrator normalization*. An important concept when deciding on the normalization procedure for Olink Target 96 panels is randomization, which in this context applies to the sample placement across the plates. For randomized studies with more than one plate, sample intensity normalization is the default normalization. For other studies, IPC normalization is the default. (See Randomization FAQ on the Olink website.)

Olink uses an arbitrary, relative quantification unit called Normalized Protein Expression (NPX). In qPCR, the x-axis value of the point where the reaction curve intersects the threshold line is called the Ct, or "threshold cycle." This indicates the number of cycles needed for the signal to surpass the fluorescent signal threshold line. NPX is derived from the Ct values obtained from the qPCR using the following equations:

Extension Control:

$$Ct_{Analyte} - Ct_{Extension\ Control} = dCt_{Analyte}$$

Inter-plate Control:

$$dCt_{Analyte} - dCt_{Inter\text{-}plate\ Control} = ddCt_{Analyte}$$

Adjustment against a correction factor:

$$Correction\ factor - ddCt_{Analyte} = NPX_{Analyte}$$

The correction factor is calculated by Olink during the validation of the panels.

4.1 IPC normalization

Three inter-plate controls (IPC) are included on each plate and run as normal samples. The inter-plate control is a pool of 92 antibodies, each with one of the pairs of unique DNA tags on it positioned in fixed proximity (i.e. 92 Extension Controls). The median of the three IPCs is subtracted for each assay, and this reduces variation between plates. This method is completely independent of the samples included on the plate and is therefore recommended for projects where complete randomization of samples cannot be guaranteed.

4.2 Intensity normalization v.2

The Intensity normalization v.2 adjusts the data so that the median NPX for a protein on each plate is equal to the overall median. Each plate is adjusted so that the median of all assays is the same on all plates. This method relies on the assumption that the true median of each plate is the same. One way of ensuring this is to randomize the samples beforehand. If there is total randomization, this method outperforms other normalization methods. If there are specific types of samples that are only available on certain plates, this normalization method should not be used.

NOTE: Only samples are included in this normalization. Negative Controls, IPCs and control samples are excluded. Compare to section 4.3 Intensity normalization.

4.3 Intensity normalization

Intensity normalization has been replaced by Intensity normalization v.2 as standard. The function remains as a tool for troubleshooting older datasets using this normalization method. This method uses the median of all samples on the plate,

including Sample Controls, Negative Controls and IPCs.

4.4 Calibrator normalization

Olink® Target 48

Calibrator normalization is used for Olink® Target 48 panels. A Calibrator is used to determine the protein concentration in the panel and randomization of samples is recommended but not mandatory. For this panel, the Extension Control, the Calibrator and the bridging factor are used to calculate the normalization. NPX is derived from the Ct values obtained from the qPCR using the following equations for Olink® Target 48 panels:

Each sample is normalized with the Extension Control:

$$Ct_{\text{Sample i, Assay j}} - Ct_{\text{Sample i, Assay Ext ctrl}} = dCt_{\text{Sample i,Assay j}}$$

Each plate is normalized with its Calibrators to generate NPX levels and is adjusted against a pre- determined bridging factor.

$$\text{Bridging factor}_{\text{Assay j}} - (dCt_{\text{Sample i, Assay j}} - \text{Median}(\text{Calibrator A}_{\text{Sample i, Assay j}})) = NPX_{\text{Sample i,Assay j}}$$

NOTE: The NPX value will differ between the same protein measured on Olink® Target 96 and Olink® Target 48. This is mainly due to the fact that Olink® Target 48 uses a bridging factor and Olink® Target 96 a correction factor, and the sample used for plate normalization differs.

The NPX unit is unique to each protein assay, meaning that even if two different proteins have the same NPX values, their concentrations in pg/mL may differ.

The bridging factor is determined by Olink during the validation of the panels, and for each new kit lot, a lot-specific bridging factor is defined.

4.4.1 Results in standard unit (pg/mL)

The method for quantification of data in standard units (pg/mL) for Olink® Target 48 is described in this section.

Method overview

The method requires fewer sample wells in each plate/run for controls, allowing more wells for customer samples.

Before the run (during product development at Olink)

1. A precise pre-defined standard curve is established for each protein.
2. The triplicate Calibrator is used to adjust the standard curve along the y-axis to normalize between plates and a second normalization, utilizing a bridging factor, is applied to ensure accuracy between production batches.
3. A four parameter logistic (4PL) model fit is performed to define the standard curve mathematically within the measurement range for each protein in the panel.

After each run

1. The median value of the Calibrator triplicates is used to adjust the measured samples along the y-axis to normalize between plates. In other words, the samples are normalized in the same way as the standard curve is during development. The samples are also adjusted against the bridging factor.
2. The measured sample values are related back to the adjusted standard curve model, which is used to translate the measured NPX value to the protein concentration in pg/mL.

Standard curve

During development, a thorough fine-tuned 32-point standard curve is developed for each protein biomarker simultaneously, including all 45 proteins. Multichannel pipetting and numerous replicates of the curves are used to minimize errors and establish an accurate immunoassay curve fitting. Due to the wide dynamic range of the PEA assays in the panel, a large spread of measurement points are required to cover the entire range. Eight measurement points, which are commonly used in many technologies, would not suffice for Olink's multiplex measurement of 45 assays. The

predefined standard curves avoid operator-dependent reconstitution and pipetting of standard curves at each lab and for each run. The figure below shows an example of a point standard curve defined for each assay during development.

Standard curves for each assay can be found via the panel product page (www.olink.com/t48cyt).

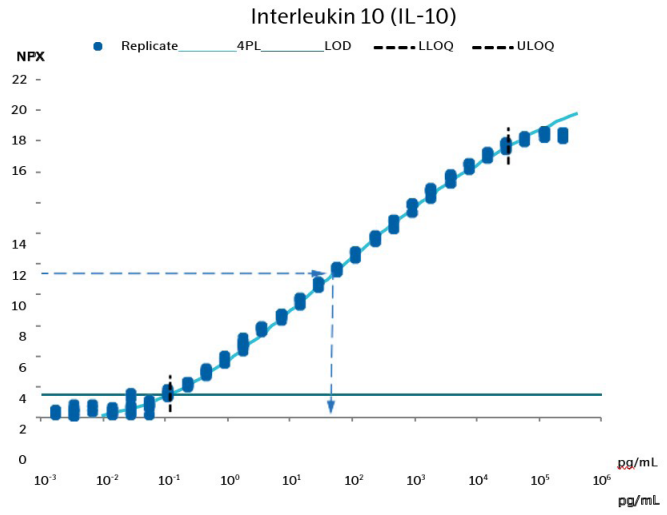


Figure 30. A 32-point standard curve defined for each assay during development.

A 4PL model curve fit is used to describe the immunoassay standard curve, indicated by the turquoise line in Figure 18. When running a project, the measured patient sample value (represented by the dotted blue arrows in *Figure 30*) is related back to the adjusted standard curve model which translates the measured value to the protein concentration in pg/mL using the equation below. Where A= asymptote (a constant value) at low doses, B= slope factor, C= EC50 (mid-range concentration) and D= asymptote at high doses. These parameters are indicated in *Figure 31.*

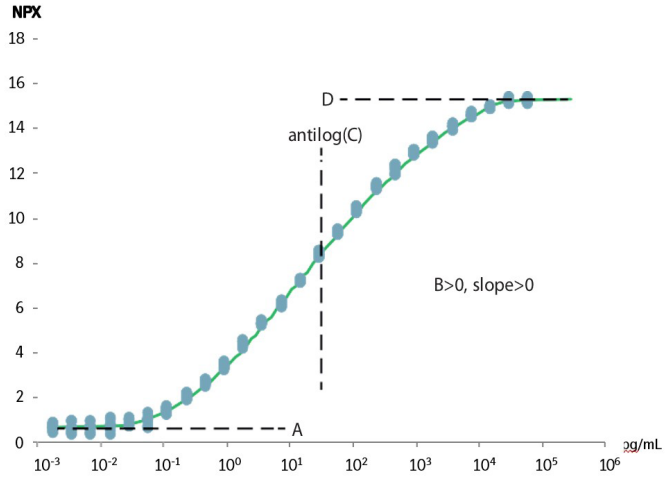


Figure 31. An example of a point standard curve defined for each assay during development.

$$\text{Concentration (pg/mL)}_{\text{Sample I, Assay J}} = \frac{10^{\wedge}\left(10^{\wedge}\left(\frac{\log_{10}\left(\frac{(A-D)}{(\text{NPXvalue}_{\text{Sample I, Assay J}}-D)}-1\right)}{B}+\log_{10}(C)\right)\right)}{1000}$$

Repeated testing and validation show that the 4PL curve fitting describes the standard curve well and can be used to correctly estimate the protein concentration in analyzed samples within the limits of quantification. The approximate lower and upper limits of quantification (ULOQ and LLOQ) are defined during the development of the panel, and specifications for LLOQ and ULOQ are established for each lot. See the panel specific Validation data document available on the Olink website (www.olink.com) for more details.

QC of assays in pg/mL

The QC of each assay is performed utilizing the Sample Controls. Sample Controls per assay are assessed for both accuracy and precision (CV%) to confirm that the concentration values in pg/mL are within the expected limits (±30%) and assure the correct functionality of the quantification method.

Results reported in pg/mL

Figure 32 and the list below provide an overview of how data in pg/mL is reported in the results file.

- Data between LQL and ULOQ is reported as a pg/mL value and shown in the white areas of the graphs.

NOTE: Lowest Quantifiable Level (LQL) is defined as the value used as the lower limit, LLOQ (default) or plate LOD (when plate LOD > LLOQ).

- Data below the lowest fitting parameter in the 4PL curve fit model cannot be calculated and is indicated as NaN in the red areas of the graphs.
- Data >ULOQ is indicated as >ULOQ in the red areas of the graphs. Values above ULOQ are not reported in pg/mL due to high risk of misinterpreting hooking data.
- Data below LQL is presented in pg/mL value in the red areas of the graphs. Values below LQL should be treated with caution due to decreased precision and accuracy in the lower range and should not be used for individual comparison to reference values.

For a more detailed list describing the results file, see *7.7 Export data*.

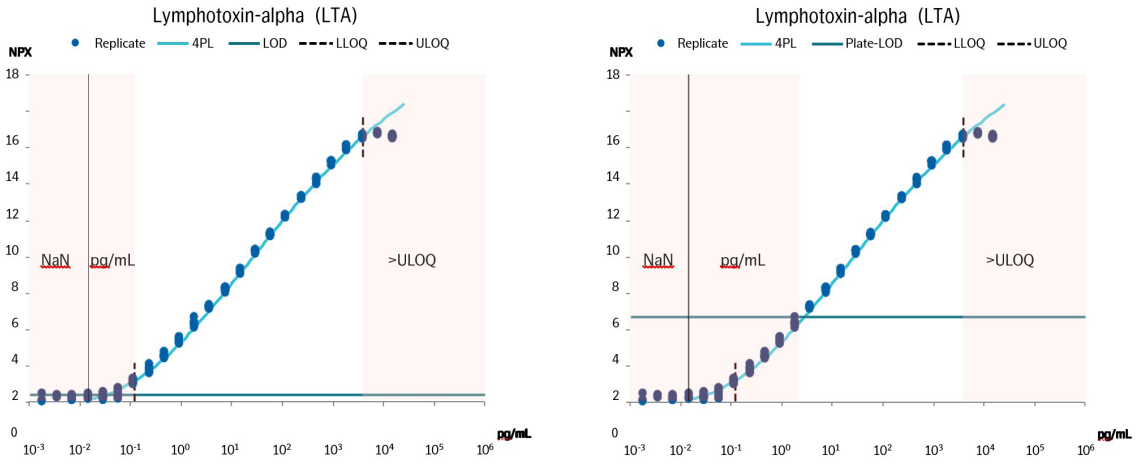


Figure 32. Overview of how data in pg/mL is reported in the results file.

4.5 Olink® Explore NPX generation and normalization

Olink® Explore

4.5.1 Converting counts to NPX

The Olink® Explore system's raw data output is counts, where each combination of assay and sample is given an integer value based on number of copies detected. These raw data counts are converted into NPX values for use in the continued analysis.

NPX generation

The NPX values are calculated in two main steps, followed by an optional additional step between-plate-normalization. First, the assay counts of a sample are divided with the Extension Control for that sample and block. The resulting scale has increasing values with increasing concentration for each assay. A log2 transformation is applied and the final step is subtracting the median of the Plate Controls. The optional additional step is intensity normalization which sets the median level of all assays to the same value for all plates. The different steps are described below.

Steps in the NPX generation described in equation form, where i refers to a specific assay, j refers to a sample, and ExtNPX defines an extension normalized NPX value.

1. `ExtNPX_i, j = log2(counts(sample_jAssay_i)/counts (ExtCtrl_j))`
   a. Relate counts to known standard (Extension Control).
   b. For all assays and all samples, including Negative Controls, Plate Controls, and Sample Controls.
   c. Log2 transformation gives more normally distributed data.
2. `NPX_i, j = ExtNPX_i, j-median (ExtNPX(Plate Controls_i))`
   a. Perform plate standardization.
   b. For all assays and per plate of samples.
3. `NPX_Intnorm_i,j = (NPX_i,j – plate median(NPX_i)) + global median(NPX_i)`
   a. Between plate normalization (Optional but default for multi plate projects).
   b. For each assay, for all plates in project.

4.5.2 LOD and CV calculation

LOD is defined as being three SDs above the median NPX of Negative Controls. The median is set using all samples annotated as Negative Controls per plate. A predefined SD is used (fixSD). Detectability is calculated per assay and plate and is defined by the percentage of samples above the LOD threshold. The overall detectability of the project is generated and reported in the CoA.

The CV is calculated per assay (i) using the assumption of a log-normal distribution. The average CV is then calculated across panels and included in the CoA output.

$$CV_i = 100 \sqrt{(e^{Sln_i^2} - 1)}, \text{ where } Sln_i = \ln(2) \times SD_i$$

4.5.3 Normalization of NPX data

The two between-plate-normalization methods are called Plate Control (PC) normalization and intensity normalization. They both adjust each assay per plate to a median, but they differ in how these values are calculated. The intensity normalization is the default for randomized studies on multiple sample plates. In this setting, the median for a random selection of samples is more stable across plates than the three PC's on each plate. Therefore, intensity normalization is the better option. When randomization of samples across plates is not possible, then PC normalization is recommended.

5. Pre-processing using Fluidigm software

Heatmaps with Ct values exported from the *Fluidigm Real-Time PCR Analysis* software are the input data for the Olink NPX Manager. The heatmaps are exported in CSV format. This chapter provides instructions for how to use the *Fluidigm® Biomark™ Data Collection* software and how to make a first evaluation of run quality in the *Fluidigm Real-Time PCR Analysis* software. Specific instructions for Olink® Target 48 are highlighted.

Quality check recommended

Olink highly recommends performing a first quality check of the data in both software as soon as possible after a finished run on the BioMark™ System. The extension product can be stored at 4°C for up to one week and after that at -20°C for four weeks, which makes it possible to rerun samples from the detection step if any issues arise.

NOTE: 5.5 Evaluation of heatmap view and 5.6 Evaluation of image view are essential for evaluating the functionality of Olink kit reagents and Fluidigm IFCs (Integrated Fluid Circuits).

Fluidigm® Real-time PCR analysis software

The *Fluidigm® Biomark™ Data Collection* software captures images of the processed runs throughout the real-time PCR cycling. The images are imported into the *Fluidigm Real-Time PCR Analysis* software for quality control and calculation of cycle threshold values (Ct values) that are exported for downstream preprocessing using Olink NPX Manager.

5.1 Open a chip run

Open a chip run with the *Fluidigm Real-Time PCR Analysis* software. The analysis requires access to automatically created *Cals and Data* folders and the file *ChipRun.bml,* which is found in the chip run folder.

1. In the *Fluidigm Real-Time PCR Analysis* software, go to **File** > **Open**.
2. Browse to the chip run folder and select the ChipRun.bml file.

5.2 Add sample information

Enter the sample names. Adding the sample names in this step ensures maximum traceability and easy annotation in Olink NPX Manager. Sample names must be unique within a project. It is recommended to give unique identifiers to Sample Controls, IPCs, and Negative Controls as well.

1. Prepare a Microsoft® Excel® spreadsheet with sample names that correspond to the sample plate layout.

Olink® Target 96

Table 2. Example plate layout for Olink® Target 96.

	1	2	3	4	5	6	7	8	9	10	11	12
A	Sample A1	Sample A2	Sample A3	Sample A4	Sample A5	Sample A6	Sample A7	Sample A8	Sample A9	Sample A10	Sample A11	Sample A12
B	Sample B1	Sample B2	Sample B3	Sample B4	Sample B5	Sample B6	Sample B7	Sample B8	Sample B9	Sample B10	Sample B11	Sample B12
C	Sample C1	Sample C2	Sample C3	Sample C4	Sample C5	Sample C6	Sample C7	Sample C8	Sample C9	Sample C10	Sample C11	Neg Ctrl
D	Sample D1	Sample D2	Sample D3	Sample D4	Sample D5	Sample D6	Sample D7	Sample D8	Sample D9	Sample D10	Sample D11	Neg Ctrl
E	Sample E1	Sample E2	Sample E3	Sample E4	Sample E5	Sample E6	Sample E7	Sample E8	Sample E9	Sample E10	Sample E11	Neg Ctrl
F	Sample F1	Sample F2	Sample F3	Sample F4	Sample F5	Sample F6	Sample F7	Sample F8	Sample F9	Sample F10	Sample F11	IPC
G	Sample G1	Sample G2	Sample G3	Sample G4	Sample G5	Sample G6	Sample G7	Sample G8	Sample G9	Sample G10	Sample G11	IPC
H	Sample H1	Sample H2	Sample H3	Sample H4	Sample H5	Sample H6	Sample H7	Sample H8	Sample H9	Sample H10	Sample H11	IPC

Olink® Target 48

For the plate layout, use unique identifiers for samples, Sample Control, Negative Control, and Calibrator A. When analyzing one panel with 40 samples or less, use a layout corresponding to columns 1-6 in the example below, and leave columns 7-12 empty. When analyzing more than 40 samples, either make a new layout using only columns 1-6, or use columns 7-12 and leave columns 1-6 empty.

Table 3. **Example plate** *layout for Olink® Target 48.*

	1	2	3	4	5	6	7	8	9	10	11	12
A	Sample A1	Sample A2	Sample A3	Sample A4	Sample A5	Sample Control						
B	Sample B1	Sample B2	Sample B3	Sample B4	Sample B5	Sample Control						
C	Sample C1	Sample C2	Sample C3	Sample C4	Sample C5	Sample Control						
D	Sample D1	Sample D2	Sample D3	Sample D4	Sample D5	Negative Control						
E	Sample E1	Sample E2	Sample E3	Sample E4	Sample E5	Negative Control						
F	Sample F1	Sample F2	Sample F3	Sample F4	Sample F5	Calibrator A						
G	Sample G1	Sample G2	Sample G3	Sample G4	Sample G5	Calibrator A						
H	Sample H1	Sample H2	Sample H3	Sample H4	Sample H5	Calibrator A						

2. In the *Real-Time PCR Analysis* software's **Chip Explorer** pane, select **Sample Setup**. In the **Task** pane, click on the **New** button.

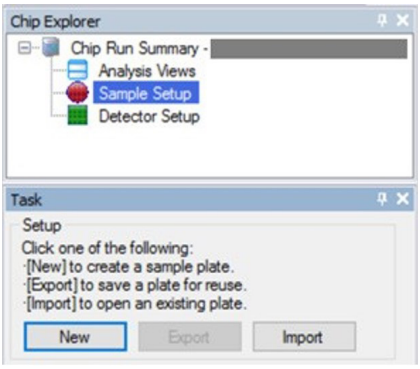


Figure 33. Left view of the Fluidigm Real-Time PCR Analysis software with Sample Setup selected.

1. Enter a name for the plate. This name will be used as the file name for the exported data file and will also be used in the final file exported from Olink NPX Manager.
2. Set the container type to "SBS Plate" and the format to "SBS96". Click **OK**.
3. The format is now the same as the sample format created in Microsoft Excel in step 1. Copy the sample names from Microsoft Excel (do not include the column and row headers).

4. Go to the *Real-Time PCR Analysis* software and select the A1 well in the sample setup. Paste the sample names and set **Data Item** to “Sample Name” when prompted. Click **Accept**.

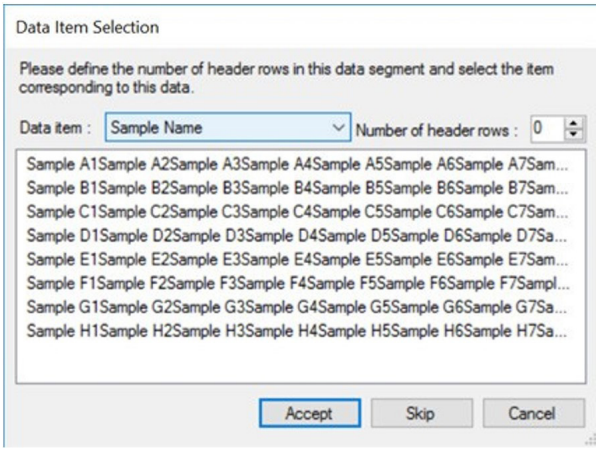


Figure 34. Data Item selection window.

5. Map the plate layout according to how the samples were loaded in the chip: In the **Plate Settings** section, go to the **Task** view and then the **Mapping** field. Press the **Map...** button and select the “M96- Sample-SBS96.dsp” file.

Olink® Target 48

For samples in columns 1-6 in the plate layout, the file “M48-Sample-SBS96-Left.dsp” should be used for mapping the samples. For samples in columns 7-12, “M48-Sample-SBS96-Right.dsp” should be used.

NOTE: This assumes that the recommended IFC loading layout was used.

Result: You have now entered the information needed to couple a reaction well to a sample.

5.3 Add detector information

It is essential for the analysis in Olink NPX Manager that you add the panel and biomarker information required for downstream data processing. The setup is similar to the sample setup, but in this case a template file from the Olink membership site is used.

1. Download the Detector Setup template file (in PLT format) that corresponds to the biomarker panel you have used, from https://www.olink.com/members-site.
2. In the *Real-Time PCR Analysis* software, go to the **Task** view under the **Chip Explorer** section. Select **Detector Setup**, then click the **Import** button in the **Setup** section.

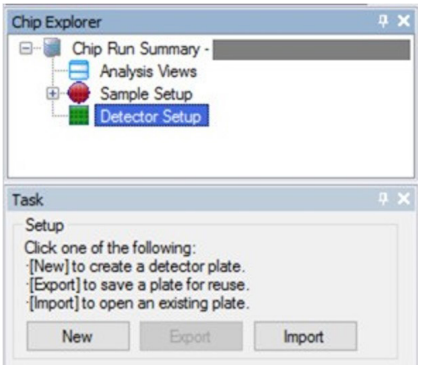


*Figure 35. Left view of the Fluidigm Real-Time PCR Analysis software with **Detector Setup** selected.*

3. Browse to the downloaded PLT template file and click the **Open** button.

Result: You have now entered the information needed to couple a reaction-well to a biomarker.

5.4 Change analysis settings and reanalyze

Before data can be evaluated and exported, some settings must be changed.

1. In the *Real-Time PCR Analysis* software, go to the **Chip Explorer** view and select **Details Views**.
2. In the **Task** view under the **Analysis Settings** section, make sure **Quality Threshold** is set to "0.5", **Baseline Correction** to "Linear" and **Ct Threshold Method** to "Auto (Global)":

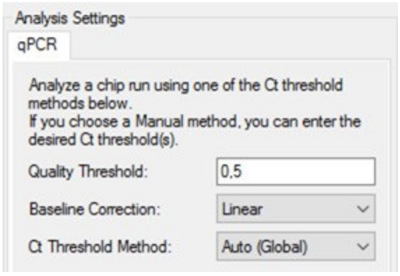


Figure 36. Analysis Settings section in the Task view of the Fluidigm Real-Time PCR Analysis software.

3. In the **Analyze** section, press the **Analyze** button. All the analysis settings and the sample and detector settings are now updated.

Result: The data is now ready to be evaluated before export.

5.5 Evaluation of heatmap view

1. Check the heatmap by choosing **Results Table** and then **Heatmap View**.
2. Assays are displayed on the x-axis and samples on the y-axis. The Negative Control samples should give a darker line from left to right and the IPCs a brighter line from left to right. Assays should give lines from top to bottom. Refer to *Figure 17*.

Olink® Target 48

For Olink Target 48, the Negative Control samples should give a darker line from left to right and the Sample Controls and Calibrators a brighter line from left to right.

5.6 Evaluation of image view

1. Check the Image View by changing to **Image View** instead of **Heatmap View**:
2. Choose ROX in the first drop-down menu and choose cycle number 4 in the next drop-down menu.

3. Click the button **Toggle Overlays** to remove the red grid pattern.
4. Check that all wells across the IFC have even coloring.
5. Select a cycle number higher than 12 and check that all wells still have even coloring with similar intensity across the IFC.
6. Choose FAM-MGB in the first drop-down menu and choose cycle number 4 in the following drop- down menu.
7. Select a cycle number higher than 12 and check that the fluorescence pattern changes across the IFC compared to cycle number 4.

5.7 Comparison of image and heatmap views

1. The **Show Dual Views** button allows you to look at two views side-by-side, such as an Image View and a Heatmap View. This can help to localize wells that failed in the Heatmap View also in the Image View. No action is needed for wells that are failed in the *Fluidigm Real-Time PCR Analysis* software. Olink NPX Manager will automatically fail these wells. However, if you detect wells in Image View with bubbles of black wells/pixels that are not failed by the *Fluidigm Real- Time PCR Analysis* software, you should note these wells and manually fail them in Olink NPX manager.
2. When comparing Heatmap View to Image View, change to chip-based view in the Heatmap View to get the samples and assays in the same order as in the Image View.

5.8 Export Ct-values in heatmap format

In the *Real-Time PCR Analysis* software, there are three ways to export data:

- In a table with each data point on a single row.
- As a 96x96 matrix (heatmap).

Olink® Target 48

- As a 48x48 matrix (heatmap).

In Olink NPX Manager, heatmaps must be used. Follow these instructions to export Ct-values in heatmap format:

1. Make sure **Inlet-based View** is selected in the **Layout** drop-down menu. This should be the default.
2. Select **File** > **Export**.
3. Under **File name**, the name assigned in sample setup will be used, refer to *5.2 Add sample information*. This name is used in the final export from Olink NPX Manager. If this field is left empty the file will be named “Undefined” in Olink NPX manager.
4. Under **File format**, select “Heatmap Results” (*.csv). Click **Save**.

6. Pre-processing Olink® Explore runs

Before analysis in Olink NPX manager is possible, a pre-processing step needs to be performed. For this purpose, software applications are installed on a local server.

6.1 bcl2counts

The *bcl2counts* software generates count files for each lane in a NGS run. A run metadata file, including run information and InterOp data, is also created. The application will output messages and errors to **/var/log/olink/bcl2counts.log**.

The application can be run manually by the command:

```
bcl2counts <ngs run folder>
```

For troubleshooting, it is possible to get more debug logs by passing arguments to the command:

```
bcl2counts -vv --stdout <ngs run folder>
```

This command will output more logs to standard output.

Use the --help option to get a list of options for bcl2counts (note two dash signs):

```
bcl2counts --help
```

For detailed installation and usage instructions, please refer to INSTALLATION_AND_USER_GUIDE.md which is distributed together with the bcl2counts software.ß

7. Operation

Input data for Olink NPX Manager are Ct values exported from the *Fluidigm Real-Time PCR Analysis* software. After quality control and normalization against the Extension Control, the IPC, and a correction factor, the output data are obtained in Normalized Protein eXpression (NPX) values. NPX is an arbitrary unit on a log2 scale, where a high NPX value corresponds to high protein concentration.

Olink® Target 48

For Olink Target 48, normalization against the Extension Control, Calibrator, and bridging factor is performed. The default output data is in standard concentration units (pg/mL).

Olink® Explore

For Olink Explore, input data is counts files and metadata files generated by *bcl2counts*. For more information on pre-processing, refer to *6. Pre-processing Olink® Explore runs*.

This section describes how you analyze data step-by-step in Olink NPX Manager. The following steps are included in the standard operating procedure:

1. Import CSV files.

Olink® Explore

- Import run metadata.
- Import sample manifest.

2. Enter project information.
3. Verify sample types and layout.
4. Calculate NPX or pg/mL values.
5. Perform quality controls including additional quality assessment.
6. Export data.
7. Create a Certificate of Analysis.

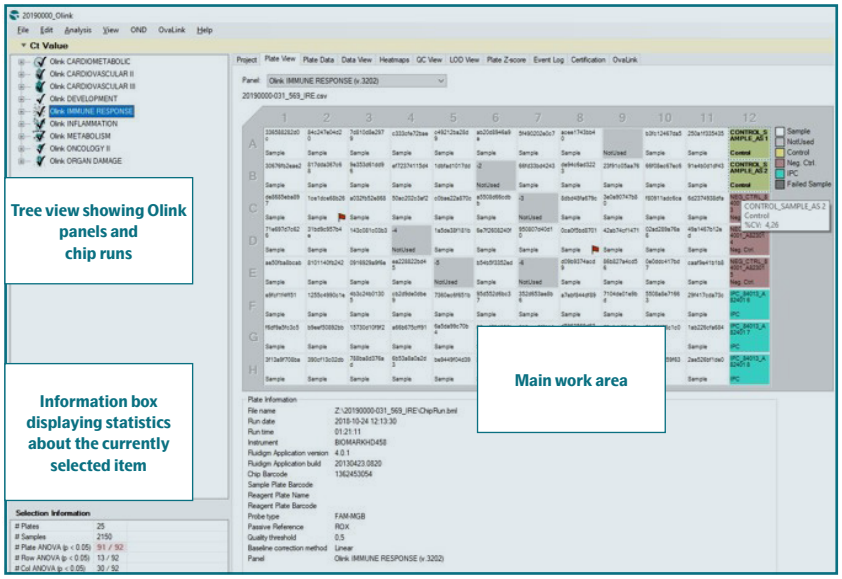


Figure 37. Olink® NPX Manager main window with the Plate View tab selected.

7.1 Import a CSV file

1. Select **File** > **Import CSV** and browse to the heatmap file (.csv) exported from the *Fluidigm Real-Time PCR Analysis* software or counts files. Multiple files within the same project can be selected for import at the same time.

TIP: You can also import files by dragging and dropping the files from Windows Explorer onto the Tree View.

NOTE: A project cannot include different panel types at the same time. It can consist of only runs from Olink Target 48 panels, only runs from Olink Target 96 panels or, only runs from Olink Explore panels.

2. During import, Olink NPX Manager alerts if duplicate sample names are found since unique sample and assay names are necessary for proper handling of the data. Change sample names if needed. Sample names can be changed directly in Olink NPX Manager by copying a plate layout from an Excel spreadsheet and pasting it onto the plate layout in the **Plate View** tab. Refer to *5.2 Add sample information*.
3. Select the panel datafile version that corresponds with the data file number written on the *Article and lot configuration* sheet found together with the Olink reagents. Click **OK**.

Result: Each imported run is annotated with the Olink panel specified by the PLT file used in the *Real-Time PCR Analysis* software and shown in the Tree View to the left, grouped by Olink panel.

Olink® Explore

Select **File > Import CSV** and browse to the run_metadata.json file and sample manifest csv file. Verify the correct panel has been selected and enter the panel's lot number as can be found on the Lot configuration sheet found together with the Olink reagents.

TIP: Drag-and-drop to reorganize plates in the Tree View.

7.2 Enter project information

For more information, refer to *2.4 Project*. In the Project view of the main window:

4. Enter a **Project Name**. This will be displayed in the data export file and in the CoA.

- Enter information in some or all the following fields to display it in the CoA:
  - **Sample Type**
  - **Customer Information**
  - **Business Development Manager**
  - **Analysis Lab**
  - **QC performed by and Reviewer**
  - **Comments for report**

NOTE: Any entered **Comments** *will be saved in the Olink NPX Manager project file (.oaf) and* **Flagged Positions** *will automatically be displayed in the CoA.*

7.3 Verify plate layout and sample annotation

1. In the upper left view, click the **+** sign next to an Olink panel name to expand the Tree View and display the imported runs for each Olink panel. Highlight the first run and click the **Plate View** tab.
2. Make sure that the correct data file version is selected in the drop-down menu above the plate layout. The same data file version will be used for all runs of the same Olink panel in the project.
3. Verify that the plate layout is correct. To change the plate layout, select one or several wells and right-click to change the well type. Available types are:

- Sample
- Control
- Negative Control
- IPC
- Not Used

Olink® Target 48

The following additional well types are available for Olink Target 48:

- Control 1-4
- Calibrator A
- Calibrator A, Negative Control, and Control 1 for Sample Control must be annotated for calibration normalization and QC to be performed. Up to four different control sample types (Control 1 – 4) can be selected, but only Control 1 should be used for Olink Target 48 Cytokine.

Olink® Explore

The following additional well types are available for Olink Explore:

- Plate Control.

4. Repeat steps 2 and 3 for all imported plates before continuing.

TIP: To add or change sample names, plate layouts may be copied directly from an excel spreadsheet. Refer to 5.2 Add sample information.

7.4 Calculate NPX values

1. Select an Olink panel in the Tree View.

2. Click **Analysis** and select the normalization type to use for the project.
   Result: NPX values are calculated for all runs in the selected Olink panel.

TIP: To view the NPX values on the Data View tab, make sure **NPX Value** *is selected from the drop-down list in the upper left corner of the screen.*

Olink® Target 48

The calculation generates values in both NPX and pg/mL. Select the **Quantified Value** setting to view values in pg/mL in the Data View tab.

Olink® Explore

NOTE: Plate control normalized data is calculated by default when importing the run.

NOTE: IPC normalization is used by default for single plate studies or non-randomized studies for Olink Target 96 panels. For randomized studies of more than one run per Olink panel, Intensity normalization v.2 should be used.

Olink® Target 48

For Olink Target 48, Calibrator Normalization is used. Refer to 4. Normalization methods for more information about the available normalization methods.

Olink® Explore

For Olink® Explore, Plate Control normalization is performed automatically when importing a run to generate the QC metrics. Intensity normalization is also available for Olink® Explore. Refer to 4. Normalization methods for more information about the available normalization methods.

7.5 Perform quality controls

1. Perform the QC for each plate separately using the **QC View**.

Olink® Explore

For Olink Explore, perform QC for each plate separately using the Quality Control tab.

2. Perform quality assessment for all plates using the other views as described in *7.6 Additional quality assessment* and then make an overall assessment.

The acceptance criteria for passing QC are described in *Table 4* and *Table 5*.

Table 4. Quality Control guidelines for Olink® Target 96.

Criteria	Recommended value
Run QC	
Std. Dev. of Incubation Control 1	< 0.2 NPX
Std. Dev. of Incubation Control 2	< 0.2 NPX
Std. Dev. of Detection Control	< 0.2 NPX
Number of flagged samples	≤ 1/6 of total number of samples on plate (15 samples on a full plate)
Sample QC	
Incubation Control 2 deviation	< ±0.3 NPX from plate median
Detection Control deviation	< ±0.3 NPX from plate median

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Table 5. Quality Control guidelines for Olink® Target 48.

Criteria	Recommended value
Run QC	
Controls Std. Dev. of Incubation Control 1	< 0.5 NPX
Samples Std. Dev. of Incubation Control 1	< 0.2 NPX
Controls Std. Dev. of Detection Control	< 0.5 NPX
Samples Std. Dev. of Detection Control	< 0.2 NPX
Number of flagged samples	≤ 1/6 of total number of samples on plate (8 samples on a full plate)
Number of failed assays	≤ 3 out of 45
Calibrator or Control replicates outside LOQ	≤ 1/3 of the Sample Control- or Calibrator replicates are outside of LOQ for < 5 assays
Number of flagged Sample Controls	≤ 1
Number of flagged Calibrators	≤ 1
Sample QC	
Incubation Control 1 deviation	< ±0.3 NPX from plate median
Detection Control deviation	< ±0.3 NPX from plate median
Assay QC	
Accuracy Sample Control	±30% of the expected concentration
Precision Sample Control	Intra-CV < 30%
Calibrator or Control replicates outside LOQ	< 1/3 of the Sample Control- and/or Calibrator replicates are outside of LOQ
Precision Calibrator	Intra- CV <30%.

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Table 6. Quality Control guidelines for Olink® Explore.

Criteria	Recommended
Run QC	
MAD of Incubation Control	≤ 0.3 NPX
MAD of Amplification Control	≤ 0.3 NPX
MAD of Deviation of assays in Plate control	< 1.5
Median Deviation of assays in Plate control	- 5 < 5
Median Deviation of assays in Negative control	< 3
Number of flagged samples	≤ 1/6 of total number of samples on plate (15/88 samples on a full plate)
Sample QC	
Incubation Control deviation	< ±0.3 NPX from plate median
Amplification Control deviation	< ±0.3 NPX from plate median
Assay QC	
Negative Control deviation	>5 standard deviations from predefined values set for each assay

TIP: If Std. Dev. is higher than the recommended value, evaluate the QC graph below to identify any extreme outlier samples. Exclude these samples from the QC analysis (right-click on the sample name in the graph and select **Exclude from QC analysis***) and re-evaluate the run QC again.*

7.5.1 Performing a quality assessment

Parameters that need to be evaluated for each run are listed in *Table 7.*

Table 7. Quality assessment for Olink® Target 96.

Information	Recommended value	Comment
Assay LOD	|LOD| < 2.5 NPX	If LOD > 2.5 please contact Olink Support for guidance.
Plate ANOVA	≤ 10 (number of assays)	No warning.
	> 10 (number of assays)	May be caused by insufficient randomization or other issues.
		NOTE: These guidelines are based on a two plate project. The larger a project is, the smaller the detectable differences become.
Row/Column ANOVA	< 30 (number of assays)	No warning.
	30 – 60 (number of assays)	Possible randomization issue. Refer to *8.5 Inconsistent results detected in Olink® Target using Olink® NPX Manager*.
	60 – 90 (number of assays)	Re-run plate or otherwise ensure that this is not the result of a poorly executed run.
		For high values, investigate the **Plate Data** view.
%CV Intra	< 15%	Refer to *8.5 Inconsistent results detected in Olink® Target using Olink® NPX Manager* and *8.3 Deviating controls for Olink® Target in Olink® NPX Manager*
%CV Inter	< 25%	Refer to *8.5 Inconsistent results detected in Olink® Target using Olink® NPX Manager* and *8.3 Deviating controls for Olink® Target in Olink® NPX Manager*
Detectability	> 75% of the samples	Refer to document on https://www.olink.com/members-site/.

If all plates pass quality control and assessment, the data analysis is finished and the data is ready for export and reporting.

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Table 8. Quality assessment for Olink® Target 48.

Information	Recommended value	Comment
Plate ANOVA	≤ 10 (number of assays)	No warning.
	> 10 (number of assays)	May be caused by insufficient randomization or other issues. If samples were not randomized, this warning can be disregarded.
		NOTE: These guidelines are based on a two plate project. The larger a project is, the smaller the detectable differences become.
Row/Column ANOVA	< 15 (number of assays)	No warning.
	15 – 30 (number of assays)	Possible randomization issue. Refer to *8.5 Inconsistent results detected in Olink® Target using Olink® NPX Manager*
	30 – 45 (number of assays)	Re-run plate or prove that this is not the result of a poorly executed run.
		For high values, investigate the **Plate Data** view.
%CV Intra	< 15%	Refer to *8.3 Deviating controls for Olink® Target in Olink® NPX Manager*
%CV Inter	< 25%, maximum 40%	Refer to *8.3 Deviating controls for Olink® Target in Olink® NPX Manager*
Detectability	> 75% of the samples	The limit for detectability is determined from observed values in plasma.

7.6 Additional quality assessment

7.6.1 Additional quality assessment Olink Target panels

NOTE: Switch between Ct and NPX in the upper left corner of the screen e.g. to evaluate the effect of normalization or to search for position effects in internal controls and assays.

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For Olink Target 48, the quantified value setting can also be used to evaluate these effects.

7.6.2 Instructions

1. On the **Plate Data** tab, view the values of internal controls and assays (change assay in the drop-down menu above the plate layout) for each sample. For example, search for specific patterns that may indicate technical errors.
2. On the **Data View** tab, evaluate patterns of data below LOD between sample plates in a project and/or remove specific assays or single datapoints from a project/sample plate.
3. On the **Heatmap** tab, search for outlier samples and/or visualize homogeneity of Negative Controls and IPCs (change sample type in the drop-down menu).

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For these panels, the homogeneity of Calibrators and Sample Controls can also be visualized using the Heatmap tab. Use the **Quantified Value** setting to provide an overview of failed assays which will be marked in black.

4. On the **LOD View** tab, evaluate the detection limit for each assay and sample plate (optimal range for LOD ±2.5 NPX). Assess if deviating LOD for one sample plate affects detectability on other sample plates.

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For these panels, some assays can be expected to have LOD values above 2.5 and this should not affect the number of samples that can be quantified above the plate LOD. The LOD View can be used to assess if such assays have a high number of samples outside LOQ.

5. On the **Plate Z-score** tab, display differences between sample plates or distributions within a plate.

7.6.3 Additional quality assessment Olink® Explore panels

Click the following tabs in the left menu to assess the quality of the data in each pane and further determine if any reruns are required (Views are further depicted in *2. Olink® NPX Manager user interface*):

1. **QC View**: View deviation from the median in all samples per plate and panel. Each block is displayed. Change the plate and panel using the drop-down menus and assess samples deviating > 0.3 NPX from the median.
2. **Plate data:** View the values of internal controls and assays (change assay in the drop-down menu above the plate layout) for each sample plate and panel. Each block is displayed. Assess if there are patterns in the plate (i.e., row or column) representing a technical error. Change the plate and panel using the drop-down menus at the top of the page.
3. **Plate Z-score:** Display differences in sample distributions within and between plates.
4. **LOD View:** Evaluate detectability for each plate, panel, and block. Assays with a high limit of detection are listed below the graph. Assess if there are deviations between sample plates within the same block.
5. **Heatmaps:** Search for extreme outlier samples.

7.7 Export data

Data can be exported to a XLSX or CSV file for use in other applications and for statistical analysis.

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Data can be exported both as NPX and pg/mL in separate reports. Default export is pg/mL.

1. Select **NPX Value** on the status bar in the upper left corner of the main window.

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Select the **Quantified Value** setting to export data in pg/mL.

2. Under **Project Option**s on the **Project** tab, select the output option for NPX values below LOD. The default is "Actual Value", which displays the actual measured value for each data point.
3. Go to **File** > **Export Data**. Data can be exported for all runs and Olink panels in the project or for the selected run or Olink panel only.

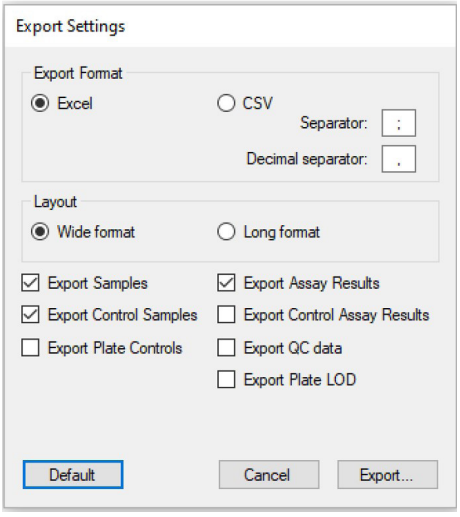

Figure 38. Olink® NPX Manager Export Settings window.

4. Figure 26 shows the **Export Settings** window with the recommended default settings. These settings may be adjusted to change the format, layout, or to add additional data to the export. See *Table 8* for more information.
5. Click **Export**.
6. Enter a filename and location in the **Save** dialog, select an export format and click the **Save** button.

Table 9. Data export options.

Setting	Description
Export format	Excel or CSV For CSV format, column separator and decimal separator can be configured.
Layout	Wide format or Long format. Wide is the default matrix format and Long format will output one line per datapoint. *NOTE: In Excel, there is a number of rows limit of 1 048 576. If this number is exceeded, Excel export will not work, and CSV format should be selected instead.*
Export Samples	Export data for customer samples.
Export Control Samples	Export Control samples.
Export Plate Controls	Export IPC, Plate Control, Negative Control or Calibrators.
Export Assay Results	Export results for assays.
Export Control Assay Results	Export results for control assays.
Export QC data	Export deviation from median for Incubation Control 2 and Detection Control.
	Olink® Target 48 Deviation from median for Incubation Control and Detection Control is exported in NPX. Data for the controls cannot be calculated in pg/mL and will be blank in the results file for quantified values.
	Olink® Explore Deviation from median for Incubation Control and Amplification Control is exported in NPX.
Export Plate LOD	Will export plate LOD for each plate as well as max LOD.
	Olink® Explore Only plate LOD is calculated and exported.

7.7.1 Considerations

- Samples that are flagged by the analysis are reported in red text. NPX-values that are below maximum plate LOD have a red background.
- Samples, assays, or data points that have been marked as failed in the software show empty cells in the data export.

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In the results file in standard units (pg/mL) the data is reported as follows:

- Data between LQL and ULOQ is reported as pg/mL value in white cells.

NOTE: Lowest Quantifiable Level (LQL) is defined as the value used as the lower limit, LLOQ (default) or plate LOD (when plate LOD > LLOQ)

- Data >ULOQ is indicated as >ULOQ in red cells.
- Data below LQL is presented in pg/mL value in red cells.
- Data below lowest fitting parameter in the 4PL curve fit model cannot be calculated and is indicated as **NaN** in red cells.
- Failed data points (either because of assay failure or sample failure) are indicated in grey cells.
- For samples and assays with QC warning, values are indicated as described above but marked in red text. Data from samples and assays that do not pass QC should be treated with caution.
- Failed data points (because of chip failure) are indicated as **No data** in grey cells.
- For each plate and assay, values for LQL, LOD, LLOQ and ULOQ, as well as results for assay QC in **Assay warning**, are presented on separate rows below the data for the samples.
- Missing data frequency is reported for each assay and indicates the percentage of samples with values < LQL, > ULOQ as well as failed data.

Table 10. Overview presentation of data in standard units (pg /mL) in results report.

Result and QC results					In Data report			
Sample QC	Calibrator QC precision	Sample Control QC precision and/ or accuracy	Calibrator and/or Sample Control QC replicates within LOQ	Data between LQL and ULOQ	Data Output Format	Sample QC	Assay QC	Notes
Pass	Pass	Pass	Pass	Yes	pg/mL	Pass	Pass	
Fail	Pass	Pass	Pass	Yes	pg/mL	Warning	Pass	Treat with caution
Pass	Fail	Pass	Pass	Yes	pg/mL	Pass	Warning	Treat with caution
Pass	Pass	Fail	Pass	Yes	pg/mL	Pass	Fail	
Pass	Pass	Pass	Fail	Yes	pg/mL	Pass	Fail	
Pass	Pass	Pass	Pass	No; < curve fitting	NaN	Pass	Pass	
Pass	Pass	Pass	Pass	No; < LQL	pg/mL	Pass	Pass	Treat with caution
Pass	Pass	Pass	Pass	No;>ULOQ	>ULOQ	Pass	Pass	

In the NPX results Excel file that can be optionally exported, data will be presented as follows:

- As NPX values.
- Values below maximum plate LOD have a red cell background.
- Data for samples with a QC warning are indicated in red text. Data from samples that do not pass QC should be treated with caution.
- The maximum plate LOD value for each assay is presented on a separate row below the data for the samples and is indicated as LOD.
- Missing data frequency is presented for each assay and indicates the percentage of samples with values below Maximum plate LOD.

The layout is described in Table 10. Parameters in brackets represent quantified data.

Table 11. Layout of exported NPX data.

Project Name	Olink® NPX Manager version																					
NPX data or Quantifed data																						
Panel	Panel 1					...					Panel x					Panel 1	...	Panel x	Panel 1	...	Panel x	
Assay	1	2	3	...	96						1	2	3	...	96	Plate ID		Plate ID	QC Warning		QC Warning	
Uniprot ID	1	2	3	...	96						1	2	3	...	96							
Olink ID	1	2	3	...	96						1	2	3	...	96							
Sample #1	1	2	3	...	96						1	2	3	...	96	plate_1. csv		plate_x. csv	Pass / Warning		Pass / Warning	
Sample #2	1	2	3	...	96						1	2	3	...	96	plate_1. csv		plate_x. csv	Pass / Warning		Pass / Warning	
Sample #x	1	2	3	...	96						1	2	3	...	96	plate_1. csv	...	plate_x. csv	Pass / Warning		Pass / Warning	
...																						
[Assay warning]	1	2	3	...	96						1	2	3	...	96	plate_1. csv		plate_1. csv				
[Lowest quantifiable level]	1	2	3	...	96						1	2	3	...	96	plate_1. csv		plate_1. csv				
Max LOD [Plate LOD]	1	2	3	...	96						1	2	3	...	96	[plate_1. csv]		[plate_1. csv]				
[ULOQ]	1	2	3	...	96						1	2	3	...	96							
[LLOQ]	1	2	3	...	96						1	2	3	...	96							
Missing Data freq. (%)	1	2	3	...	96						1	2	3	...	96							
Normalization type	1	2	3	...	96						1	2	3	...	96							

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Table 12. Layout of the NPX-file.

SampleID	Index	OlinkID	UniProt	Assay	Missing Freq	Panel	Panel_ Lot_Nr	PlateID	QC_ Warning	LOD	NPX	Normalization	Assay_ Warning
A1-2	1	OID20321	O00584	RNASET2	0.25	CARDIO-METABOLIC	B04405	CSF-validering_ Cardio_Inf	PASS	5.6733	8.4576	Plate control	PASS
A1-2	1	OID20206	Q07108	CD69	0.44	CARDIO-METABOLIC	B04405	CSF-validering_ Cardio_Inf	PASS	5.7325	7.0288	Plate control	PASS
A1-2	1	OID20195	P35754	GLRX	0.44	CARDIO-METABOLIC	B04405	CSF-validering_ Cardio_Inf	PASS	4.4524	6.3161	Plate control	PASS
A1-2	1	OID20094	Q9H5Y7	SLITRK6	0.36	CARDIO-METABOLIC	B04405	CSF-validering_ Cardio_Inf	PASS	3.5921	3.655	Plate control	PASS

Table 13. Specification for column content in the NPX file.

Column	Description	Type
SampleID	The annotated sample ID	String
Index	Well index in 96 plate	Integer
OlinkID	OlinkID for assay	String
UniProt	UniProt ID for assay	String
Assay	Gene name for assay	String
MissingFreq.	Frequency of missing data (below LOD or NaN)	Float
Panel	Panel name assay belongs to	String
Panel_Lot_Nr	Lot number for the panel	Integer
PlateID	Name of the plate the sample was run on	String
QC_Warning	Indicates whether the sample passed QC or not	String PASS, WARN or FAIL
LOD	LOD value for assay	Float
NPX	NPX value	Float
Normalization	Type of normalization used in project	String Plate control or Intensity
Assay_Warning	Indicates whether the assay passed QC or not	String PASS or WARN

7.8 Create a Certificate of Analysis

Once all data has been thoroughly checked, Olink NPX Manager can generate a Certificate of Analysis (CoA) for the project. The CoA includes information and QC parameters for the project.

Select **File** > **Save Certificate of Analysis** to save the CoA as a PDF document.

8. Troubleshooting

This chapter describes issues that may arise during use of Olink NPX manager, or data issues during analysis, and how to solve these issues (sections 8.2-8.4). *8.1 QC in Fluidigm Real-time PCR Analysis software* covers the issues that may arise when using the *Fluidigm Real-time PCR Analysis* software. *8.2 Warning messages in Olink® NPX Manager* covers the issues that may arise when running Olink Explore.

8.1 QC in Fluidigm Real-time PCR Analysis software

ISSUE		EXPLANATION	REASON	ACTION
	Software message "Corners in the IFC were not detected".	Chamber finding algorithm cannot locate the four corner cells of the IFC.	Insufficient amount of ROX dye in corner wells. Contrast not optimal. Edge sample or assay is not loaded (e.g. air bubbles or particle clogging of the IFC).	Manually set the corners and analyze the IFC. Make sure to zoom in to clearly see where the wells should be (if empty). All wells will not fit optimally, the important ones are the four corner wells.
	Black lane or part of lane in Image View (assay or primer lane) both ROX and FAM – line in IFC.	Failed sample or assay.	• Air bubble during loading of IFC.	• Sample/assay will automatically be failed in Olink NPX Manager. • Rerun from detection step.
			• Precipitation in samples that clog channels.	• Carefully inspect extension product after centrifugation. Spin again if precipitation is visible. Pipette from surface. • Rerun from detection step. Contact Olink for order of detection kit (and IFC).
			• IFC failure.	• Extremely rare, this IFC should not pass QC at Fluidigm. • If IFC purchased from Olink, contact Olink. Otherwise contact Fluidigm.
Image View	Black pixels in Image View (ROX and FAM).	Failed reaction.	• Single wells: Most likely IFC failure.	• Datapoint will automatically be failed in Olink NPX Manager. Fluidigm do not guarantee 100% functionality of all wells.
			• Large number of wells.	• Entire lanes; see line in IFC.
	Large white dots in wells.	Particles that fluoresce.	• Dust on the central area of the IFC.	• Exclude wells from analysis. • Remove dust from IFC using adhesive tape before starting the Biomark run.
	Bright dots between wells.	Particles that fluoresce.	• IFC construction.	• Does not affect data; continue analysis.
	Larger permeable circle.	Particles that fluoresce.	• From production of the IFC.	• Does not affect data; continue analysis.
			• Control line fluid on the IFC surface.	• Rerun IFC from detection step.

	Brighter line in IFC; sample or assay orientation.	Increased fluorescent signal.	• Reagent mixing factor. • Degrading Taqman probes that bleed into the ROX channel and give some increased ROX signal. • Sample specific, e.g. inherent fluorescence or fluorescent contaminants in the sample.	• Does usually not affect data. • Evaluate if sample deviates from the remaining sample set.

8.2 Warning messages in Olink® NPX Manager

The following warnings can be displayed during the import of Fluidigm CSV files:

Could not identify panel

This warning indicates that the assay names and order do not correspond to any Olink panel. This could be caused by using an incorrect PLT file or wrong layout (CSV exported in tabular format or heatmap CSV exported in chip-based view) when creating the CSV file in *Fluidigm Real-Time PCR Analysis* software.

Quality Threshold does not meet specified criteria 0.5

This warning indicates that the setting for Quality Threshold was not set to 0.5 in the *Fluidigm Real Time PCR* software. Refer to *5.4 Change analysis settings and reanalyze*.

Baseline Correction Method is not set to Linear.

This warning indicates that the settings for the **Baseline Correction Method** was not set to “Linear” in the

Fluidigm Real Time PCR software. Refer to *5.4 Change analysis settings and reanalyze*.

Some sample names were not unique

This indicates that there are samples with identical names within the project. It is recommended that all samples within the project have unique names, e.g. by adding plate number and position to sample name. Refer to *7.1 Import a CSV file*.

The following warnings can be displayed during analysis:

Please note that normalization is performed again

When adding or removing a Negative Control, IPC, Calibrator, Plate Control, or Control sample from the sample layout, this message will be displayed as a reminder that normalization is performed again.

No Negative Controls found on plate

This indicates that no samples on the plate have been annotated as Negative Controls. Switch to the **Plate View** tab to assign the positions where the Negative Control samples are located.

8.3 Deviating controls for Olink® Target in Olink® NPX Manager

If the normalized value for internal controls or a specific sample deviates from the rest of the sample set, the sample is flagged. A flagged sample should not be excluded from the data export but may be excluded from the QC analysis. Refer to *7.5 Perform quality controls* for evaluation of the data.

Flagged samples

ISSUE	EXPLANATION	REASON	ACTION
A sample is flagged	A sample is flagged when one or several internal controls deviate for that specific sample. All flagged samples in a project are shown on the Project tab in the table Flagged Positions.	The behavior of the internal controls makes it possible to understand why the sample is flagged. See the rest of this section for more information.	For more detailed information about flagged samples, click the QC View tab (*Figure 39,* refer to *Table 14*) and the Plate Data tab (*Figure 40*).

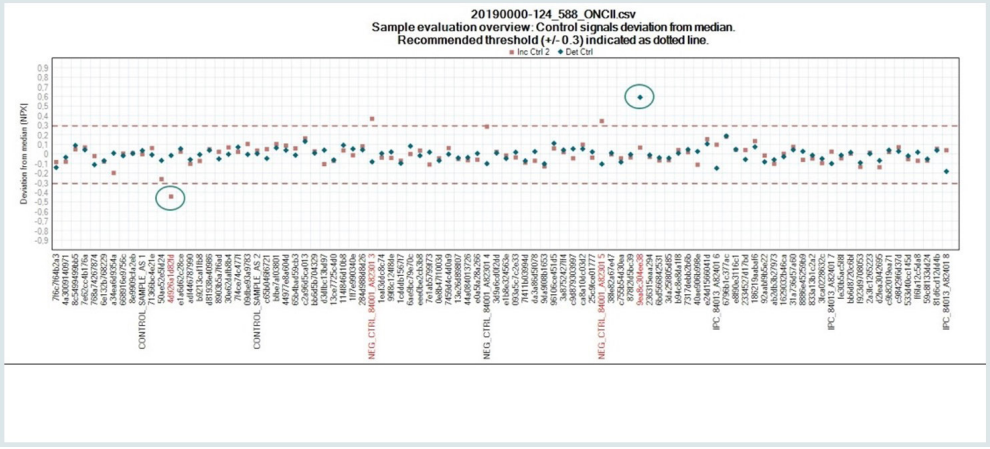


Figure 39. The QC View tab, where the first circled sample is flagged by Incubation Control 2, and the second circled sample is flagged by the Detection Control.

Figure 40. Plate Data tab for samples shown in figure 36. The circled sample to the left has a lower NPX for Incubation Control 2. The circled sample to the right has a higher NPX for the Detection Control and lower NPX for the Extension Control.

8.3.1 Sample flagged by Incubation Controls

ISSUE	EXPLANATION	REASON	ACTION
A sample is flagged by the Incubation Control.	The Incubation Controls deviate.	If the two Incubation Controls deviate, but not the Extension and Detection Controls, this is an indication that something, most likely in the sample, is affecting only the Incubation Controls.	Refer to *Table 14* for examples.

Table 14. Common reasons for deviating Incubation Controls.

Possible cause	Explanation	Solution
Sample matrix	If the sample matrix for the flagged sample is different from the others on the same plate, the incubation environment can be different. This can make the reaction slightly more or less efficient.	Do the QC evaluation for one sample type at a time.
Sample volume or concentration	The sample volume is too high or too low.	Mark the sample as failed, if the sample is rerun within the same project, either alter the name of the sample or set the failed sample as sample type: "Not used". Refer to *Figure 39* for example.
Sample quality	If the sample is stored in a freezer for long periods of time, it can evaporate. A more concentrated sample can increase possible matrix effects, and thus make the internal controls more or less efficient.	
Sample type	Olink assays are validated for serum and plasma. Other sample matrices can contain factors that interfere with the immuno reaction step.	Standardize samples as much as possible. (E.g.: for lysed cells and tissue, use similar concentration of protein in each sample)

8.4 Sample flagged by Detection Control

ISSUE	EXPLANATION	REASON	ACTION
A sample is flagged by Detection Control.	The NPX value of the Detection Control for this specific sample deviates more than 0.3 NPX from the plate median.	One example can be seen in *Figure 39* and *Figure 40*. Data is normalized using the Extension Control. Since both Incubation Controls and the Extension Control display decreased Ct-values, the normalization step will adjust the data. The Detection Control that did not show decreased Ct-values will be overcompensated by normalization against the Extension Control and thereby deviate from the rest of the sample set after normalization.	This type of flag is generally caused by the same reasons as flagged Incubation Controls. It is not uncommon that both the Incubation Control 2 and Detection Control flag simultaneously, but with opposite “direction” in the QC View. This is seen when the flagged sample affects both the extension and immunoassay step, but to different extent. Refer to *Table 14* for examples.

8.4.1 Deviating external controls

ISSUE	EXPLANATION	REASON	ACTION
Olink® Target 96 High Intra-CV value	Intra-CV value is larger than 15%.	The control samples differ within the sample plate.	Check sample annotation of the control samples and that they pass QC. Check the row pattern for rows A and B to make sure that none of the rows is an outlier (e.g. issue with a channel on one of the multichannel pipettes). Fail one or both of the control samples if remaining data on the plate pass all QC analysis and assessment.
Olink® Target 48 High Intra-CV value	Intra-CV value is larger than 15%.	The Sample Control replicates differ within the sample plate.	Check sample annotation of the Sample Controls and that they pass QC. Check the row pattern for rows A, B and C to make sure that none of the rows is an outlier (e.g. issue with a channel on one of the multichannel pipettes). If one Sample Control replicate deviates from the other two and this pattern can be confirmed for several assays, this could indicate a manual issue with this replicate. In such case, that replicate can be failed and the remaining used for CV calculation. Contact support@olink.com for further guidance.
Olink® Target 96 High Inter-CV value	Inter-CV value is larger than 25%.	The control samples differ between sample plates.	Check sample annotation of the control samples and that they pass QC. If intensity normalization has been used, ensure that randomization assumption is valid by comparing with IPC normalization.
Olink® Target 48 High Inter-CV value	Inter-CV value is larger than 25%.	The Sample Control replicates differ between sample plates.	Check sample annotation of the control samples and that they pass QC. If one Sample Control replicate deviates from the other two and this pattern can be confirmed for several assays, this could indicate a manual issue with this replicate. In such case that replicate can be failed and the remaining used for CV calculation. Contact support@olink.com for further guidance.

ISSUE	EXPLANATION	REASON	ACTION
Olink® Target 96 High LOD (> 2.5 NPX)	Background levels, values in Negative Controls, are increased above expected levels	• Sample and/or primer columns double pipetted during loading of IFC	• Rerun from Detection step.
		• Technical issue (increased background may occur sporadically due to random interaction of probes or airborne contamination). Seen for all three Negative Control wells for specific assays.	• If high LOD does not affect the detectability for the assay, continue data analysis. • If detectability is affected in a multi plate project, the affected assay can be excluded from the affected plate and data analysis continues for remaining plates or rerun from immuno reaction step. • Contact support@olink.com for further guidance.
		• Sample/IPC contamination of Negative Control	• If one well affected: Exclude contaminated Negative Control. • If two or more Negative Controls are affected: Rerun samples.
		• Sample and/or primer columns switched during loading of IFC	• If noted during lab work, switch data in CSV file before importing Ct values to Olink® NPX Manager.
Olink® Target 96 Large number of high and/or low LODs (outside ±2.5 NPX)	Calculated LODs are out of expected range	• Wrong datafile • Wrong panel • Contamination of Negative Controls • Wrong annotation of negative controls or IPCs	• Confirm PLT file and datafile; reanalyze data. • Contact support@olink.com for further guidance.
Olink® Target 96 Large deviation in LOD between plates in project	LOD values differ more than 1 NPX between sample plates in the same study	• Contamination of one or several wells • Pipetting error • Technical issue (increased background may occur sporadically due to random interaction of probes). Seen for all three Negative Control wells for specific assays.	• Evaluate which plate and/or well is contributing to the large deviation. • Exclude affected well if that can be defined. • Exclude assay on affected plate or rerun from immuno reaction step. • Contact support@olink.com for further guidance.
Olink® Target 48 High LODs that affect the number of samples that can be quantified above LOD.	Calculated LODs are out of expected range	• Wrong datafile • Wrong panel • Contamination of Negative Controls • Wrong annotation of Negative Controls, Sample Controls or Calibrators.	• Confirm annotation of Negative Controls, Sample Controls and Calibrators. • Confirm PLT file and datafile; reanalyze data. • Contact support@olink.com for further guidance.
Olink® Target 96 Large deviation between triplicates of IPC and/or Negative Controls on the same plate	NPX values for triplicate samples deviate more than 0.5 NPX	• Contamination of one or several wells • Pipetting error	• If one well affected: Exclude that well. • Rerun if several wells are affected.

ISSUE	EXPLANATION	REASON	ACTION
Olink® Target 48 Sample Control intra CV > 30% and/or accuracy > ±30% causing many failed assays	Sample Control intra CV>30% and/or accuracy > ±30%	Sample Control replicates differ within the same plate.	Check sample annotation of the Sample Controls and that they pass QC. Check the row pattern for rows A, B and C to make sure that none of the rows is an outlier (e.g. issue with a channel on one of the multichannel pipettes). If one Sample Control replicate deviates from the other two and this pattern can be confirmed for several assays, this could indicate a manual issue with this replicate. In such case that replicate can be failed and the remaining used for CV and accuracy calculation. Contact support@olink.com for further guidance.
Olink® Target 48 Calibrator intra CV>30% resulting in many assays with warning	Calibrator CV > 30%	Calibrator replicates differ within the same plate.	Check sample annotation of the Calibrators and that they pass QC. Check the row pattern for rows F, G and H to make sure that none of the rows is an outlier (e.g. issue with a channel on one of the multichannel pipettes). If one Calibrator replicate deviates from the other two and this pattern can be confirmed for several assays, this could indicate a manual issue with this replicate. In such case that replicate can be failed and the remaining used for CV calculation. Contact support@olink.com for further guidance.

8.4.2 Missing sample data

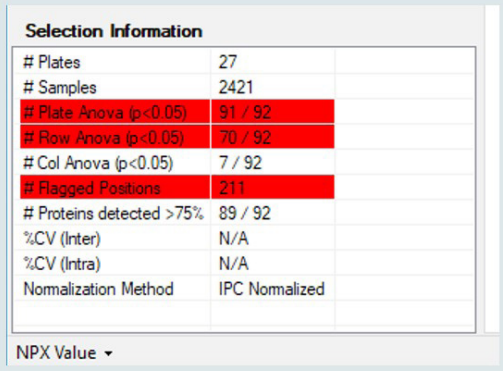
ISSUE	EXPLANATION	REASON	ACTION
Missing sample data in exported results file	Data for some samples are not exported.	This can be caused by not using unique sample names.	Ensure that sample names are unique or change sample type to **Control** for non- unique samples to force export of data for these samples.

8.5 Inconsistent results detected in Olink® Target using Olink® NPX Manager

8.5.1 Variation between plates

ISSUE	EXPLANATION	REASON	ACTION
The "Plate ANOVA" value in the Selection Information box (*Figure 41*) shows a high value. The variation is also shown on the Plate Z-score tab (*Figure 42*).	The total NPX varies between plates.	May be caused by insufficient randomization, meaning that the distribution of samples from different groups is not the same for all plates in the project.	If the samples are completely randomized, make sure that intensity normalization has been used. The results will then look like the example in *Figure 43*.

ISSUE	EXPLANATION	REASON	ACTION
High Inter-CV value	Inter-CV value is larger than 25%.	The control samples differ between sample plates.	Check sample annotation of the control samples and that they pass QC. If intensity normalization has been used, ensure that randomization assumption is valid by comparing with IPC normalization.



NOTE: Make sure that the deviation cannot be explained with biological variation, i.e. that a unique sample group is placed on one column/row.

Figure 41. The Selection Information window, with a Plate ANOVA value that indicates variation between plates.

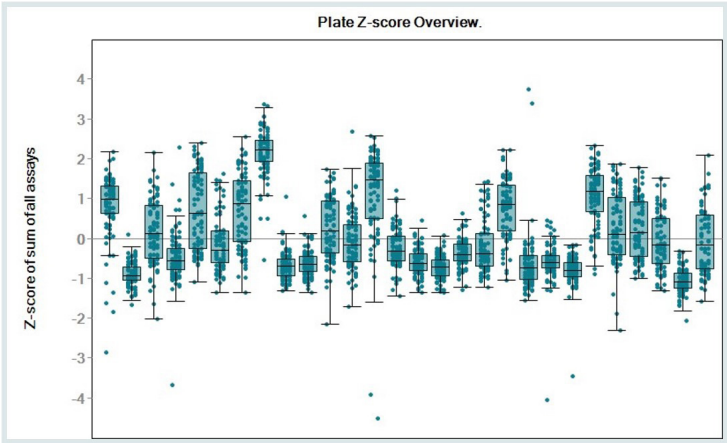


Figure 42. Plate variation that should be solved by intensity normalization.

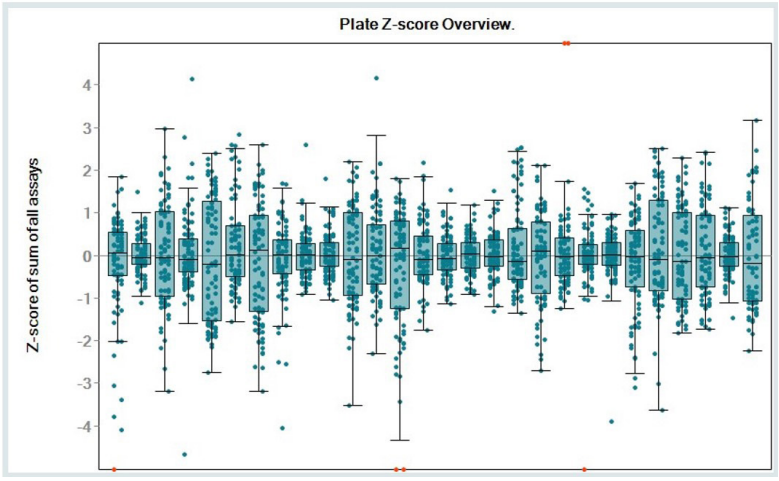


Figure 43. The same results as in Figure 30, after intensity normalization.

8.5.2 Variation within a plate

ISSUE	EXPLANATION	REASON	ACTION
The columns or row ANOVA in the Selection Information box show high values The effects are visualized in the Plate Data tab.	Areas of a plate (rows and/or columns) have systematically higher or lower values (Ct and/or NPX) than the rest of the plate.	The variation is caused by the sample position on the plate. Within-plate effects are often caused by laboratory mistakes.	Refer to *Table 15* for examples.

Position effects/patterns: visible only for Internal controls

ISSUE	EXPLANATION	REASON	ACTION
All internal controls are affected on Ct-level in a similar manner (all increased or decreased)	One sample column or row consistently show deviating values	Pipetting error, e.g. pipette not pre- conditioned	Exclude data from analysis and/or rerun.
		Mistake during pipetting of the incubation solution, extension solution, detection solution or samples	Refer to *Figure 44.*
		Evaporation due to incomplete sealing of plate during incubation or PCR steps	Exclude data from analysis and/or rerun.
		Different sample matrices	QC matrices separately.
Incubation Controls and Extension Control differ from Detection Control on Ct-level	Incubation and Extension Controls show the same pattern, Detection Control shows another pattern	PCR instrument not good at keeping 50°C, Detection Control not affected by extension step	Rerun from immuno reaction step.
		May be pre-analytical (interfering factors may affect all controls but the Detection Control)	Please contact support@olink.com for further guidance.
Incubation Control/s differ from Extension and Detection Controls	One or both Incubation Controls show deviating pattern	Pre-analytical factors in samples that interfere with the immuno reaction	Few deviating samples: Exclude samples from subsequent statistical data analysis. > 1/6th of samples deviating: Contact support@olink.com for further guidance.
Position effects/patterns: Visible for samples and internal controls			
Columns/rows/parts of sample plate	Patterning that follows sample naming/sample matrices	Different sample matrices	QC matrices separately.
Gradient over the sample plate	Upper or lower part or left and right part of the plate affected with clearly deviating values	IFC issue Poor vortexing in extension step Poor mixing of detection mix Uneven temperature in PCR block	Rerun. Contact support@olink.com for further guidance.

Table 15. Common problems and reasons for within-plate effects.

Problem	Possible cause	Figure reference
First column different than the rest of the plate (observed for internal controls)	The pipette was not preconditioned.	
A specific column or columns is different than for the rest of the plate (observed for internal controls)	A mistake when the incubation solution, extension solution, detection solution or samples were pipetted.	*Figure 45*
One row consistently different from the rest of the plate (observed for internal controls and/or samples)	The pipette tip in that specific position was not tight enough.	
A gradient from row A to row H	Pipetting was done at an angle. The plates were not sufficiently vortexed.	*Figure 48*
Deviating Z-score distribution for single assays on one plate	Primer contamination.	*Figure 47*

8.5.3 Extreme outliers

ISSUE	EXPLANATION	REASON	ACTION
Extreme blue or red lines in heatmap (when only selecting "samples" in the drop-down menu) or outlier dots in z-score view	Sample with extremely low or high protein quantification	Incorrect dilution of sample No sample added.	Fail sample.

NOTE: Extreme outliers can be discovered in different views (Plate Z-score, Plate Data, Heatmap). In Figure 32, two types of outliers are illustrated.

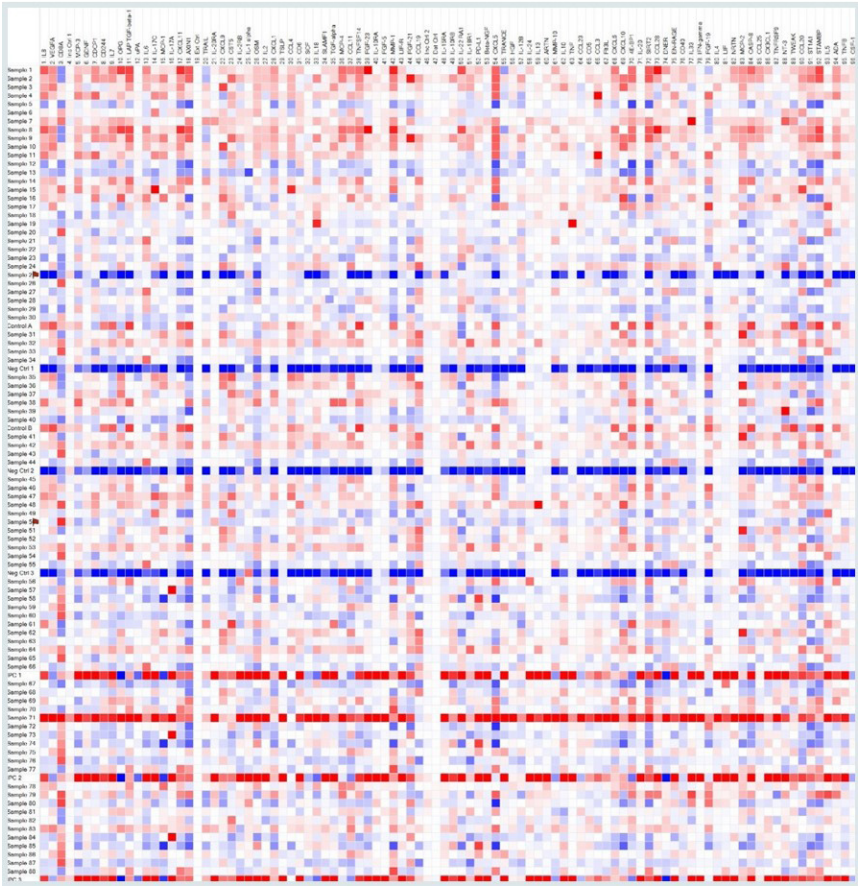


Figure 44. Heatmap View with NPX values from a single plate run which contains two outliers. Sample 25 has the lowest NPX values for all assays and Sample 71 has the highest NPX values for all assays.

For Sample 25, most NPX values are below LOD and one conclusion for this outlier is that there is no biological sample present in that well.

For Sample 71 in this example, the NPX values are higher than for the rest of the samples. This can arise from poor dilution of a sample when running one of the high-abundant panels.

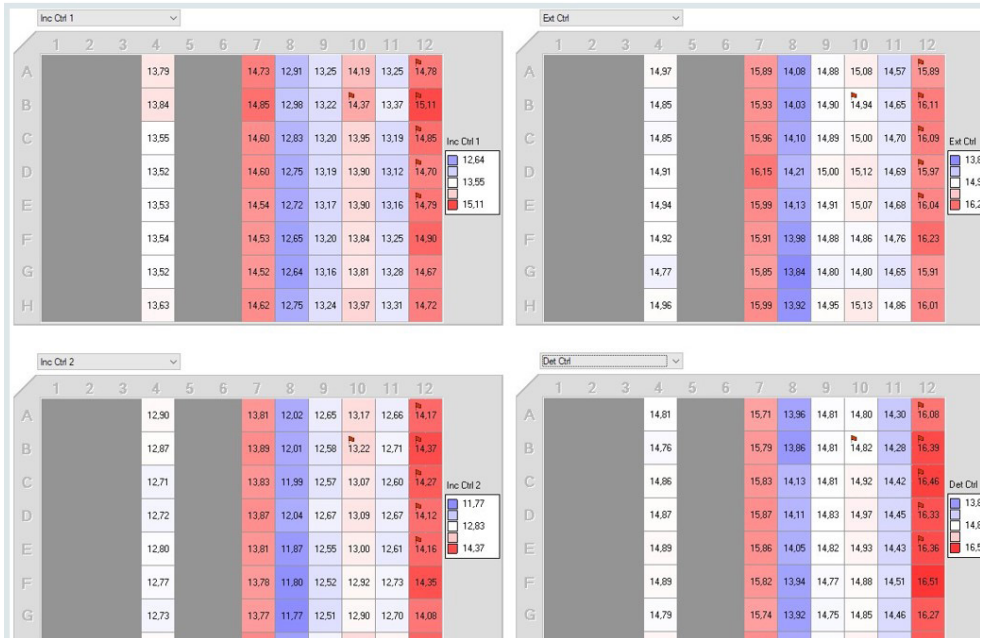


Figure 45. Ct-values that show results of laboratory mistakes. See Table 16 for a description of errors performed on this run.

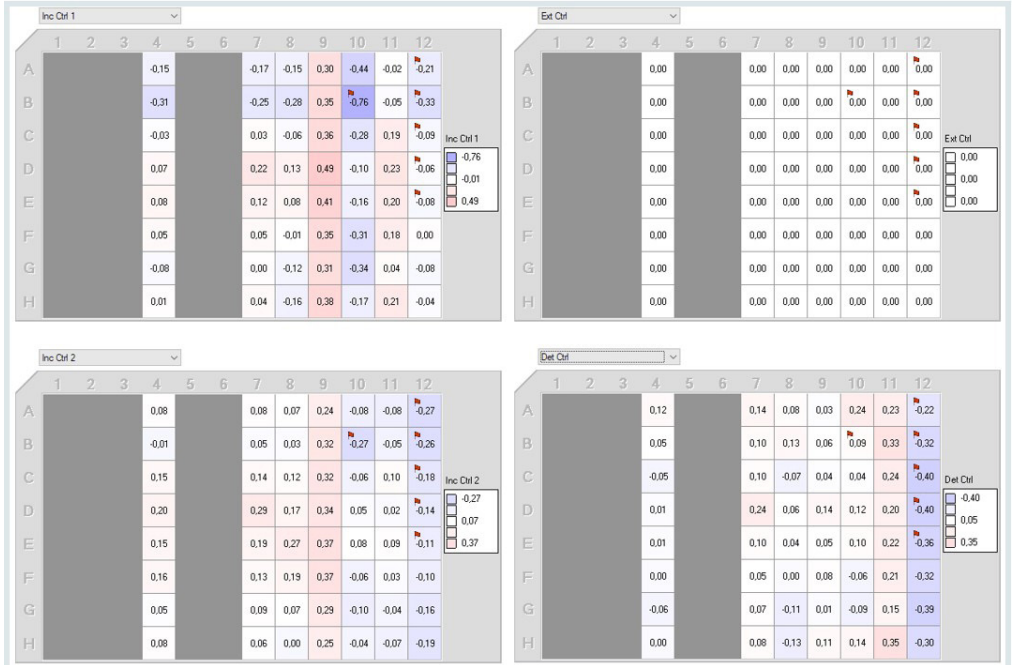


Figure 46. NPX values that show results of laboratory mistakes. See Table 16 for a description of errors performed on this run.

Table 16. Explanation of columns in Figure Figure 45 and Figure 46. Red numbers indicate laboratory mistakes.

	Column 4 (correct)	Column 7	Column 8	Column 9	Column 10	Column 11	Column 12
Incubation mix (µL)	3	3	3	3	3	6	3
Sample volume (µL)	1	1	1	0	2	1	1

Extension mix (µL)	96	96	192	96	96	96	96
Detection mix (µL)	7.2	14.4	7.2	7.2	7.2	7.2	7.2
PCR product (µL)	2.8	2.8	2.8	2.8	2.8	2.8	5.6

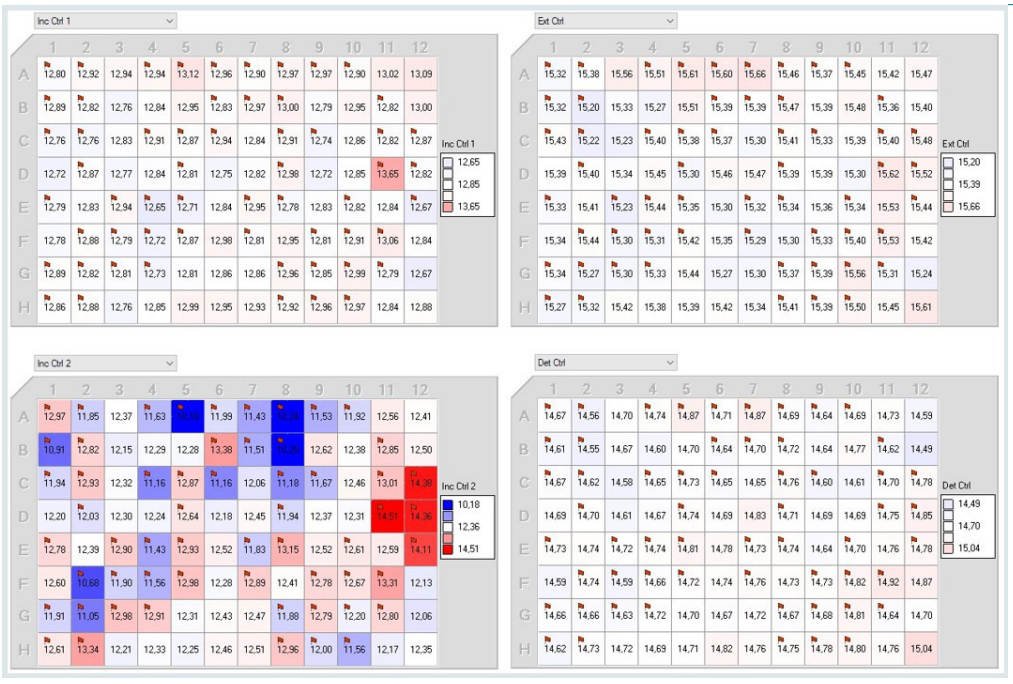


Figure 47. Ct values that show primer contamination of Incubation Control 2.

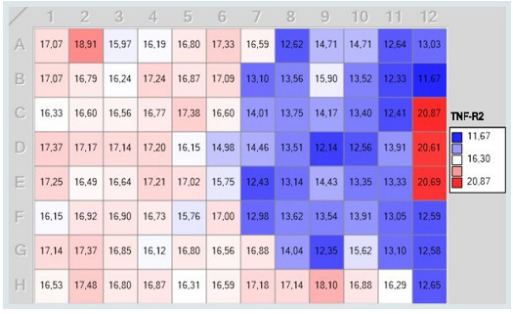


Figure 48. Within plate effects due to incorrect or insufficient vortexing of the sample plate during sample dilution.

8.5.4 High NPX signals

ISSUE	EXPLANATION	REASON	ACTION
Unexpectedly high NPX signals in diluted panel for the majority of samples	Low variation between samples Saturated assays (may be visualized in Z-score tab)	Samples were not diluted	Rerun from sample dilution step

8.6 Olink® Explore troubleshooting

This chapter describes possible root causes and recommended actions for obtained non-expected or erroneous results. Reruns are not recommended due to failed individual samples, but only if a project has failed to pass formal QC.

The chapter describes issues that may occur using the *Olink® NPX Manager* software. The issues are described as well as

the UI tabs where they occur. The table columns Possible causes and Actions can, for example, refer to instruments and steps in the laboratory work (described in detail in the Olink® Explore User Manual).

8.6.1 Low counts / Low NPX values

No	Issue	Possible cause	Recommended action
1	For one Sample, only internal controls have counts above background in all blocks for all panels. Instead, the Sample is like the Negative Control with very low NPX levels, visible in the *Heatmaps* or in the *Plate Data* tab.	1. The original sample was diluted. 2. The sample was missing from the sample plate. 3. The sample was present in the sample plate but was not transferred to the sample source plate.	Set manual warning for the sample in all panels.
2	Same as issue no 1 but affecting only diluted blocks for one sample. The undiluted blocks are unaffected.	The sample was present in the Sample source plate (containing undiluted samples) but was not transferred to the Sample dilution plate (containing serial sample dilutions). A Mosquito® tip may have caused the failure in a position when pipetting the dilution plate.	Set manual warning for the sample in all panels
3	Same as issue no 2 but affecting diluted blocks for all samples.	Sample dilution protocol on the Mosquito® was not performed. The samples are present in the Sample source plate (containing undiluted samples) but not in the Sample dilution plate (containing serial sample dilutions).	Make sure to dilute the samples before a possible rerun.
4	In the *Quality control* tab, all samples have warnings. Plate Control and Negative Controls, Amplification Control and Incubation Control criteria fail for all blocks and panels. In the *Plate data* tab, there are very low NPX levels or NaN for all samples and controls.	No counts or very few counts for both samples and controls when sequencing. If the Library QC analysis showed a correct Library peak at 150bp, it could have been caused by an over-dilution of the library (wrong volumes pipetted), or the library was not added at all in the dilution step. If there are few or no counts/reads, this should also be visible when reviewing the quality metrics in Illumina's Sequencing Analysis Viewer.	Repeat the Library preparation before sequencing, starting with the purified Olink Library that passed in QC. A possible rerun of the sequencing.
5	In the *Quality control* tab, all samples in a block have a warning and a failed Plate Control for that block (Negative Control might also fail). The sample warning is caused by a failed counts criterion. High variation for Amplification Control can be observed in the *QC View* tab. In the *Plate data* tab, all samples will have low NPX values or NaN for the block controls and assays.	Reverse probes for a block were not added when the incubation mixes were prepared. Then there are no counts for the block (both assays and controls).	This issue can be discovered during the laboratory steps and corrected: The probe incubation mix contains 90 µL instead of 100 µL, and after transferring the mix to the reagent source plate, there is only 10 µL left instead of 20 µL.
6	In the *Quality control* tab, there is a failed Plate Control criterion in a block. In the *Plate data* tab very low NPX values will be seen for all assays and Incubation Control.	Forward probes for a block were not added when the incubation mixes were prepared. This means that the block has counts only for the extension and amplificiation controls.	This issue can be discovered during the laboratory steps and corrected: The probe incubation mix contains 90 µL instead of 100 µL, and after transferring the mix to the reagent source plate, there is only 10 µL left instead of 20 µL.

No	Issue	Possible cause	Recommended action
7	In the *Quality control* tab, a sample has a warning for one or more blocks. The sample warning is caused by a failed counts criterion. Incubation control, Amplification Control, and all assays will show low levels or NaN in these samples in the *Heatmaps* tab and the *Plate data* tab.	An epMotion® tip failure during the PCR1 pooling, implying there will be no counts for one or several blocks in a sample	Contact support@olink.com for guidance.
8	In the *Quality control* tab, a failed counts criterion is indicated as a warning for a sample for all blocks in one panel. These samples will have NaN for all assays and controls in the *Plate data* tab and *Heatmaps* tab, and several samples can be affected.	An epMotion® tip failure in 10µl tool during the PCR2 setup. The addition of PCR1 pool for one or more samples fails, which causes that no Library is created for one or more samples, resulting in no counts for either the sample or the controls. This can be compared to the case with missing samples in the sample plate, when there are no counts for the controls.	Contact support@olink.com for guidance.
9	In the *Quality control* tab, there is a failed counts criterion for all blocks in a panel in a sample/index, indicated as a warning for a sample. In the *Plate data* and *Heatmaps* tabs, incubation and control and Amplification Controls, and all assays, will show low levels or NaN in these sample(s) with high variation compared to the rest of the plate. Potentially in several panels for the same index. If several samples are affected, then row or column effects could be seen on the *Plate data* tab.	1. An epMotion® tip failure in 10 µl tool during PCR2 set up during Index primer adding to a single sample. This issue can affect several samples. There was no or too low volume index primer added in the PCR2 reaction, affecting one or several samples. 2. Forgot to centrifuge index plate. 3. One or several wells of the index plate were empty.	Contact support@olink.com for guidance. • Make sure to centrifuge the Index Plate before starting the EpMotion® protocol. • Ensure liquid in every well of the Index Plate before starting the EpMotion® protocol (20 µL). If a well is empty or contains a lower volume, contact Olink for a free charge replacement Index Plate.
10	In the *Quality control* pane, a counts criterion is failed for one or more (up to 12 in a row) sample(s) for all blocks in one panel, indicated as a warning for these samples. If Plate Control is affected, this can be detected in the *Control Strip* pane QC. If several samples are affected, the *Plate data* tab will show systematic effects for Amplification Control, Incubation Control, and other assays with very low levels or NaN. There are no reads for one or up to 12 samples in a row in a panel. If several samples are affected, row effects can be seen in the *Plate data* pane.	An epMotion® one tip failure in 10µl tool during PCR2 pooling, affecting one to 12 samples in a row in a panel. The samples will be missing from the library pool, and there will be no counts generated.	This issue can be avoided by checking the volume after PCR2 pooling in the PCR2 Pooling plate (should be 36 µL).
11	In the *Quality control* tab, a failed counts criterion is indicated as a warning for several samples in a row. In *QC View* tab, incubation and Amplification Controls are failed for a block (one panel) in several sample in a row. Up to 12 samples in a row or several rows have failed criteria. The *Plate data* tab show systematic row effects for Amplification control, Incubation Control, and other assays with very low values or NaN.	One or several Mosquito® tip(s) fails in a position when dispensing incubation mix, resulting in a missing block for up to 12 samples in a row of samples (12 samples). One or more rows of samples can be affected and lacks both assays and controls for a block.	This issue can be avoided during the Mosquito® protocol: make sure to verify that there is a volume in all incubation plate wells after adding the Incubation mix (0.6 mL).

8.6.2 Inconsistent results

No	Issue	Possible cause	Recommended action
1	Systematic column effects. • The first eight columns in the A & C blocks • The last eight columns in the B & D blocks • The four last columns in the A & C blocks, and the first four columns in B & D blocks The effects are visible on the *Plate data* tab and vary in NPX values between columns compared to the rest of the plate for all assays and Incubation Control. Refer to schematic figures below.	During PCR1 setup, the Dragonfly will dispense PCR1 Mix using three different syringes. If the dispensation from one of the syringes fails (lower volume), this could affect the results column-wise, as shown by the figures below.	Make sure the Dragonfly is calibrated correctly and functional before possible rerun. After the Dragonfly protocol, always inspect the PCR1 plate. Every well should contain the same amount of liquid (19.8 µL).

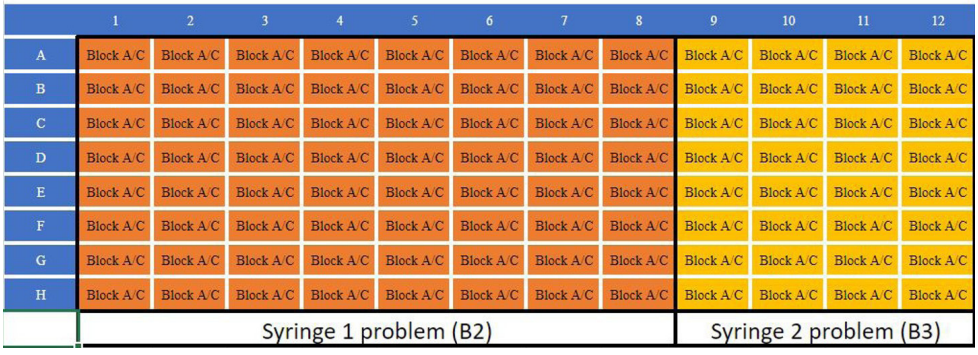


Figure 49. Pattern that would be observed in Blocks A and C in NPX Manager if there is an issue with one syringe.

NOTE: Syringe 3 pattern would not impact blocks A and C, Syringe 2 would be visible on all blocks.

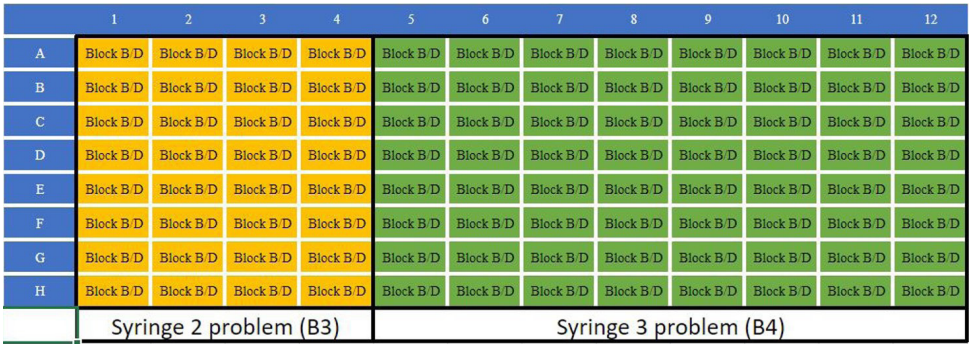


Figure 50. Pattern that would be observed in Blocks B and D in NPX Manager if there is an issue with one syringe.

NOTE: Syringe 1 pattern would not impact blocks B and D, Syringe 2 would be visible on all blocks

No	Issue	Possible cause	Recommended action
2	In the *Plate data* tab, all assays show a pattern with lower or higher values than the samples in the rest of the plate. A systematic effect is repeated on the left and right sides of the plate. Refer to the schematic figure 48 below.	The samples in some wells are less diluted than intended. This issue is likely to be caused by uneven vortexing of the sample dilution plate between dilutions. As a result, the assays will be higher, and controls will be lower than expected in affected samples. This causes systematic effects that are repeated on the left and right side of the plate.	This sample plate needs to be rerun for affected panels. Make sure to vortex the sample dilution plate thoroughly between dilutions during the Mosquito® protocol.

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

Figure 51. Example pattern that would be observed in all blocks of same dilution My Data if vortexing was skipped or insufficient.

NOTE: If the issue is in 1:10 you would also see a pattern at further dilutions, but it would fade with each dilution if vortexted.

3	Systematic effects between plates are visible in the *Plate Z-score* tab	The samples were not randomized between plates. Instead, different sample groups were systematically placed on different plates, causing plates to deviate from each other.	Change from Intensity normalization to Plate Control normalization.
4	Systematic effects between rows and columns are visible in the *Plate data* tab.	The samples were not randomized on the plate. Instead, different sample groups were systematically placed in different wells, causing rows/columns to deviate from each other.	Contact support@olink.com

8.6.3 Deviating external controls

No	Issue	Possible cause	Recommended action
1	In the *Quality control* tab, plate control criteria fail on all blocks in all panels. Systematic effects in Plate Control normalized data in the *Plate Z-score* tab if several plates are used.	The external controls/control strip was not added or added at the wrong positions (Plate Control and Negative Control switched places). This issue will cause incorrect normalization and calculation of NPX.	If the Plate control was not added, a rerun is necessary. If the Plate Control and Negative Control switched places, this issue could be corrected in the Plate layout.
2	In the *Quality control* tab, Plate control criteria fail on diluted blocks. Undiluted blocks are unaffected.	The sample dilution plate was not vortexed between dilutions. This issue will result in samples less diluted than intended: The Mosquito® will add a sample on top of the sample diluent in each well and take a sample from the bottom of each well during the protocol. Thus, the assays will be higher, and the controls will be lower than expected for all samples in the plate.	This sample plate needs to be rerun for all panels. Make sure to vortex the sample dilution plate thoroughly between dilutions during the Mosquito protocol.

9. Revision history

Version	Date	Description
6.1	22-04-11	MAD for negative controls removed. *Table 6* updated. *2.12 Quality Control (Olink® Explore projects)* figure changed.
6.0	22-02-02	*2.12 Quality Control (Olink® Explore projects)* figure changed. *3.2 Run QC* QC critera changed.
5.0	21-12-13	Added chapter *2.6 QC Warnings (Olink® Explore projects).* Failure function for Olink® Explore removed. Not used function added.
4.2	21-10-13	Updated information in *7.7.1 Considerations.* Added values to *Table 10.* Updated information in *Table 14.*
4.1	21-08-31	Added chapter *2.10 Control strip (Olink® Explore projects).* Added NOTE in *1. Introduction* .
4.0	21-04-28	Added instructions needed for data processing when running Olink Explore panels with a locally installed set up of software. Added Revision history.

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Olink Proteomics, Dag Hammarskjölds väg 52B , SE-752 37 Uppsala, Sweden

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