Olink® NPX Explore

User Manual

1251, v1.5, 2023-01-18

Table of contents

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

This User Manual provides you with the instructions needed for data processing when running Olink® Explore panels with a locally installed set up of software.

NOTE: The information in the Olink® NPX Explore 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 Explore is a data analysis software that is designed for the Olink Explore platform. It allows for importing data, validating data quality, and normalizing Olink data for subsequent statistical analysis.

Olink NPX Explore 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 Explore is intended to be used by staff certified to run the Olink Explore platform. Quality control should be performed by trained users that determine whether data from a run can be approved for further analysis.

1.2 Limitations

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

1.3 Process

1.3.1 NGS Readout

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 Explore for generating NPX data, data QC and normalization.

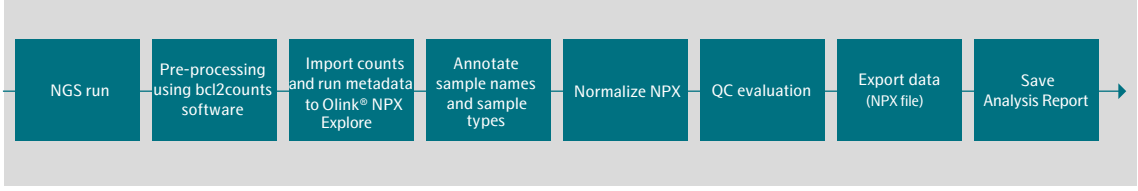


Figure 1. Flowchart describing steps involved in the analysis of Olink data.

1.4 Hardware and software requirements

1.4.1 System requirements

Components	Minimum	Recommended
Operating System	Windows® 10 or higher	
Processor	Intel® Core™ i5	Intel® Core™ i7 or higher
Memory	8 GB RAM	16 GB RAM or more
Disk Space	500 GB	500 GB

1.4.2 Requirements for analysis

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)

1.5 List of abbreviations

%CV	Coefficient of Variance
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

Olink documentation

- Olink® Explore Overview User Manual
- Olink® Explore 384 User Manual
- Olink® Explore 4 x 384 User Manual
- Olink® Explore 1536 & Expansion User Manual
- Olink® Explore E3072 User Manual
- Olink® Explore Sequencing using NextSeq 550 User Manual
- Olink® Explore Sequencing using NextSeq 2000 User Manual
- Olink® Explore Sequencing using NovaSeq 6000 User Manual

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

1.7 Technical support

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

2. User interface

The Olink NPX Explore user interface consists of several views on separate tabs where information and results are displayed. These views are described in this chapter.

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 2. Search field.

2.1.2 Help

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

2.2 Tree view

The Tree View displays the imported runs, grouped by plate in alphabetical order. Panels in red are panels with outdated calculations.

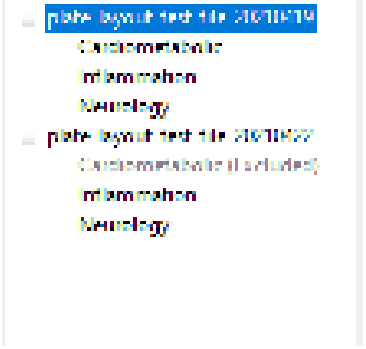


Figure 3. Olink Panels Tree View.

- Click on the **+** next to the Plate name to display the imported runs for each plate.
- Mark an individual run to see information about that specific run. If a plate is highlighted in the Tree View, information for the first panel is displayed on the run specific tabs.
- Change the order of the runs in the Tree View by dragging and dropping a run to its desired location
- Right-click on a panel to **recalculate**, **delete** or **exclude** a panel.
  - Excluded panels will be excluded from all calculations of the export files and data, and will not be shown in the QC views. Excluded panels will be greyed out and gets a suffix (Excluded).
  - When deleting a run or panel, a pop-up audit window will be prompted. Enter the reason and click **OK**.

NOTE: If there is panel plates withmissing data, a window will be prompted. Contact support@olink.com for support.

2.3 Selection information

The **Selection Information** view in the lower left corner displays information about the currently selected item in the Tree View. This information gives you an indication of the overall data quality.

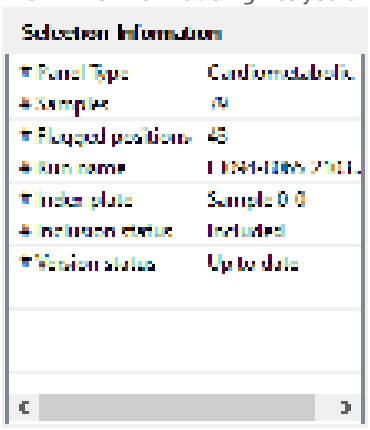


Figure 4. Selection Information view.

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.

Table 1. Description of Selection Information.

Item	Description
# Panel Type	Selected panel type.
# Samples	Number of samples, excluding control samples, in selected Olink panel or run.
# Flagged Positions	Number of flagged positions (samples that did not pass the quality control, including control samples) in the selected Olink panel or plate.
#Run name	Run specific name.
# Index plate	Panel specific index plate.

Item	Description
# Inclusion status	Shows included or excluded status of the panel.
# Version status	Shows if the version is up to date or outdated.

2.4 Project

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

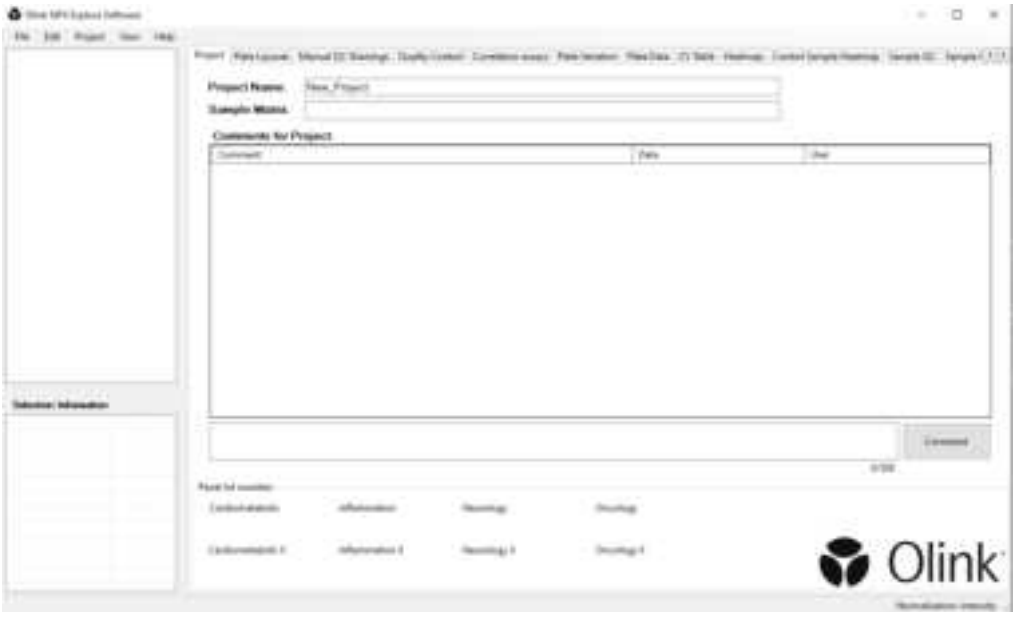


Figure 5. The Project tab.

2.4.1 Project Name

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

2.4.2 Sample Matrix

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

2.4.3 Comments for Project

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

2.4.4 Comment

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

2.5 Plate Layouts

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

Figure 6. The Plate Layout tab.

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. Each sample position is automatically annotated to one of the following content types, based on the sample naming:

- Sample
- Negative Control
- Plate Control
- Control
- Not Used

2.5.1 Incorrect annotation

If a position has been annotated incorrectly, right-click on the selection and select the correct content type.

2.5.2 Flagged samples

Wells with QC warnings are marked with flags; red for automatic and yellow for manual set QC warnings. Refer to *2.6 Manual QC Warnings.*

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 7. 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 *6.5 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 has been set as not used.

2.6 Manual QC Warnings

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

All manual warnings are listed below the plate layout.

Figure 8. The Manual QC Warnings overview dislaying automatic (red flag) and manual (yellow flag) QC warnings.

To add or edit a manual QC warning:

1. Right-click on preferred sample well. To mark a cluster of wells, click and drag over preferred wells.
2. Click Set manual warning.
3. Choose block and fill in reasons for creating a QC warning.

Figure 9. Edit manual QC warning menu

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

2.7 Quality Control

The **Quality Control** pane displays an overall quality assessment for the selected plate(s). A green checkmark indicates that the block fullfilled the corresponding QC criteria, a yellow exclamation that the block is flagged and may need further investigation and red cross indicates that the block does not meet the specification and further assessment is needed. Refer to sections *3.1 Sample QC* and *3.2 Run QC* for description of the QC criteria.

Figure 10. The Quality Control tab.

2.8 Correlation Assays

On following Explore panels: Cardiometabolic, Inflammation, Neurology and Oncology, there are three assays present on all panels: IL6, CXCL8 and TNF.

On following Explore panels: Cardiometabolic II, Inflammation II, Neurology II and Oncology II these three assays are present: LMOD1, SCRIB and IDO1.

These overlapping assays will be measured four times each in studies where all panels are included. The **Correlation assays** view shows data for these overlapping assays.

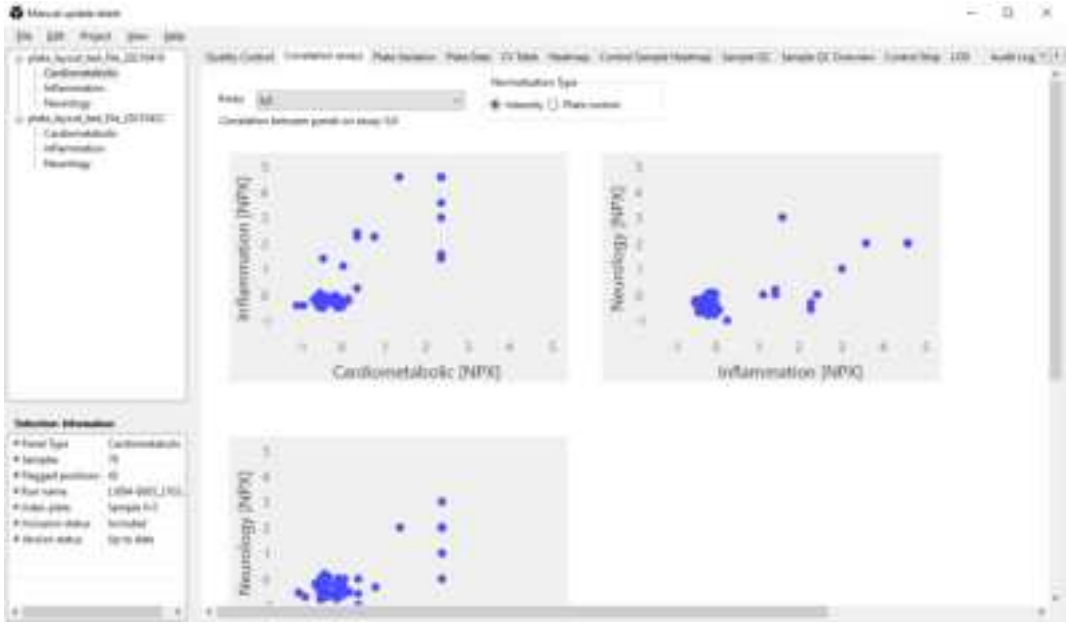


Figure 11. The Correlation Assays view.

- To display a specific correlation plot, use the drop-down menus to select a assay.
- Option for intensity normalized projects: Choose which data to view by selecting *Plate control* or *Intensity.*
- Hover over a data point to show the name and NPX values.

2.9 Plate Variation

The **Plate Variation** 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.

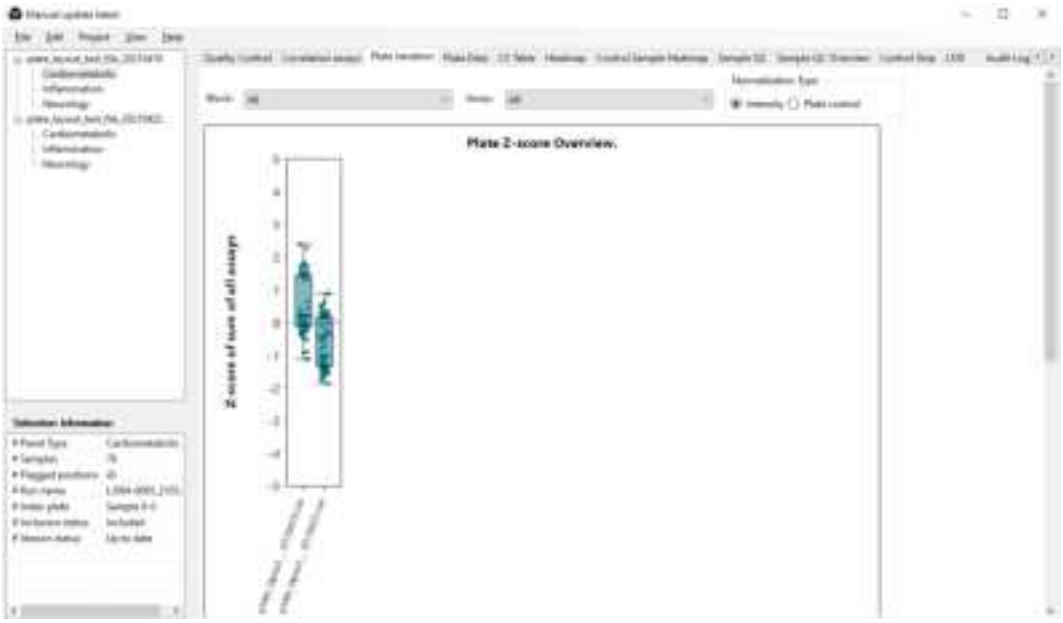
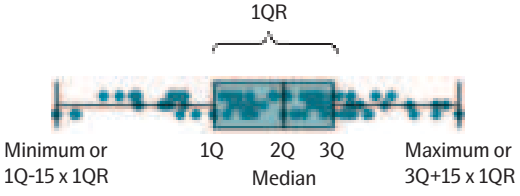


Figure 12. The Plate Variation tab displaying z-score box plot for the sum of all assays per plate.

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.

2.10 Plate Data

The **Plate Data** tab shows by default heatmaps of the three internal control assays: extension control, incubation control and amplification 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). The heatmaps show assays available in each abundance block.

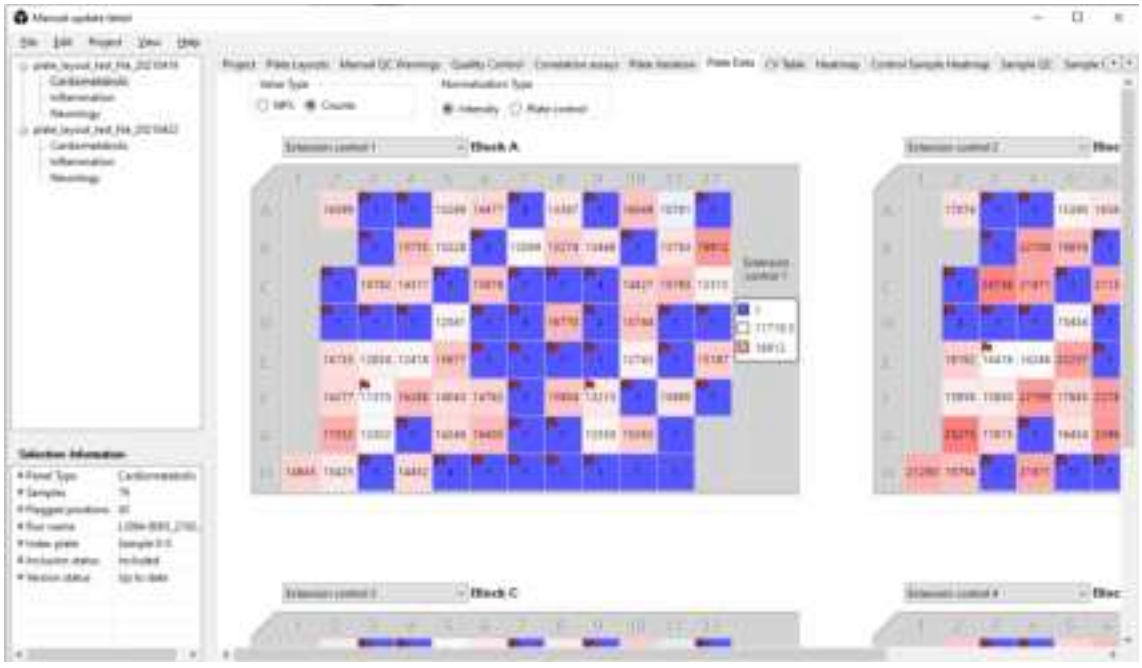


Figure 13. The Plate Data tab.

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 count or NPX-values depending on the selection in the upper left corner of the screen.

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

2.11 CV Table

The **CV Table** tab shows the CV information per block and per panel.

The tabels are per default empty, to show the information, click **Calculate**.

The information in the upper table, CV per panel, is also displayed in the Analysis of Report. The lower table, CV per block, replaces the former CV Table File that could be exported.

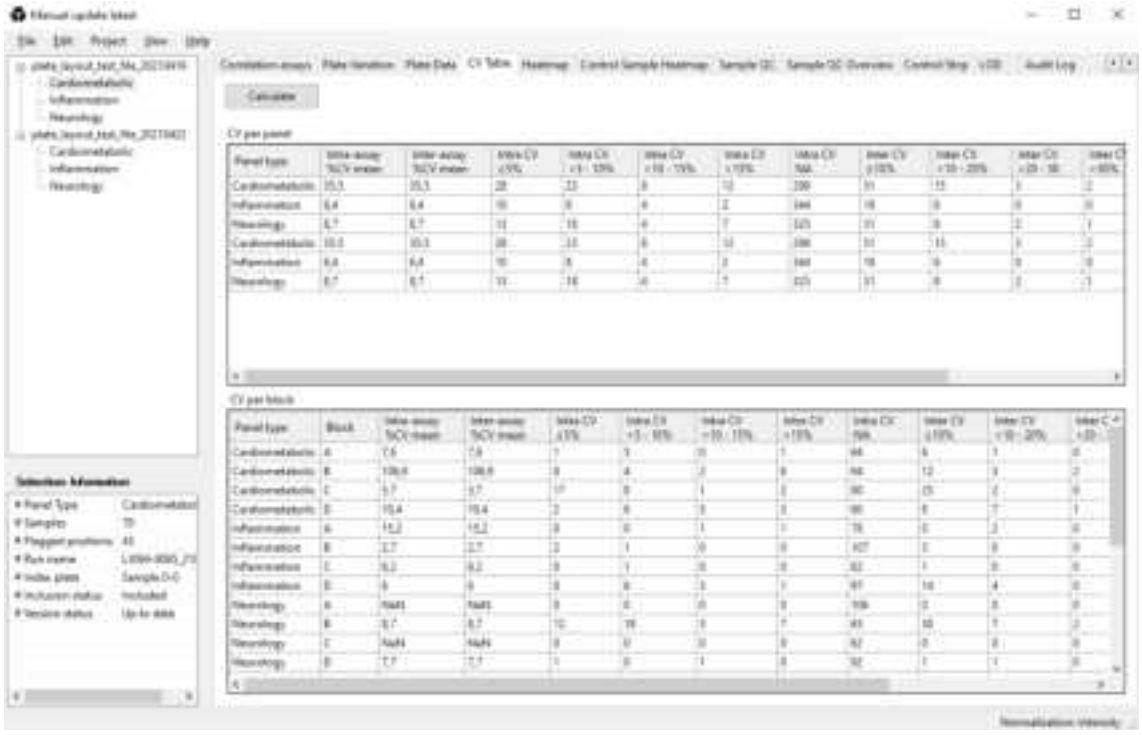


Figure 14. The CV Table tab.

- To export the information, mark whole or parts of the table, right-click and choose **Copy.** Go to preferred program, for example Microsoft Excel, and paste the information.
- If changes are done, for exemple, a well is set as Not used, a warning is shown beside the **Calculate** button: *Data is outdated, please calculate CV*. Click Calculate to update the information.

2.12 Heatmap

The **Heatmap** 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 count or NPX values, depending on the selection in the upper part of the screen. For both count and NPX, blue values are below the median and red 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.

The normalization type is shown below the upper part of the screen.

Figure 15. Heatmap view showing samples' deviation from mean per assay.

- Use the **Display** drop-down list to select the sample type to display (all samples, sample, plate control, negative control or control).
- Use the **Block** drop-down list to select block.
- 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.

2.13 Control Sample Heatmap

The **Control Sample Heatmap** shows a compilation of the control samples of all the plates, one plate at the time. Red wells indicates NPX or counts values higher than the median, and blue indicates values lower than the median.

Figure 16. Control sample heatmap

- Select the type of control, sort order and block to view in the drop-down menues in the upper left corner.
- Select value type and normalization type in the radio buttons in the middle of the upper part of the view.
- Hover over a well to display the NPX or counts value for selected sample and assay.

2.14 Sample QC

The **Sample QC** 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.

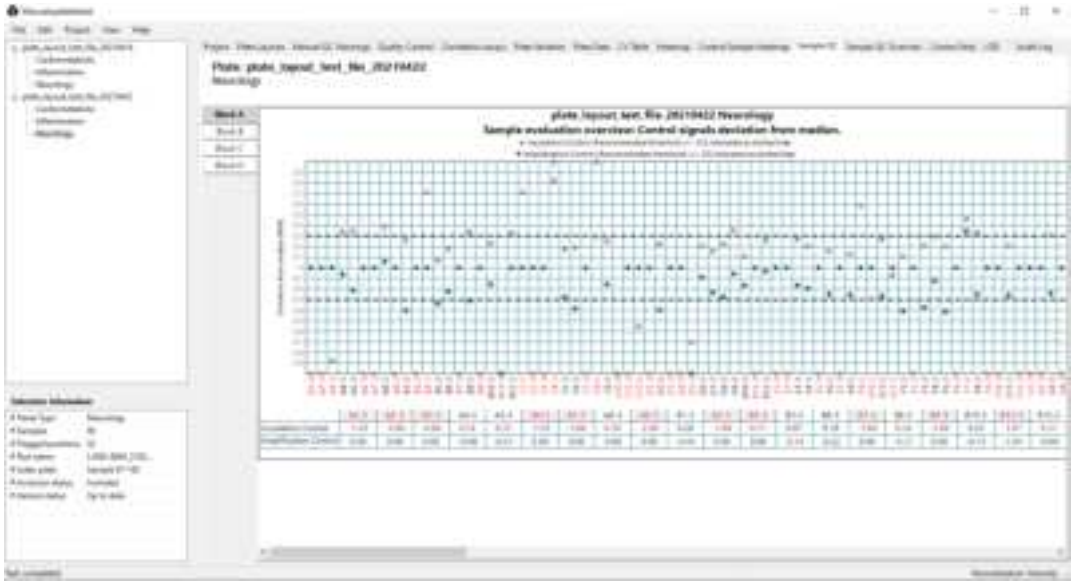


Figure 17. The Sample QC tab

The **Sample QC** 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 **Sample QC**, Olink NPX Explore offers several tools for troubleshooting and issue detection. Guidelines for what is considered an issue are available for the **LOD** and the **Selection Information** view and can serve as a good starting point. For more information about the **Selection Information** pane, refer to *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.

2.15 Sample QC Overview

The **Sample QC Overview** displays a heatmap with passed (blue), automatic QC warning (red), or manual QC warning (yellow) status for each sample in all blocks and panels.

Figure 18. Heat map example for a complete run in Sample QC overview pane

- Hovering over the cells will give information about the sample and warnings.

2.16 Control Strip

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 and the median Z-score of Negative Controls must not exceed 3.

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

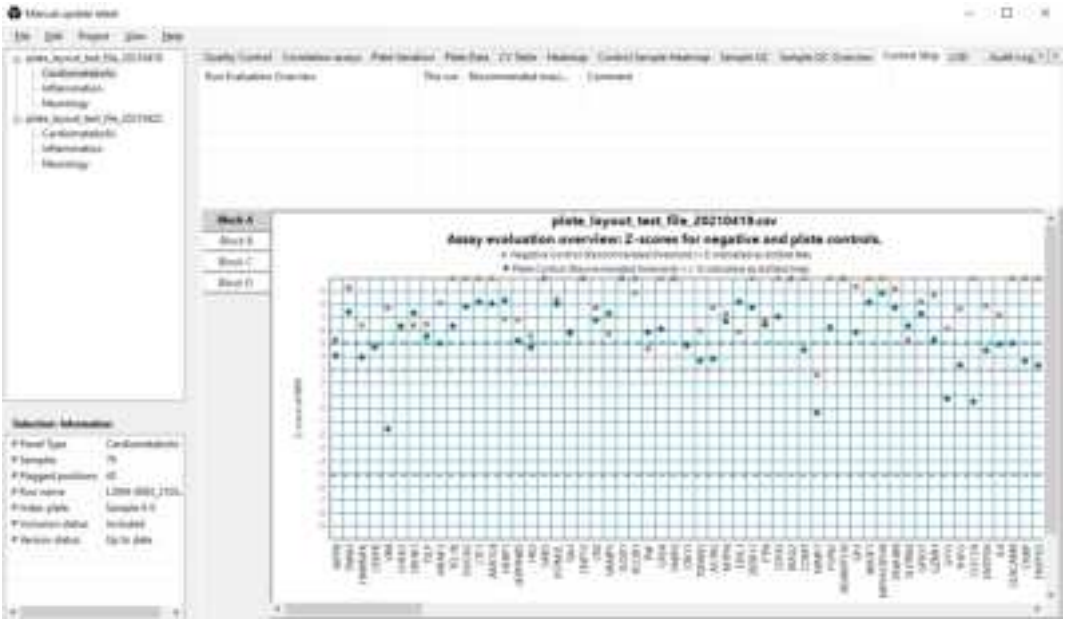


Figure 19. The Control Strip View tab

2.17 LOD

The **LOD** 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 assays with deviating negative controls.

LOD is estimated using NPX values and the following formula:

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

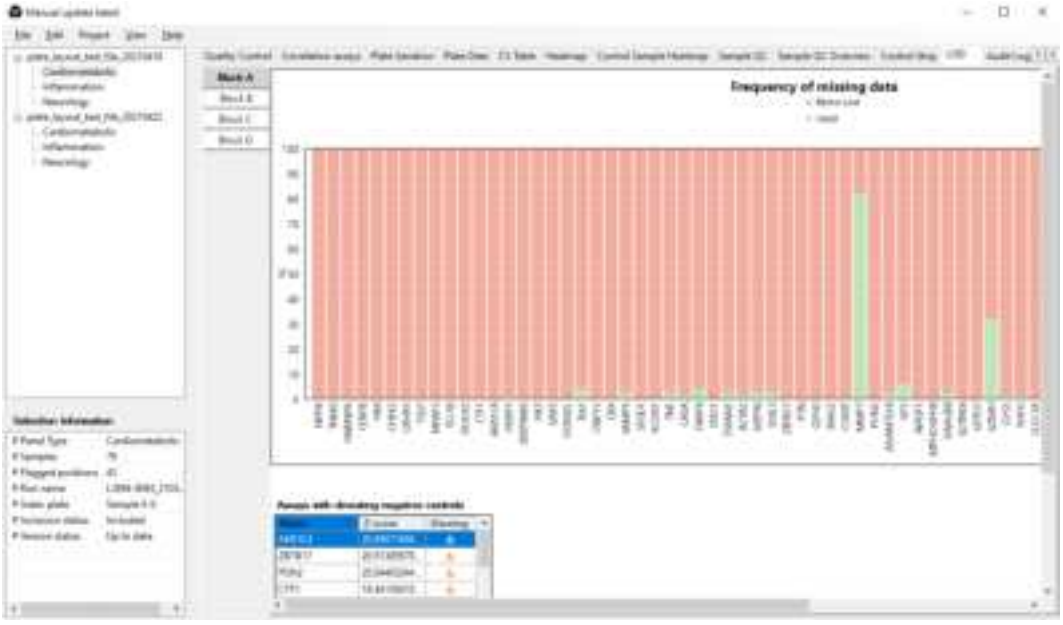


Figure 20. The LOD tab

- 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.

2.18 Audit log

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

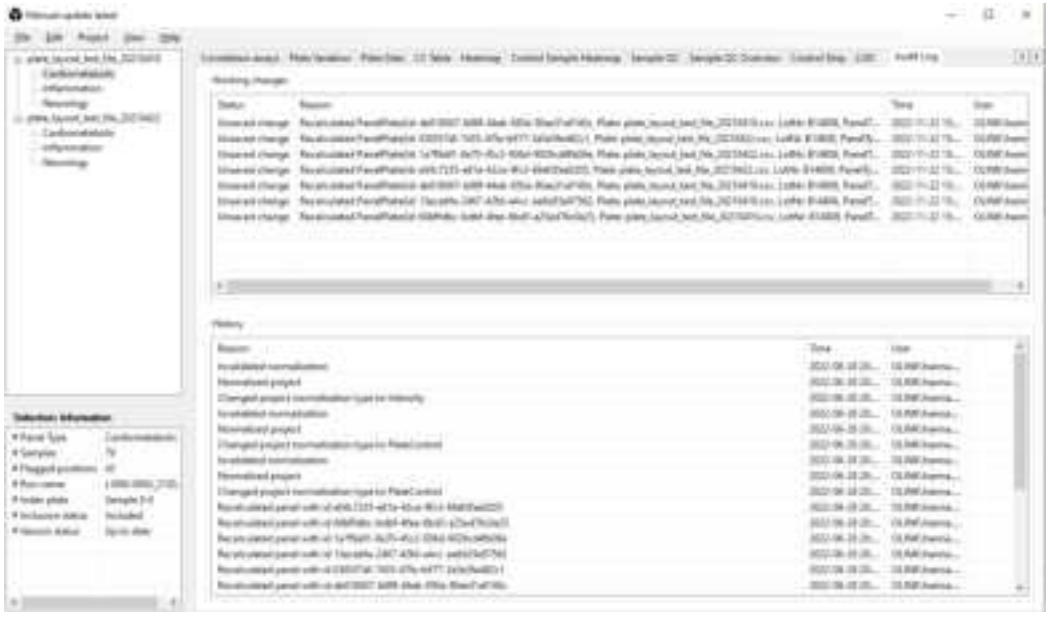


Figure 21. The Audit Log tab displaying all changes performed on the project.

3. Quality control

Three internal controls are added to each sample to monitor the quality of assay performance, as well as the quality of individual samples:

- Incubation Control
- Extension Control
- Amplification Control

The Extension Control is used to calculate the NPX, refer to *4.2 NPX generation and normalization*, and the other two are used in Quality Control (described below).

3.1 Sample QC

Sample QC is performed based on verage counts per samples

Acceptance criteria for passing a sample is an average counts > 500. Deviating values for counts per samples can be caused by, for example, errors in pipetting or pre-analytical factors in the samples that affect the performance of assays. Average counts dropping below 500 within a sample may impact variability in that sample.

For acceptance criteria for passing a sample, refer to *Table 2*.

3.2 Run QC

Plate QC is based on overall performance of the plate utilizing both total samples passing QC and Olink external controls. Criteria for plate controls and negative controls is based on both accuracy and precision in comparison to expected lot specific reference values.

For acceptance criteria for passing a run, refer to *Table 2*.

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.

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%

3.3 Assay QC

For acceptance criteria for passing an assay, refer to *Table 2*..

3.4 Additional quality assessment

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, refer to 4. Normalization.

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

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 medians are calculated. An important concept when selecting normalization procedure is randomization, which in this context applies to the sample placement across the plates. For details, refer to Randomization FAQ on the Olink website.

For randomized studies with more than one plate, intensity normalization is the appropriate method to use. In this setting, the median for a random selection of samples is more stable across plates than the three Plate Control samples on each plate. For projects where complete randomization of samples cannot be guaranteed, or where there is only one plate, Plate Control normalization should be used.

4.1 Bimodal distribution

Plate Control normalization must be used for assays with bi-modular distribution. The following assays uses bimodal distribution:

- TDGF1 (Teratocarcinoma-derived growth factor 1, Uniprot number: P13385)
- FOLR3 (Folate receptor gamma, Uniprot number: P41439)
- PNLIPRP2 (Pancreatic lipase-related protein 2, Uniprot number: P54317)

4.2 NPX generation and normalization

4.2.1 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))`
   a. Between plate normalization (Optional but default for multi plate projects).
   b. For each assay, for all plates in project.

4.2.2 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 Analysis Report.

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 Analysis Report output.

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

5. Pre-processing runs

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

5.1 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.txt which is distributed together with the bcl2counts software.

6. Operation

Input data is counts files and metadata files generated by *bcl2counts*. For more information on pre-processing, refer to *5. Pre-processing runs*.

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

1. Import run files (Plate layouts and NGS run folder) or open or clone a project.
2. Enter project information.
3. Verify sample types and layout.
4. Calculate NPX.
5. Perform quality controls including additional quality assessment.
6. Export data.
7. Create an Analysis Report.
8. Finalize the project

Figure 22. NPX Explore main window with the Plate View tab selected.

1	Information box displaying statistics about the currently selected item
2	Tree view showing Olink panels
3	Main work area

6.1 Import run files or open a project

6.1.1 Import run files

To create a project, following files must be imported:

- Plate layouts
- NGS run folder

1. Select **File > Import -> Import Plate Layouts** and browse to the preferred plate layouts.
2. Select **Project -> Change Lot Numbers** to verify that the correct panels has been selected.
3. Select **File > Import -> Import NGS Run Folder** and browse to the preferred folder.
4. Select plate layouts and click **Ok.**

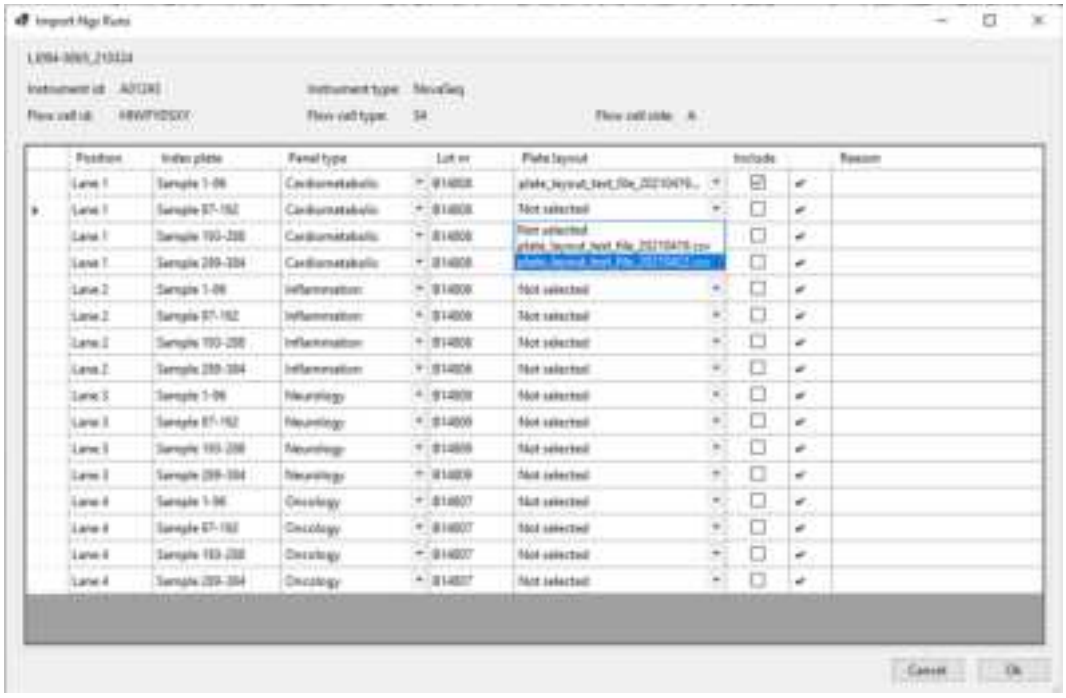


Figure 23. The Import NGS Run window

NOTE: A plate layout can only be selected once for each panel type.

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

6.1.2 Open or clone a project

To open an existing project, either:

- Select File > Open a Project and browse for preferred project.

or

1. Select File > Clone a Project and browse for preferred project.
2. Browse for a folder to place the cloned project in. A folder with the same name with _CLONE added at the end will be created.

6.2 Enter project information

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

3. Enter a **Project Name**. This will be displayed in the data export file and in the Analysis Report.

- Enter information in some or all the following fields to be included in the Analysis Report.
  - **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 Explore project file (.oaf) and Flagged Positions will automatically be displayed in the Analysis Report.

6.3 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
- Not Used
- Plate Control

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

6.4 Calculate NPX values

1. Select an Olink panel in the Tree View.
2. Click **Project** -> **Normalization** and select the normalization type to use for the project: plate control or intensity. The choosen normalization type will be used in the export data files.

NOTE: The project will not be recalculated.

Plate Control normalization is performed automatically when importing a run to generate the QC metrics. Also intensity normalization is available. Refer to *4. Normalization* for more information about the available normalization methods.

6.5 Perform quality controls

1. Perform review overall QC for each plate separately using the Quality Control tab.
2. Perform quality assessment for all plates using the other views as described in *6.6 Additional quality assessment* and then make an overall assessment.

NOTE: If more than one person are working on the same plate at the same time, changes will not be shown until the project has been saved.

The acceptance criteria for passing QC are described in *Table 2.*

Table 2. Quality Control guidelines for Olink® Explore.

Criteria	Recommended value
Sample QC	
Average counts per sample	> 500 counts
Run QC	
MAD of Plate Control	≤ 1.5 NPX
Median of Z-score Plate Control	≤ 5, ≥ -5
Median of Z-score Negative Control	≤ 3
Number of samples with warning flags	≤ 1/6 of total number of samples on plate (15/88 samples on a full plate)
Assay QC	
Median of the triplicates of the Negative Control	< 5 SD from the 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.

6.5.1 Performing a quality assessment

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

6.6 Additional quality assessment

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. 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.

6.7 Export data

Data can be exported as NPX, Extended NPX and file for use in other applications and for statistical analysis.

To export data, go to **File -> Export** and select preferred file type. Browse for a folder to save the file to.

NOTE: The CV table function is replaced by the table CV per block. For more information and how to export the table, refer to 2.11 CV Table.

Table 3. Data export options.

Setting	Description
Export format	CSV. Column separator and semicolon separator can be configured. The files will be saved as a ZIP file.
Layout	Extended format will output one line per datapoint.
Export Samples	Export data for customer samples.
Export Control Samples	Export Control samples.
Export Plate Controls	Plate Control or Negative Control
Export Assay Results	Export results for assays.
Export Control Assay Results	Export results for control assays.
Export QC data	Deviation from median for Incubation Control and Amplification Control is exported in NPX.
Export Plate LOD	Only plate LOD is calculated and exported.

Table 4. Specification for column content in the NPX extended file.

Column	Description	Type
SampleID	The annotated sample ID	String
Sample_Type	Type of sample	String PLATE_CONTROL, NEGATIVE_CONTROL, CONTROL, SAMPLE
Index	Well index in 96 plate	Integer 1-96
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
WellID	Id for well	String Capital letter A-H followed by number 1-12
QC_Warning	Indicates whether the sample passed QC or not	String PASS, WARN, MANUAL_WARN
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
Intra CV	Intra CV for sample	Float
Inter CV	Inter CV for sample	Float
Processing_StartDate	Start date for processing	Timestamp
Processing_EndDate	End date for processing	Timestamp
AnalyzerID	The analyzer ID	String
ExploreVersion	Software version of the module in Olink NPX Explore used for panel calculations and normalization	String

Table 5. Specification for column content in the NPX file.

Column	Description	Type
SampleID	The annotated sample ID	String
Sample type	Type of sample	CONTROL, SAMPLE
Index	Well index in 96 plate	Integer 1-96
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, MANUAL_ WARN
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
ExploreVersion	Software version of the module in Olink NPX Explore used for panel calculations and normalization	String

NOTE: All assays for each Olink panel will be visible in NPX and extended NPX file. Assays that do not pass QC will not be included in the calculations. Please reach out to your contact person at Olink for further information.

6.8 Create an Analysis Report

Once all data has been thoroughly checked, Olink NPX Explore can generate an Analysis Report for the project. The Analysis Report includes information and QC parameters for the project.

Select **Project > Analysis Report input.** Fill in the information and click Submit.

6.9 Finalize the project

When the QC is done, the project can be marked as finalized.

Select **Project > finalize Project.**

When the project is finalized, all output files are generated. The project can stil be viewed but cannot be modified.

7. Troubleshooting

This chapter includes a trouble shooting guide, describing 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 Explore software. The issues are described as well as the UI panes where they occur. The panes are described in detail in section *2. User interface*, in this manual. The table columns *Possible causes* and *Actions* can, for example, refer to instruments and steps in the laboratory work (described in detail in the applicable Olink Explore User Manual).

7.1 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 *Heat map* or in the *Plate data* pane.	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 fail 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* pane, 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* pane, 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.

No	Issue	Possible cause	Recommended action
5	In the *Quality control* pane 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. It is visible in the *Sample QC overview* pane when hovering over cells with a warning. High variation for Amplification Control can be observed in the *Sample QC* pane. In the *Plate data* pane, 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* pane there is a failed Plate Control criterion in a block. In the *Plate data* pane 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.
7	In the *Quality control* pane, a sample has a warning for one or more blocks. The sample warning is caused by a failed counts criterion. It is visible in the *Sample QC overview* pane, when hovering over cells with a warning. Incubation Control, Amplification Control, and all assays will show low levels or NaN in these samples in the *Heat map* pane and the *Plate data* pane.	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 support
8	In the *Quality control* pane, a failed counts criterion is indicated as a warning for a sample for all blocks in one panel. In the *Sample QC* pane, Amplification and Incubation Controls are out of specifications for a sample for all blocks in one panel. The QC failures will also be seen in the *Sample QC overview* pane. These samples will have NaN for all assays and controls in the *Plate data* pane and *Heat map* pane, 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 support

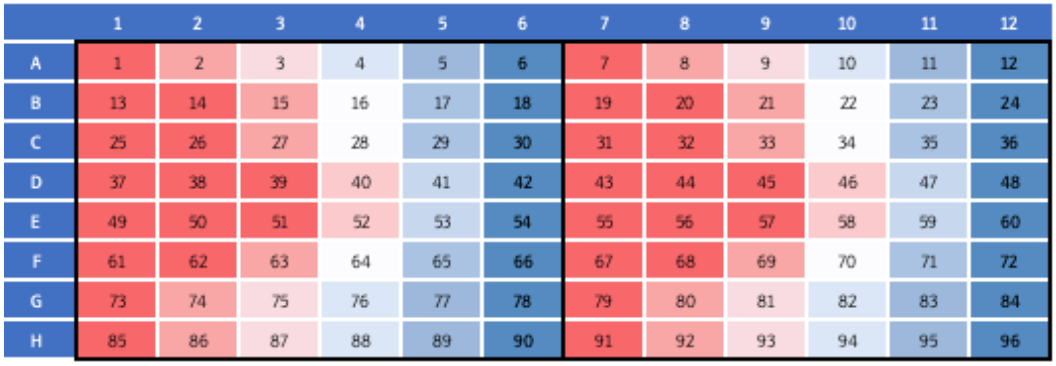
No	Issue	Possible cause	Recommended action
9	In the *Quality control* pane, there is a failed counts criterion for all blocks in a panel in a sample/index, indicated as a warning for a sample. This issue can also be seen in the *Sample QC overview* pane, indicating counts criteria as the cause for the warning. In the *Plate data* and *Heat map* panes, Incubation 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* pane.	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* pane 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 10 µl 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* pane, a failed counts criterion is indicated as a warning for several samples in a row. In *Sample QC* pane, 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* pane show systematic row effects for Amplification Control, Incubation Control, and other assays with very low values or NaN. Rows of samples will have a warning for Amplification Control, Incubation Control, and counts criteria in the *Sample QC overview* pane. Row effects can be seen in the *Plate data* pane.	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).

Inconsistent results

No	Issue	Possible cause	Action
1	Systematic column effects in every 4 columns in all blocks, column 1-4, column 5-8 and column 9-12. The effects are visible on the Plate data pane 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).
2	In the *Plate data* pane, 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 3 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.

Figure 1. Pattern that would be observed in Olink MyData Cloud if there is an issue with one syringe.

NOTE: Any Syringe Issue will be visible on all blocks with same pattern.

No	Issue	Possible cause	Action
	 *Figure 2. Example pattern that would be observed in all blocks of same dilution MyData 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 variations* pane.	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* pane.	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.

7.2 Deviating External Controls

No	Issue	Possible cause	Action
1	In the *Quality control* pane, plate control criteria fail on all blocks in all panels. Systematic effects in Plate Control normalized data in the *Plate variations* pane 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.

No	Issue	Possible cause	Action
2	In the *Quality control* pane, Plate Control criteria fail on diluted blocks. In the *Controls strip* pane, the Plate Control will exceed the upper limit while the Negative Control's confidence interval will be OK. 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.

7.3 Reload Issues

No	Issue	Possible cause	Action
1	Failures in data validity checks.	Errors during project save or if data is otherwise changed outside of the application.	Restore the project files to a previous version backup on disk made before data corruption occured. If no backups are available, the entire project folder can be sent as a zipped archive to support@olink.com so that data recovery may be performed.

8. Revision history

Version	Software version	Date	Description
1.5	v1.7	2023-01-18	*2.4* updated according to new view. *2.15* updated with new hovering information. *3.1* and *3.2* updated with reference to new QC criteria. *3.3* new. *Table 2, Table 4* and *Table 5* updated. *Figure 5* and *Figure 17* updated.
1.4	v1.6	2022-11-29	*2.2* updated with new Tree view and new functions. *2.3* updated with new Section information content. *2.4*–*2.18* Rearranged and updated figures. *2.11* added. *6.4* updated with new Normalization function. *6.5* updated. *6.7* Export of CV Table File removed, updated and two Notes added. *Table 3* and *Table 5* updated. *Table 4* added. Former Table 4 deleted. Editorial changes.
1.3	v1.4	2022-10-17	*Figure 10* updated.
1.2	v1.3	2022-10-03	*2.2* updated *6.9* added. Former sections 2.4.6 Flagged positions and 2.4.8 Project options removed.
1.1	v1.1	2022-09-05	*2.13* added and related pictures updated. *Table 2* corrected. *7.3* added.
1.0	v1.0	2022-07-04	New

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