Olink® NPX Explore HT

User Manual

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Part 1: Introduction

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

1. About this manual

This user manual provides you with the instructions needed for data processing when running Olink® Explore HT with a locally installed set up of software.

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

1.1 Intended use

Olink NPX Explore HT is a data analysis software that is designed for the Olink Explore HT platform. It allows for importing data, validating data quality, and normalizing Olink data for subsequent statistical analysis.

Olink NPX Explore HT 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*.

1.2 Intended target group

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

2. List of abbreviations

%CV Coefficient of Variance

NGS Next Generation Sequencing

PCR Polymerase Chain Reaction

PEA Proximity Extension Assay

QC Quality Control

SD Standard Deviation

3. Associated documentation

3.2.1 Olink documentation

Olink documentation

- Olink® Explore Preprocessing Technical information, v4.0

- Olink® Explore CLI Technical information, v.2.0

- Olink® Explore HT 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

- Olink® Explore Sequencing using NovaSeq 6000 S4 User Manual

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

3.2.2 Other documentation

- Article & lot configuration sheet - delivered together with the analysis kit.

4. Technical support

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

5. Process

5.1   Hardware and software requirements

5.1.1   System requirements for Olink® Explore HT

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

5.1.2   Requirements for analysis

Files and information needed for analysis:

- Count files in CSV format and run metadata in JSON format (NGS raw data pre-processed using ngs2counts)

- Plate layout with sample names

- Data analysis reference ID number of reagents used (provided on the Lot configuration insert delivered with the kit)

5.2   Pre-processing runs

Preprocessing is a necessary step in all Olink® Explore analyses. During preprocessing, the Next Generation Sequencing (NGS) output from the sequencing instrument is converted to counts files containing the number of reads for each Olink® sequence and a run metadata file containing additional information which is necessary for importing the counts files into the analysis software Olink® NPX Explore HT, or Olink Explore CLI.

For more information, refer to Olink® Explore Preprocessing Technical information and Olink® Explore CLI Technical information.

5.3   CLI

QC and normalization of Olink Explore HT data can be performed either using the Olink NPX Explore HT software or using the Olink Explore CLI software or using a combination of the two.
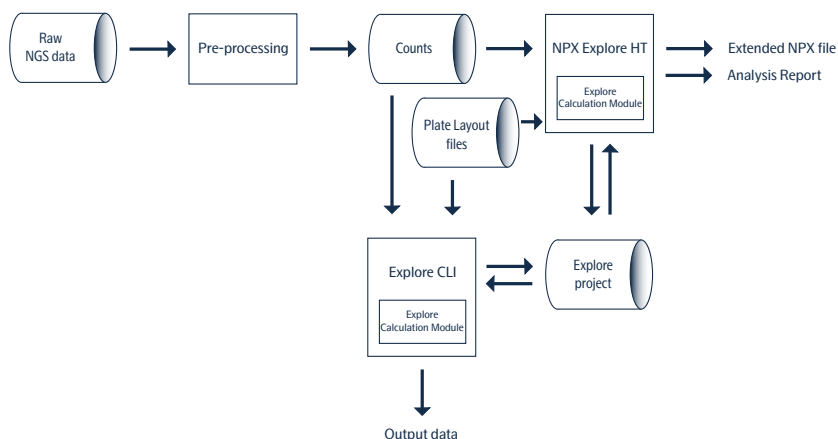
Olink® Explore CLI is a command-line interface (cli) for the Olink® Explore HT product. The application is capable of performing normalization, quality control (QC) and CV computations on NGS data and exporting the results on several supported formats. For more information, refer to the Olink® Explore CLI Technical Information.

Part 2: User Interface

6. General

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

6.1 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**.
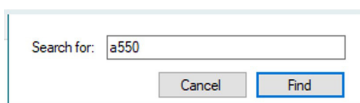
6.2 Help

Press F1 to open the Olink® NPX Explore HT User Manual as a PDF file. The manual can also be downloaded from *www.olink.com/downloads*.

6.3 Tree view

The tree view displays the plates and the imported data for each plate.

- Click on the **+** next to the Plate name to display the imported data for each plate.

- Mark an individual block to see information about that specific block. If a plate is highlighted in the Tree View, information for the first block is displayed on the specific tabs.

- Right-click on a block to **recalculate**, **delete, include** or **exclude** a block.

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

6.4   Selection information

The Selection Information view in the lower left corner displays information about the currently selected item in the Tree view.

Selection Information

# Block	Block 1
# Samples	63
# Experiment name	LJ129-3042-re...
# Index plate	A
# Inclusion status	Included
# Version status	Up to date

Item	Description
# Block	Selected block.
# Samples	Number of samples, excluding control samples, in selected Olink panel or run.
#Experiment name	Experiment specific name.
# Index plate	Panel specific index plate.
# Inclusion status	Shows included or excluded status of the panel.
# Version status	Shows if the version is up to date or outdated.

7. Tabs

7.1 Project

The **Project** tab contains fields for entering and saving project information.

7.1.1 Project Name

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

7.1.2 Sample Matrix

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

7.1.3 Comments for Project

Write a message in the box next to the Comment button. Click the **Comment** button to add the message in the **Comments for Project** field. The comments will only be visible in the project tab and not be generated into the Analysis Report.

Copy a comment by right-clicking on the comment and selecting **Copy**.

7.1.4 Data Analysis Reference ID

This field displays data analysis reference ID for the blocks included in the project.
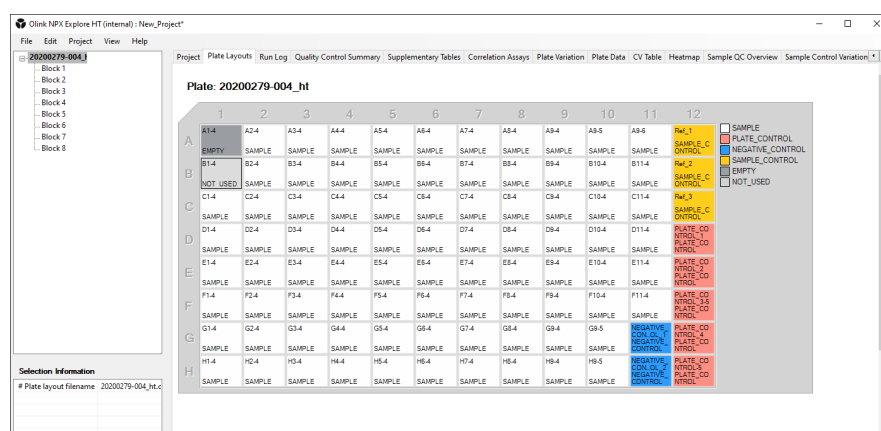
7.2   Plate Layouts

General:

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

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

- White: SAMPLE

- Red: PLATE_CONTROL

- Blue: NEGATIVE_CONTROL

- Yellow: SAMPLE_CONTROL

- Dark grey: EMPTY

- Light grey: NOT_USED

Neither EMPTY or NOT_USED wells are covered by the NPX calculations. EMPTY shows counts while NOT_USED shows nothing.

Useful for:

- Getting an overview of the plate setup, one block at the time.

Functions:

- Choose block to display in the tree view.

- Information about the plate is shown below the plate layout.

7.3   Run Log

General:

The Run Log tab shows a detailed list of all run units at either plate or block level. Each run has a comment window where users to make notes in.

Useful for:

- Making comments on run unit level for others working on the same project.
- Getting an overview of run unit details.

Functions:

- Write a comment in the comment window for a run. Click **Submit** to save the comment or **Reset** to remove it.
- Add a rerun and edit its information.
- To submit comments and/or changes in more than one run unit, click **Submit all**, found above the list.
- For each plate, the following information is displayed:
    - Sequencing date
    - Sample source (SS) plate name
    - Run name
    - Data analysis Ref ID
    - Index plate
    - Instrument ID
    - Count file
    - Flowcell information: ID, type and side
    - Library
    - Plate control and negative control yes/no
- Editable information:
    - Rerun of
    - Manual assessment
    - Primary cause
    - Step failed
    - Rerun needed no/yes
    - Inclusion status and reason

7.4   Quality Control Summary

General:

The Quality Control Summary tab displays a summary of the QC criteria per block. Refer to *14. Overview* for description of the QC criteria.

Useful for:

- Reviewing failed blocks/samples.
- Finding systematic effects.

Functions:

- For each plate, the following information is displayed:
    - Plate ID
    - Panel
    - Block
    - Failed samples
    - Failed PC
    - Failed NC
    - Failed Sample control
    - Warned assays
    - Block QC
    - Systematic effects
    - Counts
    - % Passed

7.5 Supplementary Tables

General:

The supplementary Tables tab contains five tables displaying samples that have failed or have warnings based on QC criteria. All data for one sample source plate is displayed at a time.

Only tables that contain data will be displayed.
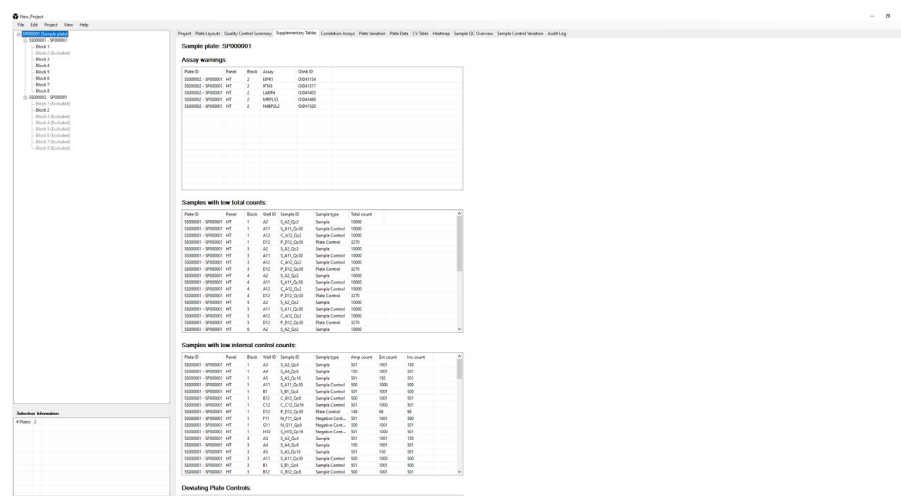
Useful for:

- Reviewing flags of all samples and assays in tables, divided into five different flag reasons:
  - Assay warnings
  - Samples with low total counts
  - Samples with low internal control count
  - Deviating plate controls
  - Failed negative controls

Functions:

- The following information will be displayed in the different tables:

Assay warnings	Samples with low total couns	Samples with low internal control count	Deviating Plate Controls	Failed Negative Controls
• Plate ID	• Plate ID	• Plate ID	• Plate ID	• Plate ID
• Panel	• Panel	• Panel	• Panel	• Panel
• Block	• Block	• Block	• Block	• Block
• Assay	• Well ID	• Well ID	• Well ID	• Well ID
• Olink ID	• Sample ID	• Sample ID	• Sample ID	• Sample ID
	• Sample type	• Sample type	• Amp	• Sample Type
	• Total count	• Amp count	• Ext	
		• Ext count	• Inc	
		• Inc count		

- The Failed Negative Controls table includes empty wells that show too high signal.

- To export the information, mark whole or parts of the table, right-click and choose **Copy.** Open preferred program, and paste the information.
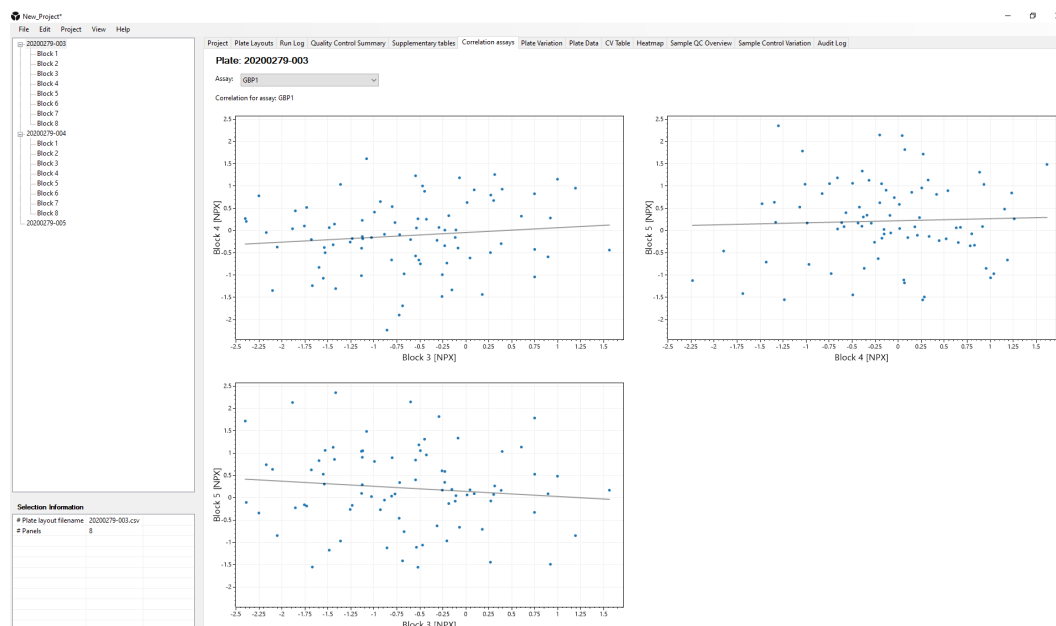
7.6   Correlation Assays

General:

The Correlation Assays tab shows correlation assays that overlaps across the blocks per sample plate.

•   A plate is chosen: all included run units are shown.

•   An excluded run unit is chosen: the text "No data display" is shown.

•   Failed samples: no data points will be shown.

•   Failed blocks: an empty graph with red text "Block QC failed [NPX] "will be shown.

Correlation assays are GBP1 (uniprot P32455) and MAP2K1 (uniprot Q02750).

Useful for:

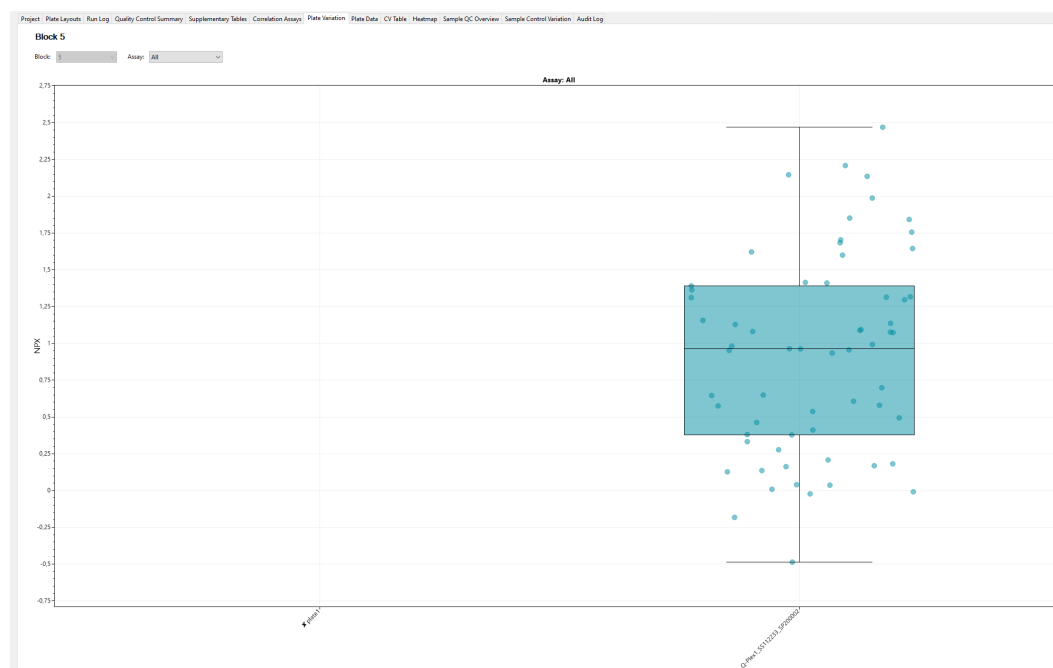•   Getting an overview of correlation assays.

Functions:

•   Use the Assay drop-down list to switch between correlation assays.

•   Zoom in and out with scroll wheel or touch pad.

7.7   Plate Variation

General:

The Plate Variation tab displays a box plot with NPX values from the Sample wells. Failed run units will be left empty, refer to the left run in the picture below.

Useful for:

•   Detecting systematic variance in total intensity between plates.

Functions:

•   Use the Assay drop-down list to show specific assay.

•   Select **All** in the Assay drop-down list to view the summary of all assays for each sample.

•   To highlight a plate, press **ctrl+f** or go to **edit -> find** and type in part of a plate name.

It is not possible to toggle between the normalization types. To change the type, go to **Project -> Normalization** and choose type.

7.8　Plate Data

General:

The Plate Data tab shows heatmaps of one unit a time, including the internal controls: extension control, incubation control and amplification control. Empty wells show counts but not NPX.

The heatmap displays deviations from the plate median for the selected assay using the colors blue and red:

- Blue: values below median

- Red: values above median.

Warnings are marked with flags in yellow and red:

- Yellow flag: indicates warning.

- Red flag: indicates fail.

Useful for:

- Inspecting counts and NPX across a plate for each assay.

- Discovering systematic patterns.

- Discovering plates swap based on data pattern across the plate (If the plate has any empty well).

Functions:

- Change values by selecting either NPX or Counts.

- Use the assay drop-down menu to change which assay to display.

- Use the Systematic effect filter to switch off or show either column or row effect.

- Hover over a well to show:

    - Sample

    - Well ID

    - Value

    - Deviation from median

7.9   CV Table

The CV Table tab shows the CV information, one row per block. CV is calculated based on the data points of Sample Controls that passed the QC criteria.

Useful for:

- Getting an overview of the CV values per block, and which blocks that have missing CV values.

Functions:

- Choose which panel to be shown in the tree menu.

- To export the information, mark whole or parts of the table, right-click and choose **Copy.** Go to preferred program, and paste the information.

- Missing values for Intra CV mean and Inter CV mean are represented by NA.

7.10   Heatmap

General:

The Heatmap tab contains a color-coded view for all assays and samples in the chosen block. The assays are displayed in the horizontal direction and samples in vertical direction. Empty wells show counts but not NPX.

The heatmap displays deviations from the plate median for the selected assay using the colors blue and red:

- Blue: values below median

- Red: values above median.

Useful for:

- Finding systematic patterns.

- Finding outlier samples for specific assays.

Functions:

- Change values by selecting either NPX or Counts.

- Use the Display drop-down list to select the sample type to display (All samples, Sample, Plate Control, Negative Control, Sample Control, or Empty).

- Hover over a well to show:

  - Assay

  - Sample ID

  - Well ID

  - Deviation from assay median

  - NPX

- Use the Sort order drop-down to sort either by row or by column.

- Use the Systematic effect filter to switch off or show either column or row effect.

- Zoom in and out with scroll wheel.

- Move the map to see different assays by clicking and holding the left mouse button and drag the heatmap.
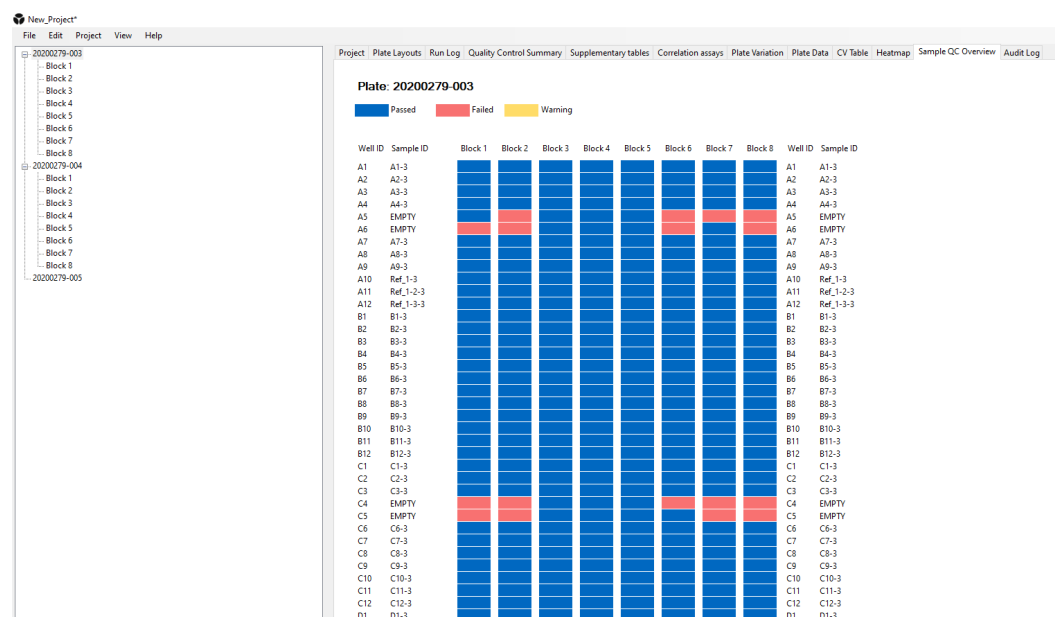
7.11 Sample QC Overview

General:

The Sample QC Overview displays a color-coded heatmap showing status for each sample in all blocks.

• Blue: Pass

• Red: Fail

• Yellow: QC warning

If a sample has both a fail and a warning, it is shown as fail.

Useful for:

• Finding systematic errors across different blocks.
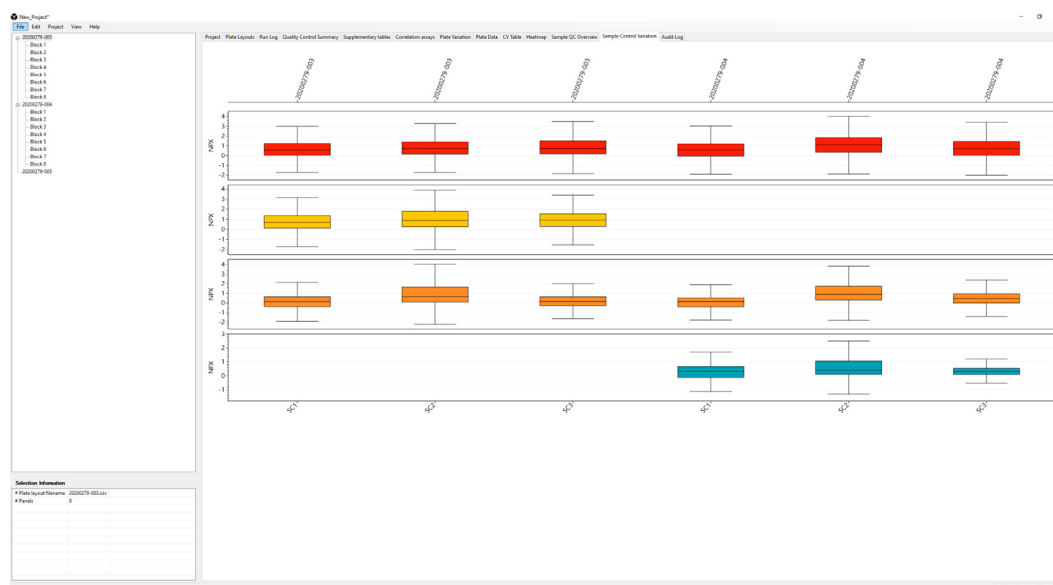
Functions:

• Hover over the cells provides information on:

  – Sample ID

  – Block

  – Well ID

  – For failed and warned samples: reason for failure or warning.

7.12   Sample Control Variation

General:

The sample control variation view displays data for all Sample Controls in the entire project. Data points for both internal control assays and regular protein assays are included.

> **NOTE:** *The range of the Y-axis differs between the blocks.*

Useful for:

- Getting an overview of the variation of the sample controls through blocks and plates.

Functions:

- Every row represents a block and every column a plate.
- Each boxplot represents non-failed Sample Control with NPX values.
- Excluded run units are not shown.
- Missing plate-block gives empty sub-boxplots.

7.13   Audit Log

General:

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

Useful for:

- Seeing what actions that has been done on the project.

Functions:

- The upper table shows changes not yet saved.
- The lower table shows changes that has been saved.

Part 3: Operation

8. Introduction

Input data is counts files and metadata files generated by *ngs2counts*. For more information on pre-processing, refer to *5.2 Pre-processing runs*.

This section describes how you analyze data step-by-step in Olink NPX Explore HT. 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. Perform quality controls including additional quality assessment.
4. Export data.
5. Create an Analysis Report.
6. Finalize the project
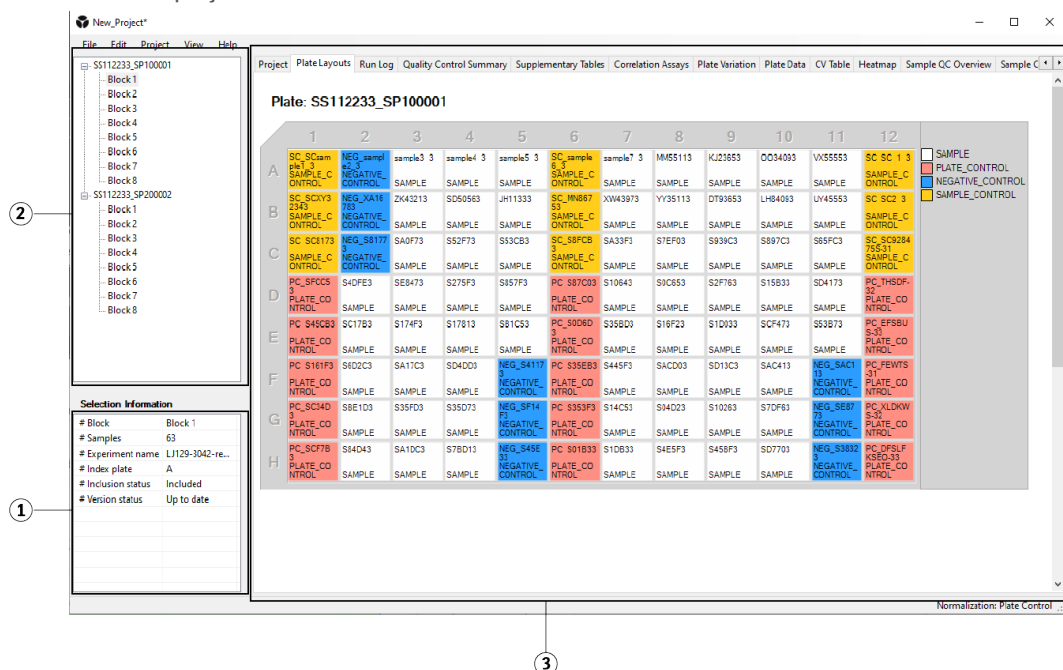
Figure 1.   NPX Explore main window with the Plate Layouts tab selected.

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

9. Start or open a project

9.1 Import run files

To create a project, the following files must be imported:

- Plate layouts
- NGS run folder

1. Select **File -> Import -> Import Plate Layouts - standard** and browse to the preferred plate layout(s).

2. Select **Project -> Select Data Analysis Reference IDs** to verify that the correct panels has been selected.

3. Select **File -> Import -> Import NGS Run Folder** or **File -> Import -> Import NGS Run Zip File,** depending on the format. Browse to the preferred folder or file.

4. Select plate layouts for both Index Plates and click **Ok.**

Figure 2.   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.*

NOTE: *Open or clone project*

To open an existing project, either:

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

or

1. Select **File -> Clone 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.

9.2 Enter project information

For more information, refer to *7.1 Project*. In the Project view of the main window:

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

2. Enter **Sample Matrix**. This will be displayed in the data export file and in the Analysis Report.

3. Enter **Comments for Project**. This will not be displayed in the data export file and in the Analysis Report.

> ✎ **NOTE:** *Any entered Comments will be saved in the Olink NPX Explore HT project file (.oaf)
> and Flagged Positions will automatically be displayed in the Analysis Report.*

10. Perform quality controls

1. Perform review of overall QC for each plate separately using the Quality Control Summary tab.

2. Perform quality assessment for all plates using the other views.

3. Make an overall assessment.

*✎ **NOTE:** If a run fails to meet the acceptance criteria, please contact support@olink.com.*

Criteria	FAIL *NPX is not calculated, exclude from statistical analysis*	WARN *NPX is calculated, assess further, use data with caution*
Sample QC		
Total counts per sample	< 10 000	N/A
Incubation control counts per sample	< 150	< 500
Extension control counts per sample	< 150	< 1000
Amplification control counts per sample	< 150	< 500
External Control QC		
Total counts per sample	< 10 000	N/A
Incubation control counts per sample	< 500	N/A
Extension control counts per sample	< 1000	N/A
Amplification control counts per sample	< 500	N/A
Plate control internal control counts relative to assay counts	< or > internal control reference range to assay counts	N/A
Negative Control internal control counts relative to assay counts	Negative control fails	N/A
Block QC		
Plate Controls passing external sample QC	< 50% (minimum 3 Plate Control must pass QC)	N/A
Negative Controls passing external sample QC	< 1	N/A
Systematic effect (NPX)	N/A	>10% of assays Systematic effect identified
Assay QC		
Assay count relative to internal control count in negative control	N/A	Assay count > any internal control count

If a run fails to meet the acceptance criteria, please contact *support@olink.com*.

10.1 Import rerun data

1. Mark the block to be excluded in the tree view.

2. Right-click and select **Exclude**. The block will be light gray and the text (Excluded) is added to the block name.

3. Select **File -> Import -> Import NGS Run Folder** and browse to the preferred folder.

4. Select plate layouts for the Index Plate.

5. To only import the rerun block unmark the imported, not excluded blocks.

6. Click **Ok**.

10.2 Additional quality assessment

Click the following tabs in the menu to assess the quality of the data in each tab and further determine if any reruns are required. For more information about the tabs, refer to *Part 2: User Interface.*

- **QC Supplementary table**
  Contains tables that display sample IDs and assays that failed or warned based on any of the QC criteria. The information will help identify the possible root cause for sample and block failure.

- **Sample QC overview**
  View and evaluate patterns of failed/warned samples within and across the blocks in each plate. Hovering over each failed/warned sample to view the criteria that have resulted in failure or warning status.

- **Plate data**
  View the values of NPX and Counts for internal controls and assays for each sample per block. Assess if there are patterns in the plate (i.e. row or column) representing a possible technical error. Filter out the list of assays that reflect any systematic effects by clicking on the type of effect in the "systematic effect filter" drop down menu.

- **Plate variation**
  Display differences in sample distributions within and between plates.

  📝 **NOTE:** *For intensity normalized projects, the assessment of this view should be performed using the Intensity option for Normalization type after normalization has been performed.*

- **Correlation assays**
  Assess the correlations of each overlapping assay between blocks. Skewed data correlation in any of the plots can indicate the technical error in the lab workflow.

- **Heat map**
  Search for extreme outlier samples and systematic patterns that reflect a possible technical error.

- **CV table**
  Assess the precision of the data based a group of pre-selected assays across different blocks on the Sample Controls. The reported %CV is the mean %CV over all pre-selected assays. A high %CV does not fail a run automatically but could indicate a technical issue or poor sample randomization and can be a cause for further investigation.

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

- **Control Sample variation**
  The box plot analysis is complementary to CV analysis to identify a sample control or plate that may be an outlier that drives high CV.

11. Export data

11.1   NPX file

To export data as NPX file, perform the following steps:

1. Go to **File -> Export**.

2. Select **NPX File**.

3. Browse for a folder to save the file.

4. Click **Save**.

11.2   Analysis Report

1. To export an analysis report, perform the following steps (Steps 1–4 are optional):

2. Go to **Project** -> **Analysis Report Input**.

3. Fill in information that will be included in the Analysis Report.

4. Click **Submit**.

5. Go to **File** -> **Export**.

6. Select **Analysis Report**.

7. Browse for a folder to save the file.

8. Click **Save**.

12. Finalize a project

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

Select **Project -> Project State -> Finalize.**

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

13. Data export files

13.1 Parquet file format

NPX data is exported from Olink NPX Explore HT in Parquet file format. Parquet is a free open source file format built to handle columnar storage data.

Parquet files provide several advantages compared to CSV, such as

- Much more efficient data compression with enhanced performance to handle complex data in bulk.

- Improved data integrity through binary encoding (files can't be opened and changed using a text editor).

The binary encoding also means the file cannot be opened in Microsoft® Excel. If required, Parquet files can be exported to CSV format.

Parquet is available in multiple languages including Java, C++, Python, C# etc. You can find further details at these locations

- https://parquet.apache.org/docs/

- https://en.wikipedia.org/wiki/Apache_Parquet

13.1.1 Recommended way of interacting with a Parquet file

Olink recommends installing and using DBeaver to view, query and export Parquet data.

Installing DBeaver

9. Download DBeaver for free from the Microsoft® Store by clicking on the **start menu** and type "Microsoft store".
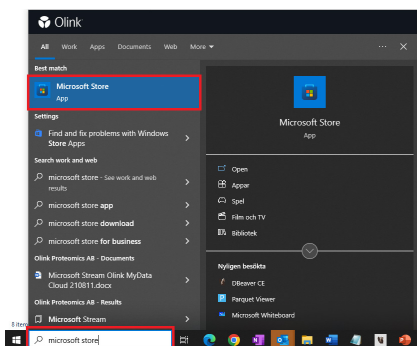
10. Type "DBeaver" in the search field, and press **Enter**.
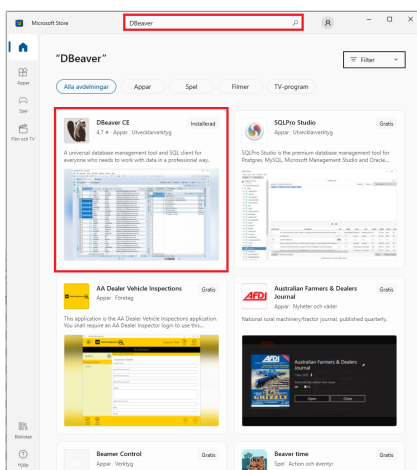
11. Once DBeaver is installed, setup a new database connection.
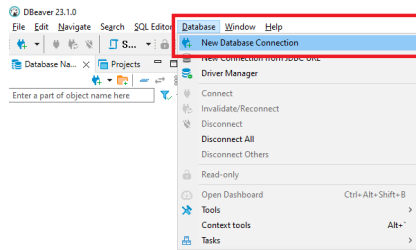
12. Choose **DuckDB** and hit the **Next** button.
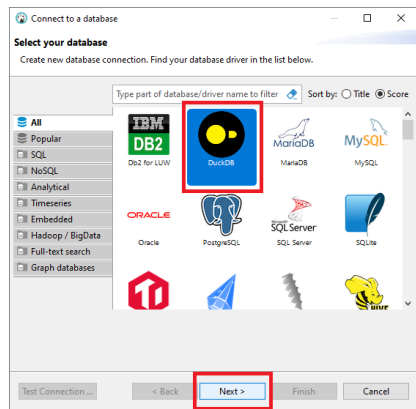
13. Type ":memory:" in the **Path** input field.
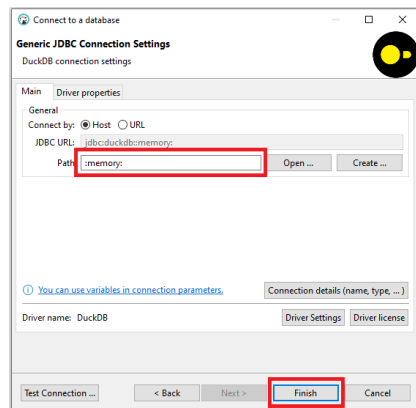
14. Click the gray arrow.
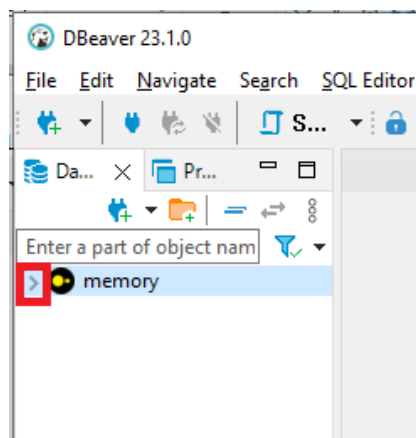
15. Download driver files.
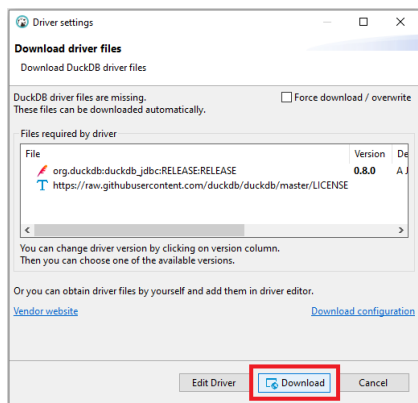
16. Open a new script window.
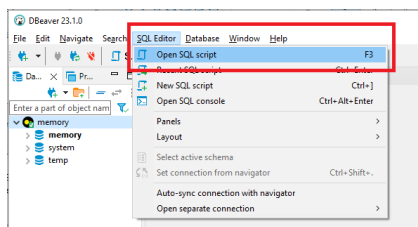
17. Type a PostgreSQL query such as

SELECT * FROM 'C:/npxfile.parquet'.

Make sure that the path in double quotes refers to the NPX file you want to query.

18. Click the "Execute SQL query" button
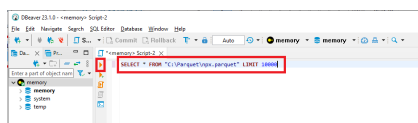
19. View the result.
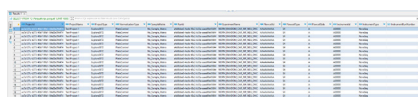
Export data to a CSV file

1. Click **Export data.**
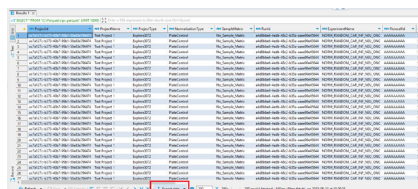
2. Highlight CSV and click **Next**.
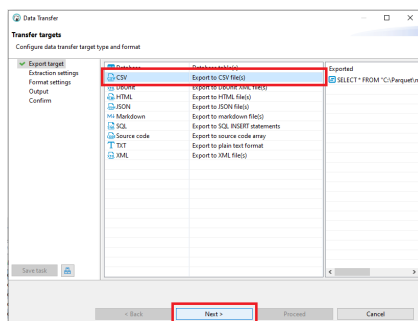
1. Click **Next**.
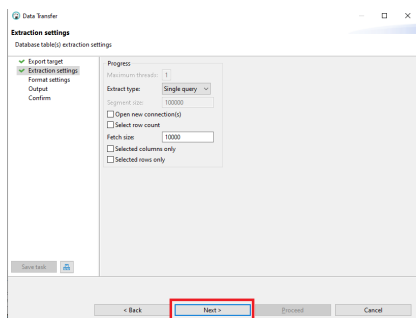
2. Click **Next.**
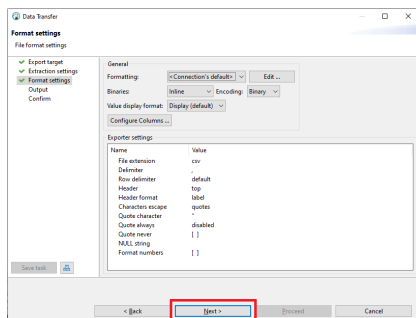
3. Set the "Directory" field to a location on your hard drive.

4. Click **Next.**
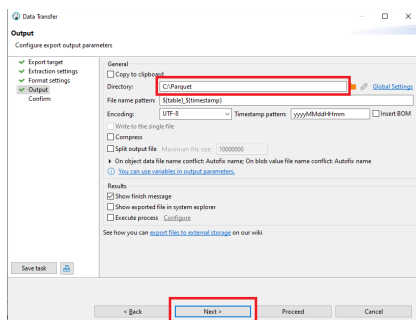
5. Click **Proceed**.
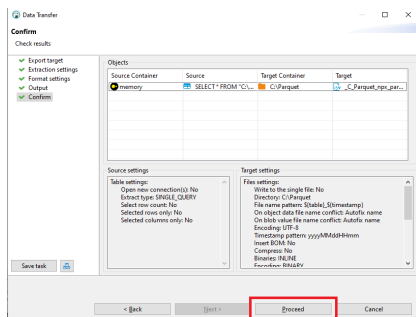
13.1.2 Quick-check parquet-viewer

To quick-check the parquet, perform the following steps:

1. Download ParquetViewer from the GitHub wepage: *https://github.com/mukunku/ParquetViewer/releases.*

2. Install ParquetViewer and open it.

3. Drag and drop the parquet file to the application window.

4. Use the query input field to filter out desired datapoints, or use the Record Offset filed to jump in the file.

5. For more information or help, refer to the GitHub homepage: *https://github.com/mukunku/ParquetViewer/wiki/Basics.*

13.2 NPX file

The NPX file is a Parquet-file that can be generated from NPX Explore HT.

The NPX file contains the following information about the project in table format:

Column	Description	Type	Typical value
SampleID	The annotated sample ID	String	
Sample Type	Type of sample	String	PLATE_CONTROL, NEGATIVE_CONTROL, CONTROL, SAMPLE
WellID	Id for well	String	Capital letter A–H followed by number 1–12
PlateID	Name of the plate the sample was run on	String	
DataAnalysisRefID	Reference ID for data analysis	String	
OlinkID	OlinkID for assay	String	
UniProt	UniProt ID for assay	String	
Assay	Gene name for assay	String	
AssayType	Type of assay	String	Amp_ctrl, inc_ctrl, ext_ctrl
Panel	Panel name	String	Explore_HT
Block	Name of the block the sample was run on	String	1, 2, 3, 4, 5, 6, 7, or 8
Count	The total number of counts	Integer	Greater than or equal to 1
ExtNPX	Intermediate value between count and NPX: log2 of the ratio between data-point Count value and the count for the Extension Control assay for the same sample.	Double	-1.94701
NPX	NPX value	Double	
Normalization	Type of normalization used in project	String	Plate control, Intensity or EXCLUDED
PCNormalizedNPX	NPX value displayed if plate control normalization has been chosen.	Double	1.735509
AssayQC	Overall QC status for an assay	String	NA, PASS, WARN
SampleQC	Overall QC status for a sample in a block	String	NA, PASS, WARN, FAIL
ExploreVersion	Software version of the module in Olink NPX Explore HT used for panel calculations and normalization	String	

13.3   Extended NPX file

The Extended NPX file is a Parquet-file generated by the Explore CLI and Olink NPX Explore HT.

The Extended NPX file contains all the columns in the NPX file, plus the set of additional columns listed below.

*   IntraCV
*   InterCV
*   SampleBlockQCWarn
*   SampleBlockQCFail
*   BlockQCFail
*   AssayQCWarn

For more information, refer to the Olink® Explore CLI Technical Information.

13.4   Analysis Report

The analysis report is a pdf-file that can be generated from NPX Explore HT and CLI.

The analysis report summarizes the quality analysis of the project. It contains basic information and descriptions of the project. It also contains a summary of the Quality control including sample passed QC.

The Analysis Report contains the following:

*   Project information
    *   Sample matrix
    *   Project specific comments
*   Quality control
    *   QC summary
    *   Data output
    *   Software version information
*   Appendix - A table including the following columns
    *   Olink Panel
    *   Block
    *   UniProt
    *   Assay Olink ID

Part 4: Quality Control

14.Overview

Olink Explore contains a built-in quality control system using three internal and three external controls, which enables full control over the technical performance of assays and samples. It is fully automated and is performed directly on Counts and therefore can detect severe technical errors.

If the QC is not accurate, please contact *support@olink.com*.

14.1 Internal controls

Three internal controls are spiked into every sample for each block. The internal controls are designed to monitor the quality of assay performance, as well as the quality of individual samples:
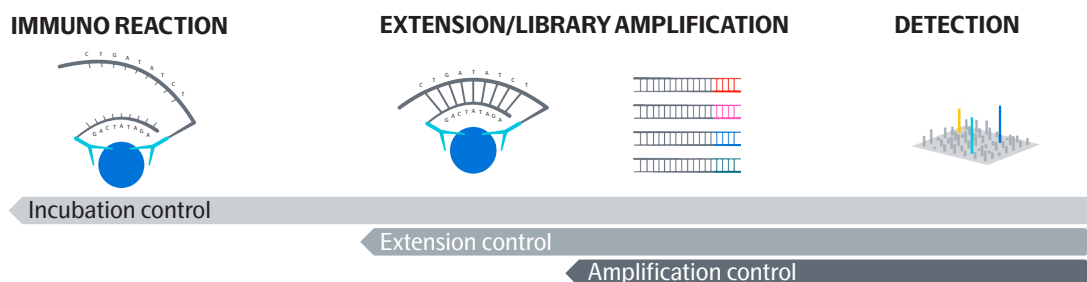
Figure 3.   Internal quality controls

Incubation Control (Immuno Control): The Immuno Control is a non-human antigen measured with PEA. This control is included in the immuno reaction (incubation) and monitors potential technical variation in all three steps of the reaction.

Extension Control: The Extension Control is composed of an antibody coupled to a unique pair of DNA-tags. These DNA-tags are always in proximity, so that this control is expected to give a constant signal independently of the immuno reaction. This control monitors variation in the extension and amplification step.

Amplification Control: The Amplification Control is a complete double stranded DNA amplicon which does not require any proximity binding or extension step to generate a signal. This control monitors the amplification/sample indexing step.

The Extension Control is used to calculate the NPX, refer to *15.4 Normalization*, and the other two are used in Quality Control.

15. The QC workflow

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

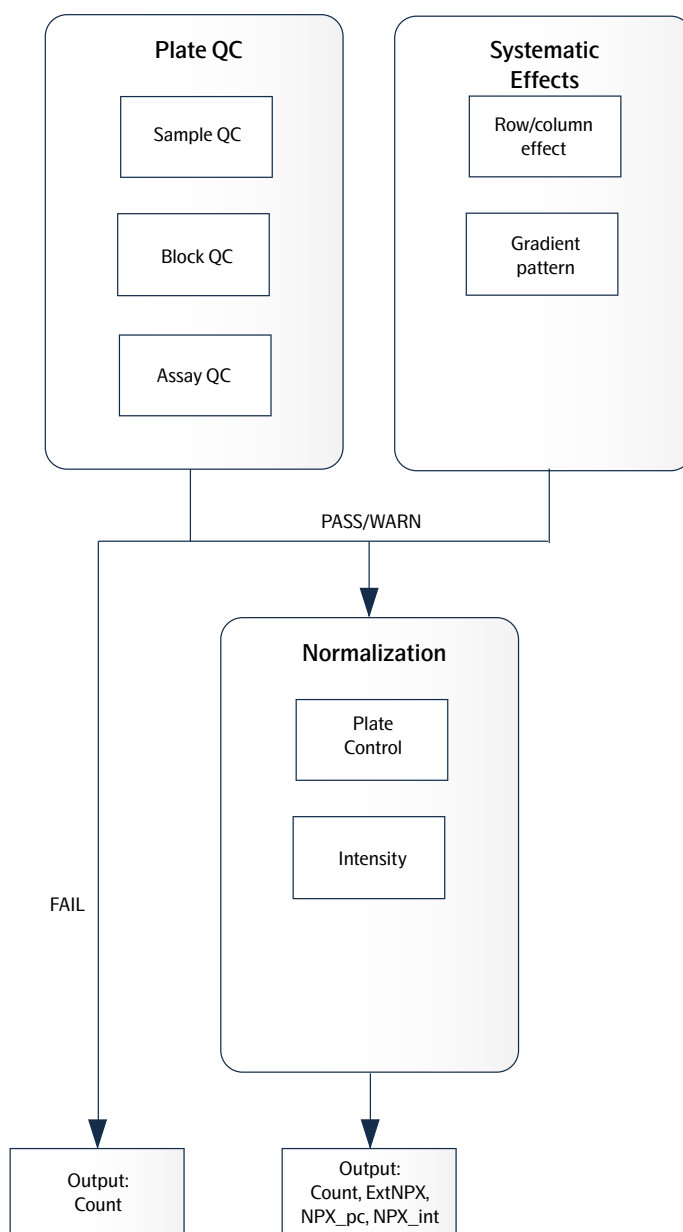*   Incubation Control
*   Extension Control
*   Amplification Control

The Extension Control is used to calculate the NPX, refer to *15.4 Normalization*, and the other two are used in Quality Control (described below).

15.1   Plate QC

The QC is performed directly on Counts, on one block per 96 samples. The Plate QC are divided into three parts:

15.1.1   Sample QC

Samples and external controls that fail Sample QC will not be considered for additional QC steps and not normalized. Only counts will be reported for those.

1. Low total number of counts per sample per block
   Too low total counts for a sample might indicate that the sample is missing or the index was not added to the sample. Each sample and external control should have a minimum number of counts in a block, otherwise they fail.

2. Low number of counts of internal controls per block
   Low counts in any of the internal controls might indicate a technical error in the workflow for the corresponding sample, for example a missing internal control.

   – Each external control should have a minimum number of counts for each of the internal controls, otherwise they fail.

   – Each sample should have a minimum number of counts for each of the internal controls, otherwise they fail or get a warning.

3. Deviation of counts in Plate Controls
   Deviation of internal control's counts from the expected ranges in Plate Controls might indicate different technical errors.

   – A Plate Control fails if the fraction of counts of all internal controls to total counts in logarithmic scale, deviates positively or negatively from the reference values. The reference ranges are block specific and kit lot related.

4. Unexpected signal in Negative Controls
   Detection of high number of counts for many assays, relative to the counts of internal controls, in any Negative Controls might indicate that signals are from other sample types or not from pure buffer.

   – A Negative Control fails if many assays get higher number of counts relative to the counts of internal controls.

15.1.2   Block QC

Block QC is to quality control whether a block-plate is affected by technical errors This step is performed only on the Plate and Negative Controls that pass Sample QC. A failed block is recommended to be rerun.

1. Based on Plate Controls:

   – A block-plate fails if <50% of Plate Controls pass the Sample QC (a minimum of 3 Plate Controls is required to pass the block-plate).

2. Based on Negative Controls:

   – A block-plate fails if none of the Negative Control pass the Sample QC.

15.1.3   Assay QC

Detection of high number of counts for any assay, relative to the internal controls, in any of the Negative Controls is considered as unexpected signal. This step is performed on Negative Controls that pass Sample QC.

• An assay gets a QC warning if it gets high number of counts relative to the counts in internal controls in all Negative Controls.

For acceptance criteria for Sample QC, Block QC and Assay QC, refer to table in *10. Perform quality controls*.

15.2   Systematic effect detection

Systematic effects are defined as a systematic pattern on a plate caused by a deviation in the workflow such as instrument failure or human error. A pattern corresponds to a particular type of failure. Detection of systematic effects is complementary to the Plate QC and is performed on NPX. All samples and data-points (passed, failed, and warned) are included in the computation.

Systematic effects are categorized into patterns that impact the full plate or impacting a row or/and column. To detect the patterns, three main criteria are considered:

- The frequency of assays representing the effect
- The intensity of the effect on NPX deviation
- Number of samples showing the effect

A pattern is detected per assay/block/plate unit. If enough assays (internal controls are not included) are affected, the block will get a systematic effects warning corresponding to that effect in NPX Explore HT Software (Quality Control Summary tab). Systematic effects may be due to either non-randomized plate design or technical errors and should be investigated further when warned.

15.2.1   Types of systematic effects

- Row
- Column
- Four_column
- Alternating_column
- Row_gradient
- Column_Gradient
- Diagonal_Gradient

15.3   Summary notes for exported data

- Data related to all assays, including the internal controls, will be presented in the output NPX file.
- Data related to all samples, including the external controls, will be presented in the output NPX file.
- Data related to any failed sample or block that do not pass QC criteria will not be normalized and therefore no NPX will be computed. Counts are reported for the failed data-points in the NPX file.
- Any data-points with a QC warning should be used with caution.

For any more information about Plate QC and systematic effects, please contact *support@olink.com*.

15.4 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.

Plate Control normalization should be selected for all Explore HT project (projects with single or multiple plates, projects where complete randomization of samples can not be guaranteed, etc). Plate Control normalized NPX will by default be reported in the output NPX file. Contact support for study design assistance and choosing the appropriate normalization type.

15.4.1 Counts

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.

15.4.2 Converting counts to NPX

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 = (ExtNPX_i,j – plate median(ExtNPX_i))`
    a. Between plate normalization.
    b. For each assay, for all plates in project.

15.4.3 CV calculation

To evaluate the reproducibility and repeatability of data related to each plate in the project, and to assess the within- and between-plate variation in the project, two types of CV are computed for each of the pre-selected assays: within plate (intra CV) and between plates (inter CV) in Sample Controls. For the inter CV calculation, the mean of Sample Controls is used to represent the plate before CV calculation.

In each block, pre-selected assays with good performance in Olink Sample Controls are used so that any deviating CV actually indicates a potential technical issue within the workflow. The CV is calculated for NPX of those selected assays (i), using the assumption of a log-normal distribution, in Sample Controls using the formula below. The CV is part of the UI assessing run quality, and is presented in a CV Table showing average CV:s over the blocks
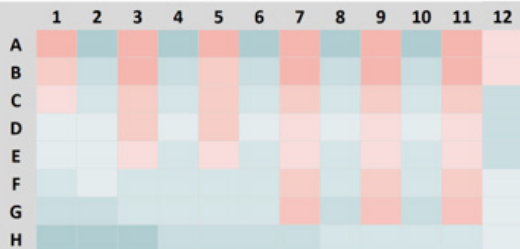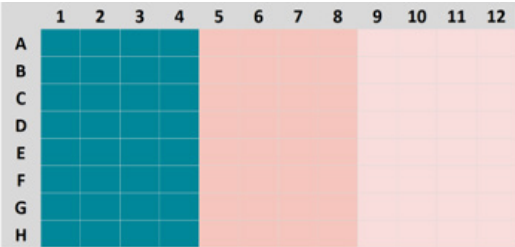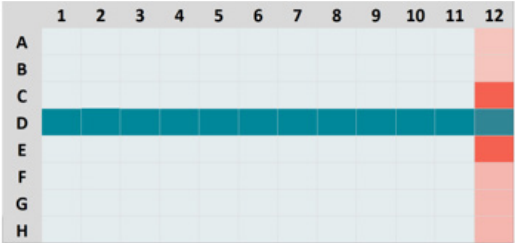
$$CV_i = 100 \sqrt{(e^{Sln_i^2} - 1)}, \text{ where } Sln_i = \ln(2) \times SDisk$$

Part 5: Troubleshooting

16.   Troubleshooting

This chapter includes a trouble shooting guide, describing QC issues, affected level (sample, block, plate) and possible root causes for some potential non-expected results. Reruns are not recommended except for the projects that have failed to pass formal QC. Please contact _support@olink.com_ for further investigations and receiving appropriate guidance for any of these cases.
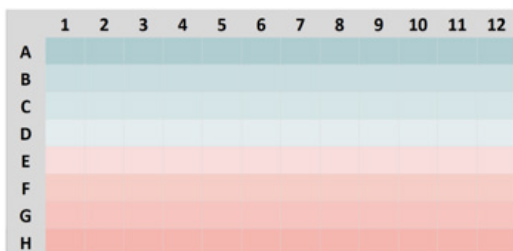
QC issue	Affected samples/blocks/Sample plates	Possible cause
Plate Controls and/or samples fail		
Failed Plate Controls: Deviation from reference values (±)	Multiple blocks (1-4 or 5-8) Both sample plates plates	− Dilution plates or Sample Plate swapped positions on Mosquito − Wrong dilution protocol was used
Plate Controls fail: Deviation from reference values (Inc & Ext -, AMP +) (Inc, Ext & AMP -)	Multi blocks in both Sample plates	Poor PCR enzyme efficiency due to not adding enough Enzyme A or Enzyme B
Plate Controls fail: Deviation from reference values (Inc -, Amp & Ext +) − Low counts for Incubation Controls − Low counts for assays − High counts for Amplification and External Controls	The same single block in both Sample Plates (e.g. Block 2 in SP1 and SP2)	1A: Forward probe was not added to the incubation reagent mix 1B: Not enough incubation solution was added while preparing the reagent mix
	The same two blocks in both Sample Plates (e.g. Blocks 2 and 5 in SP1 and SP2)	2A: Forward probes were mixed up for the two blocks when preparing incubation mix (wrong pair of probes) 2B: Not enough incubation solution was added while preparing the reagent mix
Samples and/or external Controls fail: Too low counts	1. One or multi samples in a block One or both Sample Plates.	1. Incubation reagents mix or index not added properly due to Mosquito pipetting error
	2. All or multi samples in multi blocks (Likely blocks 1-4) Both Sample Plates.	2. Poor sequencing if %PF is low
Samples and Plate Controls fail: Too low counts − Low counts for assays − Low counts for all internal controls	1. One same block in both Sample plates (e.g. Block 2 in SP1 and SP2)	1. Reverse probe was not added into incubation reagent mix
	2. The same two blocks in both Sample plates (e.g. Blocks 2 and 5 in SP1 and SP2)	2. Reverse probes were mixed up for the two blocks when preparing incubation mix (wrong pair of probes). Amplification Control might have some counts.

Negative Control fail		
Negative Controls fail (Plate Controls might fail too)	1. Multi blocks in one Sample Plate (likely blocks 5-8)	1A. The plate layout does not match with the uploaded run. 1B. NCs related to one of the Sample Plates were pipette into wrong wells on Sample Source Plates. 1C. Negative Controls are contaminated
	2. The same multi blocks in both Sample Plates (blocks 1-4 or 5-8)	2A. Sample Source Plates was rotated in the workflow 2B. Samples added instead of Negative Controls in Sample Source Plates 2C. Index plate A and B were swapped positions on mosquito between the two stages of adding indexes 2D. Negative Controls are contaminated

Systematic effect		
Alternating column effect: NPX value deviates in every other column	One or multiple blocks in one or both Sample Plates	Mosquito pipetting error while dispensing reagent mix into incubation plate
Every 4 columns effect: NPX values deviate between every 4 columns	Two blocks in both Sample Plates	− Dragonfly faulty syringe during dispensation of PCR mix − Not well vortex of PCR mix
Row effect: NPX values deviate between samples in one row and samples in all other rows	Same row in the same block in both Sample Plate	− EpMotion pipetting error when pooling the samples − Human error missing one well during pooling step

Gradient patterns (row/column/diagonal wised): NPX Values deviate gradually in samples across the plate in row, column, or diagonal pattern	One or multi blocks in both Sample Plates	– Not well vortex of reagent mix/plates – Not well adjusted/calibrated instruments

17. Revision history

Version	Software version	Date	Description
2.2	v2.2	2023-12-01	*7.3* added. *13.1.2* added. Version number updated to match the software version(v.1.1−v2.1 does not exist)
1.1	v2.1	2023-10-09	*7.2, 7.7,* and *7.10* supplementary information added. *9.1* supplementary information added. *11.2* supplementary information added. *13.4* supplementary information added. *15.2.1* new.
1.0	v2.0	2023-08-14	New

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