NPX™ Signature for Olink® Target 48

Software User Manual

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Part 1: Introduction

1. About this manual

The NPX™ Signature User Manual provides you with the instructions needed for processing the raw data and performing quality control on the data from Olink® Target 48 panels with the NPX Signature software.

This manual contains step-by-step instructions for analysis in NPX Signature and troubleshooting.

1.1   Intended use

NPX Signature is a data analysis software that is designed for use with the qPCR-based kits from Olink, including Olink® Target 48, Olink® Target 96, Olink® Focus as well as for Olink® Flex panels. It allows for importing run data, validating data quality, normalizing and exporting Olink data for subsequent statistical analysis.

1.2   Intended target group

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 Olink Focus panels, contact Olink Support at _support@olink.com_. NPX Signature is intended to be used by trained users of the Olink Target analysis platform

2. List of abbreviations

%CV	Coefficient of Variation
IFC	Integrated Fluidic Circuit (also called "chip" in this document)
LLOQ	Lower Limit of Quantification
LOD	Limit of Detection
LOQ	Limit of Quantification
LQL	Lowest Quantifiable Level
PCR	Polymerase Chain Reaction
QC	Quality Control
ULOQ	Upper Limit of Quantification

3. Safety

This chapter contains important safety information.

3.1   Definition of alert levels

The following alert levels are used in the Olink Explore manuals:

IMPORTANT: _Indicates an important action that may impair the results if not performed correctly._

NOTE: _Contains information that can make it easier to understand or perform a certain task._

4. Associated documentation

- Olink® Signature Q100 User manual, doc no 1172
- Olink® Target 48 User Manual, doc no 1141

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

5. Technical support

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

6. Process

NPX Signature is a standalone Windows software application that lets you import data from Olink® Signature Q100, validate its quality and normalize it. The data is not required to be annotated or processed before importing it into NPX Signature. The software can also process Fludigim® Biomark™ data obtained by running Olink kits.
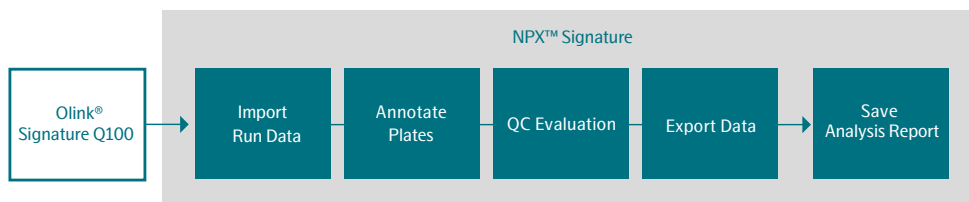
7.Software and file requirements

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

7.1   Files and information

NPX™ Signature Study File (.npx)

IMPORTANT: *To enable NPX Signature's remote data functions to Olink Signature Q100, ensure that the instrument is connected to the network and TCP port 8085 (enabled by default) is not blocked. For instructions on how to securely connect the Olink Signature Q100 to a network, refer to the Olink Signature Q100 User manual.*

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 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.

7.2   Files and Information Needed for Analysis

Q100 Run Data

This is the Olink Signature Q100 run data that can be imported into 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);
- NPX Manager project file (.oaf); or
- Fluidigm Real-time PCR exported heatmap or table file (.csv).

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

Plate Manifest File
Microsoft Excel file (.xlsx) that can be used to define the sample plate layout.

Panel Data File

File needed to properly process and quality control your data. This file is unique to your panel and kit lot. The file is provided by Olink.

NOTE: *The size of the .zip file that is transferred via USB from Olink 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 Olink Support. If possible, email only the NPX Signature file with the .npx extension.*

8.Installing

8.1   License agreement

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

8.2   Install software

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 latest version of the NPX Signature software to your computer from *olink.com/software*.

2. Open the zip folder.

3. Double-click the installer (.msi) file.

4. Follow the on-screen instructions.

5. NPX Signature will be displayed in the Windows Start menu for future use. To open the application, double-click on the icon.

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

8.3   Uninstall software

If you need to uninstall NPX Signature, it can be uninstalled using the Windows Add found in Windows Settings, or Remove Programs in the Control panel. Select NPX Signature from the program list and click **Uninstall**.

All user data is retained where it was saved before the application was uninstalled, and your custom NPX Signature settings are retained on the system.

Part 2: User Interface

9. General

9.1 Start view
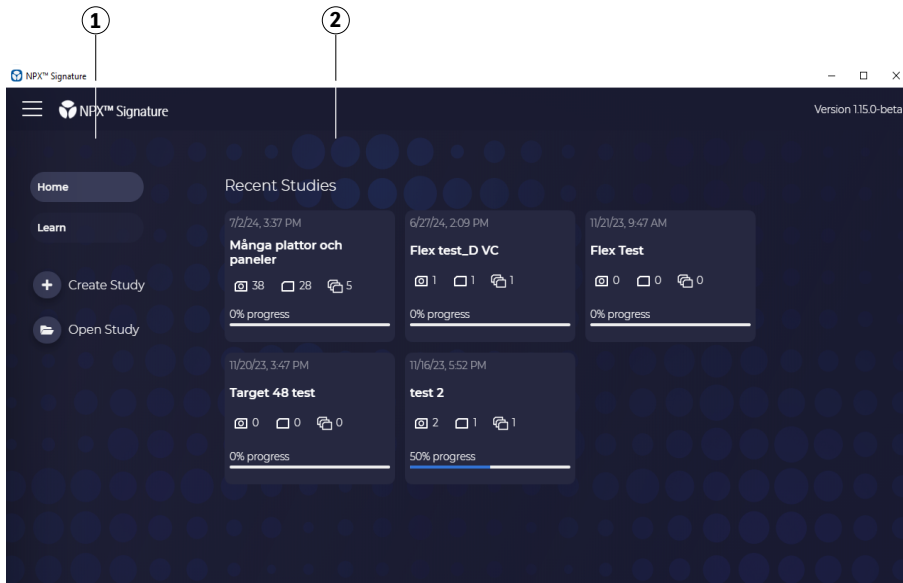
No	Function	Description
1	Main menu	Access main functions. Click on the three lines in the top left corner to expand the main menu.
2	Recent studies	Show recent opened studies. Click on a project to get to the Study Overview.

9.2 Main menu
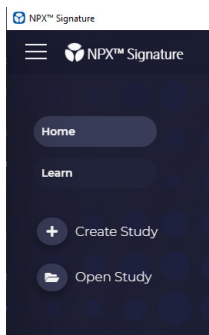
Function	Description
Home	Access the main level of the application, where you see your most recently opened studies.
Learn	Access all the integrated tutorials. Refer to *11. Tutorials and in-line help* for more information.
Create Study	Create a new study.
Open Study	Open an existing study.

The expanded menu lists three functions in the lower part of the menu.

9.2.1 Lower part of main menu
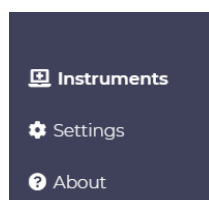
Function	Description
Instruments	Show a list of Olink Signature Q100 instruments available to the NPX Signature software over the network.
Settings	Access the application settings. Refer to *10. Settings* for more information.
About	For instructions on how to securely connect the Olink Signature Q100 to a network, refer to the Olink® Signature Q100 User Manual.

10. 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 Analysis Report 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 Panel Data Files in the application.
- Import or remove Olink Flex Panel Data Files.

11. Tutorials and in-line help

NPX Signature contains integrated tutorials and in-line help.

11.1 Tutorials

Click **Learn** on the main menu to access integrated tutorials meant to assist you in learning the various features of NPX Signature. Click a tutorial and navigate through the pages using **Next** or **Previous**.
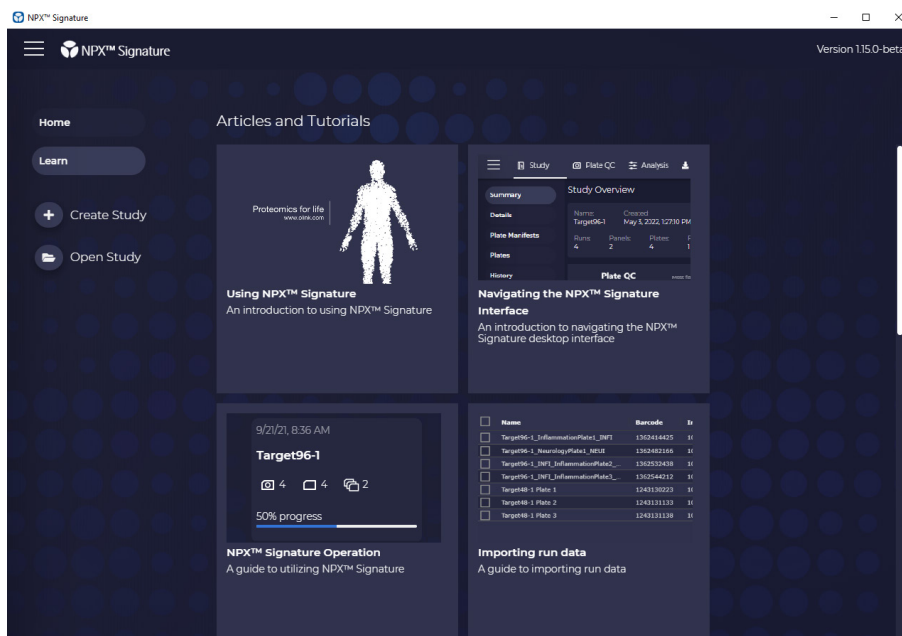
You can also launch a tutorial by clicking [TUTORIAL] in the end of the in-line help content.

11.2 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.

Part 3: Operation

12. Introduction

Input data for NPX Signature are run files from Olink Signature Q100, 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 bridging factor, the output data are obtained in NPX values.

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 NPX Signature. Olink Target 48-specific information is highlighted. The following steps are included in the standard operating procedure:

1. Create an Olink Target 48 Study.

2. Import run data.

3. Verify panel and plate layout information.

4. Under Details, enter optional study information.

5. Perform quality controls including additional quality assessment.

6. Export Data and Analysis Report under Export tab.
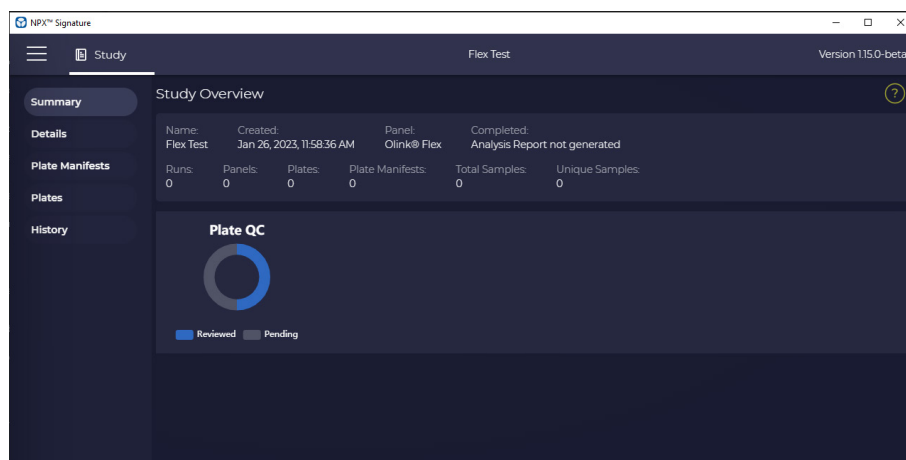
13. Start a project

13.1 Import panel data files

Every panel has its own panel data file, which is provided by Olink. These panel data files are included in the software. To ensure that you have the panel data files corresponding to the Target 48 kit you have run, make sure to always use the latest version of NPX Signature.

13.2 Create a study

To create a study, perform the following steps:

1. Click Create a study.

2. Select Product.

3. Enter Study Name (required information), Sample Type and Notes.

4. Click Create.

13.3 Import run data

Add run data to a new study

When prompted, select your Run Data Source:

- To import run data directly from an Olink Signature Q100 instrument, select Olink Q100 Connect. Select the desired run(s) and press Next.

   NOTE: *Run data cannot be saved in the npx folder. Please make sure that the data is securely saved on disk/ server/computer.*

   – Select Next again and verify panel, data file version and plate layout information. Click Add. The Study Overview will appear.

- To import run data files from your computer or a local server, select Run Data Files.

   – Select the desired run data file(s). Valid options are Olink Signature Q100 export files, .q100 or .zip, exported heatmap or table results files in .csv format from Fluidigm Real-time PCR analysis software.

   – To add more run data files, simply click on Add again and select the next file. Please note that more run data files can be added to the project at later stages as well.

   – If the wrong file is included, mark the file and click Remove.

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

If duplicate sample names are found, NPX Signature prompts an alert message box. During data handling, 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 NPX Signature by importing a sample manifest in the Plate Manifests view. Refer to the built-in help for more information.

When the selected runs have been validated, click Next. Select the correct Panel and Panel Data File for each of the imported runs, and select if the plate should have the default plate layout or if the layout should be mapped based on a plate manifest. Click Add.

For instructions on how to securely connect the Olink Signature Q100 to a network, please refer to the Olink® Signature Q100 User Manual.

Add run data to existing study

Open your already saved study. Expand the main menu by clicking the three horizontal lines in the top left corner. Select Import Run Data, and follow the above instructions to add data directly from an Olink Signature Q100 instrument or from run data files on your computer.

13.4   Enter study information

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

- Enter information in some or all the following fields to be displayed in the Analysis Report:
    - Sample Type
    - Customer Info
    - Business Development Manager
    - Lab Info
    - Report Notes
    - QC Notes for Analysis Report

13.5   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 panel 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)
- Negative Control (NEG)
- Sample Control (SC)
- Calibrator (CAL)
- N/A

Calibrator, Negative Control, and Sample Control must be annotated for calibration normalization and QC to be performed.

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

14. 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 and then make an overall assessment.

The acceptance criteria for passing QC are described below.

Criteria	Recommended value	Note in results file
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 of samples	
Detection Control deviation	< ±0.3 NPX from plate median of samples	
Assay QC		
Accuracy Sample Control	±30% of the expected concentration	Warning
Precision Sample Control	Intra-CV <30%	Warning
Control replicates outside LOQ	≤ 1/3 of the Sample Control replicates are outside of LOQ	Warning
Calibrator replicates outside LOQ	≤ 1/3 of the Calibrator replicates are outside of LOQ	Fail (results are replaced by "No Data")
Precision Calibrator	Intra-CV <30%	Warning

💡 **TIP:** *If Std. Dev. is higher than the recommended value for internal controls, evaluate the Control signal deviation from median/sample QC graph 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.*

14.1   Perform a quality assessment

Parameters that need to be evaluated for each run are listed in the table below. The corresponding information in NPX Signature can be found in the lower left corner under Plate Metrics.

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

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	< 7 (number of assays)	No warning.
	7–14 (number of assays)	Possible randomization issue. Refer to section *21.*
	14–21 (number of assays)	Re-run plate or verify that this is not the result of a poorly executed run.
		For high values, investigate the **Assay QC** view.
%CV Intra	< 15%	Refer to Section *19.*
%CV Inter	< 25%, maximum 40%	Refer to section *19.*
Detectability	> 75% of the samples	The detectability should be comparable across plates if the samples run are randomized.

14.2   Additional quality assessment

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

To perform additional quality assessment, follow these steps:

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 the control replicates (change sample type in the drop-down menu).

4. On the Detectability tab, evaluate the detection limit for each assay and sample plate. For the Olink Target 48 panels, some assays can be expected to have LOD values above 2.5 NPX 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.

5. The Z-scores tab displays differences in data distribution between sample plates as well as within plates.

15. Export data

In the Export -> Data tab, Data can be exported for use in preferred applications. It can be exported both as CT, NPX, and pg/mL (default) in separate reports, by selecting the desired Data type.

1. Under Panels, select from which panels in the study data should be exported.

2. To export data according to the recommended default settings, click Defaults.
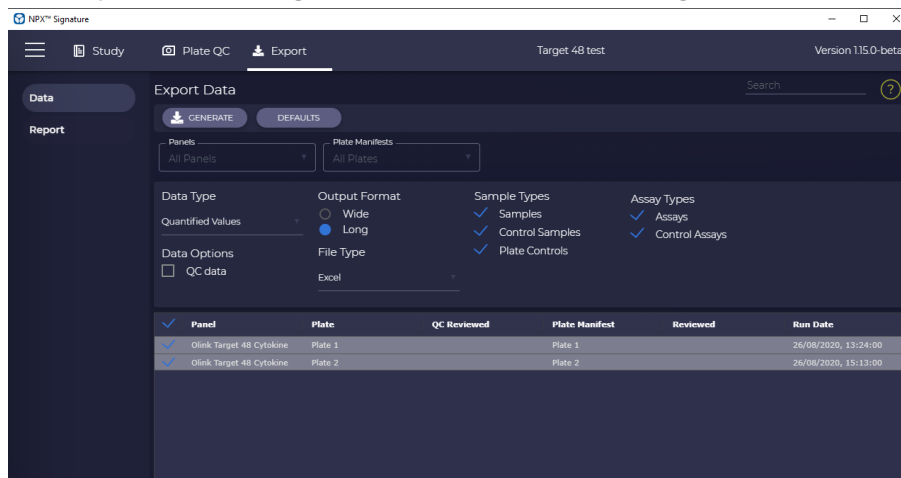
> **NOTE:** *The settings in the* **Export Data** *view may be adjusted to change the format, layout, or to add additional data to the export. Refer to the table below for more information.*

3. When the desired options have been selected, click **Generate**.

4. Enter a filename and location in the **Save** dialog, select an export format and click the **Save** button.

Setting	Description
Data Options: QC data	Deviation from plate median for Incubation Control and Detection Control is exported in NPX values for each sample. Data for the controls cannot be calculated in pg/mL and will be exported in NPX in the results file for quantified values.
Data Options: Plate LOD	Will export plate LOD for each plate.
Output format	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. Select Text format instead.*
File Type	Microsoft Excel (.xlsx) or Text (.csv) For CSV format, column separator and decimal separator can be configured
Sample types: Samples	Export data for customer samples.
Sample types: Sample Control	Export results from the Sample Controls.
Sample types: Plate Controls	Export results from Negative Control and Calibrators.
Assay types: Assay	Export results for assays.
Assay types: Control Assay	Export results for internal control assays. Data for internal controls cannot be calculated in pg/mL and will be blank if quantified values are selected for export.

15.1 Considerations

Solor indications noted below are valid for Microsoft Excel exports only.

- Data from assays and samples that have not passed the sample QC are reported in red text and are marked with Warning.

- Samples, assays, or data points that have been marked as failed in the software are reported as No Data.

For panels with absolute quantification, the data are given in standard units (pg/mL) and presented 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 but above the lowest fitting parameter in the 4PL curve fit model, 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.

- 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.

Result and QC results						In Data report			
Sample QC	Calibrator QC precision	Sample Control QC precision and/or accuracy	Sample Control replicates within LOQ	Calibrator replicates within LOQ	Data between LQL and ULOQ	Data Output Format	Sample QC	Assay QC	Notes
Pass	Pass	Pass	Pass	Pass	Yes	pg/mL	Pass	Pass	
Fail	Pass	Pass	Pass	Pass	Yes	pg/mL	Warning	Pass	Treat with caution
Pass	Fail	Pass	Pass	Pass	Yes	pg/mL	Pass	Warning	Treat with caution
Pass	Pass	Fail	Pass	Pass	Yes	pg/mL	Pass	Warning	Treat with caution
Pass	Pass	Pass	Fail	Pass	Yes	pg/mL	Pass	Warning	Treat with caution
Pass	Pass	Pass	Pass	Fail	–	No Data	Pass	Fail	Data is unreliable and not shown
Pass	Pass	Pass	Pass	Pass	No; < curve fitting	NaN	Pass	Pass	
Pass	Pass	Pass	Pass	Pass	No; > curve fit, but < LQL	pg/mL	Pass	Pass	Treat with caution
Pass	Pass	Pass	Pass	Pass	No; > ULOQ	>ULOQ	Pass	Pass	

The NPX results can be exported as an XLSX file. The NPX data are presented as follows:

• As NPX values.

• Values below LQL have a red cell background.

• Data for samples with a QC warning are indicated in red text. Data from samples that do not pass QC should be treated with caution.

• The LOD value for each assay is presented on a separate row below the data for the samples and is indicated as LOD.

• Missing data frequency is presented for each assay and indicates the percentage of samples with values below Maximum plate LOD.

The layout is described in the table below for wide format and for long format in the table after.

Study Name	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 quantifiablelevel]	1	2	3	...	96				1	2	3	...	96	plate_1. csv		plate_1. csv			
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						

| 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

15.2   Create an Analysis Report

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

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

Part 4: Quality control

16. Overview

Olink has built-in quality controls in all multiplex panels. Each 48-plex panel contains 45 assays. Three 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.
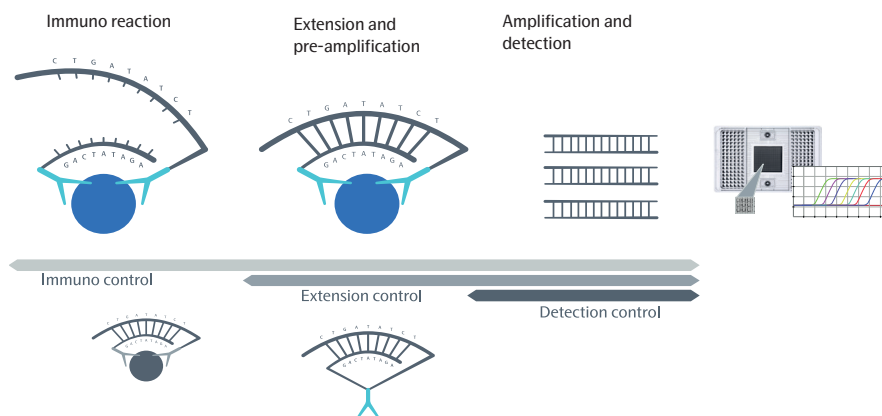
- The Incubation Control consist of non-human antigen measured with PEA assays. This control monitors potential variation in all three steps of the reaction.

- The Extension Control is an antibody coupled to both DNA-tags (hence always in proximity). This control monitors the extension/pre-amplification and amplification/detection step and is used for normalization of the data.

- The Detection Control is a complete double stranded DNA amplicon which does not require any proximity binding or extension step. This control monitors the amplification/detection step.

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

16.1 Sample QC

Each of the internal controls are 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. 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 gets a QC Warning and the sample is flagged in NPX Signature. 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 and Detection Control deviates < ±0.3 NPX from the median of the samples on the plate for the corresponding internal control.

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 assay. For more information on troubleshooting samples that do not pass QC. Refer to *19. Deviating controls* for more information.

For reference values for passing Sample QC, refer to *14. Perform quality controls.*

16.2   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 and the Detection Control. If the variation for one of the controls is too large (see below) the entire run is considered unreliable.

For acceptance criteria for passing a run, refer to *14. Perform quality controls..*

If a too large variation is observed for either of the controls, go to the Plate QC -> Assay QC and/or Sample 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, refer to *19. Deviating controls*.

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

For reference values for %CV, refer to *14. Perform quality controls..*

16.3   Assay QC

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 of the Sample Control replicates as well as the precision of the Calibrator replicates are evaluated for each assay.

Acceptance criteria for passing an assay:
- The accuracy of the calculated concentration mean for the Sample Control for each assay must fall within ±30% of the expected concentration. If this criterion is not met, the assay is noted with "Warning" in the results file.
- The precision of the calculated concentration for the Sample Control Calibrator must be < 30%. If the criterion is not met, the assay is noted with "Warning" in the results file.
- A maximum of one of the Calibrator replicates can fall **outside** of the limits of quantification (LOQ). If the criterion is not met, the assay is reported with "Fail" in the results file, and "No Data" will replace the results for the affected assay.
- A maximum of one of the Sample Control replicates can fall **outside** of the limits of quantification (LOQ). If the criterion is not met, the assay is noted with "Warning" in the results file.

For acceptance criteria for passing an assay, refer to *14. Perform quality controls..*

17. Normalization methods

Olink has developed its own arbitrary, relative quantification unit called NPX™. In qPCR, the x-axis value of the point where the reaction curve intersects the threshold line is called the Cycle threshold (Ct). This indicates the number of cycles needed for the signal to surpass the fluorescent signal threshold line. NPX as well as the results available in standard concentration units are derived from the Ct values obtained from the qPCR using the steps below:

Extension Control:

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

Inter-plate Control:

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

Adjustment against a correction factor:

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

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

17.1   Calibrator normalization

Calibrator normalization is used for Olink Target 48 panels. The calibrators are used to determine the protein concentration in the panel. The calibrators consists of a pooled plasma, with a spike-in of recombinant antigen for a select number of assays. Randomization of samples across and within plates is recommended but not mandatory.

For this panel, the Extension Control, the Calibrator and the bridging factor are used for the normalization calculation. 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. 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.

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

> 🖉 **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.

17.1.1   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 compared to e.g. running a full calibration curve on each plate, 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. A bridging factor, utlized to ensure accuracy between production batches, is calculated.

3. A four parameter logistic (4PL) model fit is performed to define the standard curve mathematically within the measurement range for each protein in the panel.

After each run:

1. The median value of the Calibrator triplicates is used to adjust the measured samples along the y-axis (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*).
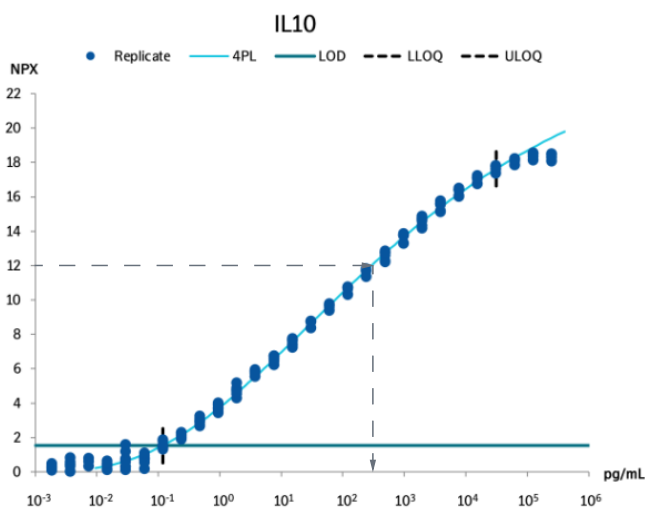
A 32-point standard curve defined for each assay during development.

4PL model

A 4PL model curve fit is used to describe the immunoassay standard curve, indicated by the turquoise line in the figure below. When running a study, the measured sample value (represented by the dashed arrows) 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 the formula below . An example of a point standard curve defined for each assay during development.
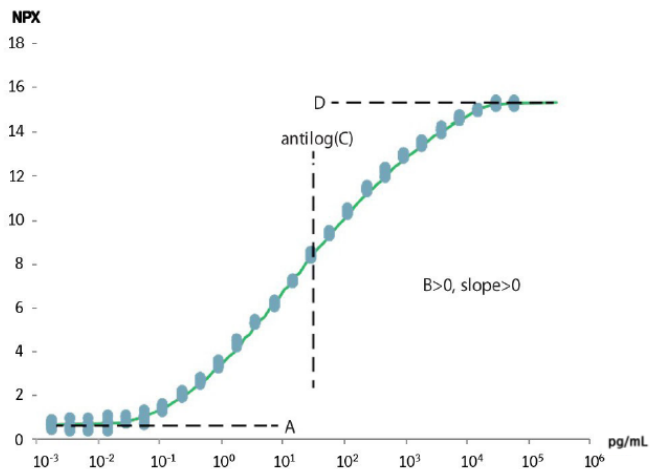
An example of a point standard curve defined for each assay during development.

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

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. This information is available in the individual Panel Validation Reports provided by Olink.
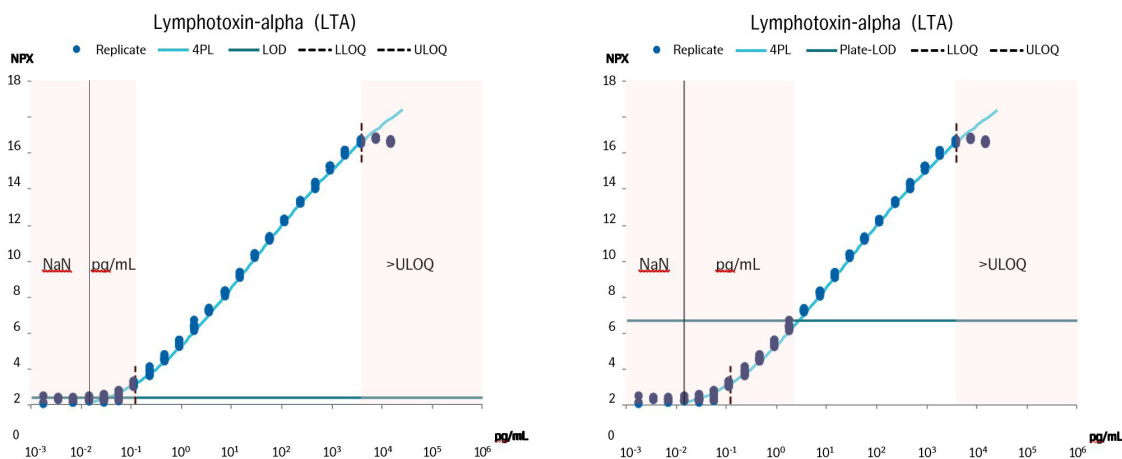
Results reported in pg/mL

The figures 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 data export and in the red areas in the left side of the graphs.
- Data >ULOQ is indicated as >ULOQ in the data export and is found in the red areas to the right of the graphs. Values above ULOQ are not reported in pg/mL due to high risk of misinterpretation of hooking data.
- Data below LQL is presented in pg/mL value s in the data export, and is shown in the red areas in the left side of the graphs. Values below LQL should be treated with caution due to decreased precision and accuracy in the lower range and should not be used for individual comparison to reference values.

For a more detailed list describing the results file, refer to *15. Export data*.

Overview of how data in pg/mL is reported in the exported results file depending on if LLOQ>plate LOD (left) or if plate LOD>LLOQ (right).

Part 5: Troubleshooting

18.Introduction

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

> ✎ **NOTE:** *The figures in troubleshooting are schematic only and might not reflect the current interface of NPX Signature for Olink Target 48.*

18.1   Warning messages

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 with a supported IFC type.

   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 Target 96, Olink Target 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 Olink 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 Olink 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 Olink Signature Q100 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

19. Deviating controls

If the normalized value for internal controls for a specific sample deviates from the rest of the sample set, the sample does not pass sample QC and is flagged in the software. A flagged sample should not be excluded from the data export but may be excluded from the QC analysis. Refer to *14. Perform quality controls* for evaluation of the data.

| Issue	Explanation	Reason	Action
A sample is flagged.	A sample is flagged when one or several internal controls deviate from the plate median 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 7*) and the Assay QC tab (*Figure 10*).
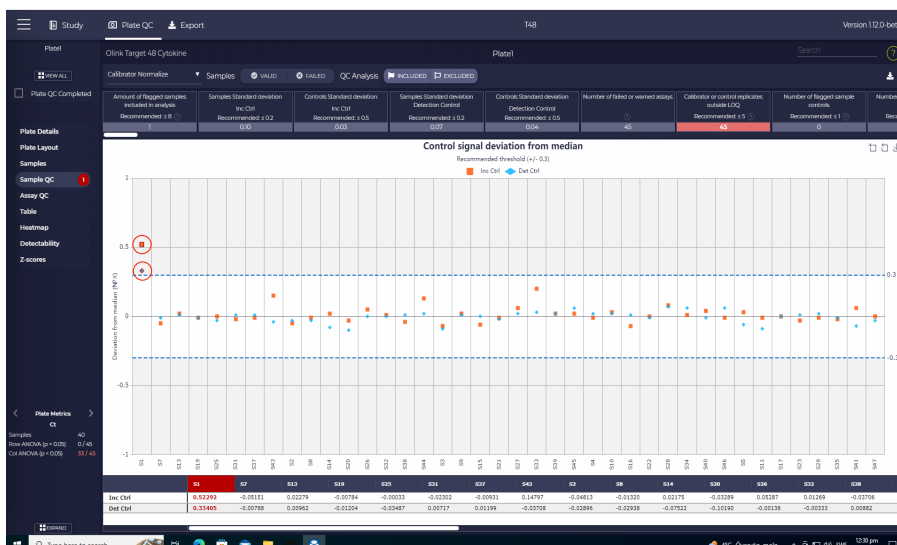
Figure 1.   The Sample QC tab, where the left circled sample is flagged by Incubation Control 2, and the right circled sample is flagged by the Detection Control. Both of these datapoints are outside the recommended threshold which is visualized with the blue dotted lines.
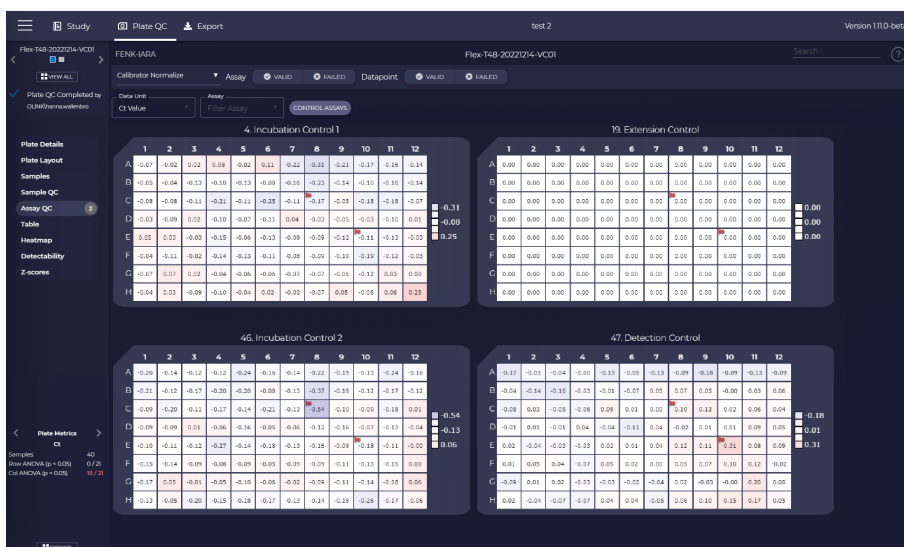
Figure 2.   Assay QC tab for samples shown in *Figure 1*. The circled sample to the left has a lower NPX for Incubation Control 2 (well C8). The circled sample to the right has a higher NPX for the Detection Control (well E10) and lower NPX for the Extension Control.

19.1   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 *19.1.1.*

19.1.1   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.	Perform 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 examples in *Figure 1*.
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).

20. 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 1* and *Figure 2*. Data is normalized using the Extension Control. Since both Incubation Controls and the Extension Control display decreased Ct-values, the normalization step will adjust the data. The Detection Control that did not show decreased Ct-values will be overcompensated by normalization against the Extension Control and thereby deviate from the rest of the sample set after normalization.	This type of flag is generally caused by the same reasons as flagged Incubation Controls. It is not uncommon that both the Incubation Control 2 and Detection Control flag simultaneously, but with opposite "direction" in the Sample QC. This is seen when the flagged sample affects both the extension and immunoassay step, but to different extent. See examples in *19.1.1*.

20.1 Deviating external controls

Issue	Explanation	Reason	Action
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.

| 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 *support@olink.com* for further guidance. |

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.
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.

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.

20.1.1 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.

21. Inconsistent results detected

21.1.1 Variation between plates - common reasons

| Issue	Explanation	Reason	Action
The "Plate ANOVA" value in the Panel metrics box (*Figure 3*) shows a high value. The variation is also shown on the Plate QC -> Z-scores tab (*Figure 4.* 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 5,* the same results as in *Figure 4* after intensity normalization.
High Inter-CV value	Inter-CV value is larger than 25%.	The Sample Control differ between sample plates.	Check sample annotation of the Sample Controls and that they pass QC. If intensity normalization has been used, ensure that randomization assumption is valid by comparing with IPC normalization.
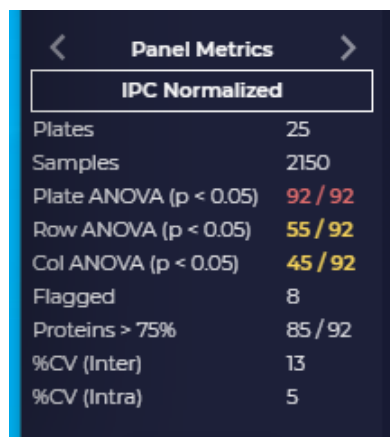
Figure 3.   The Panel metrics field, with a Plate ANOVA value that indicates variation between plates.
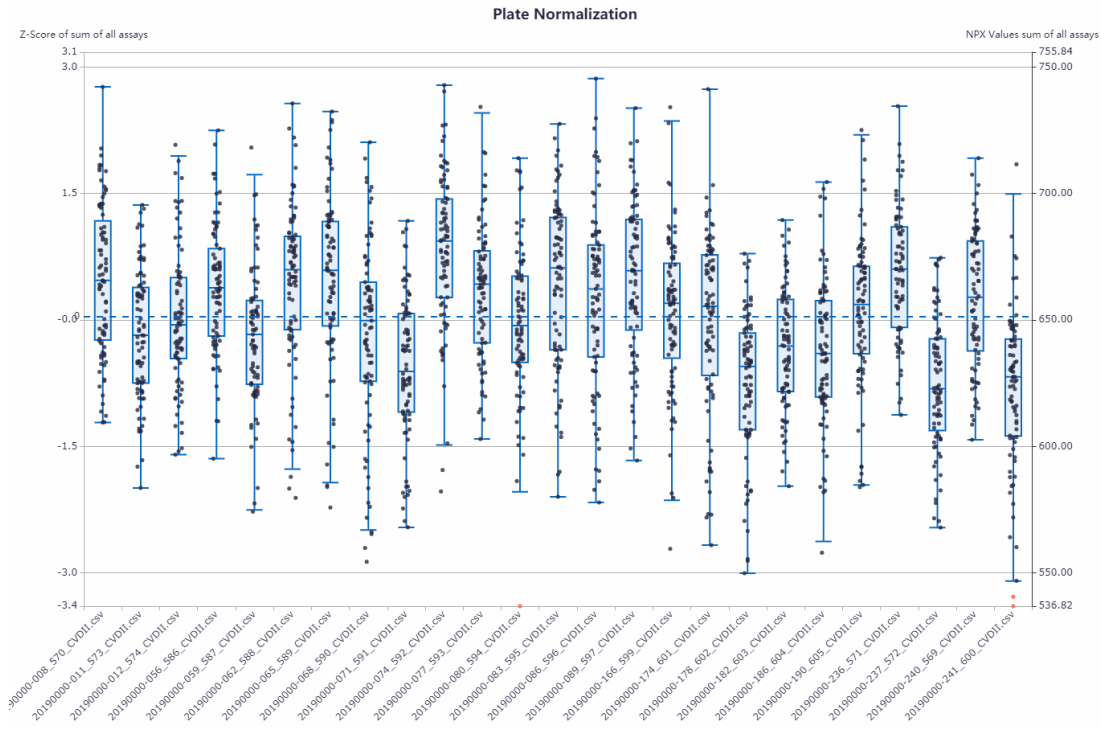
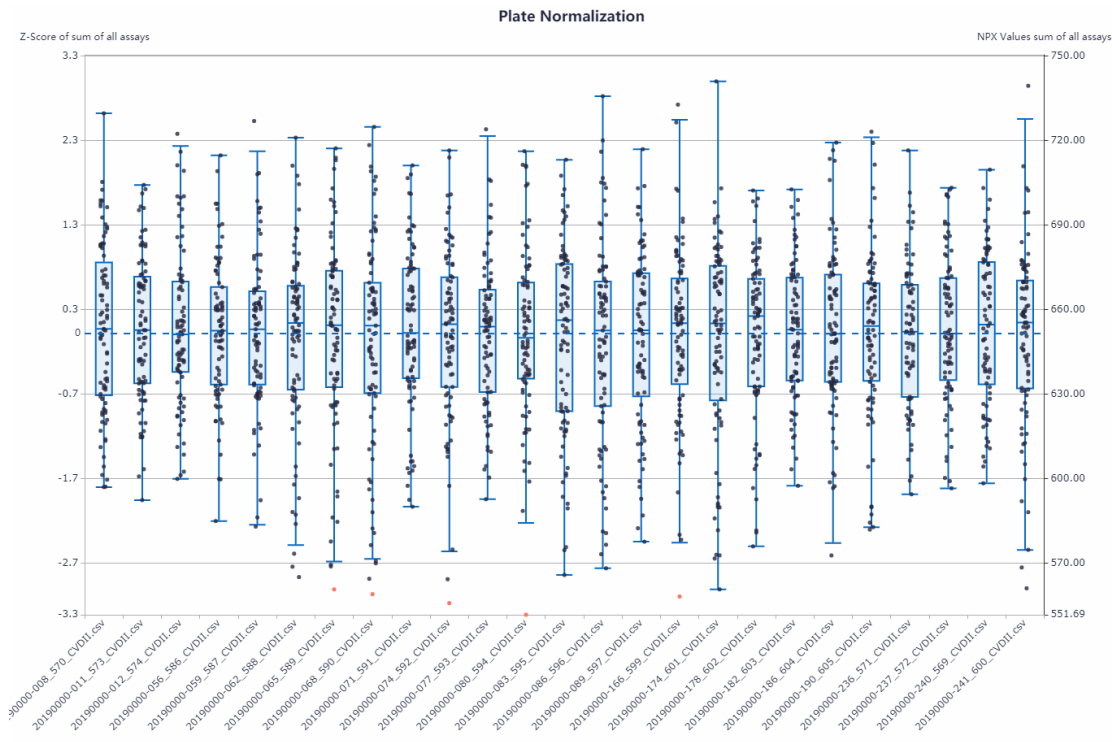*Figure 4.   Plate variation that should be solved by intensity normalization.*

Figure 5.   The same results as in Figure 4, after intensity normalization.

21.1.2 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 the next table. 📝 **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 6*.
		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).	Contact *support@olink. com* for further guidance.
Incubation Control(s) differ from Extension and Detection Controls.	One or both Incubation Controls show deviating pattern.	Pre-analytical factors in samples that interfere with the immuno reaction.	Few deviating samples: Exclude samples from subsequent statistical data analysis. > 1/6th of samples deviating: Contact *support@olink.com* for further guidance.
Position effects/patterns: visible for samples and Internal controls			
Columns/rows/parts of sample plate.	Patterning that follows sample naming/sample matrices.	Different sample matrices.	QC matrices separately.
Gradient over the sample plate.	Upper or lower part or left and right part of the plate affected with clearly deviating values.	IFC issue. Poor vortexing in extension step. Poor mixing of detection mix. Uneven temperature in PCR block.	Rerun. Contact *support@olink. com* for further guidance.

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.	*Figure 7*.
One row consistently different from the rest of the plate (observed for internal controls and/or samples).	The pipette tip in that specific position was not tight enough.	
A gradient from row A to row H.	Pipetting was done at an angle. The plates were not sufficiently vortexed.	*Figure 10*.
Deviating Z-score distribution for single assays on one plate.	Primer contamination.	*Figure 9*.

21.1.3 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 6, two types of outliers are illustrated.*	Sample with extremely low or high protein quantification.	Incorrect dilution of sample No sample added.	Fail sample.
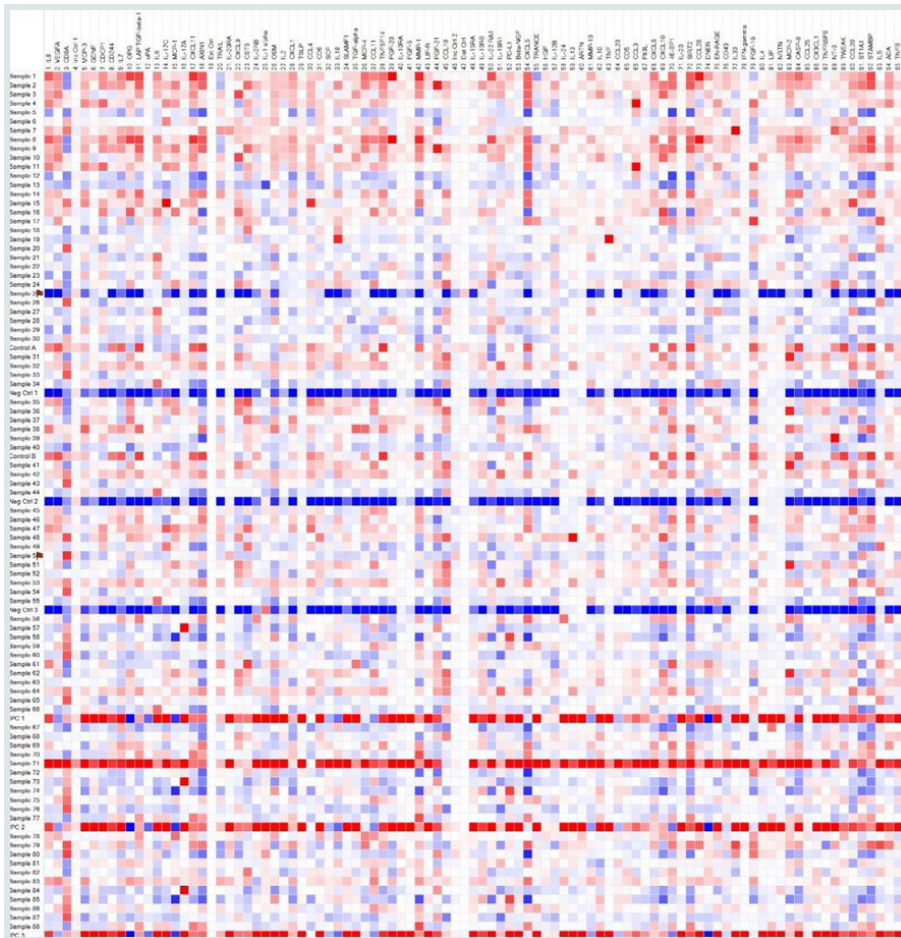
Figure 6.   Heatmap with display type Delta with NPX values from a single plate run which contains two outliers. Sample 25 has the lowest NPX values for all assays and Sample 71 has the highest NPX values for all assays.

For Sample 25 in *Figure 6*, 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 *Figure 6*, the NPX values are higher than for the rest of the samples. This can e.g. arise from poor dilution of a sample.

Figure 7.   Ct-values that show results of laboratory mistakes. Refer to table below for a description of errors performed on this run.

Figure 8.   NPX values that show results of laboratory mistakes. Refer to table below for description of errors performed on this run.

Explanation of columns in *Figure 7* and *Figure 8*. 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**

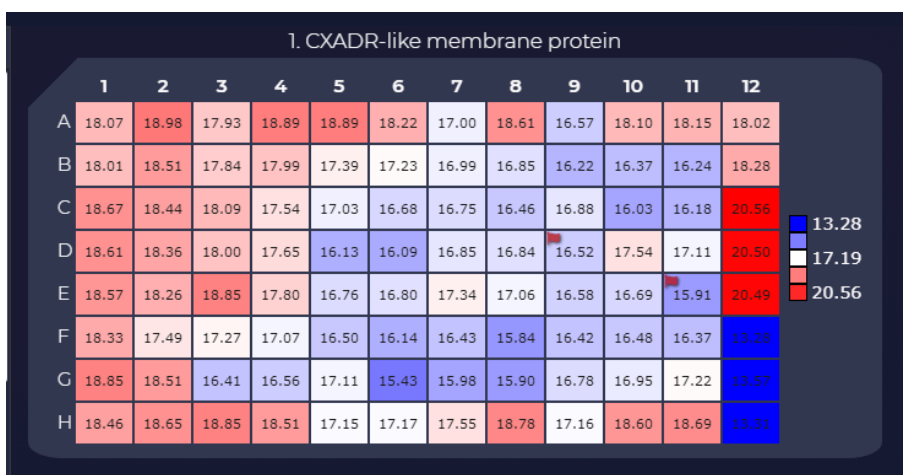Figure 9.   Ct values that show primer contamination of Incubation Control 2.

Figure 10.   Within plate effects due to incorrect or insufficient vortexing of the sample plate during sample dilution.

21.1.4 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.

22.Revision history

Version	Date	Description
1.15	2024-09-11	Changed product name to NPX™ Signature. Updated disclaimer.
1.11	2023-11-21	*6*, flowchart updated. *13.3*, Note added. Updated version number to match software version (version 1.2−1.10 does not exist). Screenshots updated. Editorial changes.
1.1	2023-03-16	New trademarks and disclaimer. Clarifications regarding how data is reported. *15.1*: tables updated. Editorial changes
1.0	2023-02-15	New

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1323, v1.15, 2024-09-11