Olink®

Olink® NPX Signature

User Manual

v1.0

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1. Introduction

1.1 About this Manual

Olink® NPX Signature User Manual provides the instructions needed for data processing when running Olink® Target 48, Olink® Target 96 panels. Specific instructions for Olink Target 48 are highlighted in the manual: Olink Target 48. This manual contains step-by-step instructions for analysis in Olink NPX Signature and software troubleshooting.

1.2 Intended Use and Intended Target Group

Olink NPX Signature is a data analysis software that is designed for the OlinkTarget analysis platform, including Olink Target 48, Olink Target 96, as well as for Olink Focus panels. It allows for importing data, validating data quality, and normalizing Olink data for subsequent statistical analysis.

Olink NPX Signature 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, including questions regarding Focus panels, contact Olink Support at *support@olink.com*. Olink NPX Signature is intended to be used by trained users of Olink Target analysis platform

1.3 List of Abbreviations

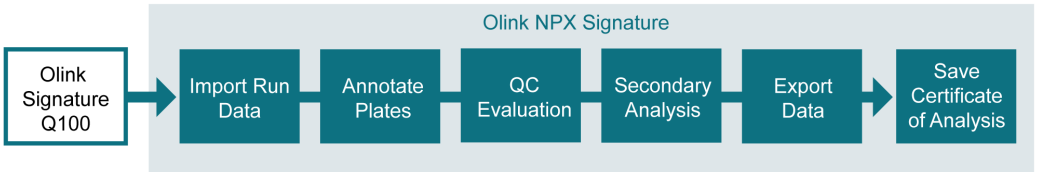
%CV	Coefficient of Variance
CoA	Certificate of Analysis
IFC	Integrated Fluidic Circuit (also called "chip" in this document)
IPC	Inter-Plate Control
LOD	Limit of Detection
NPX	Normalized Protein eXpression
PCR	Polymerase Chain Reaction
QC	Quality Control

1.4 Associated Documentation

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

1.5 About the Software

Olink NPX Signature is a standalone Windows software application that lets you import data from Olink Signature Q100, validate its quality, normalize it, and perform statistical analysis. The data is not required to be annotated or processed before importing it into Olink NPX Signature. The software can also process Biomark data.



2. Computer 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 MB	500 MB

3. Install the NPX Signature Software on Windows

The installer performs a first-time installation or an update to the current installation if it is already installed on your local computer. Any updates to the software retain all existing user settings and custom data.

1. Download the Olink NPX Signature Software to your computer from https://www.olink.com/.
2. Click the installer Olink NPX Signature Setup.exe. Olink NPX Signature is displayed in the Windows Start menu for future use, and the following icon is displayed on your desktop:

   To open the application, double-click the icon.

IMPORTANT! Before using Olink NPX Signature for the first time or upon installation of an updated version, you are required to accept the end-user license agreement.

3.1 Uninstall the NPX Signature Software

If you need to uninstall Olink NPX Signature, it can be uninstalled using the Windows Add or Remove Programs application.

1. In the Control Panel, click **Programs** and **Programs and Features**.
2. In the **Uninstall or change a program** window, navigate to Olink NPX Signature, select it, and select **Uninstall**. The application is uninstalled. All user data is retained where it was saved before the application was uninstalled, and your custom Olink NPX Signature settings are retained on the system.

4. Olink® NPX Signature Study File (.NPX)

IMPORTANT! To enable NPX Signature's remote data functions to Signature Q100, ensure that the instrument is connected to the network and TCP port 8085 (enabled by default) is not blocked.

Olink NPX Signature stores all study data in a file with the extension .NPX. This file is the single repository for all study-related data required by Olink NPX Signature to display, review, and export data for a study.

Place this NPX file on your local computer or in a folder on the network. NPX files are typical ~5–10 MB in size but can be larger if you save the run data in the .npx file.

5. Files and Information Needed for Analysis

Q100 Run Data. This is the Signature Q100 run data that can be imported into Olink NPX Signature without the need for annotation or processing in advance. The file types and information accessible for analysis are:

- Olink Signature Q100 export file (.q100 or .zip) or
- Olink Signature Q100 instrument with enabled remote connectivity or
- Olink NPX Manager project file (.oaf) or
- Fluidigm Real-time PCR exported heatmap or table file (.csv)

See the tutorials and inline help in Olink NPX Signature for more information.

Plate manifest file. This is the MS Excel file (.xlsx) needed to define the sample plate layout.

Datafile version. This is the datafile version provided on the insert that is delivered with the Reagent kit.

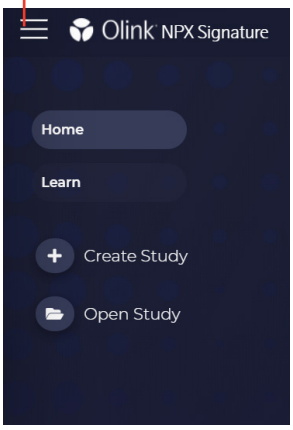
NOTE: *The size of the .zip file that is transferred via USB from Signature Q100 might be larger than 20 MB. An NPX Signature study that includes run data in the .npx archive might be too large to email to Tech Support. If possible, email only the analysis data with the .npx extension.*

6. Use the Olink® NPX Signature Software

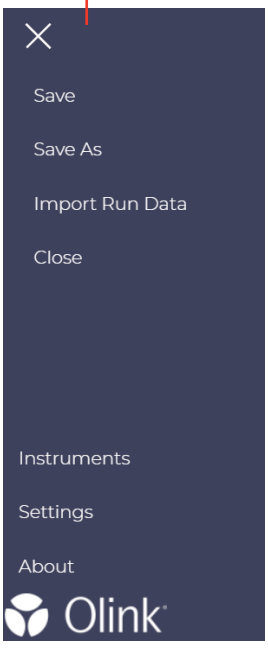
You are required to accept the end-user license agreement the first time you install Olink NPX Signature or before using an updated version for the first time.

Click **Olink NPX Signature** in the Windows Start menu to see the top-level view:

Expand the main menu.



The expanded main menu.



Home. Access the main level of the application, where you see your most recently opened studies.

Learn. Access all the integrated tutorials. See section *8. Tutorials and In-Line Help* for more information.

Create Study. Create a new study.

Open Study. Open an existing study.

Instruments. Show a list of available Olink Signature Q100 instruments to the Olink NPX Signature software over the network to import run data.

Settings. Access the application settings. See section *7. Settings* for more information.

About. View the installed version of the application, copyright dates, and Open Source license information.

7. Settings

Click **Settings** on the main menu to customize the view.

General

- Change the color theme of the application and charts.
- Enable or disable the advanced user features.
- Customize the certificate of analysis by adding a company name and logo.

Layout Templates. - Review or create new sample plate layouts to define the sample types for each well.

Panels.

- Review all data files in the application.
- Import or remove custom data files.

8. Tutorials and In-Line Help

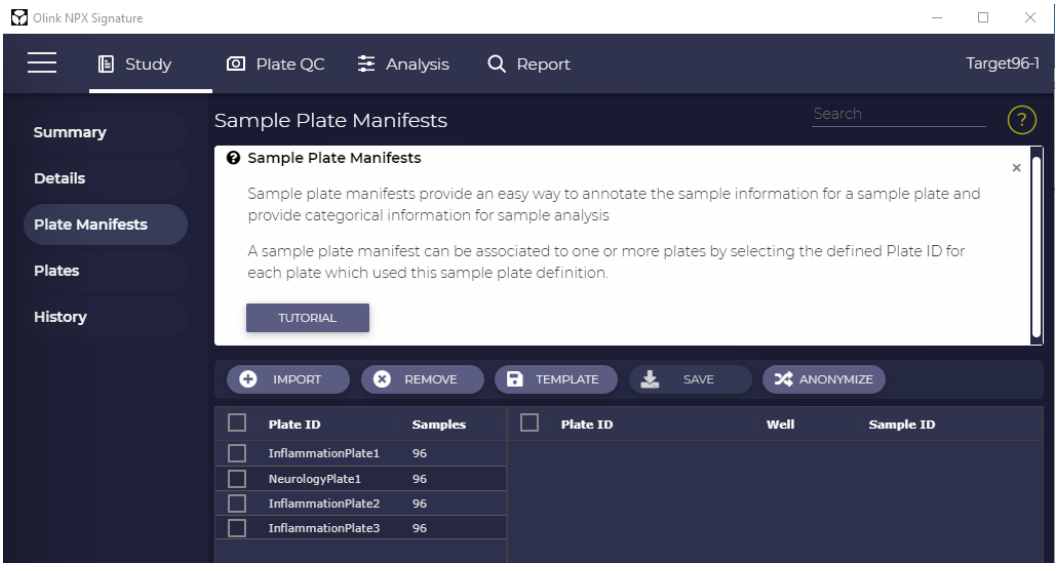
Olink NPX Signature contains browsable, integrated tutorials and in-line help.

Tutorials. Click **Learn** on the main menu to access integrated tutorials meant to assist you in learning the various features of Olink NPX Signature. Click a tutorial and navigate through the pages using **Next** or **Previous**.

You can also launch a tutorial by clicking TUTORIAL if you see it at the end of the in-line help content.

In-Line Help. Click the ? icon in the upper-right corner of a view to access the in-line help, which contains information about the usage of the current view. If the contextual help is longer than the allowed space, a scroll bar appears that lets you read the full text.

The following example shows a tutorial link to the current subject:



9. Plugins

Custom functionality may be added through a plugin library. Plugin libraries are delivered as .zip files and must be uncompressed into the user plugins folder before launching the software.

9.1 Install a plugin

1. Close all instances of Olink NPX Signature.
2. In Windows File Explorer, navigate to the following location:

   ```
   %appdata%\olink-npx-signature\UserData\Plugins
   ```

3. In the Plugins folder, unzip the entire plugin .zip to a subfolder in the Plugins folder so that all the contents of the zip are contained in their folder. For example, extract the MyPlugin.zip into the following folder:

   ```
   %appdata%\olink-npx-signature\UserData\Plugins\MyPlugin)
   ```

4. Open Olink NPX Signature.
5. Locate the plugin for a particular study in the Analysis or Report views.

9.2 Remove a plugin

1. Close all instances of Olink NPX Signature.
2. In Windows File Explorer, navigate to the following location:

   ```
   %appdata%\olink-npx-signature\UserData\Plugins
   ```

3. Delete the folder for the plugin you wish to remove.

10. Quality control

Olink has built-in quality controls in all multiplex panels. Each 92-plex panel contains 96 assays. Four of these are internal controls that allow for an in-process quality control designed to monitor different steps of the protocol: immuno reaction, extension and amplification/detection. The controls included are illustrated in *Figure 1*.

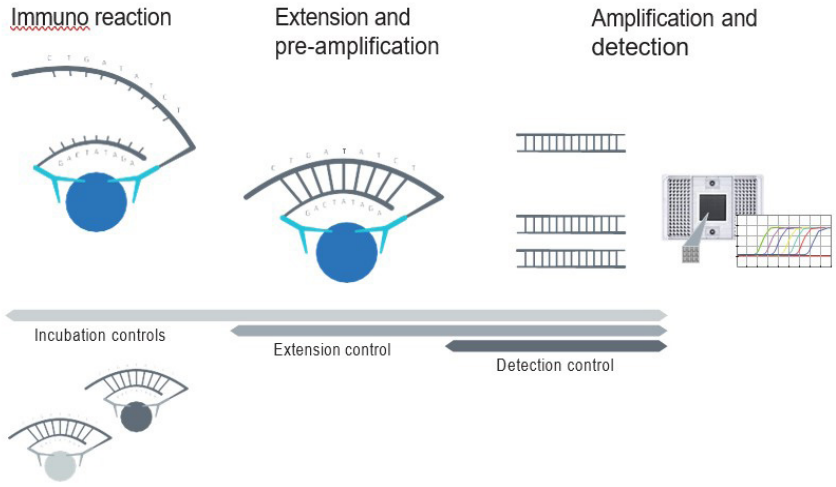


Figure 1. Outline of Olink internal controls for Olink® Target 96 panels.

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

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

Olink® Target 48

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

10.1 Sample QC

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

Acceptance criteria for passing a sample:

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

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

Olink® Target 48

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

10.2 Run QC

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

Acceptance criteria for passing a run:

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

Olink® Target 48

- Standard deviation of Incubation Control and Detection Control in samples: < 0.2 NPX
- Standard deviation of Incubation Control and Detection Control in controls: < 0.5 NPX
- Number of flagged samples: ≤ 1/6 of the total number of samples (i.e. ≤ 8 in a full plate)
- Number of failed assays: ≤3 out of 45 (see section *10.3 Assay QC*)
- ≤1/3 of the Sample Control or Calibrator replicates are outside of LOQ for < 5 assays
- Number of flagged Sample Controls: ≤1
- Number of flagged Calibrators: ≤1

If a too large variation is observed for either of the controls, go to the Plate QC -> Assay QC tab to evaluate the data. For example, if individual samples show extreme values or if a certain sample column is affected, these samples can be marked as failed, and the QC redone and re-evaluated. For more information on troubleshooting of this step, see section *13.2 Deviating controls for Olink Target in Olink® NPX Signature*.

In addition to passing or failing individual plates, ensure that no systematic bias is present in the data. The

Plate metrics view in the bottom left corner alerts you to such issues.

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

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

Olink® Target 48

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

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

10.3 Assay QC

Olink® Target 48

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

Acceptance criteria for passing an assay:

- The accuracy of the calculated concentration mean for the Sample Control and Calibrator for each assay must fall within +/-30% of the expected concentration. If this criterion is not met, the assay isnoted as “No Data” in the results file.
- The precision of the calculated concentration for the Sample Control must be <30% for intra-CV. If the criterion is not met, the assay is noted as “No Data” in the results file.
- A maximum of one of the Calibrator replicates and/or the Sample Control replicates can fall **outside** of the limits of quantification (LOQ). If the criterion is not met, the assay is noted as “No Data” in the results file.
- The precision of the calculated concentration for the Calibrator is evaluated and should be <30% forIntra-CV. If the criterion is not met, the assay is noted as “Warning” in the results file.

11. Normalization methods

Olink® NPX Signature supports three methods for between-plate normalization. Between-plate normalization adjusts median NPX levels based either on customer samples, IPC controls or Calibrators. Calibrator normalization is used for Olink® Target 48, see section *11.4 Calibrator normalization*. An important concept when deciding on the normalization procedure for Olink® Target 96 panels is randomization, which in this context applies to the sample placement across the plates. For randomized studies with more than one plate, sample intensity normalization is the default normalization. For other studies, IPC normalization is the default. (See Randomization FAQ on the Olink website.)

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

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

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

Adjustment against a correction factor:
$Correction\ factor - ddCt_{Analyte} = NPX_{Analyte}$

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

11.1 IPC normalization

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

11.2 Intensity normalization v.2

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

NOTE: *Only samples are included in this normalization. Negative Controls, IPCs and control samples are excluded. Compare to section "11.3 Intensity normalization".*

11.3 Intensity normalization

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

11.4 Calibrator normalization

Olink® Target 48

Calibrator normalization is used for Olink® Target 48 panels. A Calibrator is used to determine the protein concentration in the panel. The calibrator consists of pooled plasma from healthy individuals, with a spike-in of recombinant antigen for assays below LOQ. Randomization of samples is recommended but not mandatory.

For this panel, the Extension Control, the Calibrator and the bridging factor are used to calculate the normalization. NPX is derived from the Ct values obtained from the qPCR using the following equations for Olink® Target 48 panels:

Each sample is normalized with the Extension Control:

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

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

$Bridging\ factor_{\ Assay\ j} - (dCt_{Sample\ i,\ Assay\ j} - Median(Calibrator\ A_{Sample\ i,\ Assay\ j})) = NPX_{Sample\ i,Assay\ j}$

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

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

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

11.4.1 Results in standard unit (pg/mL)

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

Method overview

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

Before the run (during product development at Olink)

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

After each run

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

Standard curve

During development, a thorough fine-tuned 32-point standard curve is developed for each protein biomarker simultaneously, including all 45 proteins. Multichannel pipetting and numerous replicates of the curves are used to minimize errors and establish an accurate immunoassay curve fitting. Due to the wide dynamic range of the PEA assays in the panel, a large spread of measurement points is required to cover the

entire range. Eight measurement points, which are commonly used in many technologies, would not suffice for Olink's multiplex measurement of 45 assays. The predefined standard curves avoid operator- dependent reconstitution and pipetting of standard curves at each lab and for each run. The figure below shows an example of a point standard curve defined for one assay during development.

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

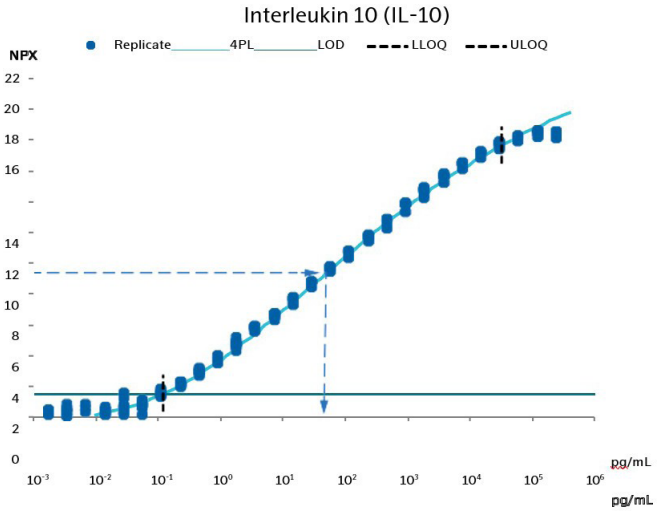


Figure 2. A 32-point standard curve defined for each assay during development.

4PL model

A 4PL model curve fit is used to describe the immunoassay standard curve, indicated by the turquoise line in *Figure 2.* When running a study, the measured patient sample value (represented by the dotted blue arrows in *Figure 2*) is related back to the adjusted standard curve model which translates the measured value to the protein concentration in pg/mL using the equation below. Where A= asymptote (a constant value) at low doses, B= slope factor, C= EC50 (mid-range concentration) and D= asymptote at high doses. These parameters are indicated in *Figure 3* . An example of a point standard curve defined for each assay during development..

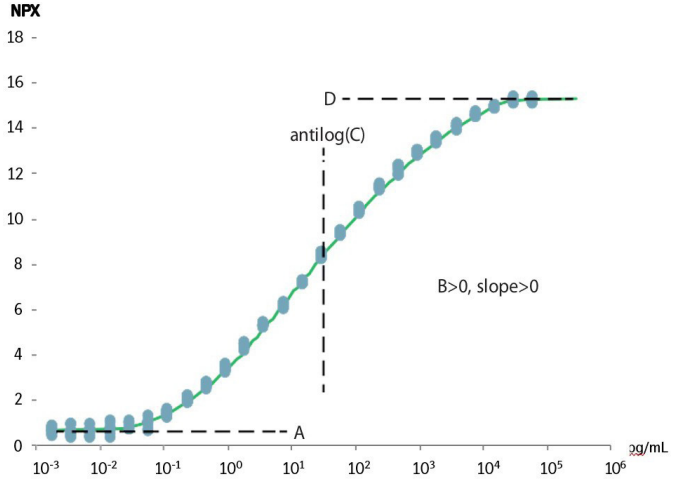


Figure 3. An example of a point standard curve defined for each assay during development.

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

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

Results reported in pg/mL

Figure 4 and the list below provide an overview of how data in pg/mL is reported in the results file.

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

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

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

For a more detailed list describing the results file, see section *12.6 Export data*.

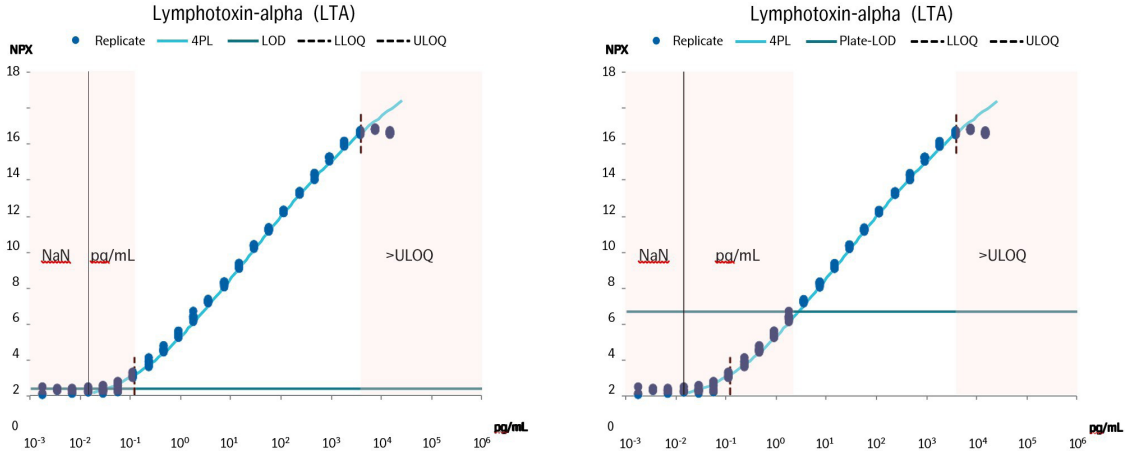


Figure 4. Overview of how data in pg/mL is reported in the results file.

12. Olink® NPX Signature - Operation

Input data for Olink® NPX Signature are run files from Q100 Signature, Ct values exported from the Fluidigm Real-Time PCR Analysis software, NPX Manager .oaf project files and NPX Signature .npx study files. After quality control and normalization against the Extension Control, the IPC, and a correction factor, the output data are obtained in Normalized Protein eXpression (NPX) values. NPX is an arbitrary unit on a log2 scale, where a high NPX value corresponds to high protein concentration.

Olink® Target 48

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

This section describes how you analyze data step-by-step in Olink® NPX Signature. Olink® Target 48-specific information is highlighted. The following steps are included in the standard operating procedure:

1. Select Create Study
2. Enter study information
3. Click Create
4. Import run data.
5. Verify panel and plate layout information
6. Under Details, enter optional study information
7. Select normalization method.
8. Perform quality controls including additional quality assessment.
9. Export data and certificate of Analysis under Report tab.

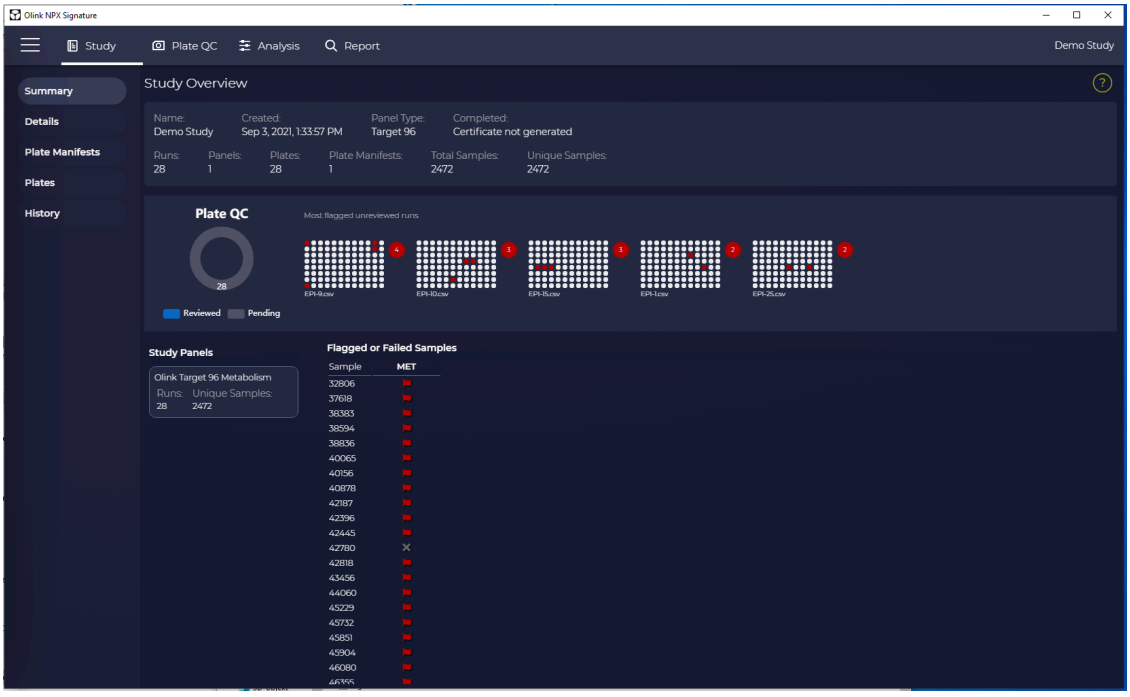


Figure 5. Olink® NPX Signature Study Overview.

12.1 Import run data

Add run data to existing study

Expand the main menu by clicking the three horizontal lines in the top left corner. Select Import Run Data.

- To import run data directly from a Signature Q100 instrument, select Olink Q100 Connect. Select the desired run(s) and press Next. Select Next again and verify panel and plate layout information. Click Add. The Study Overview will appear.
- To import run data files from a local server, select Import Run Data, and click Run Data Files. Select files the desired run data file(s). Valid options are Olink Signature Q100 export files, .q100 or .zip, Olink NPX Manager study files, .oaf, Fluidigm Real-Time PCR exported heatmap or table files, .csv.

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

During import, Olink® NPX Signature alerts if duplicate sample names are found since unique sample and assay names are necessary for proper handling of the data. Change sample names if needed. Sample names can be changed directly in Olink® NPX Signature by importing a manifest in the Plate Manifests view. For more information see built-in help in the Plate Manifests view.

12.2 Enter study information

Study information can be entered in the Study -> Details view

- Enter information in some or all the following fields to display it in the CoA:
  - Sample Type
  - Customer Information
  - Business Development Manager
  - Lab Info
  - Report Notes
  - QC Notes for CoA
  - Internal Notes

NOTE: *Any entered Notes will be saved in the Olink® NPX Signature study (.npx) file.*

12.3 Verify plate layout and sample annotation

1. Select Plate QC-> Plate Layout to display the imported runs for each Olink panel and plate.
2. Under Plate Details, verify that the correct data file version is selected in the drop-down menu.The same data file version will be used for all runs of the same Olink panel in the study.
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 (S)
- Control (C)
- Negative Control (Neg)
- Inter-plate Control (IPC)
- N/A

Olink® Target 48

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

- Control (C)
- Calibrator (Cal)

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

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

12.4 Perform quality controls

1. Perform the QC for each plate separately using the Sample QC tab.
2. Perform quality assessment for all plates using the other views as described in section 7.5 and then make an overall assessment.

The acceptance criteria for passing QC are described in *Table 1. Quality Control guidelines for Olink® Target 96.* and *Table 2. Quality Control guidelines for Olink® Target 48.*.

Table 1. Quality Control guidelines for Olink® Target 96.

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

Table 2. Quality Control guidelines for Olink® Target 48.

Olink® Target 48

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

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

12.4.1 Perform a quality assessment

Parameters that need to be evaluated for each run are listed in *Table 3. Quality assessment for Olink® Target 96.*.

Table 3. Quality assessment for Olink® Target 96.

Olink® Target 96:

Information	Recommended value	Comment
Assay LOD	|LOD| < 2.5 NPX	If |LOD| > 2.5 please contact Olink Support for guidance. Plate ANOVA
	≤ 10 (number of assays)	No warning.
	> 10 (number of assays)	May be caused by insufficient randomization or other issues.
		NOTE: *These guidelines are based on a two-plate study. The larger a study is, the smaller the detectable differences become.*

Row/Column	ANOVA < 30 (number of assays)	No warning.
	30 – 60 (number of assays)	Possible randomization issue. See section 8.5 Inconsistent results detected in Olink Target using Olink® NPX Signature.
	60 – 90 (number of assays)	Re-run plate or otherwise ensure that this is not the result of a poorly executed run.
		For high values, investigate the Assay QC view.
%CV Intra	< 15%	See section *13.4 on page 38* and section *13.2 on page 30.*
%CV Inter	< 25%	See section *13.4 on page 38* and section *13.2 on page 30.*
Detectability	> 75% of the samples	See document on *https://www.olink.com/members-site/*.

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

Table 4. Quality assessment for Olink® Target 48.

Olink® Target 48

Information	Recommended value	Comment
Plate ANOVA	≤ 10 (number of assays)	No warning.
	> 10 (number of assays)	May be caused by insufficient randomization or other issues. If samples were not randomized, this warning can bedisregarded.
		NOTE: *These guidelines are based on a two-plate study. The larger a study is, the smaller the detectable differences become.*
Row/Column ANOVA	< 15 (number of assays)	No warning.
	15 – 30 (number of assays)	Possible randomization issue. See section 8.5 Inconsistent results detected in Olink Target using Olink® NPX Signature
	30 – 45 (number of assays)	Re-run plate or verify that this is not the result of a poorlyexecuted run.
		For high values, investigate the **Assay QC** view.
%CV Intra	< 15%	See section *13.2 on page 30.*
%CV Inter	< 25%, maximum 40%	See section *13.2 on page 30.*
Detectability	> 75% of the samples	The limit for detectability is determined from observedvalues in plasma.

12.5 Additional quality assessment

12.5.1 Additional quality assessment Olink Target panels

NOTE: *Switch between Ct and NPX in each view e.g. to evaluate the effect of normalization or to search for position effects in internal controls and assays.*

Olink® Target 48

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

12.5.2 Instructions

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

Olink® Target 48

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

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

Olink® Target 48

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

On the Z-scores tab, display differences between sample plates or distributions within a plate.

12.6 Export data

In the Report -> Data tab, Data can be exported to a Microsoft Excel XLSX file or CSV file for use in other applications and for statistical analysis.

Olink® Target 48

Data can be exported both as NPX and pg/mL in separate reports, by selecting the desired Data type. Default export is pg/mL.

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

Olink® Target 48

Select Data type: Quantified Values to export data in pg/mL.

2. Under Study Options on the **Study** tab, select the output option for NPX values below LOD. The default is “Actual Value”, which displays the actual measured value for each data point.

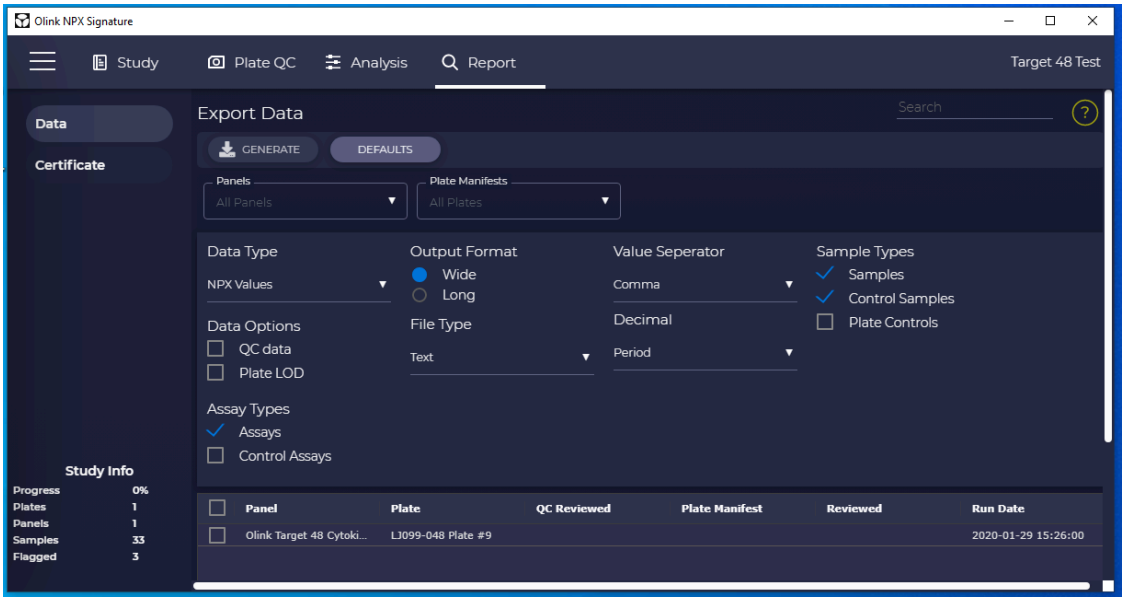


Figure 6. Olink® NPX Signature Export Data view.

3. *Figure 6* shows the **Export Data view** with the recommended default settings. These settings may be adjusted to change the format, layout, or to add additional data to the export. See *Table 4* for more information.
4. In the Report -> Data tab, mark the desired runs to be included in the report and click Generate.
5. Enter a filename and location in the **Save** dialog, select an export format (XLXS files (Excel) or CSV files) and click the **Save** button.

Table 5. Data export options.

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

12.6.1 Considerations

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

Olink® Target 48

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

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

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

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

Table 6. Overview presentation of data in standard units (pg /mL) in results report.

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

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

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

The layout is described in *Table 7.* Parameters in brackets represent quantified data.

Table 7. Layout of exported NPX data.

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

Table 8. Specification for column content in the NPX file.

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

12.7 Create a Certificate of Analysis

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

Go to Report -> Certificate and select all panels to be included in the CoA. Click Create to save the CoA as a PDF document. A preview of the document will appear.

13. Troubleshooting

This chapter describes issues that may arise during use of Olink® NPX Signature, or data issues during analysis, and how to solve these issues.

13.1 Warning messages in Olink® NPX Signature

The following warnings can be displayed:

1. Opening Study
   a. Could not identify the panel
   – The panel datafile associated to plates in the study cannot be located and thus not identified. Navigate to Settings -> Panels and import the required panel data file.
2. Importing Run Data
   a. Quality Threshold does not meet specified criteria 0.5.
   – The Quality Threshold defined for analysis was not set to 0.5. Open *Run data* in Fluidigm Real-Time PCR and export the data after analyzing with a quality threshold of 0.5.
   b. Baseline Correction method is not set to Linear.
   – The Baseline Correction method defined for analysis was not set to Linear. Open run data in Fluidigm Real-Time PCR and export the data after analyzing with a baseline correction method of Linear.
   c. Unsupported plate format
   – The run data was not acquired for a supported IFC.
   d. Unsupported probe-type count, expected 1.
   – The run data was not acquired with a supported protocol
   e. Panel types must match in study
   – The study already contains a plate for one-panel type (Olink 96, Olink 48, etc.). All plates in the study must be run for the same panel type.
   f. Run barcode already exists in a study for the panel
   – The run data for this IFC has already been imported to the study.
   g. Data integrity file not found in the archive
   – The run data checksum file was not found in the Q100 Signature export. The run data must be analyzed and exported from Real-Time PCR before importing.
   h. Could not locate data file in file
   – The run data analysis export was not located in the Q100 Signature export file. This may be due to a corner finding error during analysis, due to:
      o Insufficient amount of ROX dye in corner wells
      o Contrast not optimal
      o Edge sample or assay is not loaded (e.g. air bubbles or particle clogging of the IFC)

Unzip the run data archive and open the ChipRun.bml in Fluidigm Real-Time PCR to analyze and export data. Manually set the corners using Fluidigm Real-Time PCR Analysis Software, export in .csv format and import in NPX Signature

Make sure to zoom in to clearly see where the wells should be (if empty). All wells will not fit optimally, the important ones are the four corner wells.

i. Datafile checksum not verified

– The run data contained in the Q100 Signature export file failed to validate using the checksum values generated when the analysis was performed. This may indicate that the run data has been modified after it was initially analyzed when the run was going to be completed Unzip the run data archive and import the results_all.csv, results_all.csvx, or results_heatmap.csv file directly.

3. Importing Panel Datafiles

a. Data file already exists

– A panel definition with the same name and version is already imported in the application.

4. Importing Sample Manifest Files

a. Unrecognized file extension for sample plate manifest

– The file format could not be processed. Supported formats are Fluidigm Real-time PCR comma value separated plate definitions and NPX Signature XLSX format.

b. No Sample-ID defined in input data

– The sample manifest import file does not define the Sample-ID column

c. No Plate-ID defined in input data

– The sample manifest import file does not define the Plate-ID column

13.2 Deviating controls for Olink Target in Olink® NPX Signature

If the normalized value for internal controls or a specific sample deviates from the rest of the sample set, the sample is flagged. A flagged sample should not be excluded from the data export but may be excluded from the QC analysis. See section *12.4 Perform quality controls on page 21* for evaluation of the data.

Table 9. Flagged samples

Issue	Explanation	Eason	Action
A sample is flagged	A sample is flagged when one or several internal controls deviate for that specific sample.	All flagged samples in a study are shown under Study - > Summary in the table Flagged or failed samples.	The behavior of the internal controls makes it possible to understand why the sample is flagged. See the rest of this section for more information. For more detailed information about flagged samples, click the Plate QC -> Sample QC tab (*Figure 13*) and the Assay QC tab (*Figure 16*).

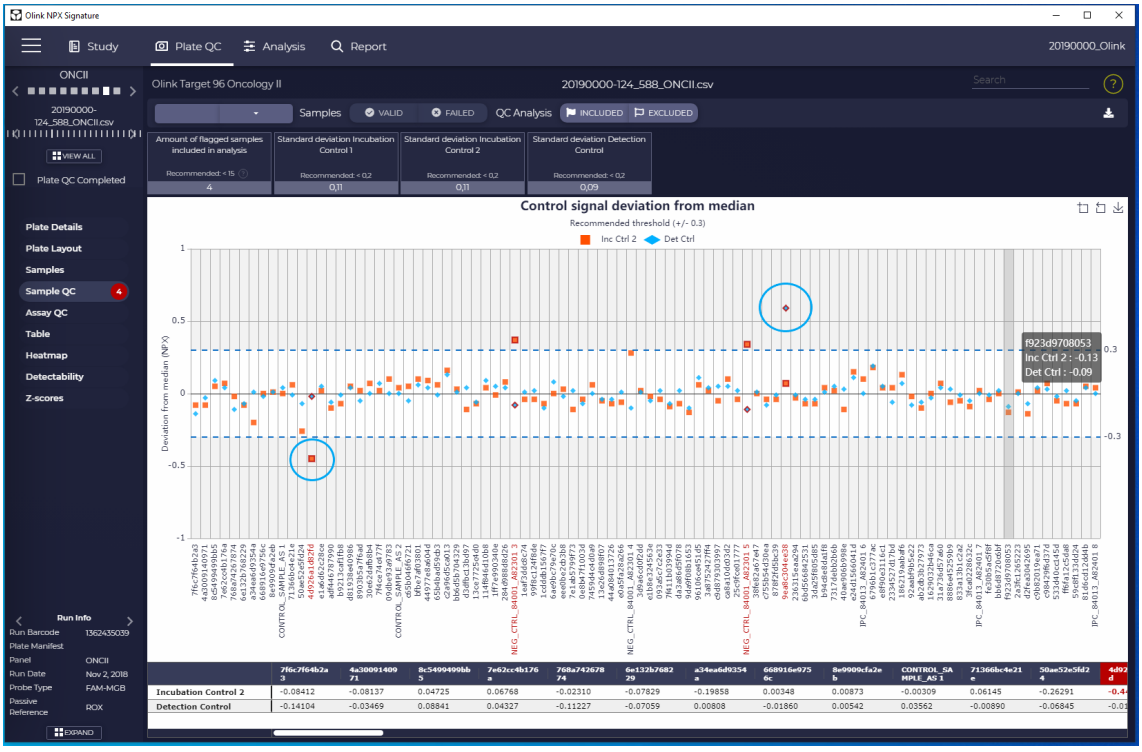


Figure 7. The Sample QC tab, where the left circled sample is flagged by Incubation Control 2, and the right circled sample is flagged by the Detection Control. Both of these datapoints are outside the recommended threshold.

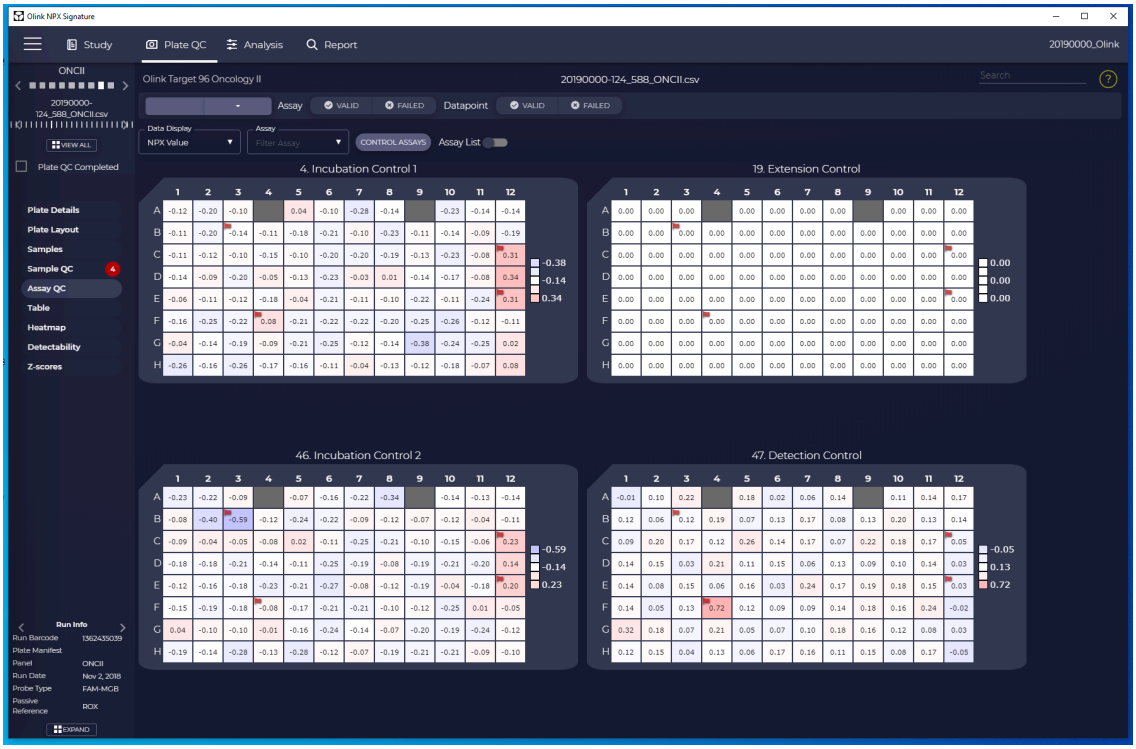


Figure 8. Assay QC tab for samples shown in Figure 7. The circled sample to the left has a lower NPX for Incubation Control 2. The circled sample to the right has a higher NPX for the Detection Control and lower NPX for the Extension Control.

13.2.1 Sample flagged by Incubation Controls

Issue	Explanation	Reason	Action
A sample is flagged by the Incubation Control.	The Incubation Controls deviate.	If the two Incubation Controls deviate, but not the Extension and Detection Controls, this is an indication that something (most likely in the sample) is affecting only the Incubation Controls.	See examples in *Table 10. Common reasons for deviating Incubation Controls.*

Table 10. Common reasons for deviating Incubation Controls.

Possible cause	Explanation	Solution
Sample matrix	If the sample matrix for the flagged sample is different from the others on the same plate, the incubation environment can be different. This can make the reaction slightly more or less efficient.	Do the QC evaluation for one sample type at a time.
Sample volume or concentration	The sample volume is too high or too low.	Set the sample as failed in the **Plate Layout** tab. If applicable, run the sample again and make sure the correct volume is used. See *Figure 7 on page 31*, column 10 for example.
Sample quality	If the sample is stored in a freezer for long periods of time, it can evaporate. A more concentrated sample can increase possible matrix effects, and thus make the internal controls more or less efficient.	
Sample type	Olink assays are validated for serum and plasma. Other sample matrices can contain factors that interfere with the immuno reaction step.	Standardize samples as much as possible. (E.g.: for lysed cells and tissue, use similar concentration of protein in each sample)

13.3 Sample flagged by Detection Control

Issue	Explanation	Reason	Action
A sample is flagged by Detection Control.	The NPX value of the Detection Control for this specific sample deviates more than 0.3 NPX from the plate median.	One example can be seen in *Figure 7 on page 31* and in *Figure 8 on page 31*. Data is normalized using the Extension Control. Since both Incubation Controls and the Extension Control display decreased NPX-values, the normalization step will adjust the data. The Detection Control that did not show decreased NPX-values will be overcompensated by normalization against the Extension Control and thereby deviate from the rest of the sample set after normalization.	This type of flag is generally caused by the same reasons as flagged Incubation Controls. It is not uncommon that both the Incubation Control 2 and Detection Control flag simultaneously, but with opposite "direction" in the Sample QC. This is seen when the flagged sample affects both the extension and immunoassay step, but to different extent. See examples in *Table 10*.

13.3.1 Deviating external controls

Issue	Explanation	Reason	Action
Olink® Target 96: High Intra- CV value	Intra-CV value is larger than 15%.	The control samples differ within the sample plate.	Check sample annotation of the control samples and that they pass QC. Check the row pattern for rows A and B to make sure that none of the rows is an outlier (e.g. issue with a channel on one of the multichannel pipettes). Fail one or both of the control samples if remaining data on the plate pass all QC analysis and assessment.

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

Issue	Explanation	Reason	Action
Olink® Target 96:			
High LOD (> 2.5 NPX)	Background levels, values in Negative Controls, are increased above expected levels	• Sample and/or primer columns double pipetted during loading of IFC	• Rerun from Detection step
		• Technical issue (increased background may occur sporadically due to random interaction of probes or airborne contamination). Seen for all three Negative Control wells for specific assays.	• If high LOD does not affect the detectability for the assay, continue data analysis • If detectability is affected in a multi plate study, the affected assay can be excluded from the affected plate and data analysis continues for remaining plates or rerun from immuno reaction step • Please contact support@olink.com for further guidance
		• Sample/IPC contamination of Negative Control	• If one well affected: Exclude contaminated Negative Control • If two or more Negative Controls are affected: Rerun samples
		• Sample and/or primer columns switched during loading of IFC	• If noted during lab work, contact Olink support
Olink® Target 96:			
Large number of high and/or low LODs (outside ± 2.5 NPX)	Calculated LODs are out of expected range	• Wrong datafile • Wrong panel • Contamination of Negative Controls • Wrong annotation of negative controls or IPCs	• Confirm datafile; reanalyze data • Please contact support@olink.com for further guidance

Olink® Target 96			
Large deviation in LOD between plates in study	LOD values differ more than 1 NPX between sample plates in the same study	• Contamination of one or several wells • Pipetting error • Technical issue (increased background may occur sporadically due to random interaction of probes). Seen for all three Negative Control wells for specific assays.	• Evaluate which plate and/or well is contributing to the large deviation • Exclude affected well if that can be defined • Exclude assay on affected plate or rerun from immuno reaction step • Please contact *support@olink.com* for further guidance

Olink® Target 48

Issue	Explanation	Reason	Action
High LODs that affect the number of samples that can be quantified above LOD.	Calculated LODs are out of expected range	• Wrong datafile • Wrong panel • Contamination of Negative Controls • Wrong annotation of Negative Controls, Sample Controls or Calibrators.	• Confirm annotation of Negative Controls, Sample Controls and Calibrators • Confirm datafile; reanalyze data • Please contact support@olink.com for further guidance
Olink® Target 96:			
Large deviation between triplicates of IPC and/or Negative Controls on the same plate	NPX values for triplicate samples deviate more than 0.5 NPX	• Contamination of one or several wells • Pipetting error	• If one well affected: Exclude that well • Rerun if several wells are affected
Olink® Target 48			
Sample Control intra CV>30% and/or accuracy > +/- 30% causing many failed assays	Sample Control intra CV>30% and/or accuracy > +/- 30%	Sample Control replicates differ within the same plate	Check sample annotation of the Sample Controls and that they pass QC. Check the row pattern for rows A, B and C to make sure that none of the rows is an outlier (e.g. issue with a channel on one of the multichannel pipettes). If one Sample Control replicate deviates from the other two and this pattern can be confirmed for several assays, this could indicate a manual issue with this replicate. In such case that replicate can be failed and the remaining used for CV and accuracy calculation. Please contact support@ olink.com for further guidance.

Olink® Target 48

Issue	Explanation	Reason	Action
Calibrator intra CV>30% resulting in many assays with warning	Calibrator CV>30%	Calibrator replicates differ within the same plate	Check sample annotation of the Calibrators and that they pass QC. Check the row pattern for rows F, G and H to make sure that none of the rows is an outlier (e.g. issue with a channel on one of the multichannel pipettes). If one Calibrator replicate deviates from the other two and this pattern can be confirmed for several assays, this could indicate a manual issue with this replicate. In such case that replicate can be failed and the remaining used for CV calculation. Please contact support@ olink.com for further guidance.

13.3.2 Missing sample data

Issue	Explanation	Reason	Action
Missing sample data in exported results file	Data for some samples are not exported.	This can be caused by not using unique sample names.	Ensure that sample names are unique by checking Plate QC -> Samples tab or change sample type to Control for non- unique samples to force export of data for these samples.

13.4 Inconsistent results detected in Olink Target using Olink® NPX Signature

13.4.1 Variation between plates - common reasons

Issue	Explanation	Reason	Action
The "Plate ANOVA" value in the Panel metrics box (*Figure 9*) shows a high value. The variation is also shown on the Plate QC -> Z-scores tab (Figure Figure 38. Plate variation that should be solved by intensity normalization.).	The total NPX varies between plates.	May be caused by insufficient randomization, meaning that the distribution of samples from different groups is not the same for all plates in the study.	If the samples are completely randomized, make sure that intensity normalization has been used. The results will then look like the example in *Figure 11 on page 39,* the same results as in *Figure 10 on page 39* after intensity normalization.
High Inter-CV value	Inter-CV value is larger than 25%.	The control samples differ between sample plates.	Check sample annotation of the control samples and that they pass QC. If intensity normalization has been used, ensure that randomization assumption is valid by comparing with IPC normalization.

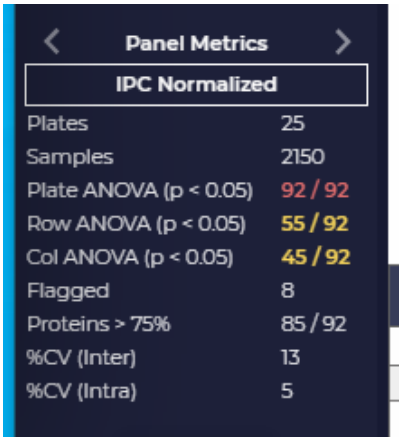


Figure 9. The Panel metrics field, with a Plate ANOVA value that indicates variation between plates.

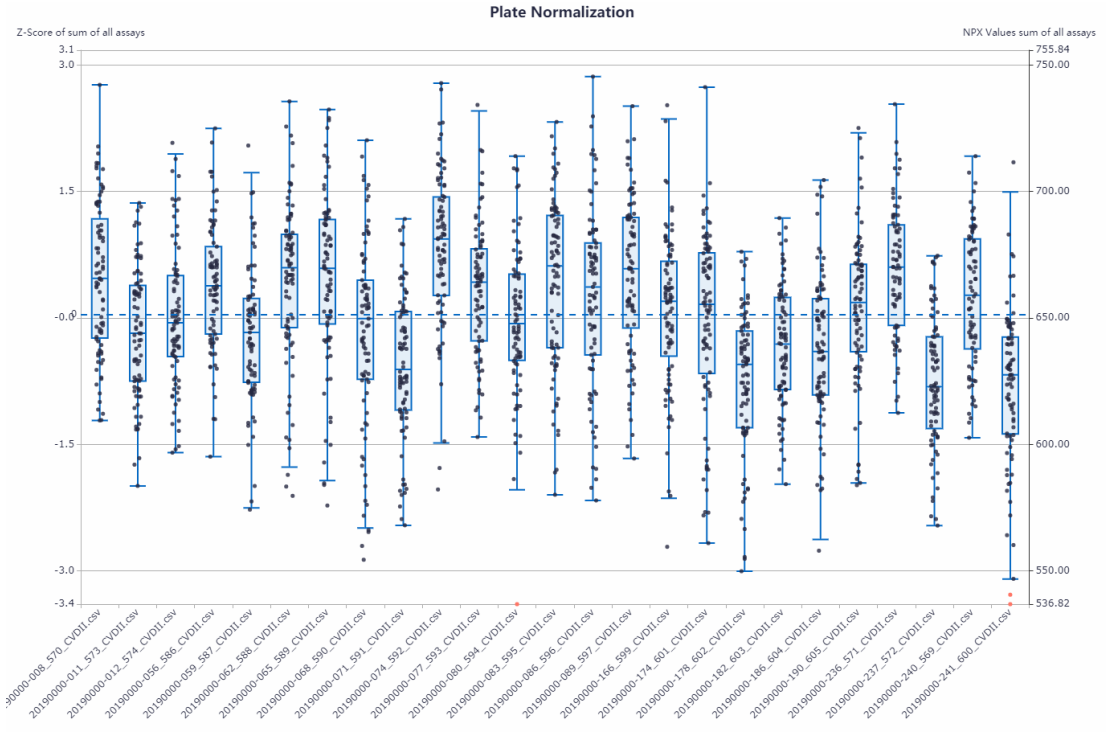


Figure 10. Plate variation that should be solved by intensity normalization.

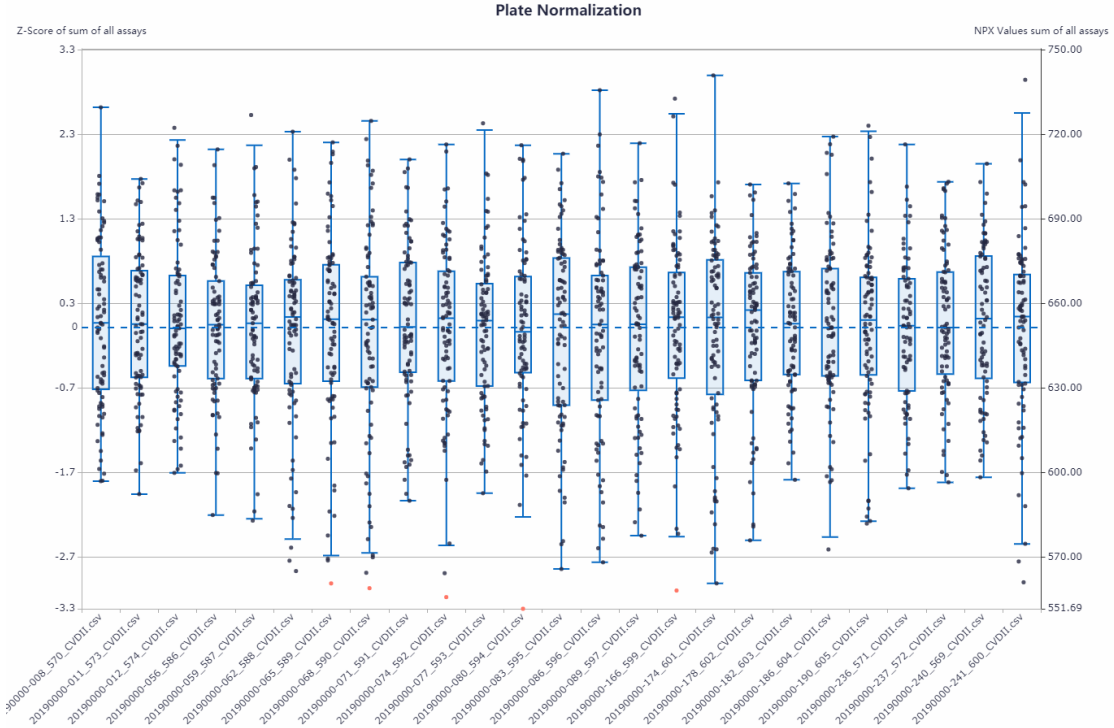


Figure 11. The same results as in Figure 38, after intensity normalization.

13.4.2 Variation within a plate - common reasons

Issue	Explanation	Reason	Action
The columns or row ANOVA in the Plate Metrics or Panel Metrics box show high values. The effects are visualized in the Assay QC tab.	Areas of a plate (rows and/or columns) have systematically higher or lower values (Ct and/or NPX) than the rest of the plate.	The variation is caused by the sample position on the plate. Within-plate effects are often caused by laboratory mistakes.	See examples in *Table 11*. **NOTE:** *Make sure that the deviation cannot be explained with biological variation, i.e. that a unique sample group is placed on one column/row.*
Position effects/patterns: visible only for Internal controls			
All internal controls are affected on Ct-level in a similar manner (all increased or decreased)	One sample column or row consistently show deviating values	Pipetting error, e.g. pipette not pre-conditioned	Exclude data from analysis and/or rerun
		Mistake during pipetting of the incubation solution, extension solution, detection solution or samples	Refer to *Figure 12 on page 42*.
		Evaporation due to incomplete sealing of plate during incubation or PCR steps	Exclude data from analysis and/or rerun
		Different sample matrices	QC matrices separately
Incubation Controls and Extension Control differ from Detection Control on Ct-level	Incubation and Extension Controls show the same pattern, Detection Control shows another pattern	PCR instrument not good at keeping 50°C, Detection Control not affected by extension step	Rerun from immuno reaction step
		May be pre-analytical (interfering factors may affect all controls but the Detection Control)	Please contact *support@olink. com* for further guidance
Incubation Control/s differ from Extension and Detection Controls	One or both Incubation Controls show deviating pattern	Pre-analytical factors in samples that interfere with the immuno reaction	Few deviating samples: Exclude samples from subsequent statistical data analysis. > 1/6th of samples deviating: Contact *support@olink.com* for further guidance
Position effects/patterns: visible for samples and Internal controls			
Columns/rows/parts of sample plate	Patterning that follows sample naming/sample matrices	Different sample matrices	QC matrices separately

Issue	Explanation	Reason	Action
Gradient over the sample plate	Upper or lower part or left and right part of the plate affected with clearly deviating values	IFC issue Poor vortexing in extension step Poor mixing of detection mix Uneven temperature in PCR block	Rerun Contact support@olink.com for further guidance

Table 11. Common problems and reasons for within-plate effects.

Problem	Possible cause	Figure reference
First column different than the rest of the plate (observed for internal controls)	The pipette was not preconditioned.	
A specific column or columns is/are different than for the rest of the plate (observed for internal controls)	A mistake when the incubation solution, extension solution, detection solution or samples were pipetted.	Refer to *Figure 13 on page 43*.
One row consistently different from the rest of the plate (observed for internal controls and/or samples)	The pipette tip in that specific position was not tight enough.	
A gradient from row A to row H	Pipetting was done at an angle. The plates were not sufficiently vortexed.	Refer to *Figure 16 on page 44*.
Deviating Z-score distribution for single assays on one plate	Primer contamination.	Refer to *Figure 15 on page 44*

13.4.3 Extreme outliers - common reasons

Issue	Explanation	Reason	Action
Extreme blue or red lines in heatmap (when only selecting “samples” in the drop-down menu) or outlier dots in z-score view **NOTE:** *Extreme outliers can be discovered in different views (Plate QC -> Z-scores, Assay QC, Heatmap). In Figure 12, two types of outliers are illustrated.*	Sample with extremely low or high protein quantification	Incorrect dilution of sample No sample added	Fail sample

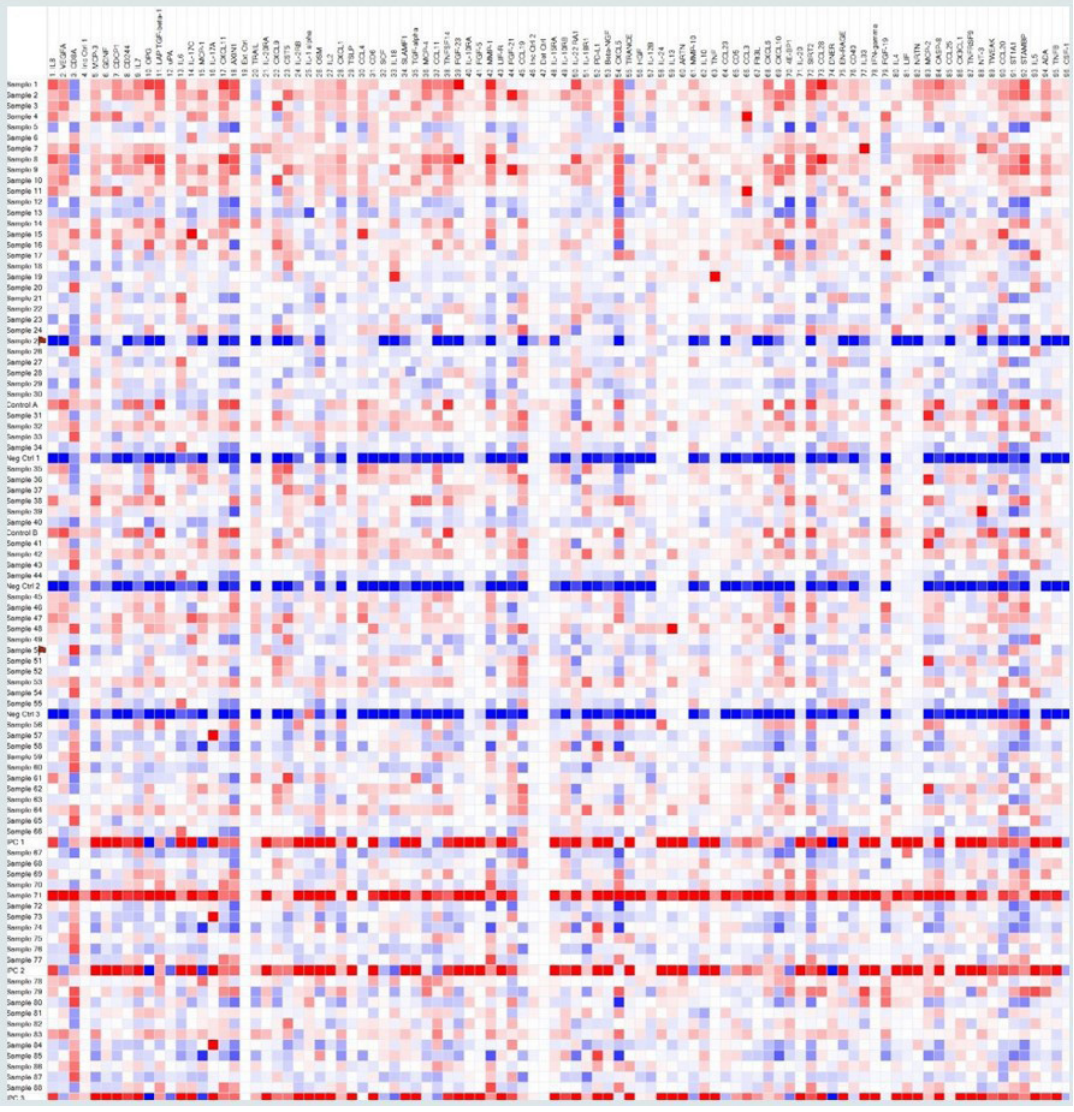


Figure 12. Heatmap with display type Delta with NPX values from a single plate run which contains two outliers. Sample 25 has the lowest NPX values for all assays and Sample 71 has the highest NPX values for all assays.

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

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

Figure 13. Ct-values that show results of laboratory mistakes. See Table 12 on page 44 for a description of errors performed on this run.

Figure 14. NPX values that show results of laboratory mistakes. See Table 12 on page 44 for a description of errors performed on this run.

Table 12. Explanation of columns in Figure 13 and Figure 14. Red numbers indicate laboratory mistakes.

	Column 4 (correct)	Column 7	Column 8	Column 9	Column 10	Column 11	Column 12
Incubation mix (µL)	3	3	3	3	3	**6**	3
Sample volume (µL)	1	1	1	**0**	**2**	1	1
Extension mix (µL)	96	96	**192**	96	96	96	96
Detection mix (µL)	7.2	**14.4**	7.2	7.2	7.2	7.2	7.2
PCR product (µL)	2.8	2.8	2.8	2.8	2.8	2.8	**5.6**

Figure 15. Ct values that show primer contamination of Incubation Control 2.

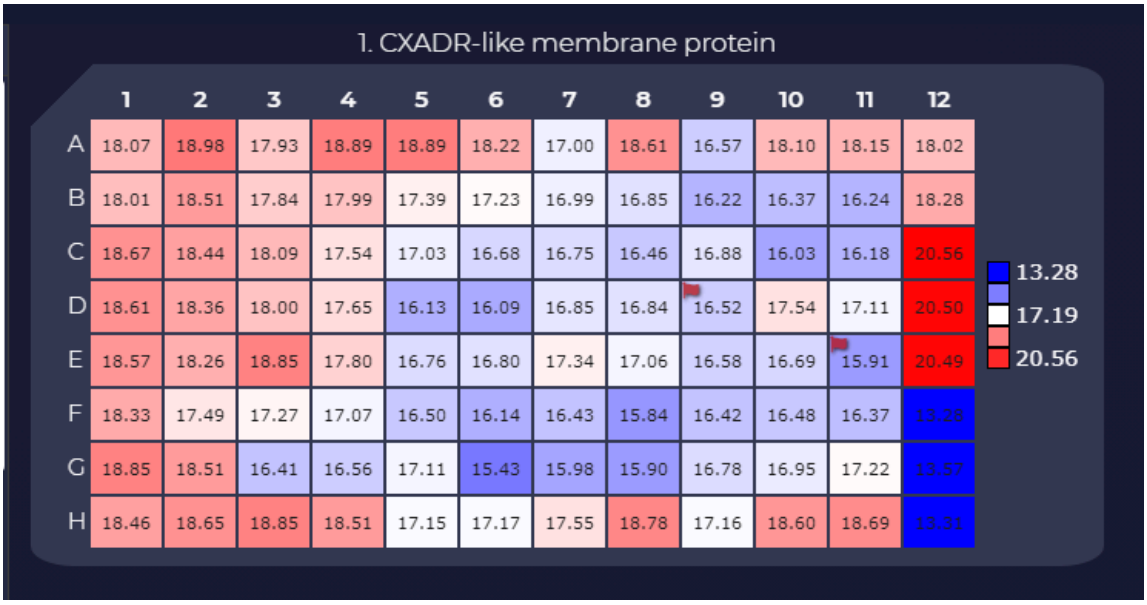


Figure 16. Within plate effects due to incorrect or insufficient vortexing of the sample plate during sample dilution.

13.4.4 High NPX signals - common reasons

Issue	Explanation	Reason	Action
Unexpectedly high NPX signals in diluted panel for the majority of samples	Low variation between samples Saturated assays (may be visualized in Z-scores tab)	Samples were not diluted	Rerun from sample dilution step

14. Revision history

Version	Date	Description
1.0		

15. Appendix A: Related Documents

Go to https://www.olink.com/resources-support/document-download-center/ to download these related documents.

Title	Document Number
Signature Q100 user manual	1172
Olink Target 48 User Manual	1141
Olink Target 96 User Manual	0935

www.olink.com

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For technical support contact *support@olink.com*.

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