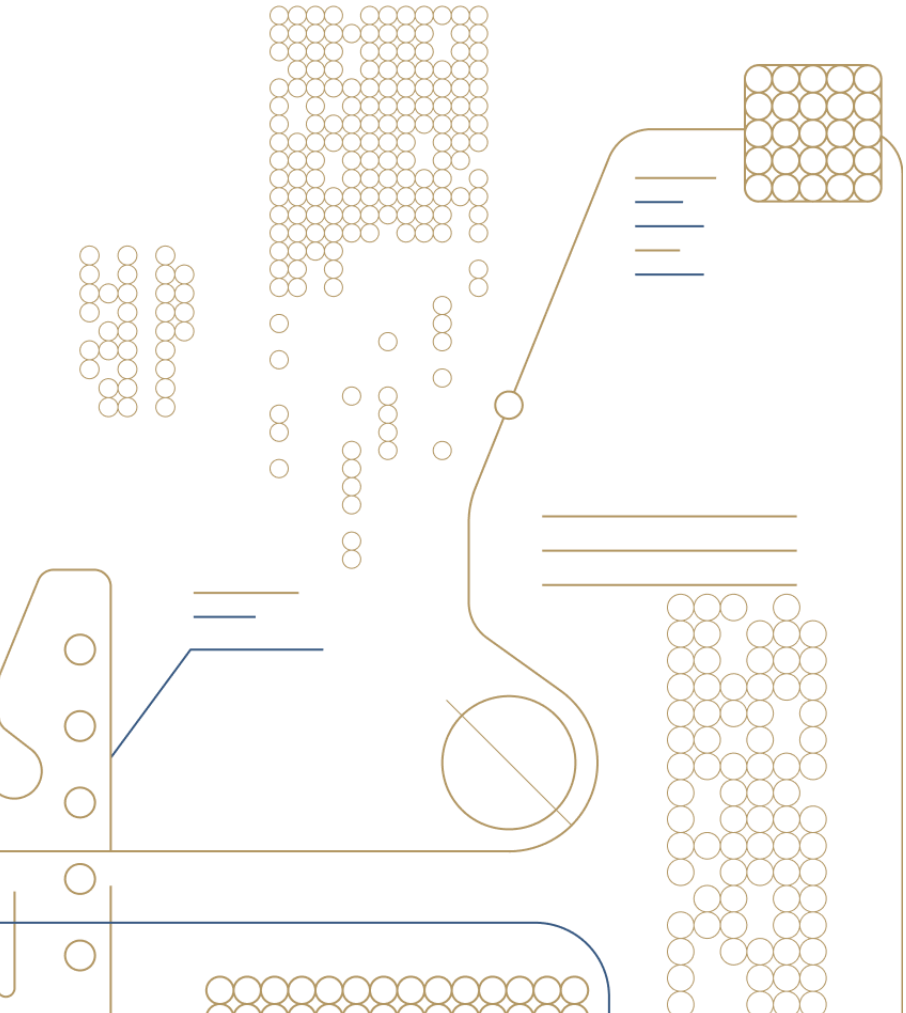
| Nio® Analyzer software V1.5

User manual _MKT-00175 rev E



Technical support

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- **United Kingdom:** lsgtechsupport.uk@bio-rad.com | 00 800 00 24 67 23
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Table of Contents

1. Introduction

Purpose of the document

This document provides detailed information for the use of the Nio® Analyzer software application. The Nio Analyzer software is the data analysis software of the Nio® Digital PCR. It is essential to read the User Manual carefully and pay attention to the safety information provided. The instructions and safety information in the User Manual must be followed to ensure the safe operation of the instrument and to maintain the instrument in a safe condition.

All documents referenced in this User Manual can be accessed here:
https://www.bio-rad.com/fr-fr/product/nio-system?ID=9d6dac6d-4584-a142-2cce-f1b84b48e731

Intended use of the Nio® Analyzer software

The Nio Digital PCR for Crystal Digital PCR® is composed of the Nio Digital PCR instrument, either in its Nio E, Nio or Nio+ configuration, which performs droplet generation and amplification, and enables imaging of the Droplet Crystal in up to 3 to 7 detection channels. The Nio Reader software is used to control and set up Crystal Digital PCR® experiments on the Nio Digital PCR.

The Nio Analyzer software is used to extract data from the images acquired using the Nio Digital PCR and to calculate the absolute concentrations of the targeted nucleic acids.

Both Nio Reader software and Nio Analyzer software are pre-installed on the Nio Digital PCR.

Both Nio software are available in two modes: Standards or Regulatory. The latter enables Nio Digital PCR users (customers) to meet the Food and Drug Administration's regulations on good laboratory practices (GLP) as well as good manufacturing practices (GMP) in pharmaceutical as well as biotechnology industries. Nio Analyzer software Regulatory provides the necessary features to permit the Nio Digital PCR to operate, in compliance with Title 21 of the U.S. Code of Federal Regulations Part 11 (21 CFR Part 11), within a closed system. A closed system is defined as "an environment in which system access is controlled by the persons who are responsible for the content of electronic records that are on the system". The customers choose between Standard or Regulatory mode when installing or updating Nio Analyzer software.

In Regulatory mode, Nio Analyzer software operates in conjunction with naica® Data Service, the latter functions as the Nio Digital PCR user account manager and ensures the compliance of all Nio Digital PCR operations with respect to regulation 21 CFR Part 11. Please refer to the Nio Digital PCR User Manual for detailed instructions of use of naica® Data Service.

Note: The security controls built into Nio Analyzer software must be properly configured and administered by the Nio Digital PCR administrator(s) in the customer's organization to be secure and in compliance with 21 CFR Part 11. Stilla® Technologies makes no claim that Nio Analyzer software is 21 CFR Part 11 compliant in and of itself, nor does the company guarantee compliance for the user. The Nio Digital PCR user organization must establish policies and standard operating procedures (SOPs) that work in conjunction with the tools provided by Stilla® Technologies to ensure compliance with 21 CFR Part 11.

The Nio Digital PCR performs Crystal Digital PCR® within microfluidic chips (Ruby Chip). The naica® PCR MIX reagents are recommended to achieve optimal Crystal Digital PCR® performance on the Nio Digital PCR.

For detailed instructions for the Nio Digital PCR and Nio Reader software, please refer to the corresponding User Manual. For detailed instructions for Ruby Chip, naica® PCR MIX, and naica® multiplex PCR MIX refer to the respective Instruction for Use (IFU).

The Nio Digital PCR is a laboratory instrument to be used by qualified personnel in a controlled environment. Before using the Nio Digital PCR, the user must be trained by a Stilla Technologies representative.

In general, Crystal Digital PCR® can be performed with all types of DNA sample on the Nio Digital PCR. However, individual sample-type compatibility for digital PCR applications may require a dedicated assay validation by the end-user. Please note that the extraction method used, and sample purity can also influence sample compatibility for digital PCR applications.

The Nio Analyzer software is intended for use by professional users trained in molecular biological techniques and in the operation of the Nio Digital PCR and Nio Reader software.

The Nio Analyzer software is part of the Nio Digital PCR.

The Nio Digital PCR is intended for Research Use Only. Not for use in diagnostic procedures.

Citing the Nio Digital PCR in scientific publications, presentations, seminars, etc.

To cite the use of the Nio Digital PCR:

- Crystal Digital PCR® (Stilla Technologies, France)
- Nio® E, Nio® or Nio®+ (Stilla Technologies, France)
- Nio® Digital PCR component names:
  - Nio® E, Nio® or Nio®+ instrument
  - Ruby Chip
  - Nio® Reader software
  - Nio® Analyzer software
- naica® PCR MIX reagents:
  - naica® PCR MIX (Stilla Technologies, France)
  - naica® multiplex PCR MIX (Stilla Technologies, France).

2. Context & Prerequisites

Definition of an experiment

An experiment is defined as a set of samples processed during an individual Crystal Digital PCR® workflow on the Nio Digital PCR:

- The samples of the respective experiment were all processed on Stilla chip consumables (Ruby Chip). Ruby is now commercialized by Bio-Rad under the product name of RDG16.
- The samples of the individual experiment were prepared using the same PCR mastermix.
- The same scanning parameters, embedded files and experimental details will be applied to all samples for an individual experiment during a Nio Digital PCR run, i.e.:
    - the same exposure times (in ms) for each of the 3 to 7 LEDs.
    - Fluorophores and target names.
    - Image Analysis Configuration.
    - Optional:
        - Spillover compensation matrix
        - Plots configuration

Once an experiment is scanned by the Nio Digital PCR, the Nio Reader software automatically records the data in a ".niodata" file. This generated ".niodata" file contains all the chamber images as well as all the contextual and pre-analysis data.

Prerequisites for using Nio Analyzer software

The experiment analysis step can be performed either directly on the Nio Digital PCR or on any other PC which fulfills the following specifications for optimal Nio Analyzer software performance:

- Operating System: Windows 10 or Windows 11 in 64 bits
- RAM: at least 16 GB
- Processors: Intel Core i5 or higher, at least 2 cores of 2 GHz or higher
- Graphical Card: recommended (equivalent to NVIDIA GeForce GT 1030 or higher)
- Screen resolution: at least 1920 x 1080; aspect ratio 16:9

To download the latest version of the Nio Analyzer software to install it on another PC, visit the Technical Resources webpage.

Note: 2 GB of storage is required for software installation.

***Warning:** For optimal performance, Stilla Technologies recommends to perform the analysis of .niodata files with Nio Analyzer software on a personal computer and not on the Nio Digital PCR instrument itself, especially when it is running.*

Installation/update instructions

Installers of the most recent versions of the Nio software are available for download, free of charge, at the following link: https://www.bio-rad.com/fr-fr/product/nio-system?ID=9d6dac6d-4584-a142-2cce-f1b84b48e731

Nio Analyzer software installer guides the user across the installation of either the Standard mode or the Regulatory mode. When launching the installer, the setup wizard pops-up:

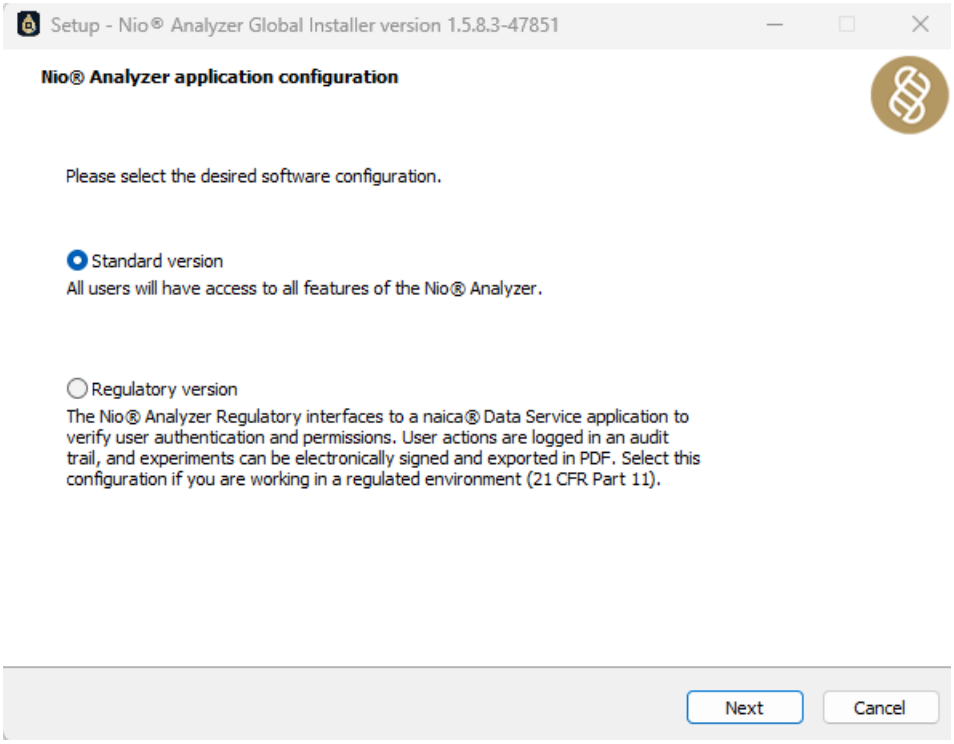
- Launch the Installer
- Allow User Account Control to launch the Installer
- Follow Installer instructions:

At this stage, the user must select to install either the Standard mode or the Regulatory mode:

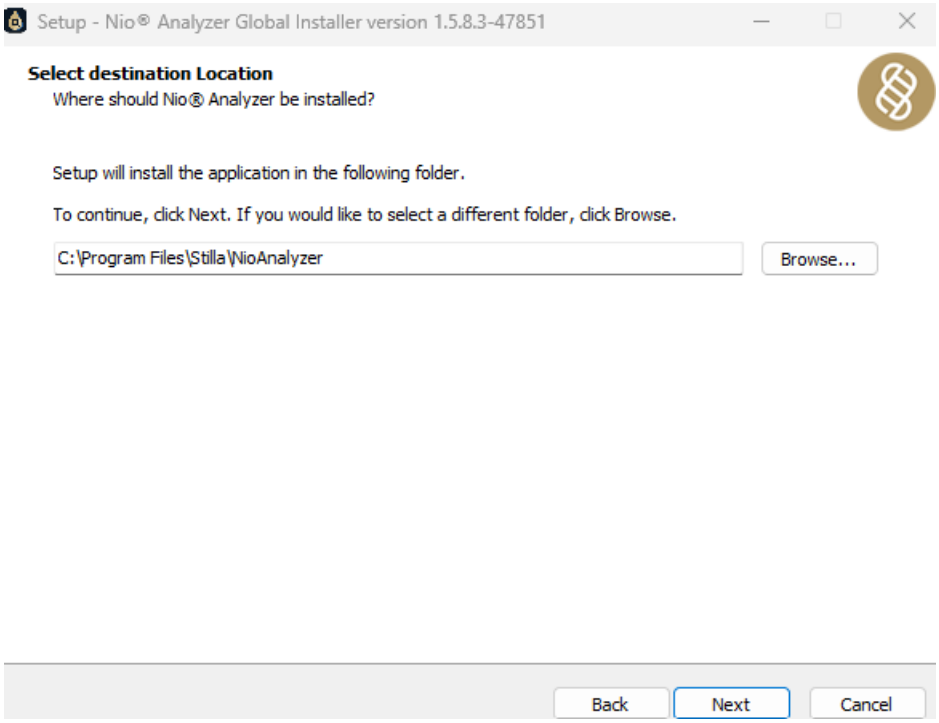
- Standard: For users who do not need to operate in compliance with Title 21 of the U.S. Code of Federal Regulations Part 11 (21 CFR Part 11).
- Regulatory: For users needing to operate in compliance with 21 CFR Part 11.

  Note: If installing the Regulatory mode, Nio Analyzer software functions in conjunction with naica® Data Service. Please refer to the Nio Digital PCR User Manual for detailed instructions on how to install and configure naica® data Service (and Nio Reader software).

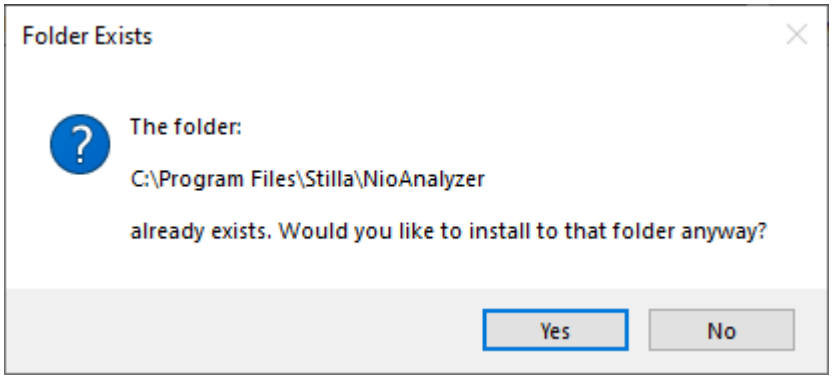
Warning: when installing or updating Nio Analyzer software on a distant personal computer, the installation mode (Standard or Regulatory) should match the one installed on the Nio instrument otherwise files generated from one end will be incompatible on the other end.



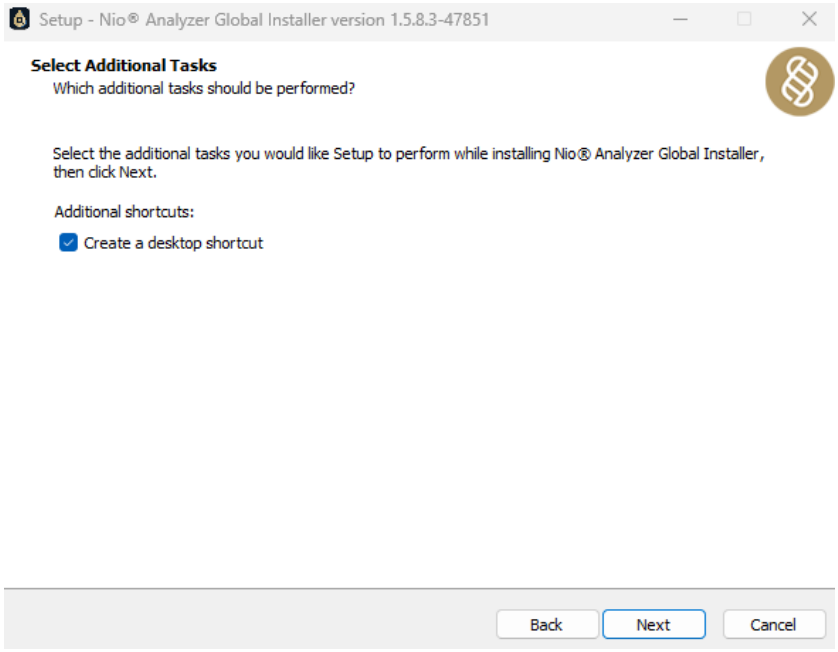
- Click on Next.
- Select an installation folder and click on “Next”



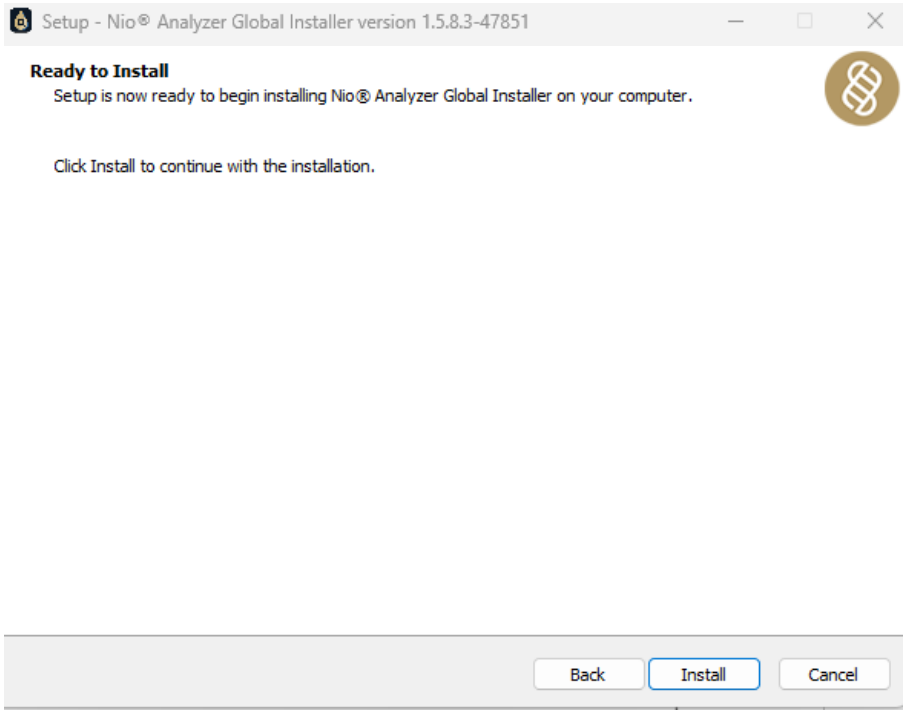
- In case of an update, the following warning popup will appear. Click on “Yes” to confirm.



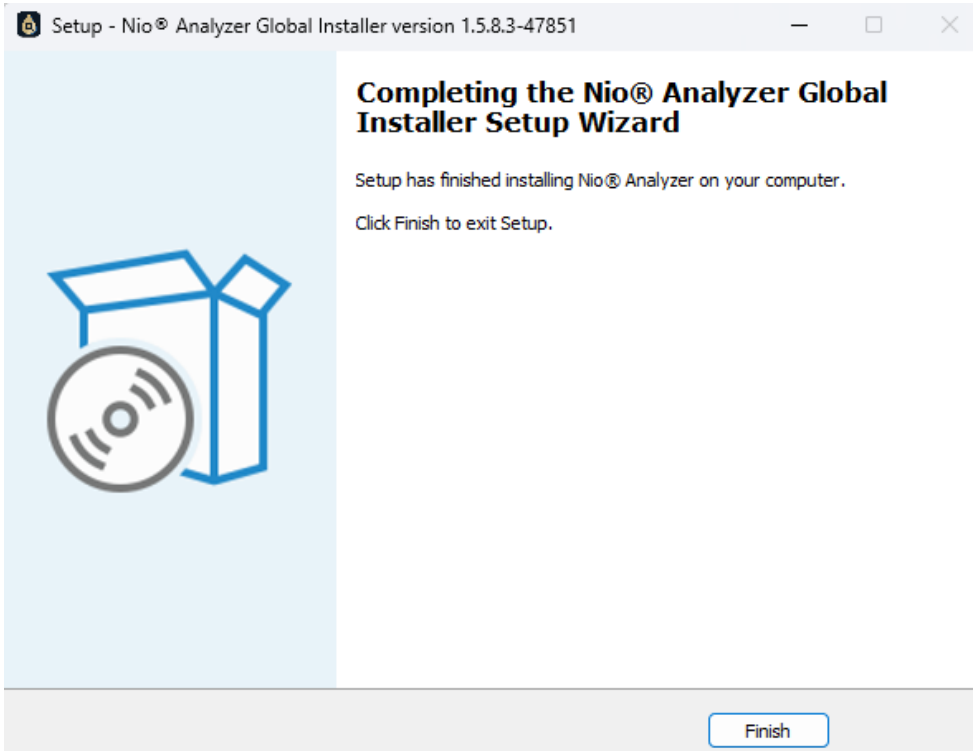
- Select “Create a desktop shortcut” and click on “Next”



- Click on "Install“



- Click on "Finish“



It is not mandatory to restart the computer.

3. Nio Analyzer user interface

Double-click on the desktop icon to launch the Nio Analyzer software application (Figure 1).

***Figure 1**: The launch icon for the Nio Analyzer software*

The different menus on the Nio Analyzer software home screen are illustrated in **Figure 2** below.

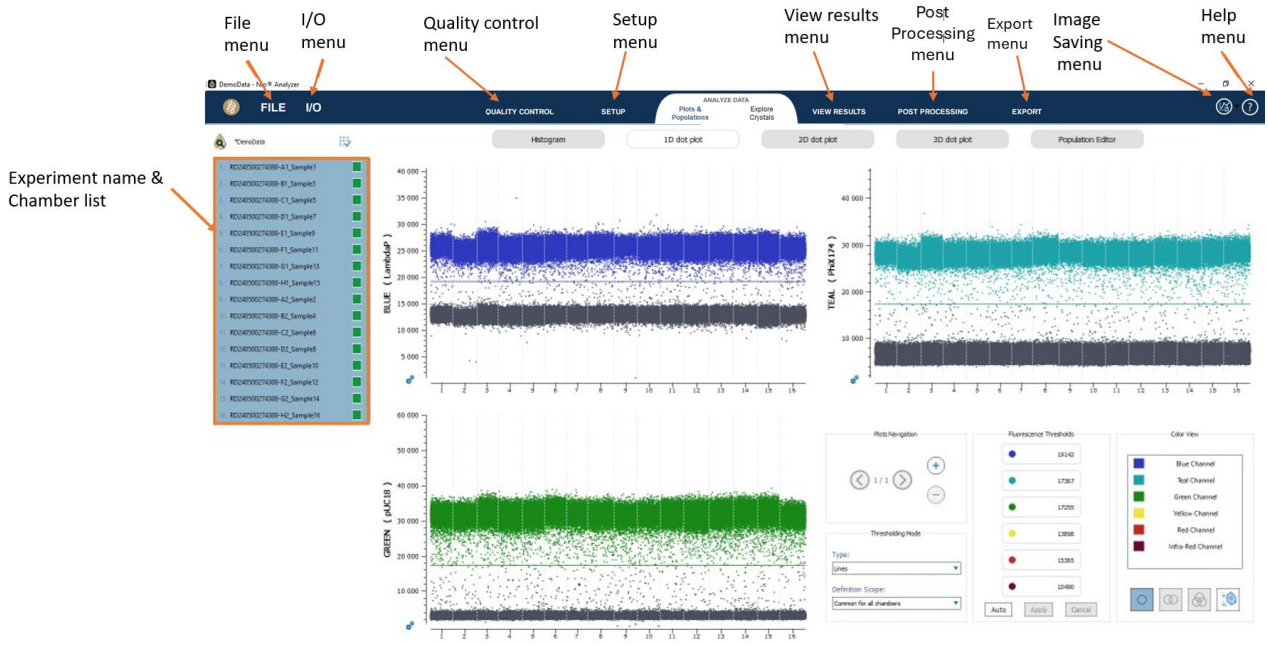


***Figure 2:** Nio Analyzer software menus displayed on the home screen.*

Menus

The purpose of each menu is as follows:

The “FILE” menu:

This menu can be used to open and save experiments, access Home, About menus, and to exit the software.

Figure 3: *Expanded view of the “FILE” menu.*

The “I/O” menu:

This menu can be used to import the different files and templates (“.ncm”, “.ncp”, and “.nca”) used by the Nio Analyzer software.

Figure 4*: Expanded view of the I/O menu.*

The “QUALITY CONTROL” menu:

This menu contains the tools required for the detailed inspection of the Droplet Crystal.

Figure 5: Expanded view of the Quality Control menu.

The display of the « Channel Selection » menu will depend on Nio Digital PCR configuration (between 3-color to 7-color display).

The “SETUP” menu:

This menu is composed of two submenus- the “Edit Experiment” menu, and the “Compensate Spillover” menu. The “Edit Experiment” submenu (**Figure 6**) provides the details about the experiment that have already been entered in the Nio Reader software, and this submenu offers the option to add further details to the experiment. Additionally, this submenu can be used to “Pool” or “Unpool” chambers for higher detection sensitivity. In the “Compensate Spillover” submenu (**Figure 7**), the user can edit the spillover compensation manually.

Figure 6: Expanded view of the “Edit Experiment” submenu under the “SETUP” menu.

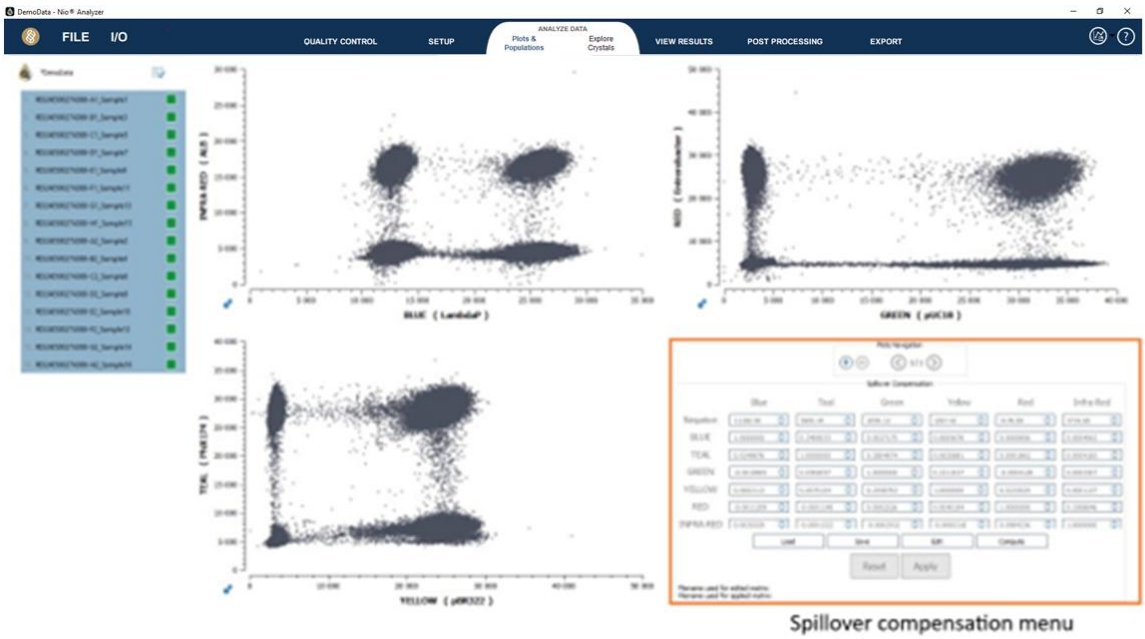


***Figure 7**: Expanded view of the “Compensate Spillover” submenu under the “SETUP” menu.*

The “ANALYZE DATA” menu:

This menu is also composed of two submenus- the “Plots & Populations” submenu and the “Explore Crystal” submenu.

The “Plots & Populations” submenu:

The “Plots & Populations” submenu (**Figure 8**) contains a wide range of tools for editing the experimental data and visualization.

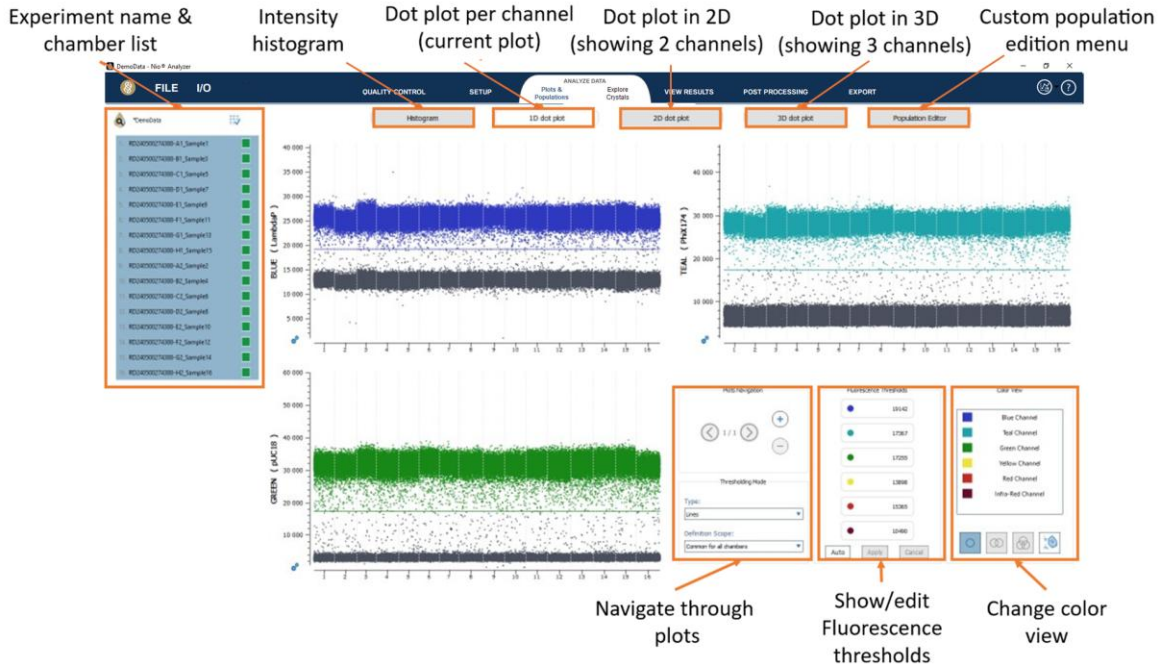


Figure 8: *Expanded view of the “Plots & Populations” submenu under the “ANALYZE DATA” menu.*

The plot navigation widget allows one to easily navigate between more than 3 dot plots, the histogram & population editor. By adding a page using the Plots Navigation “+” icon, a "page" with 3 additional plots with their settings is created. For a 6-plex experiment, for example, it will allow navigating between two pages, each displaying one channel.

Note: The dot plot settings (displayed channel, axis min/max values) are reloaded when navigating between pages.

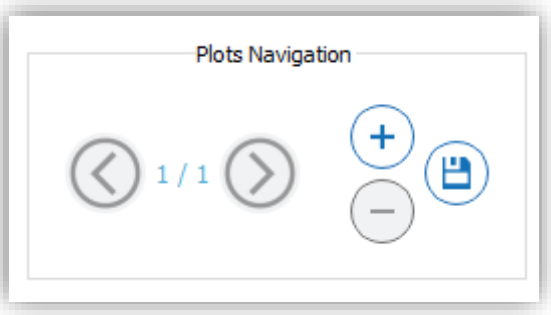


***Figure 9**: Plot navigation between several pages.*

Please refer to the I/O menu (section III.2) to see how to save/load a dot plot configuration file (".ncp"), to be able to use it throughout different experiment files.

The « Explore Crystals » submenu :

This submenu allows users to explore the Droplet Crystal images using several different features such as Color Mode, Channel Selection, Contrast Adjustment, and it allows users to manually select and exclude droplets, if necessary.

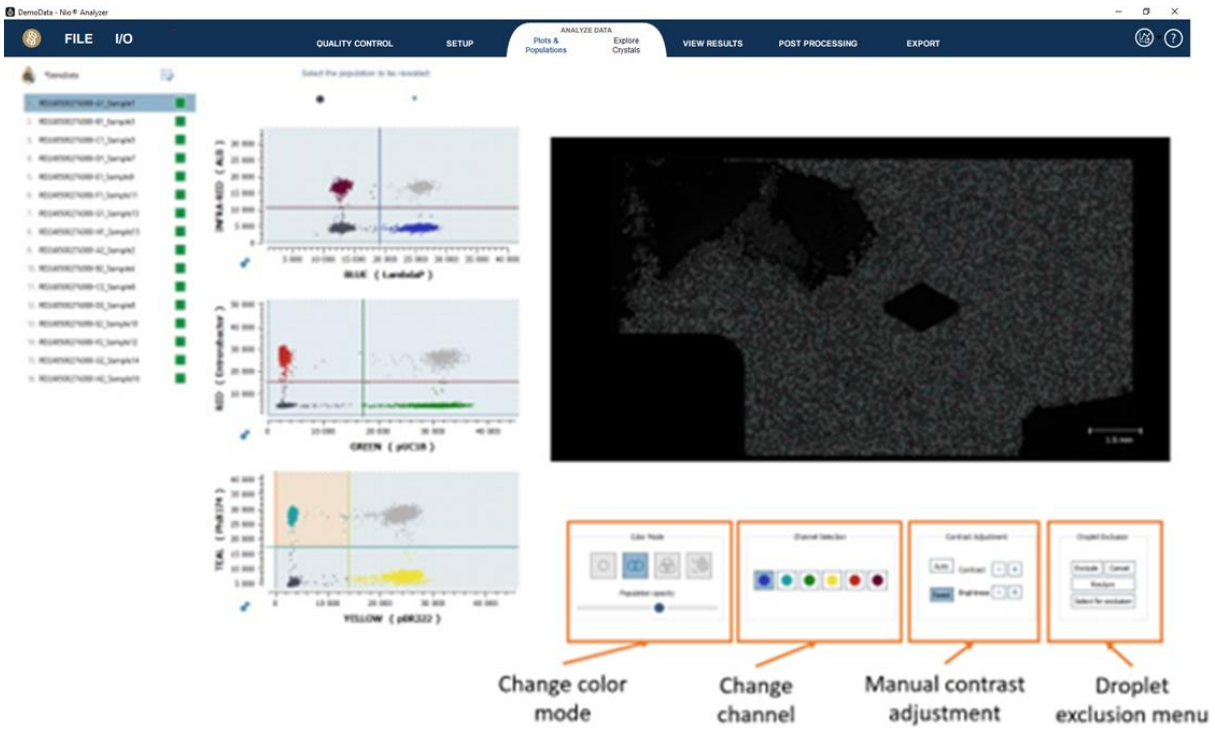


***Figure 10**: Expanded view of the "Explore Crystals" submenu under the "ANALYZE DATA" menu.*

The “VIEW RESULTS” menu:

This menu also consists of two submenus- the “Results Table” submenu, and the “Advanced Graphs” submenu. The “Results Table” submenu (**Figure 11**) contains all the experimental details as well as the experiment result details including the target concentration. On the other hand, the “Advanced graphs” submenu provides advanced result visualization options- the “Concentration Graphs” (**Figure 12**) and the “Uncertainty Curves” (**Figure 13**).

***Figure 11**: Expanded view of the “Results Table” submenu under the “VIEW RESULTS”.*

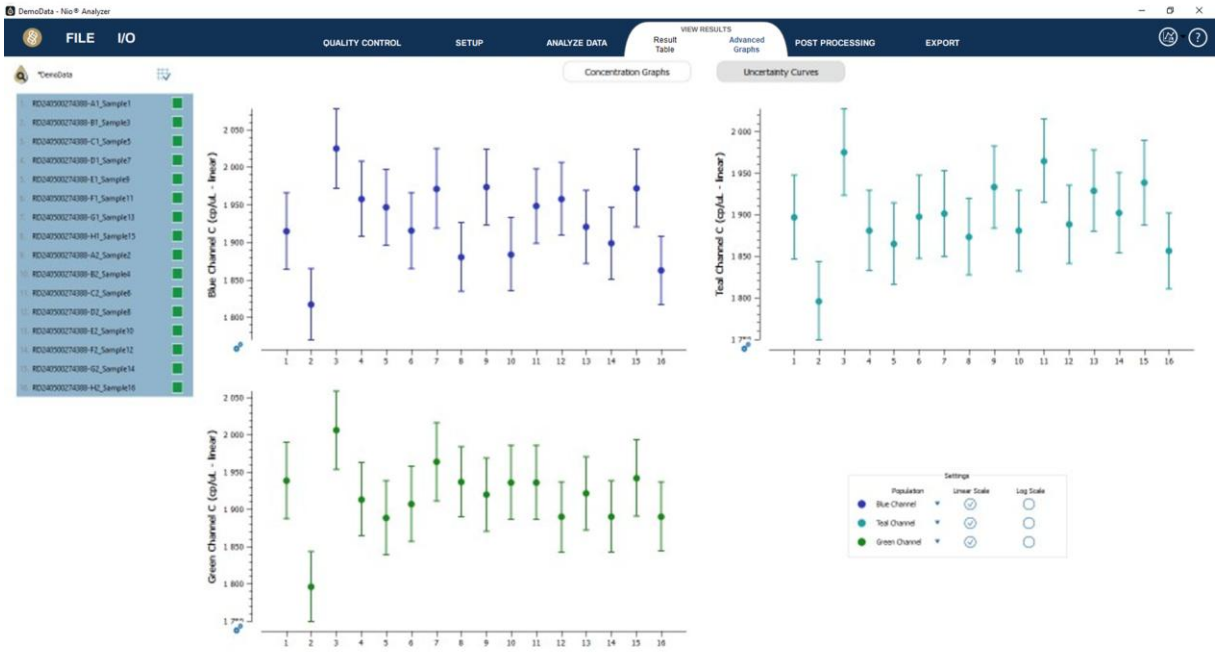


***Figure 12**: Expanded view of the “Advanced Graphs”> “Concentration Graphs” submenu under the “VIEW RESULTS” menu.*

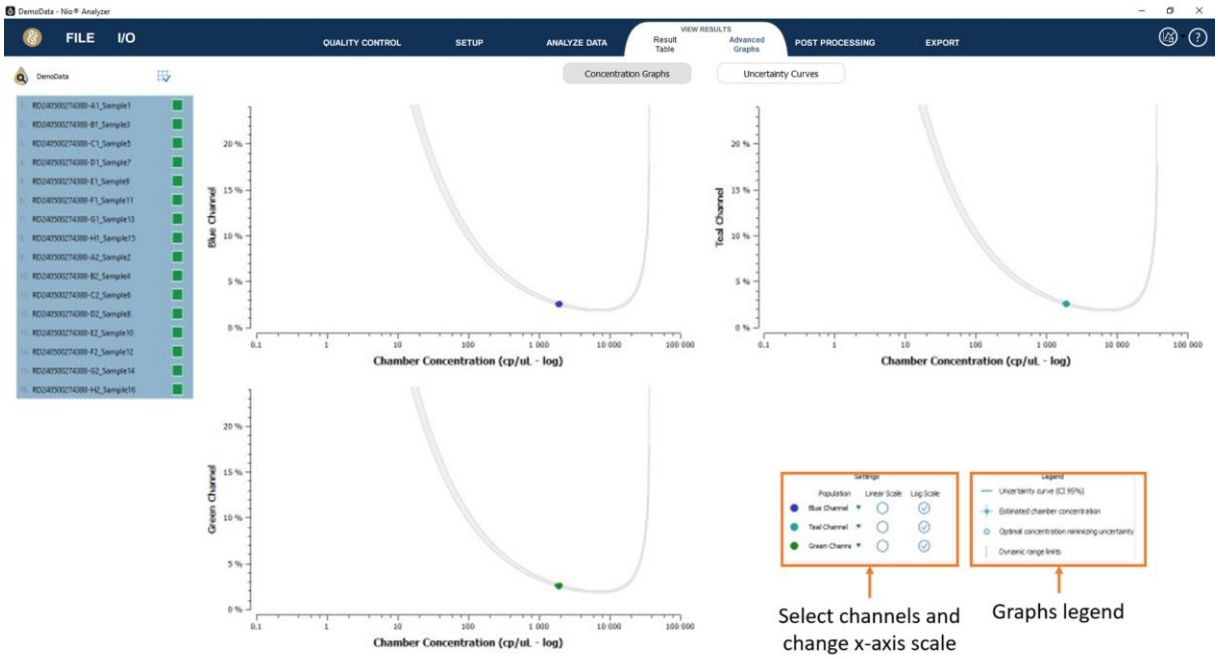


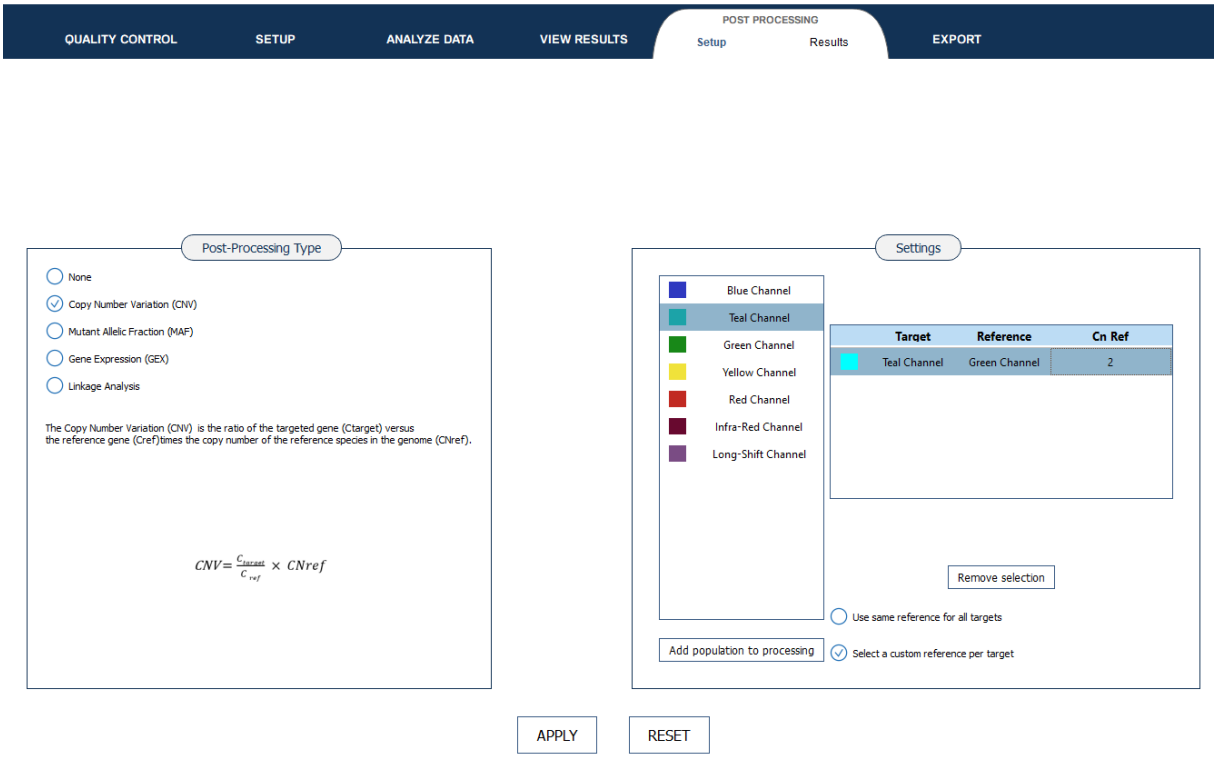
Figure 13: Expanded view of the “Advanced Graphs”> “Uncertainty Curves” submenu under the “VIEW RESULTS” menu.

The “POST PROCESSING” menu:

This menu consists of two sub-menus: the “Setup” sub-menu and the “Results” sub-menu.

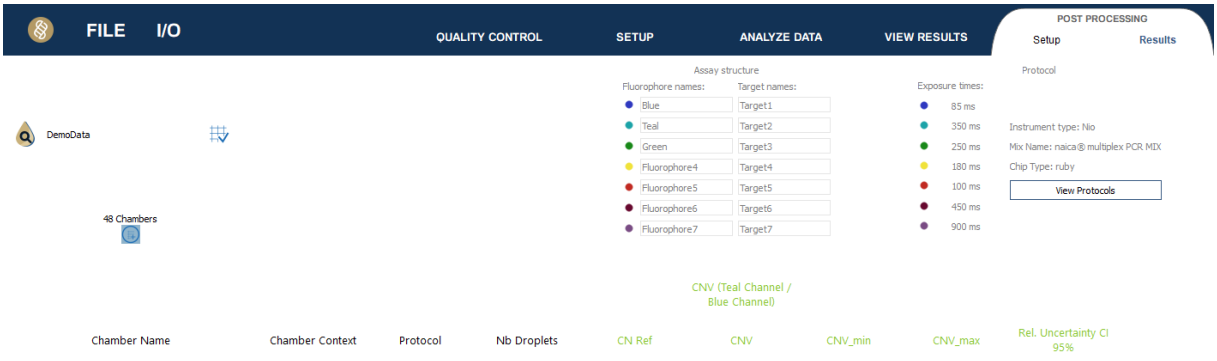
The “Setup” sub-menu allows the user to select the type of post-processing analysis required: Copy Number Variation (CNV) or Mutant Allelic Fraction (MAF) or Gene Expression (GEX) or Linkage Analysis.

Example of the “Setup” sub-menu of the POST PROCESSING menu for CNV analysis:



The “Settings” tab on the right allows the user to specify the reference gene for each type of post-processing assay and for each target population or gene. This can be identical for all target genes, or unique to each target gene.

The “Results table” submenu contains experimental details, as well as the results of the post-processing analysis:



The “EXPORT” menu:

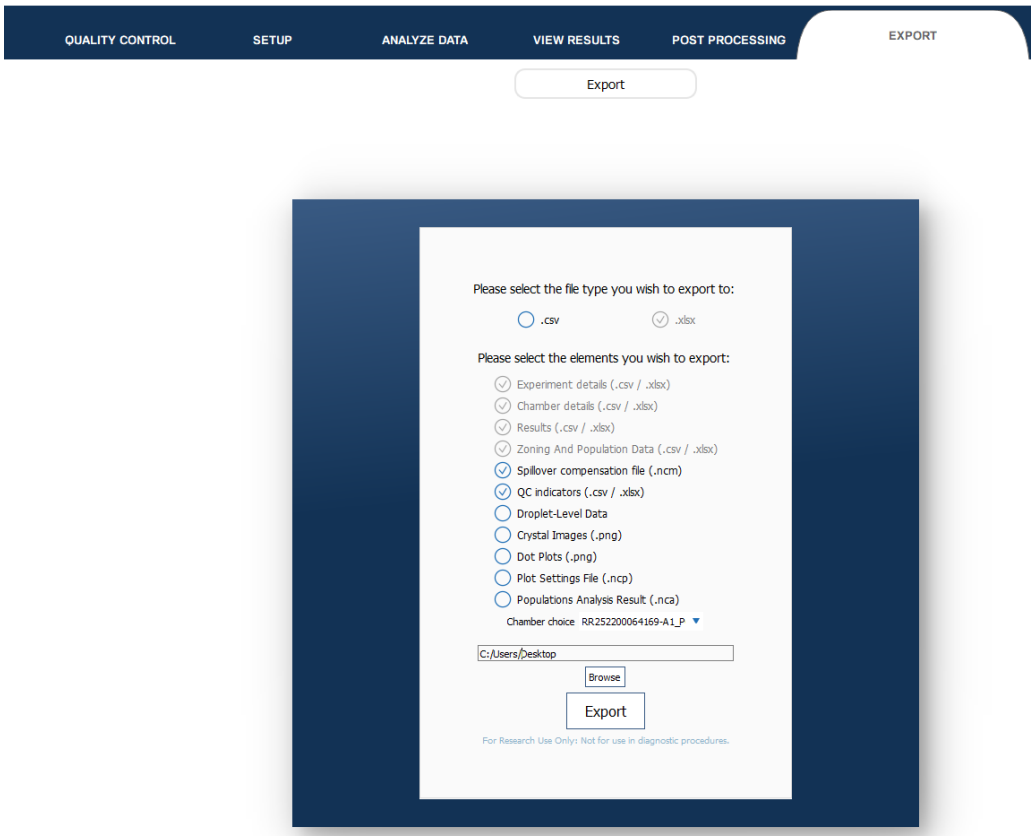


Figure 14: Expanded view of the “EXPORT” menu.

This menu allows the export of experimental data and the selection of the elements required to be exported (**Figure 14**).

The “Image Saving” menu:

This menu allows users to save or copy images or plots from any menu/submenu of the Nio Analyzer software (**Figure 15**).

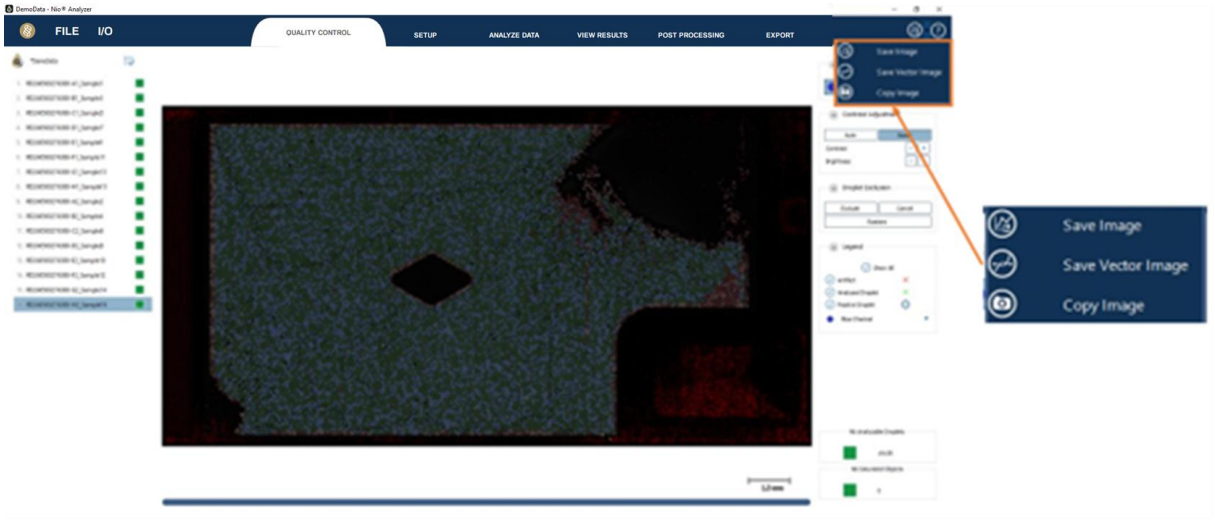


Figure 15: Expanded view of the “Image Saving” menu.

The “Help” menu:

This menu (**Figure 2**) provides help in navigating various submenus such as the “QUALITY CONTROL” menu; the “Compensate Spillover” submenu; the “Plots & Populations” submenu (**Figure 16**), the “Population Editor”; the “Explore Crystals” submenu; and the “Advanced Graphs” submenu.

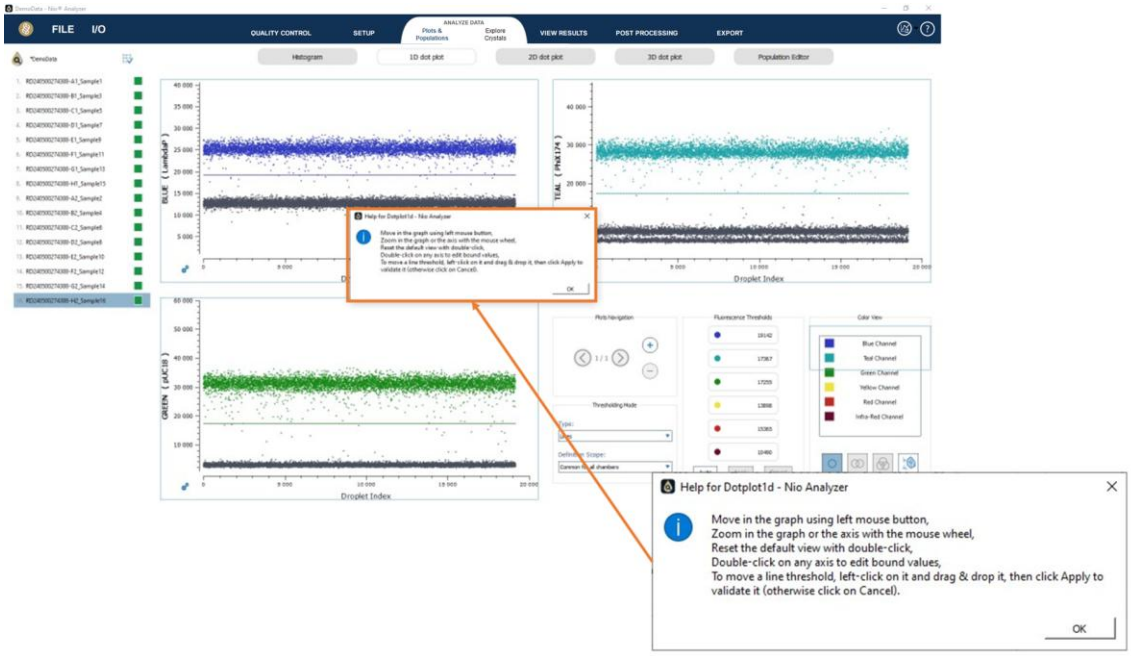


***Figure 16**: The “Help” menu provides detailed help and information about how to navigate in a specific submenu.*

4. Summary of workflow

The 6 main steps of the experiment analysis workflow, using Nio Analyzer software, are as follows:

Load an experiment.

Check the fluorescence spillover compensation.

Check the fluorescence thresholds.

Get the concentration results.

Perform the post-processing analysis.

Export the data.

Step (1) - Load an experiment

At the launch of the Nio Analyzer software, a welcome pop-up with two options appears:

- “EXPERIMENTS”: use this menu to open a new experiment or a saved experiment for data analysis.

“GET STARTED”: this is the resource menu for the Nio Analyzer software to aid new users. It includes a link to the “User Guide”.

To proceed with the “EXPERIMENTS” menu and to load an experiment, click on the “Open” button and select the experiment file to load. It is also possible to drag and drop a “.nioresult” file or a “.niodata” file in the Nio Analyzer software application window to load it.

***Figure 17**: Load an experiment.*

Once the experiment is loaded, the “ANALYZE DATA” > “Plots and Populations” menu is displayed. By default, the 1D dot plots, concatenated for all the chambers of the experiment, are shown:

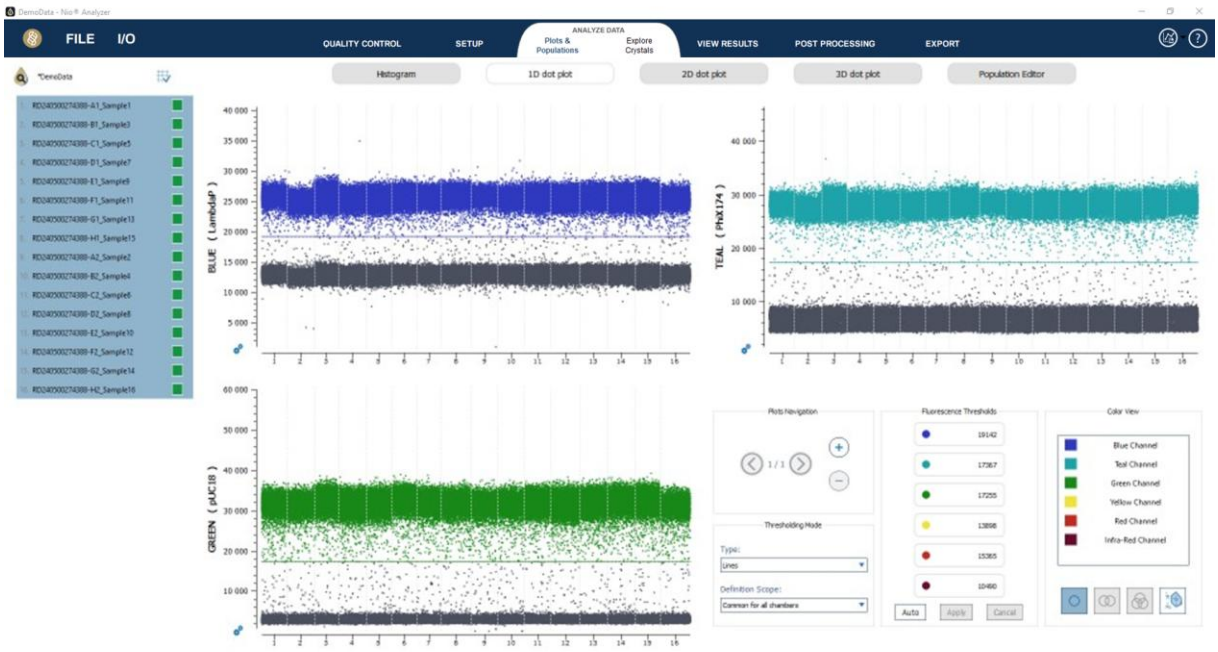


Figure* 18: Default view: concatenated 1D dot plots of all the chambers of a 6-plex experiment (sequential view).*

Input file format options

The input file format can be either a ".niodata", ".nioresult", a ".ncp" or a ".nca"

The ".niodata" *format*:

The "Nio Data" format (".niodata") is the original file generated by the Nio Reader software. The ".niodata" file contains the original high-quality images required for the image analysis process.

For an experiment that is analyzed for the first time, the ".niodata" file format is used to load an experiment.

Note: The ".niodata" files must be edited using the Nio Analyzer software

The ".nioresult" format:

The "Nio Result" format (".nioresult") is the post-analysis file generated by the Nio Reader software from the original ".niodata" file. ".nioresult" files are smaller than their original ".niodata" files.

The indicative sizes for the individual ".nioresult" files are: 1.0-1.2GB (for a 3-chips experiment)

For an experiment that was already previously analyzed, the ".nioresult" file format can be used to load an experiment as an alternative to a ".niodata" file.

Note: The ".nioresult" files must be edited using the Nio Analyzer software

Optional file import/export formats in the "I/O" menu:

- ".ncp" format: The "naica Crystal Plot Configuration" format (".ncp") is a file including the Histogram, 1D, and 2D dot plot configurations. The configuration includes for each plot, the axis labels, the scale type (linear or logarithm) when applicable and the plot page sequence.
- ".nca" format: The "naica Crystal Analysis" format (".nca") file includes the information for all data analysis parameters:
  - manual or automated thresholding strategy
  - thresholding mode and scope
  - list of the populations (default or custom) with their name, color, and zones
  - coordinates defining each zone (i.e., threshold values for lines or vertex coordinates for polygons).

Additional file formats

".ncm" format: The "Naica Compensation Matrix" format (".ncm") is a file containing the compensation parameters applied to the signal of each channel. This file is specific to the assay and assay conditions, but it is independent of exposure time.

Experiment status

The name of the loaded experiment is displayed at the top left side of the application window. The displayed experiment name is the name of the experiment file.

There are 3 experiment statuses displayed next to the experiment name:

- Modification status: if the character is displayed just before the experiment name, it means that the experiment has unsaved changes.
- In absence of the character, the experiment has not been either modified, or the last modification has been saved.
- Compensation status: if an icon showing a "validated grid" is displayed just after the experiment name, it means that a fluorescence spillover compensation has been applied (if a compensation file has been loaded in the Nio Reader software), otherwise, it means that the current experiment is not compensated for fluorescence spillover.

Chamber list

All the chambers of the experiment are listed in the left side panel (**Figure 18:** Default view: concatenated 1D dot plots of all the chambers of a 6-plex experiment (sequential view).). The chamber name details should be entered in the Nio Reader software. However, if the Sample name needs to be edited in the Nio Analyzer software, it can be edited in the "SETUP" menu > "Edit Experiment" submenu and by double-clicking on the respective sample name field. Each chamber is named using the following convention:

< Chip ID >_< Chamber ID >_< Sample Name >

Where the values are as follows:

- *"Chip ID"* is the unique barcode of the chip to which the chamber belongs. This information should have been previously filled by the Nio Digital PCR in the Nio Reader software as the Chip Barcode.
- *"Chamber ID"* is the position of the chamber in the chip (A, B, C, etc. from top to bottom) followed by the column index of the chamber on the chip (1 or 2 from left to right). This information is automatically defined by the software (e.g., "A1" or "D2").
- "Sample Name" is the name of the sample in the chamber.

CAUTION!

All the chambers of an experiment should share the same scanning protocol and the same assay, otherwise the experiment should be divided into smaller experiments including subsets of chambers (for this purpose, the "Save as" button in the "FILE" menu and the chamber removal icon "-", above the chamber list in the "Edit Experiment" submenu, can be used).

Chamber Quality Flag

A chamber quality flag is displayed next to each chamber's name on the top left side of the application window. This chamber quality flag provides visual feedback for chamber quality control. The color of the quality flag depends on quality indicators such as the presence of saturated objects and the number of analyzable droplets in the chamber:

- "Number of analyzable droplets": the higher the number the better the confidence in predicted concentration results.
- "Number of saturated objects": the lower the number the better the chamber quality. Note: Saturated objects are either saturated droplets caused by a high exposure time or saturated artifacts (this can be corrected when using Nio Reader software by checking that exposure times are not too high, or if the chip should be cleaned using an appropriate wipe to remove dust or fluorescent elements on the foil -- Please check Nio Digital PCR User Manual for instructions).

Each indicator can be either green, yellow or green with an eye icon inside.

A green flag indicates that all quality indicators are within expected specifications.

A green flag with an eye icon on it means that Stilla Technologies recommends inspecting visually the droplet crystals to decide whether the result should be taken into account. Refer to the Ruby Chip troubleshooting section of the Nio Digital PCR User Manual for more details.

A yellow flag indicates that there is either too many saturated artefacts on the picture or there are less than 1000 detected droplets in the chamber. When saturated artefacts are caused by dust particles, Stilla Technologies recommends cleaning the bottom of the Ruby Chip with a dust free tissue and read the chambers again (refer to the Nio Digital PCR User Manuel for detailed instructions).

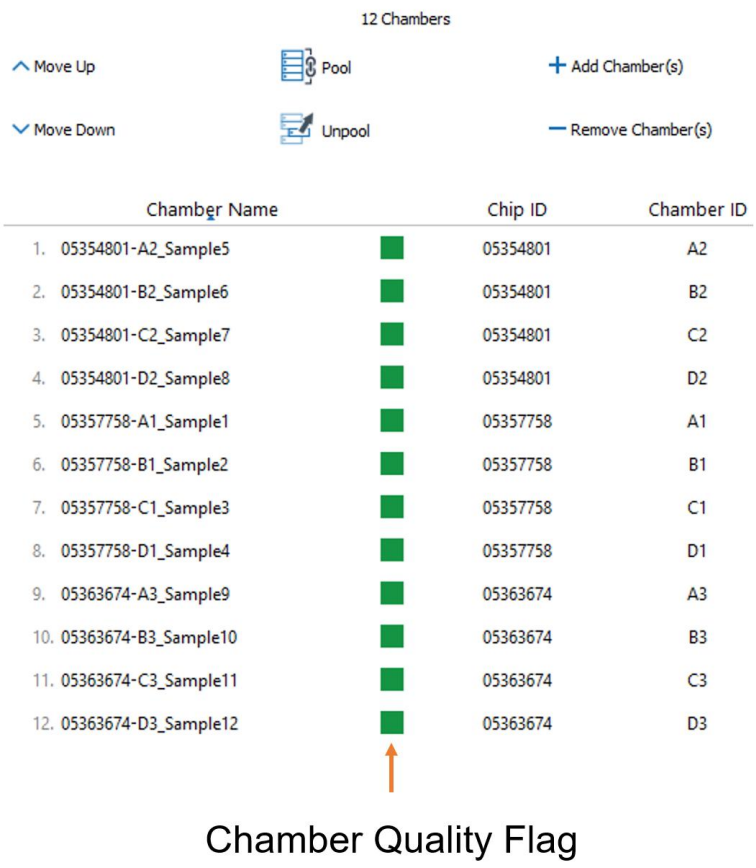


Figure 19: *Example of chamber quality flag displays in Nio Analyzer software.*

For more details about Quality Control, see the section "How to perform quality control?".

Data visualization and interpretation

To visualize the graphs generated by Nio Analyzer software, click on the "ANALYZE DATA" > "Plots & Populations" submenu, then click on one of the widgets: "1D dot plot", "2D dot plot", or "3D dot plot".

The unit of the fluorescence axis in the dot plots is RFU (Relative Fluorescence Unit). Each point in the dot plot graph represents a particular droplet in the selected crystal. If the fluorescence of the droplet in each channel is higher than the fluorescence threshold defined for this channel, then the droplet is considered "positive", otherwise it is considered "negative".

3-color experiment

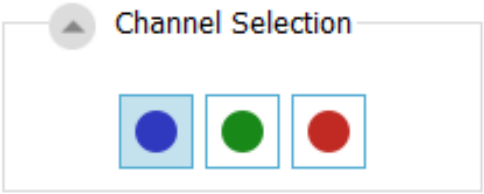


Figure 20A: *The Channel Selection available for 3-color experiment and up to 7-color experiment (Blue, Teal, Green, Yellow, Red, Infra-Red, Purple) in Nio Analyzer software.*

As each droplet may be negative or positive in each of the 3 channels ("Blue", "Green", and "Red" channels), there are 2^3 (8) possible classes for a Crystal Droplet. The Nio Analyzer software application uses the colors below for each of the droplet

classes. To support 3-color experiments with an easy-to-understand multiplex mode, the Nio Analyzer software uses the following rosace (**Figure 21**) with specific color codes that help the user to understand the classes (of positivity combinations) of the droplets. This mode only works for the Red-Blue-Green combination.

7-color experiment

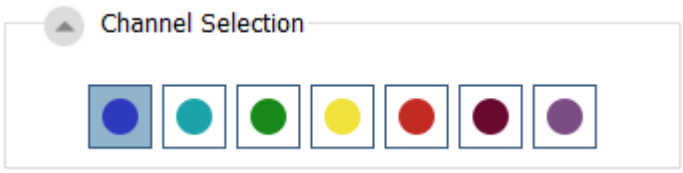


Figure 20B: *The Channel Selection available for 3-color experiment and up to 7-color experiment (Blue, Teal, Green, Yellow, Red, Infra-Red, Purple) in Nio Analyzer software.*

As each droplet may be negative or positive in each of the 7 channels (“Blue”, “Teal”, “Green”, “Yellow”, “Red”, “Infra-Red” and “Purple” channels), there are 2^7 = 128 possible classes for a Crystal Droplet.

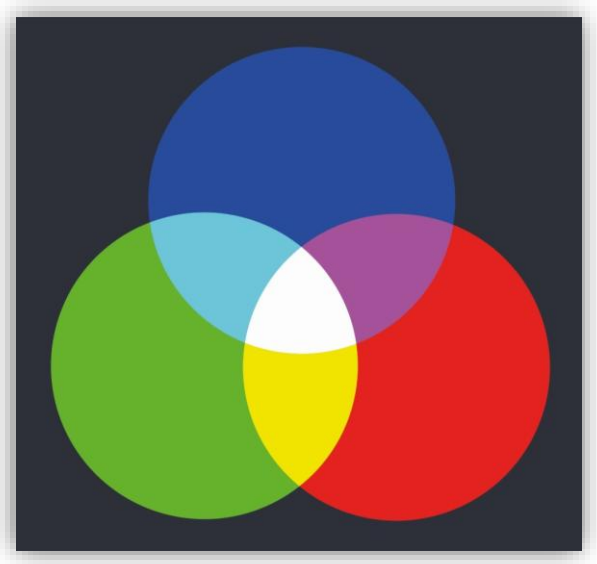


Figure 21: *The 8-color diagram used for 3D data visualization in Nio Analyzer software (the background of this diagram corresponds to the black/dark grey color, which is negative in across channels).*

The number of classes is 2^3=8 possibilities, each represented by the following colors:

1. Black/dark gray: negative in “Blue”, negative in “Green”, negative in “Red”.
2. Blue: positive in “Blue”, negative in “Green”, negative in “Red”.
3. Green: negative in “Blue”, positive in “Green”, negative in “Red”.
4. Red: negative in “Blue”, negative in “Green”, positive in “Red”.
5. Cyan: positive in “Blue”, positive in “Green”, negative in “Red”.
6. Yellow: negative in “Blue”, positive in “Green”, positive in “Red”.

7. Magenta: positive in “Blue”, negative in “Green”, positive in “Red”.
8. White/Light grey: positive in “Blue”, positive in “Green”, positive in “Red”.

Along with the dot plots, the following color modes are available and will affect the color used to draw the droplets in the dot plots:

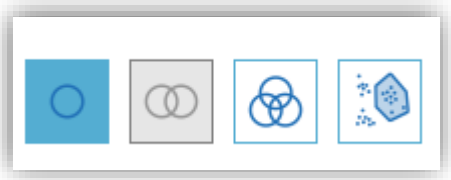


Figure 22: *The coloring modes available in the “Plots & Populations” submenu.*

To visualize in simplified color mode, go to the “Color View” widget and click on:

- The icon representing a single circle in the “1D dot plot” tab.

Figure 23: *Icon for visualization mode in 2 colors.*

This color mode displays the droplets, in the color of the considered channel, if their fluorescence value is higher than the channel threshold, or in grey otherwise. No information is given about the droplet positivity in the other channels. This color mode is available for all experiments from 3 to 7 colors.

- The icon representing two overlapping circles in the “2D dot plot” widget.

Figure 24: *Icon for visualization mode in 4 colors.*

This 2D dot plot color mode is the same as the 1D dot plots.

Note: For 3-color (RGB) experiments, the double-positive droplets are displayed in the color of the intersection on the rosace diagram. For experiments using other color combinations, the double positives appear in light grey; the double negative droplets are displayed in dark grey.

To visualize in 8 color-mode, click on the icon representing 3 overlapping circles in the “Color view” widget. This color mode is only available for the 3-color RGB experiments.

Figure 25: *Icon for visualization mode in 8 colors for a triplex experiment.*

RGB mode provides the different colors for single-, double-, or triple-positive droplets, according to the corresponding color code in the rosace diagram.

To visualize the droplets that are included in a population, in either a 1D or 2D dot plot, you can use the Color view widget in the bottom right-hand corner and click on Population Selection.

Figure 26: *Icon for visualization mode "Population Selection".*

The 1D or 2D dot plots in the "Population Selection" color mode, by default, provide a color mode for each channel, to help visualize the negative and positive droplets for the populations defined in the specific channel. This mode allows the user to distinguish between different populations or combinations of populations. For example, if the user is viewing the Teal channel, all positive droplets are displayed in teal within the Teal channel dot plots. All the other populations of droplets that are not positive in Teal will be grey.

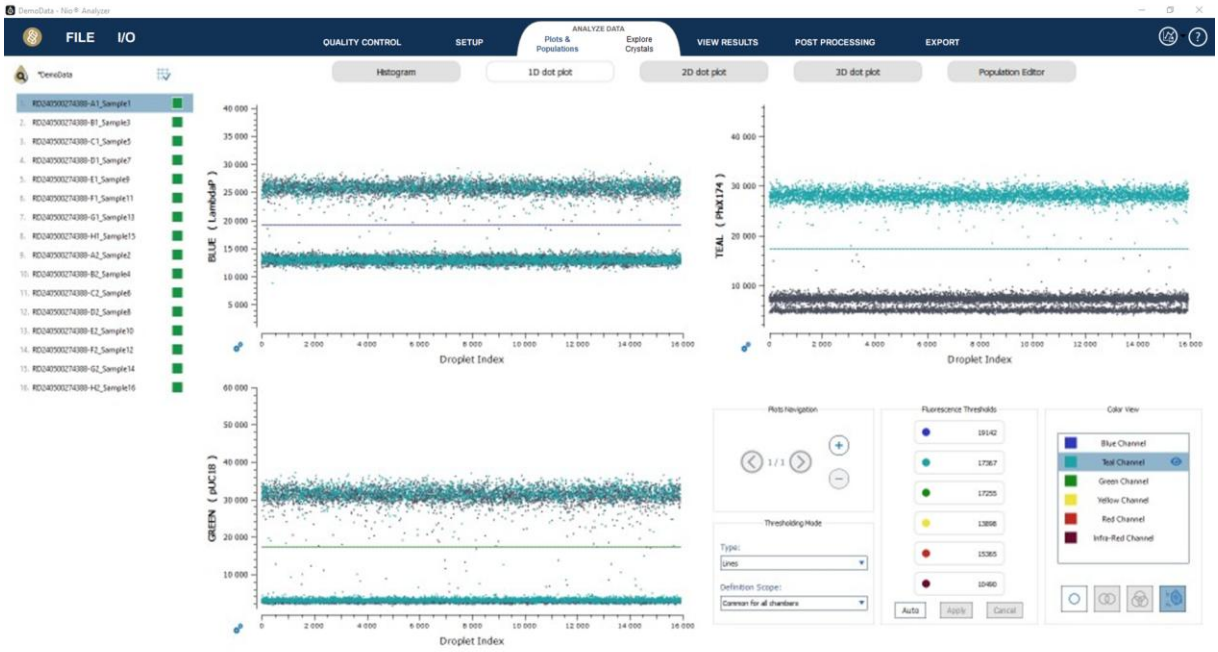


Figure 27: *Positive droplets displayed in teal in the 1D dot plot using the "Population Selection" color mode.*

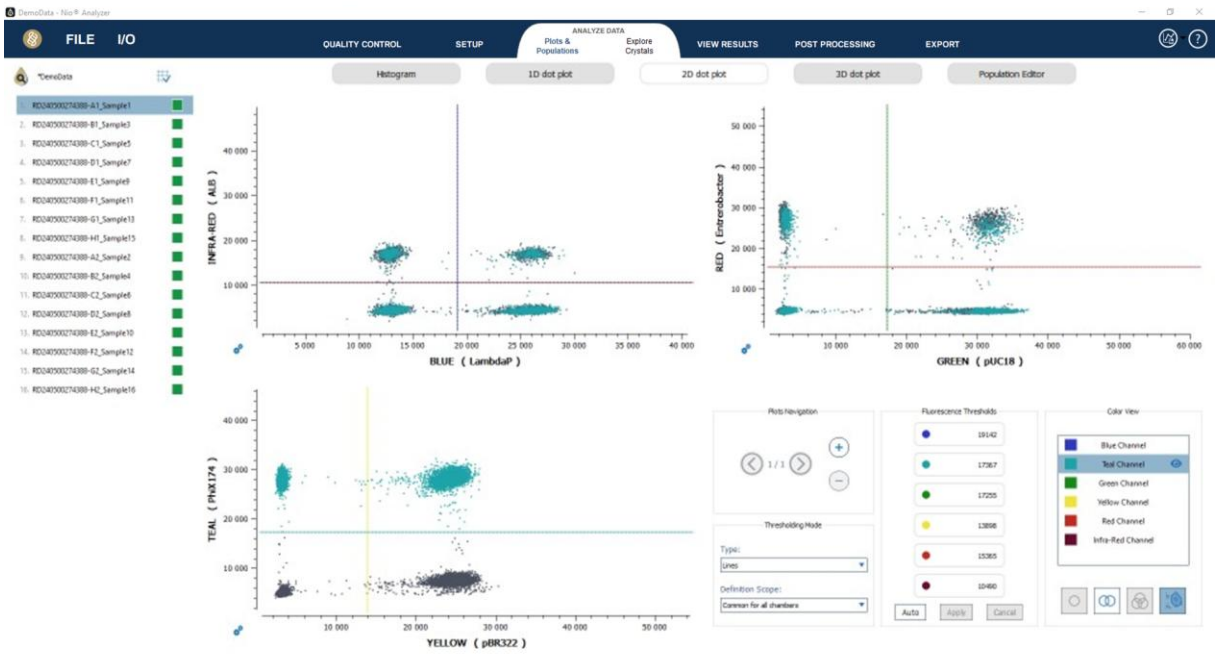


Figure 28: *Positive droplets displayed in yellow in the 2D dot plot using the "Population Selection" color mode.*

- In any given 1D graph, the vertical axis shows the fluorescence value of the droplets in the detection channel of interest ("Blue", "Teal", "Green", "Yellow", "Red", "Infra-Red" and "Purple" channel) and the horizontal axis shows the droplet index in the crystal, if only one chamber is selected, or the chamber names, if multiple chambers are selected. If more than three channels have signal, use the "+" and the arrow icons in the "Plots Navigation" panel to navigate between the different 1D graphs.

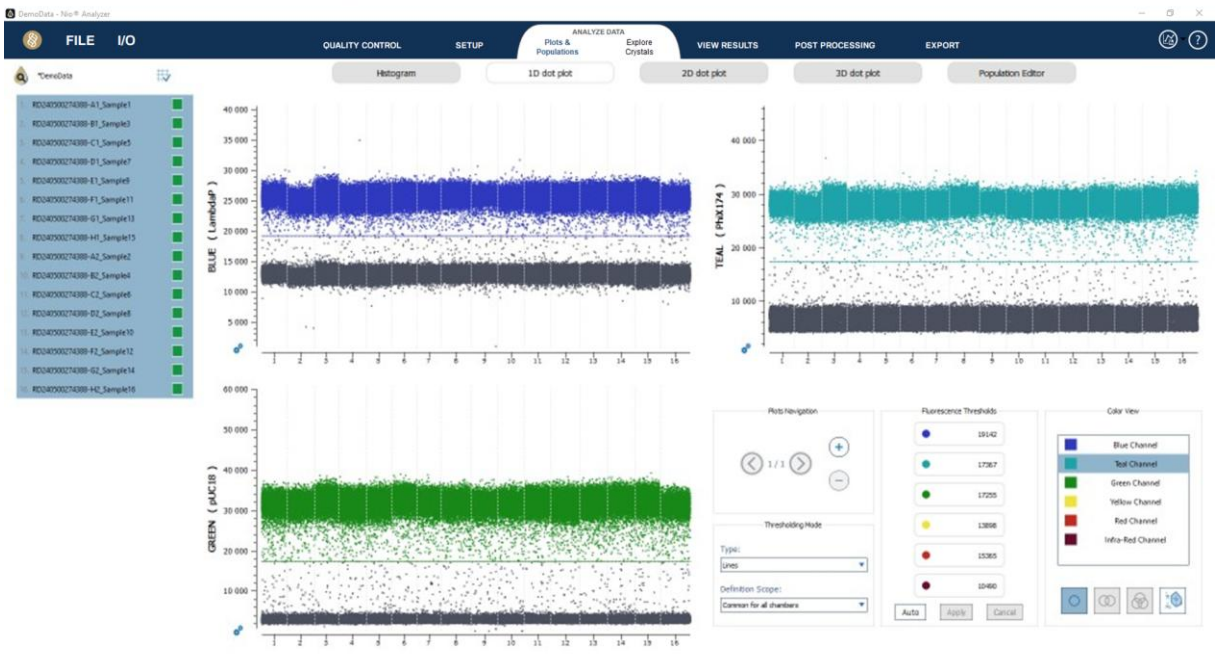


Figure 29*: 1D dot plots in simplified colors for analysis of a 7-plex experiment.*

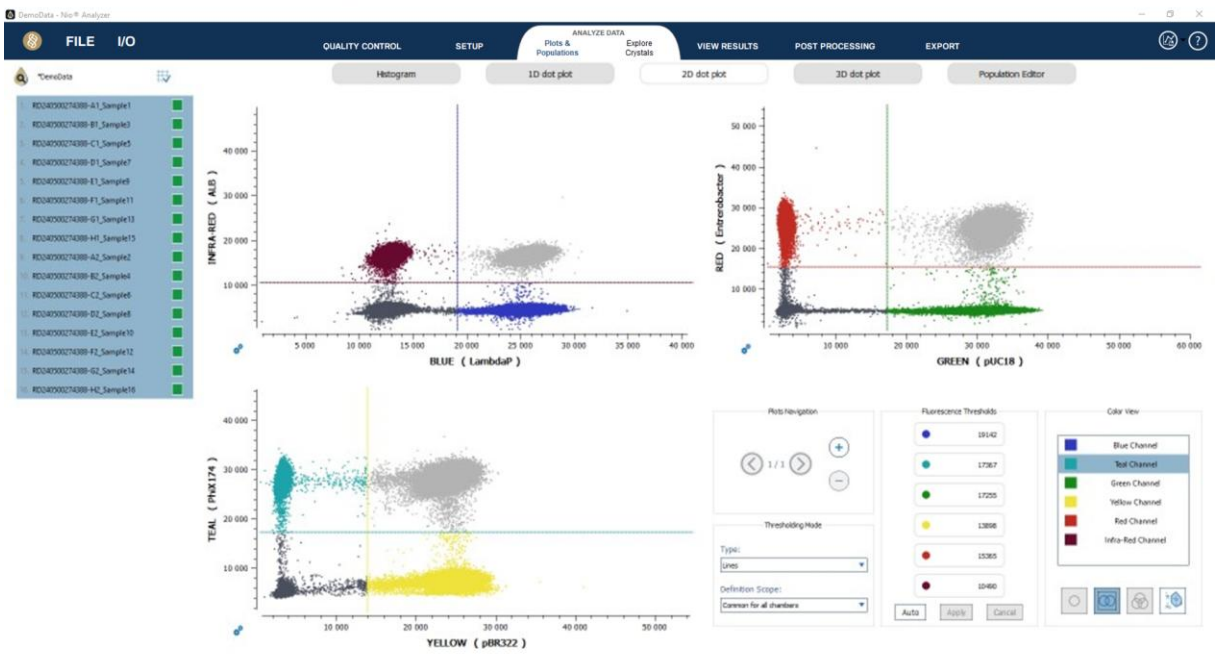


Figure 30: 2D dot plots in simplified colors.

- In any given 2D graph, the vertical axis displays the fluorescence value of the droplets in one channel of interest and the horizontal axis displays the fluorescence value of the droplets in another channel of interest.
- Customizing a graph:
  - Scroll mouse to zoom in or out in the graph.
  - Use Left-Click to move the graph.
  - Double Left-Click inside the graph to reset the view.
  - When applicable, double-click on any axis to edit the graph settings (for example min and max limits, dot size, or dot opacity).

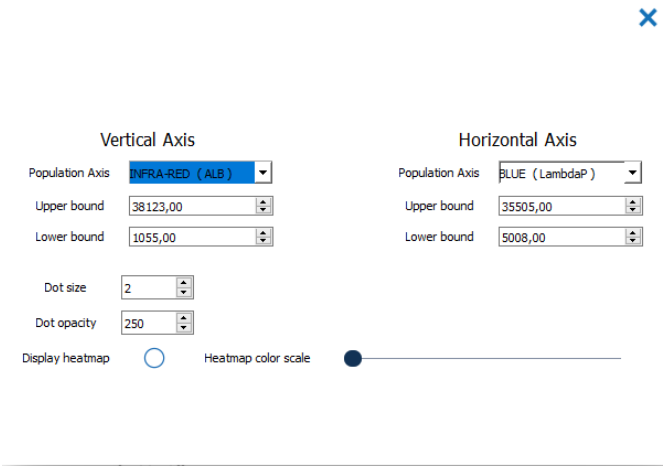


Figure 31: *Editing graph axis parameters.*

Step (2) - Check the fluorescence spillover compensation

When using different types of fluorophores on the Nio Digital PCR, absorption/emission spectra can overlap between two or more color detection channels. For example, if using two probes, one labeled with the RED fluorophore and the other labeled with the INFRA-RED fluorophore, the INFRA-RED signal will be detected partially in the RED signal, altering the RED image result. This phenomenon of spectral overlap is called fluorescence “spillover”, and in this example the INFRA-RED signal spills over to the RED signal.

To compensate for a spillover signal to obtain the results, users must create and then apply a compensation matrix for each assay.

A compensation matrix can be automatically or manually created using the Nio Analyzer software. A compensation matrix corresponds to specific thermocycling conditions and to a specific multiplex assay composed of oligonucleotides (probes and primers) and targets. If any change to primers, probes or targets is made (for example, a change in primer or probe sequence or concentration) or to the thermocycling program, a new matrix should be created. If no changes are made to an assay and to the thermocycling conditions, the same compensation matrix can be saved and repeatedly applied.

To save a compensation matrix, use the “EXPORT” menu to save it as a “.ncm” file or by using the “I/O” menu.

Once the compensation matrix is applied to the data, the separability between each cluster should be improved.

To compensate the fluorescence spillover, click on the menu “SETUP” > “Compensate Spillover”.

Depending on the assay and the number of color detection channels, the Nio Analyzer software will display 2D dot plots (**Figure 32** for 6-plex):

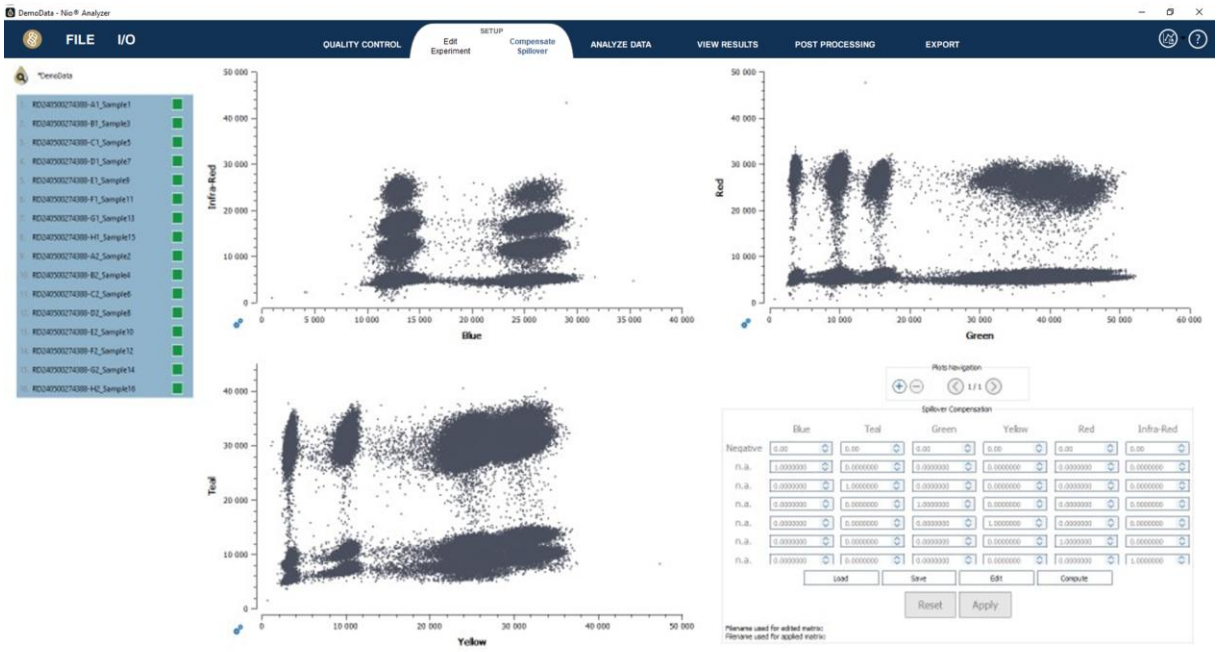


Figure 32: *Non-compensated data in the Nio Analyzer "Compensate Spillover" submenu, for a 6-plex detection.*

The compensation matrix includes:

- n color channels to be used for the assay (e.g.. "Blue, Green, Red, etc.").
- n fluorophores corresponding to each channel and a negative fluorescent cluster of droplets, defined as negative by the threshold line (for example, "Negative to Infra-Red" in **Figure 33**).

A 3-plex compensation matrix has 3 color channel indications, 3 fluorophore indications and 1 negative indication; a 7-plex matrix has 7 color channels, 7 fluorophores and 1 negative indication.

If changing the fluorophore or channel indication labels of the matrix is required, this can be performed in the "SETUP" > "Edit Experiment" submenu (please refer to How to edit the experiment?)

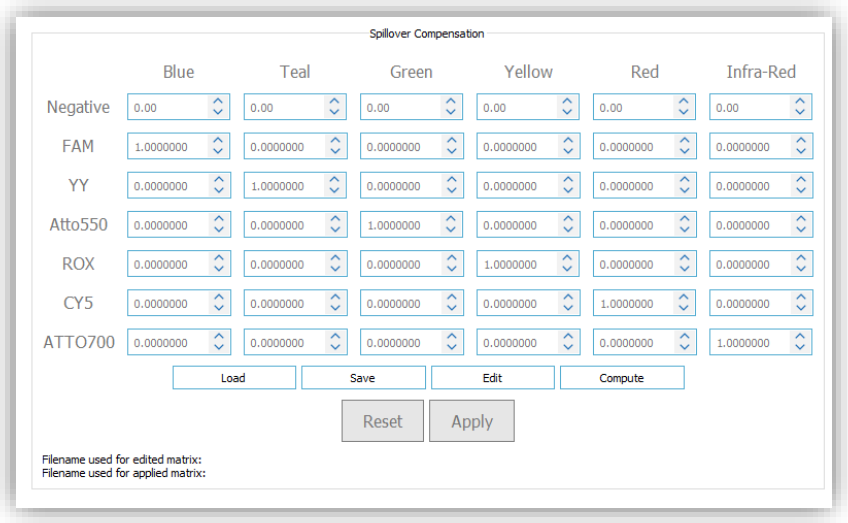


Figure 33: *Examples of default compensation matrices for 6-plex assays. Indicated are the negative fluorescent clusters in line 1 ("Negative"), and the excitation matrix coefficients in all other lines where the fluorophores are associated with their channels.*

By default, the original experiment is not compensated, which means that:

- The negative fluorescent cluster ("Negative") is "0".
- the excitation matrices are equal to the identity matrices (values "1" for identity and "0" for others).

To compensate the spillover of an assay, 3 methods are available:

- the automated method.
- the manual method (not recommended).
- the template-based method.

Automated method

This method is recommended to create a compensation matrix for a multiplex assay.

For a specific assay, a “monocolor control” run should be performed first. The goal of this control run is to have only one fluorescent signal per channel per chamber. A monocolor control is composed of all the reagents of the PCR reaction, for a given assay, with the presence of only one of the targets (see below). To achieve only one fluorescent signal per chamber, the preferred technique is to remove all but one target from the mix to have only the target of the fluorescence of interest in any given chamber. By doing this systematically for each color channel, only a signal target will be amplified and will produce a fluorescence signal higher than the background signal coming from the probes.

Compensation matrix computation for 7 channels experiments 8 chambers are needed:

- A 7-plex negative control (NNNNNNN) with no positive signal (no target).
- A mono-color control positive only for fluorophore 1 (PNNNNNN) (target for fluorophore1 and none of the 6 other targets).
- A monocolor control positive only for fluorophore 2 (NPNNNNN) (target for fluorophore2 and none of the 6 other targets).
- A monocolor control positive only for fluorophore 3 (NNPNNNN) (target for fluorophore3 and none of the 6 other targets).
- A monocolor control positive only for fluorophore 4 (NNNPNNN) (target for fluorophore4 and none of the 6 other targets).
- A monocolor control positive only for fluorophore 5 (NNNNPNN) (target for fluorophore5 and none of the 6 other targets).
- A monocolor control positive only for fluorophore 6 (NNNNNPN) (target for fluorophore6 and none of the 6 other targets).
- A monocolor control positive only for fluorophore 7 (NNNNNNP) (target for fluorophore7 and none of the 6 other targets.

Once the data are generated as described previously, the automated method can be used by clicking on the “Compute” button in the Spillover Compensation tab:

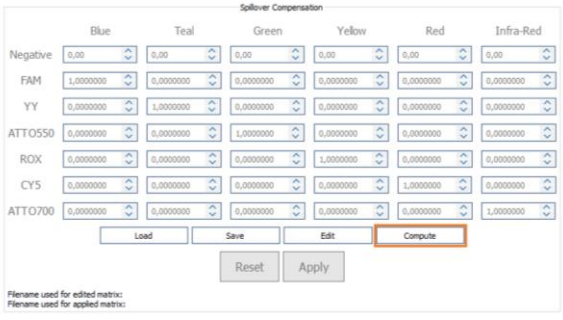


Figure 34: *Compute button in the Spillover Compensation tab to generate a compensation matrix for a 6-plex assay.*

Then, match the appropriate monocolor controls to the right chambers in the pop-up window by clicking in the boxes:

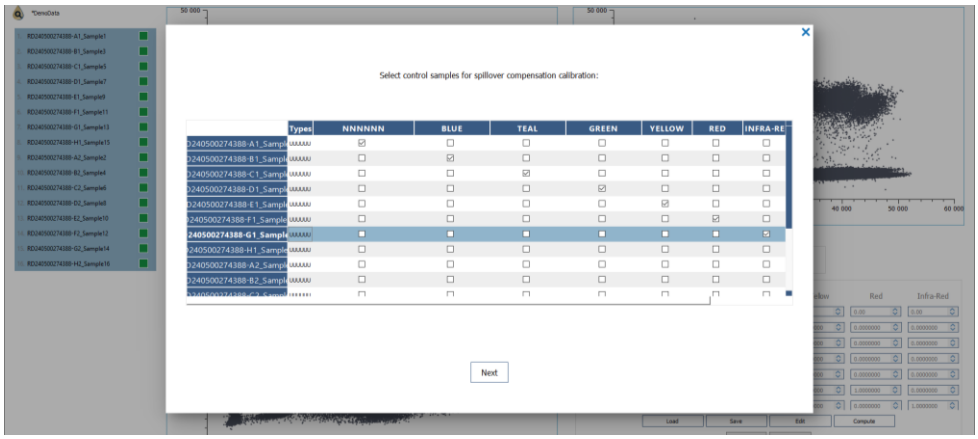


Figure 35: *Pop-up window to indicate monocolor and negative controls for a 6-plex assay.*

Now, click on “Next”, and the Nio Analyzer software will automatically place a threshold between two clusters in the channel selected (these can be adjusted by editing their value or by right-clicking in the dot plots to drag and drop the threshold lines), then click on “Compute compensation”. The threshold is used to indicate to the Nio Analyzer software that everything below it is considered negative, and everything above is positive.

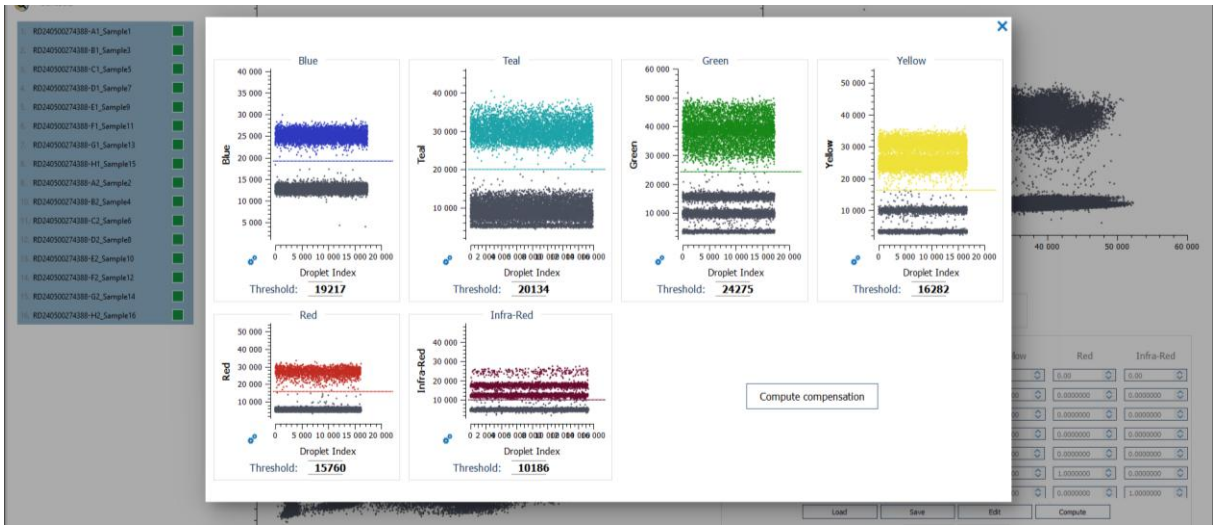


Figure 36: *Checking of the matrix automated computation to generate the compensation matrix in a 6-plex assay.*

- Click on “Compute compensation”
- The compensation matrix will be applied to the data and the software will show you the difference, between the uncompensated data (in light gray) and the compensated data (in dark grey), as a preview.
- If the cluster positions are correct (orthogonal to each other), click on “Apply” to apply the compensation matrix to all the chambers.

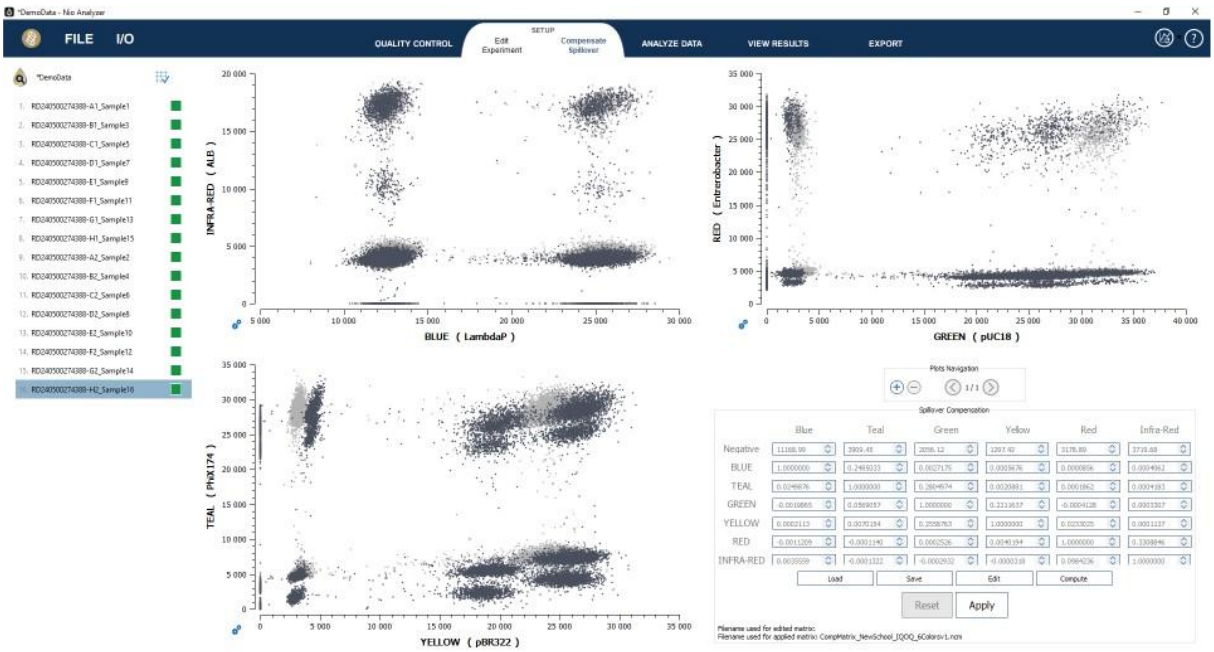


Figure 37: *Example of a compensation matrix applied to a 6-plex assay. Compensated and non-compensated data are in dark and light grey respectively.*

The Compensation matrix is now created and can be saved by clicking on the “Save” button of the “Spillover compensation” matrix. This compensation matrix is generated as a “.ncm” file and can be used for another experiment using the same assay (please see the section “template based method”).

Manual method

This method can be applied if neither the template-based method (see below) nor the automated method (above) can be applied.

CAUTION!

The manual spillover compensation method is an expert functionality and is not recommended unless you understand it’s impact. It’s specifically designed to address non-specificity issues.

- To use the manual spillover compensation method, click on the “Edit” button of the “Compensate Spillover” submenu (and edit the coefficient values individually in the compensation matrix.

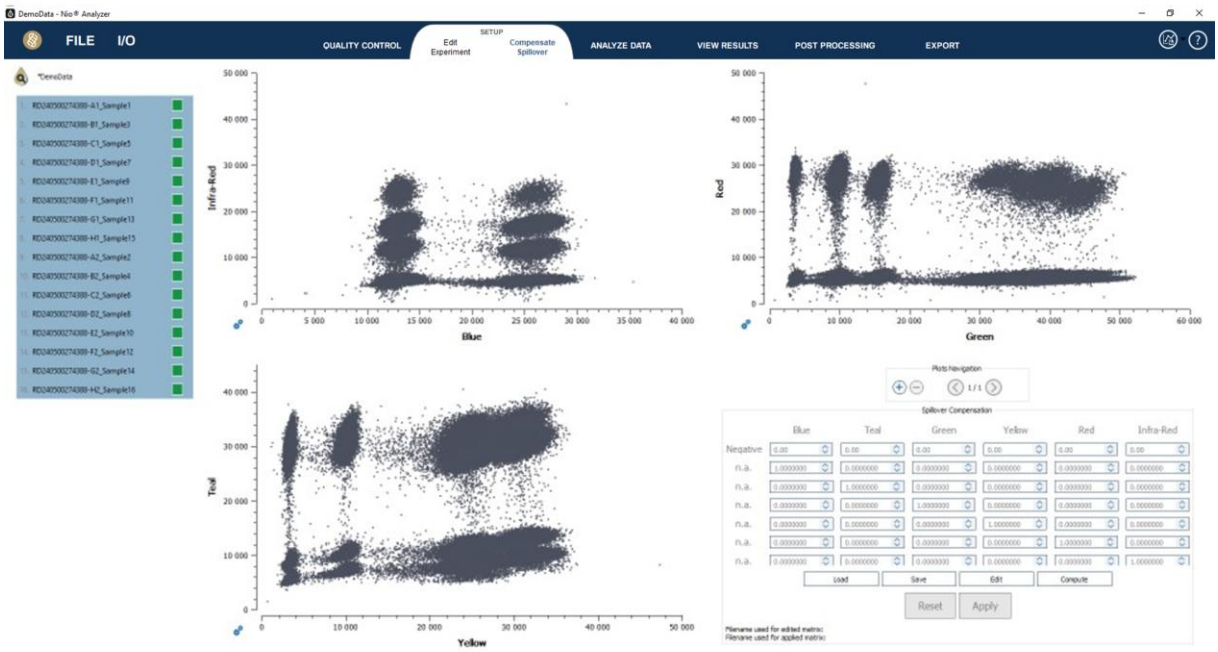


***Figure 38**: Example of a non-compensated 6-plex experiment.*

- The compensation matrix representation is divided in columns, each column corresponds to a specific color channel and each line to a specific fluorophore.
- By default, all values are set to zero, except for the diagonal, which corresponds to the matching fluorophore of a given color channel (for example, the FAM row in the Blue column).
- The goal of manual compensation is the same as the automated compensation method: i.e. to place the clusters in positions that are orthogonal to each other.

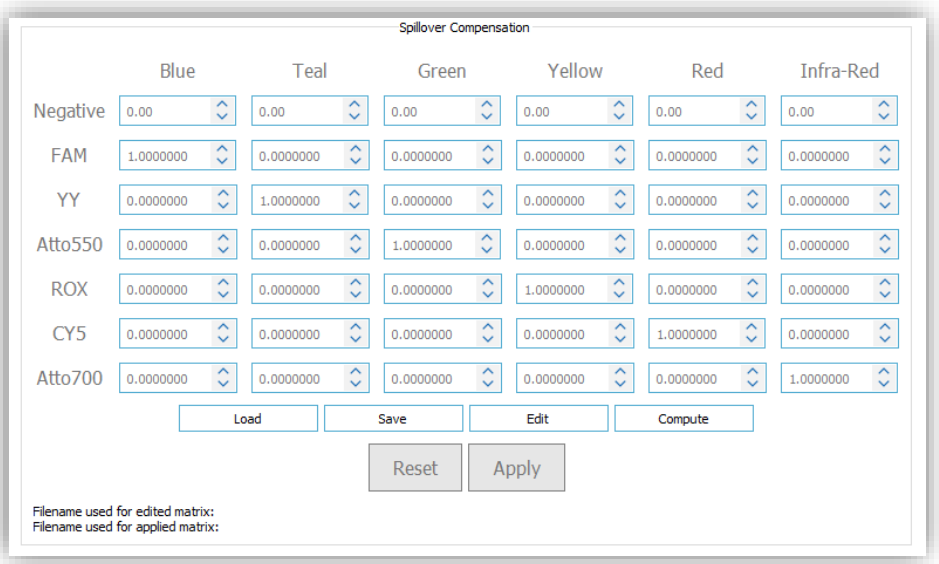


***Figure 39:** Default display of the Spillover Compensation tool.*

- Set the negative values in the first line by entering the mean RFU value of the negative cluster in each channel.

- If a cluster is not correctly placed, change the value of the corresponding box to move its position in the plot graphs. For example, to compensate the spillover from the Atto®550 fluorophore in the Yellow channel, the value of the box located at the intersection of the Atto®550 and the Yellow column has to be manually modified.
- Its value can be either increased or decreased by clicking the up or down arrow in the box, or by changing the value in the box manually by clicking on the value in the box and entering a desired value.

Once the compensation matrix has been modified manually, the compensated droplet populations then overlap in the graphs where they appear darker (dark grey) than the non-compensated droplet populations (light grey).

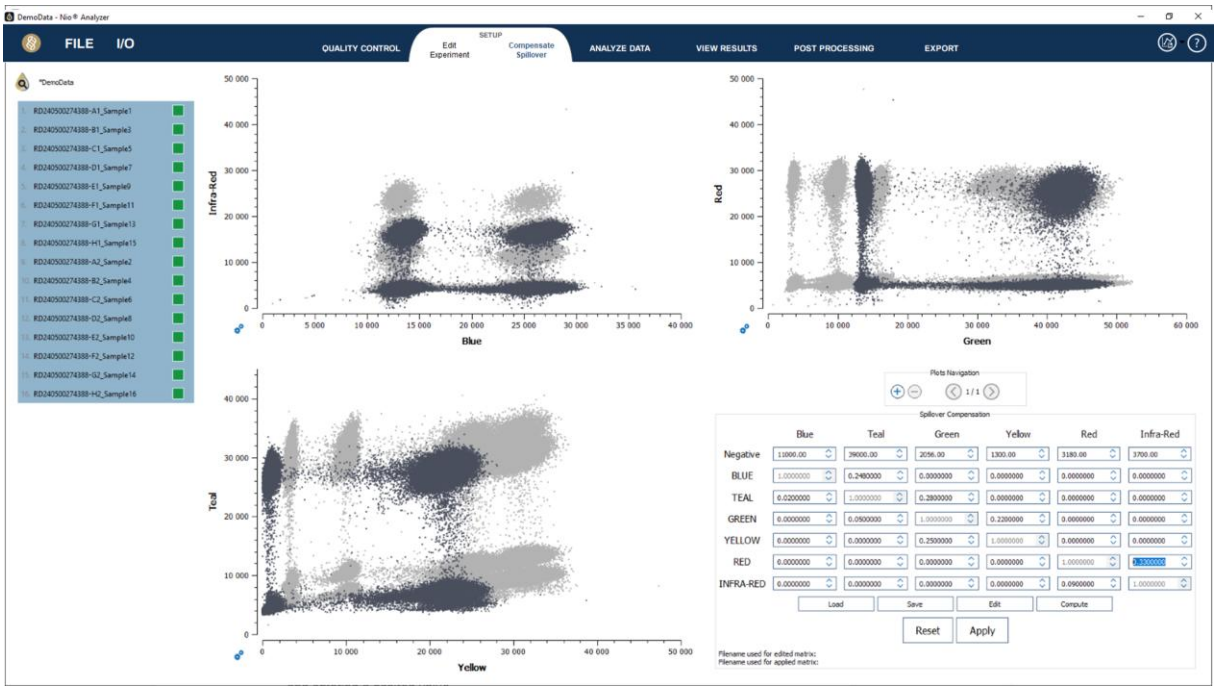


Figure 40: *Modification of the fluorescence spillover coefficients, with the non-compensated populations in light grey and the compensated populations in dark grey.*

- To apply the spillover compensation to the full experiment, click on the “Apply” button.
- Click “Yes” to confirm the update of the automated fluorescence thresholds.

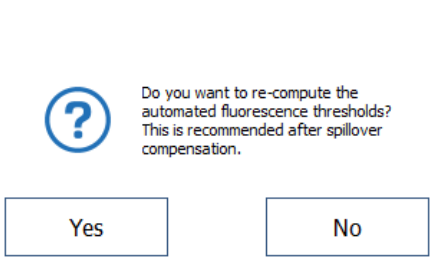


Figure 41: *Automated fluorescence thresholds should be updated after spillover compensation.*

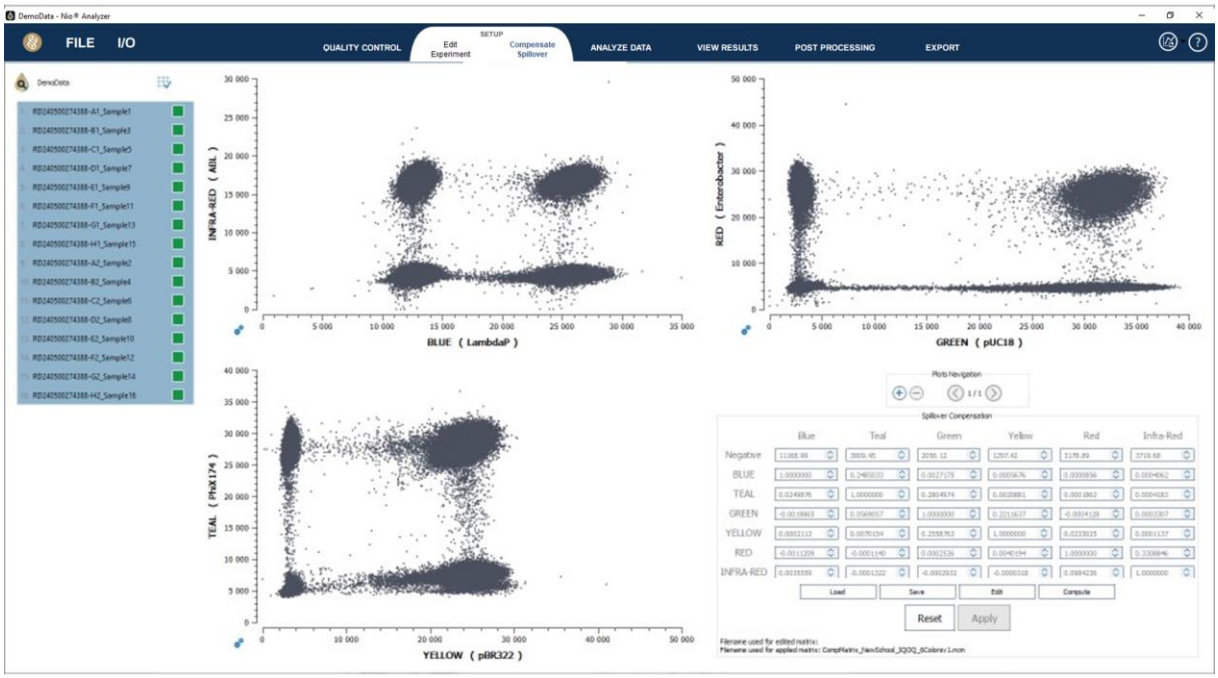


Figure 42: *Dot plot graphs correctly compensated for a 6-plex experiment.*

- To reset the spillover compensation and return to a non-compensated experiment, click on the “Reset” button.

Below are examples of 1D fluorescence graphs, before and after spillover compensation:

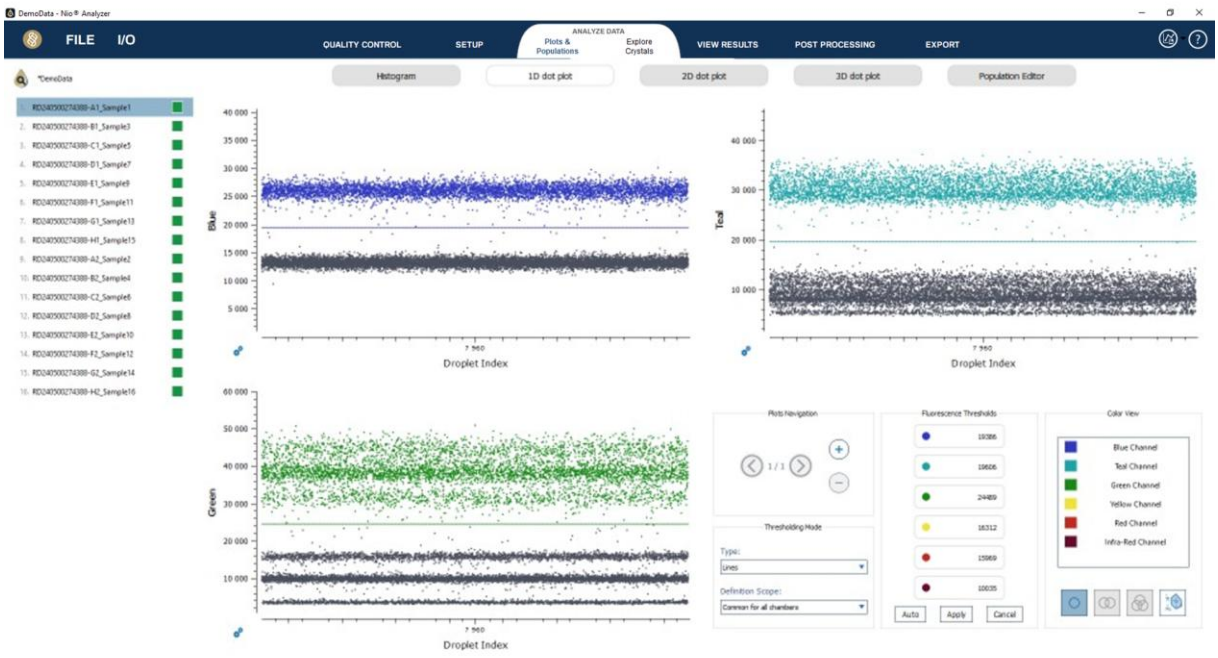


Figure 43: *Example of non-compensated 1D dot plots.*

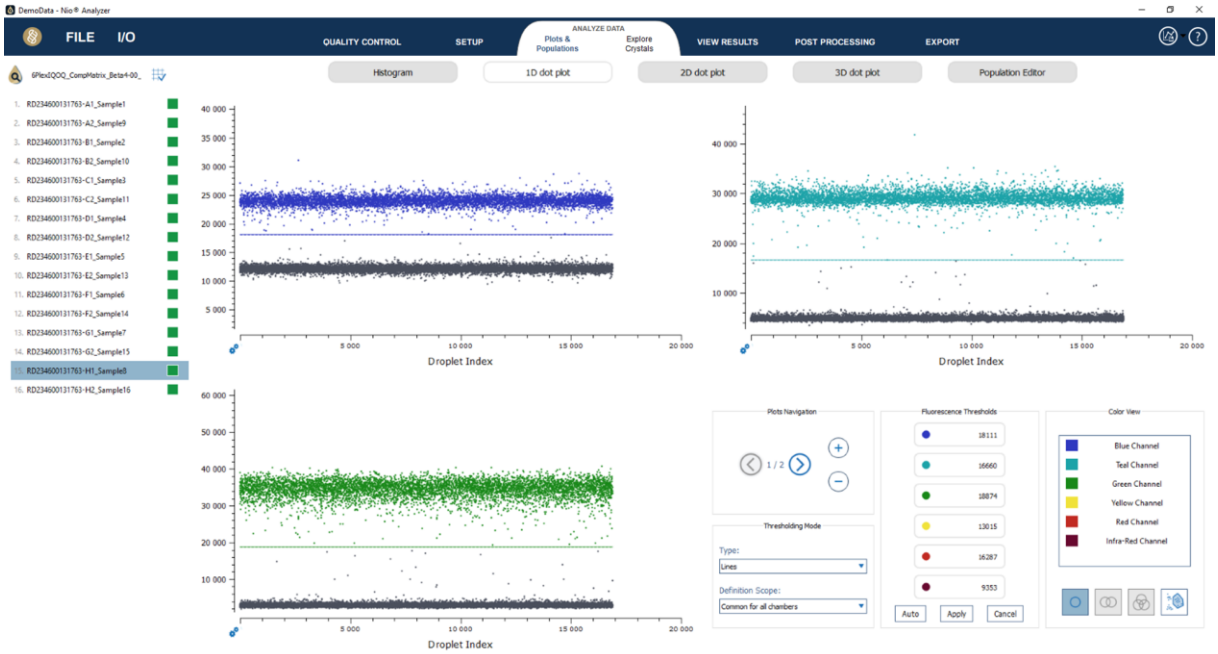


Figure 44: Example of compensated 1D dot plots in 8-color mode.

Below are examples of 2D fluorescence graphs in 8-color mode, before and after spillover compensation:

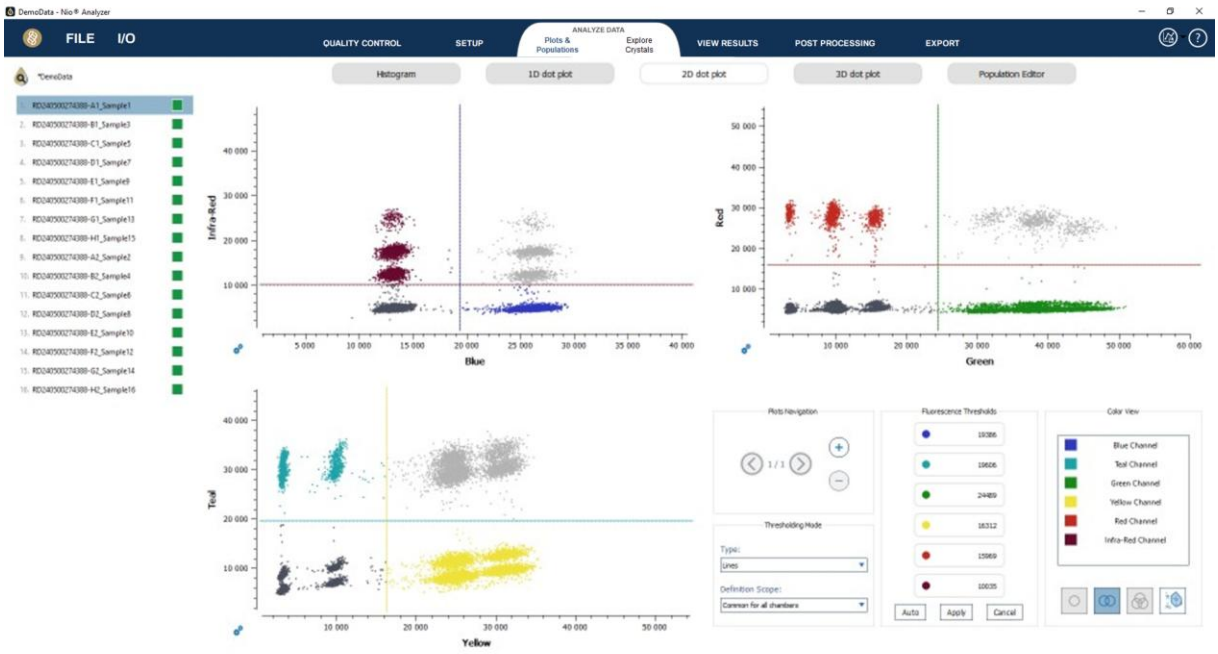


Figure 45: *Example of a non-compensated 2D dot plot in 8-color mode.*

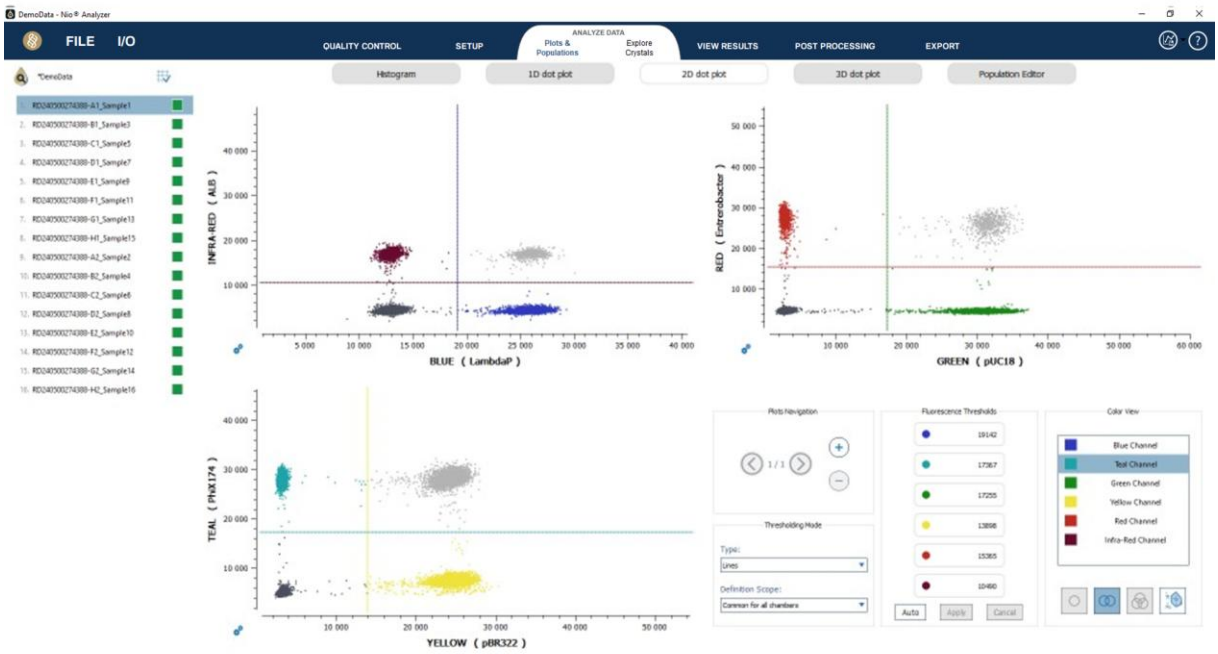


Figure 46: *Example of a compensated 2D dot plot in 8-color mode.*

Template-based method:

The template-based method can be applied if the fluorescence spillover compensation has already been performed on a previous experiment using the same conditions as the current experiment, i.e., the same fluorophores and targets for example.

Note: Two experiments with different scanning times can use the same compensation matrix. Compensation matrices are normalized by the exposure time. However, the two experiments must share the same assay conditions and thermocycling program.

To use the template-based spillover compensation method, click the “Load” button, then select the corresponding “.ncm” file.

Apply the Compensation matrix to the data.

A modification on the existing matrix can be done by editing it and the modified matrix can then be saved as a new “.ncm” file, as previously explained.

Note: The spillover compensation file can also be exported in the “EXPORT” menu (see the step “*Export the data*” for more details)

Note: Once the spillover compensation is done, the spillover signal is removed from the dot plot. As the crystal image displayed is the one acquired by the instrument, this correction is not done on the image. This means that a droplet can be seen as positive in one channel (red in this example, due to spillover) but it won’t be present on the red dot plot as the compensation will remove this spillover signal from it.

Step (3) - Check the fluorescence thresholds:

Automated thresholding is done by default at experiment load. The fluorescence threshold values are automatically estimated by the Nio Analyzer software, in the “Lines” thresholding mode, by considering all the samples of the experiment and the current spillover compensation values. See the section “*How to define custom populations*” for more details on the alternative thresholding mode “Polygons”.

The automated estimation is performed such that the fluorescence thresholds best discriminate between the positive and negative droplet populations in each channel and for the whole experiment. Specifically, the automated threshold maximizes the inter-class variance and minimizes the intra-class variance by considering the fluorescence points aggregated from all chambers.

The default automated threshold estimation is more accurate if both negative and positive droplet populations are represented in the experiment and in each channel, which is, for example, the case if the experiment includes at least one negative control chamber and one positive control chamber for each of the7channels.

To get automated estimation of the fluorescence thresholds, click on the “ANALYZE DATA” menu, then click on any of the dot plot tabs (“1D dot plot”, “2D dot plot”, “3D dot plot”) and click on the “Auto” button in the “Fluorescence Thresholding” widget (**Figure 47**).

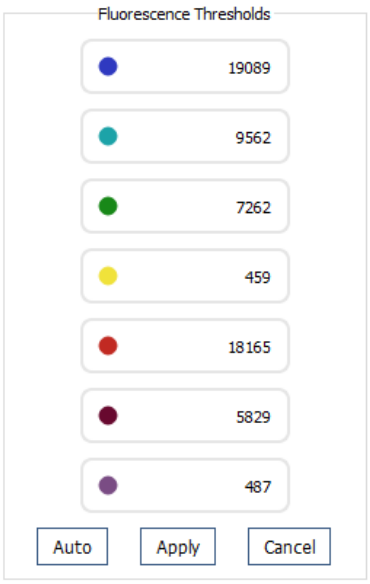


***Figure 47**: Check the fluorescence thresholds.*

- For an efficient adjustment of the fluorescence thresholds, it is recommended to inspect the sequential view of the 1D fluorescence graphs including all the chambers, by clicking on the “1D dot plot” and selecting all the experiment chambers listed in the left panel (Shift+Click or Ctrl+A).

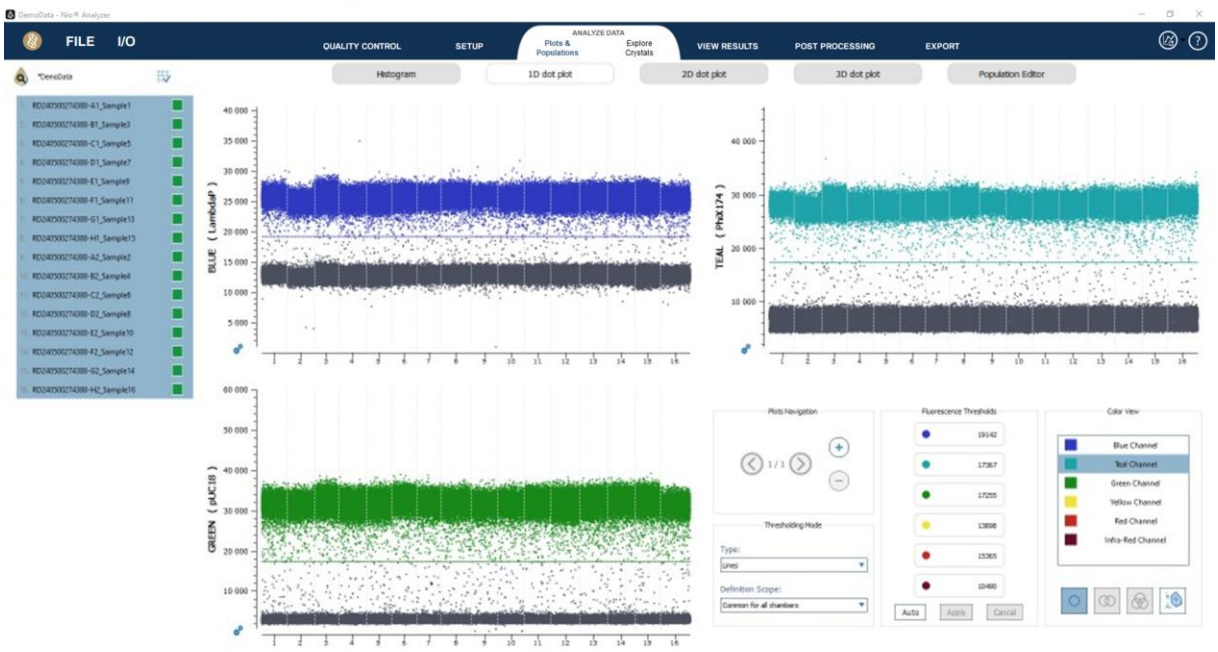


Figure 48: *Sequential view of all the experiment test chambers in the 1D dot plots, for efficient verification of the 6 fluorescence thresholds.*

To manually adjust the fluorescence thresholds, click on the dot plot thresholds to drag & drop the lines, or edit the value of the fluorescence thresholds in the “Fluorescence Thresholding” widget (unit: RFU standing for Relative Fluorescence Unit).

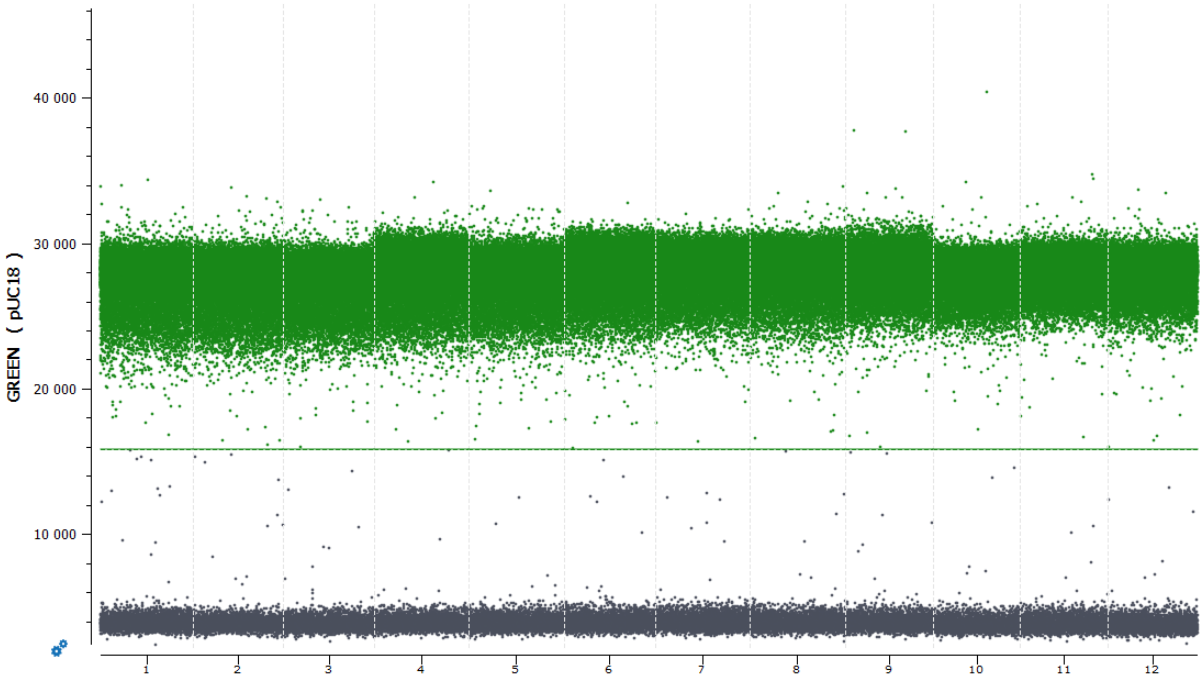


Figure 49: *Manually adjust the fluorescence threshold if desired using Left-Click to drag & drop.*

- To apply the modified values to the whole experiment, click on “Apply”.
- To recover the threshold values set before editing (i.e., the last time “Apply” has been clicked), click on “Cancel”.

- To restore the automated fluorescence thresholds, click on the “ANALYZE DATA” menu, then click on any of the dot plot tabs (“1D dot plot”, “2D dot plot”, “3D dot plot”) and click on the “Auto” button in the “Fluorescence Thresholding” widget.
- The fluorescence separability between “positive droplets” and “negative droplets” is automatically computed for each population of interest and exported as an advanced quality control file. For more details about separability scores, see the section *“How to quantify fluorescence separability for the evaluation of amplification efficiency?”* in the Advanced Functionalities.

How to adjust thresholds at chamber level?

By default, the fluorescence thresholds automatically computed by Nio Analyzer software, for each detection channel, are 1D “Line” thresholds that are common to all the chambers of the experiment.

Any manual adjustment of these thresholds is, by default, also applied commonly to all chambers. This ensures both objectivity and repeatability of the quantification results, assuming that all the chambers of the experiment share the same scanning protocol and assay.

If common thresholds are not relevant, due to observed chamber-to-chamber variability, it is possible to adjust the fluorescence thresholds for individual chambers.

- For this purpose, go to “ANALYZE DATA” > “Plots & Populations” > “2D dot plot”, then go to the “Thresholding Mode” widget and select “Individual per chamber” (instead of “Common for all chambers”) in the “Definition Scope” combo box.
- Thereafter, it is possible to manually adjust the thresholds at the level of each chamber (click on “Apply” to validate any adjustment).

Note: The easiest way to view the “Line” thresholds of all the chambers is to use the concatenated view in the 1D dot plots by selecting all the chambers in the left panel:

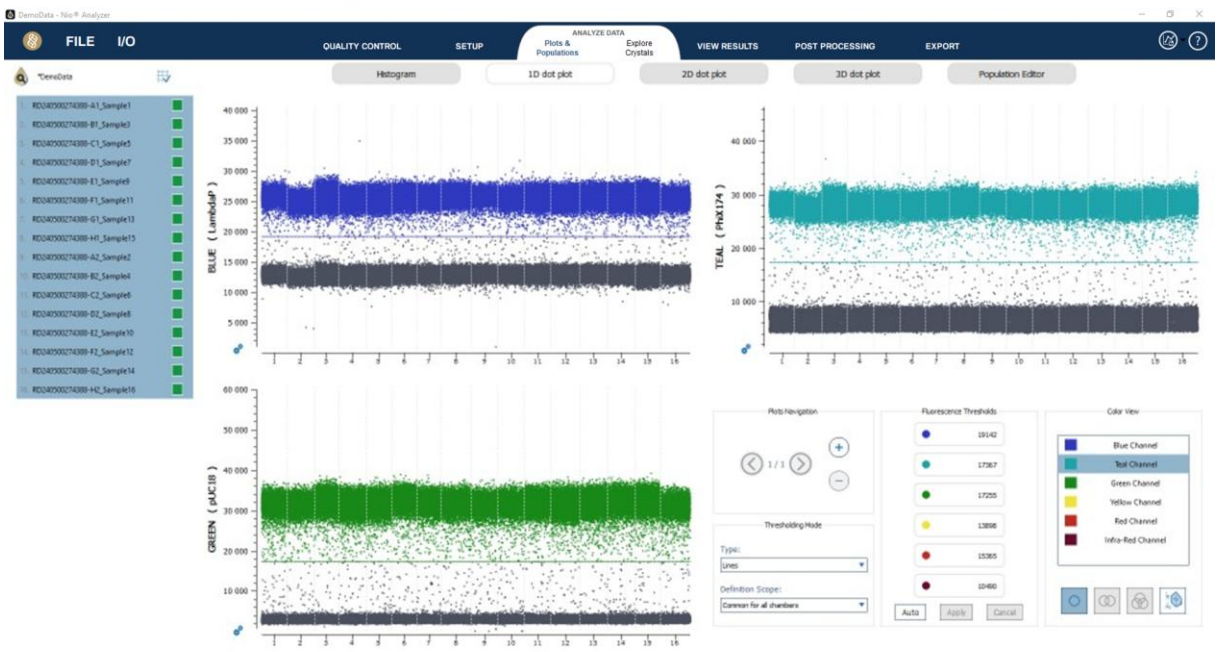


Figure 50: *Visualization of all the individual thresholds in the 1D dot plots.*

Step (4) - Obtain the concentration of the targets:

To get the concentration results, click on the “VIEW RESULTS” > “Result Table” submenu.

If the dilution factor of the samples in the PCR mix has been specified, the estimated concentrations are the concentrations of the target molecule in the initial stock in copies/µL.

CAUTION!

If the dilution factor has not been specified, it is set to the default value “1”, meaning the estimated concentration is not the stock concentration but the concentration in the loaded mix (i.e., the concentration of the target molecule in the chamber).

Note: If the sample has been diluted 10-fold in the PCR mix, then the value “10” should have been entered for the dilution factor, and the stock concentration is equal to the chamber concentration multiplied by 10.

The absolute nucleic acid concentrations are computed based on the standard Poisson statistics that are commonly used in digital PCR.

Note: The total number of chambers belonging to the experiment is indicated above the chamber list in the left panel.

Click on the “Result Table” submenu to see the table of results.

The colors of the column titles indicate the color of the corresponding channel:

- In blue color: the results for the fluorophore-target couple associated with the “Blue” LED.
- In teal color: the results for the fluorophore-target couple associated with the “Teal” LED.

- In green color: the results for the fluorophore-target couple associated with the “Green” LED.
- In yellow color: the results for the fluorophore-target couple associated with the “Yellow” LED.
- In red color: the results for the fluorophore-target couple associated with the “Red” LED.
- In infra-red color: the results for the fluorophore-target couple associated with the “Infra-Red” LED.
- In purple color: the results for the fluorophore-target couple associated with the “Purple” LED.

Figure 51: View the concentration results in a table representation; scroll right using the bar at the bottom of the page to view results for all the populations.

Click on the “expand icon”  located above the chamber list in the left panel to see more detailed results, including the number of negative droplets, the confidence intervals, and the relative uncertainties at 95% confidence level.

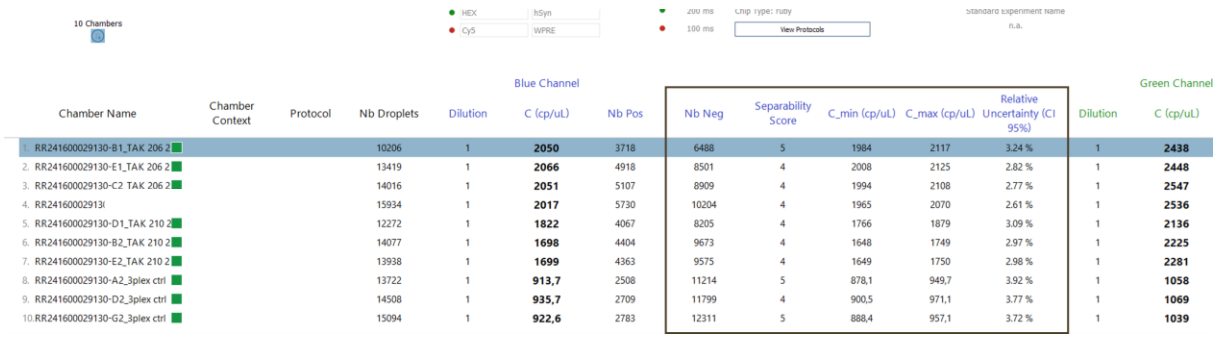


Figure 52: *Expanded view of the result table: for each population, the additional columns highlighted in the orange box show-up. Scroll right using the bar at the bottom of the page to view results for all the populations.*

Note: It is possible to copy-paste the result table shown in “VIEW RESULTS” > “Result Table” by selecting the rows and using Ctrl+C/Ctrl+V (for example, to copy the results into an Excel document).

Clicking on the ‘View Protocol’ button will open a pop-up window presenting the details of the thermocycling program(s). If there are more than one protocol in the experiment, the pop-up contains several tabs, one per protocol, between which it is possible to navigate.

Click on the “Advanced Graphs” submenu, and the “Concentration Graphs” tab to view the graphs of concentrations (use the checkboxes to switch between “Linear” and “Log” modes).
Note: For the display of populations created as part of a linkage analysis study, please refer to the information note on page 66 of this user manual.

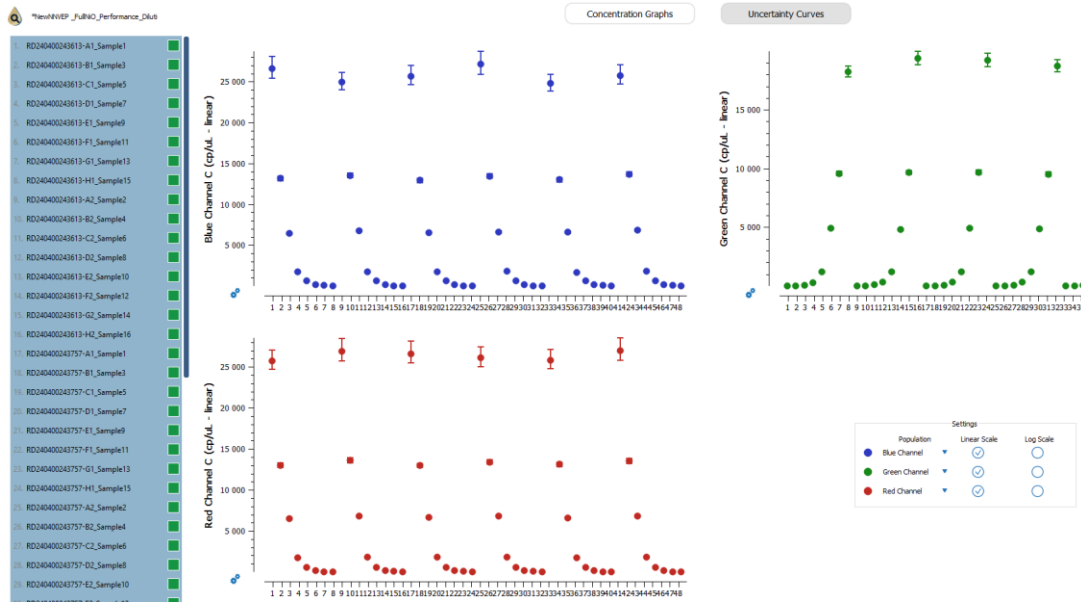


Figure 53: *Graph view of the concentrations (linear view).*

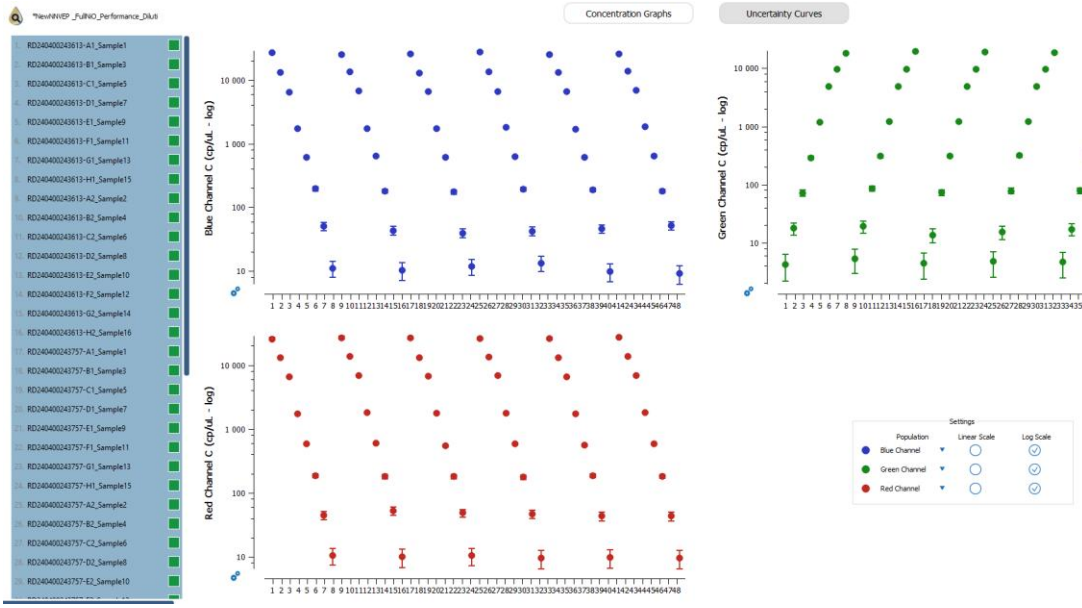
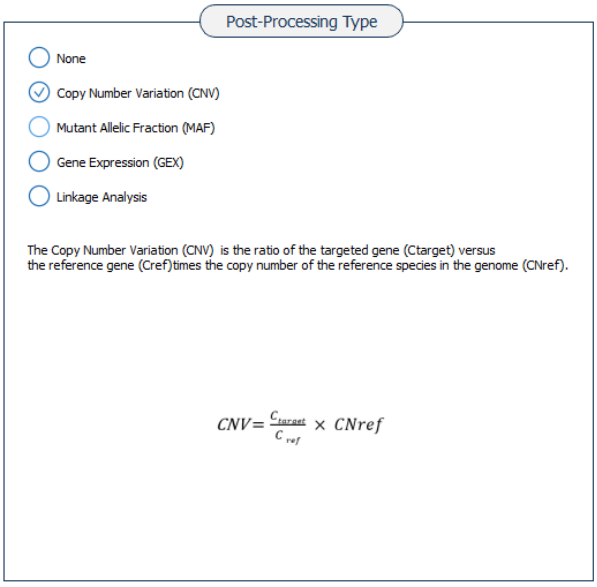


Figure 54*: Graph view of the concentrations (log view).*

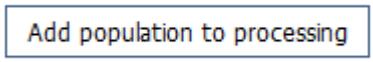
Step (5) – Perform the post-processing analysis

To perform a post-processing analysis of the results, click on “Setup” in the “POST PROCESSING” menu and select the appropriate analysis:

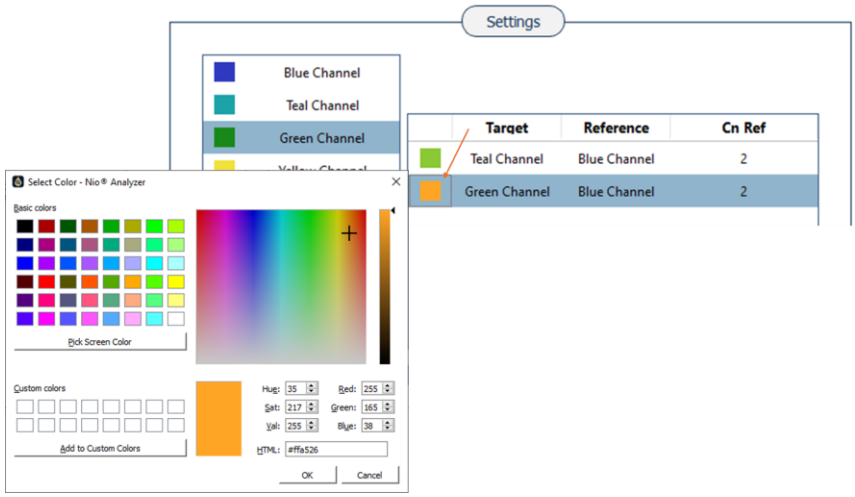
- **None**: no further analysis will be performed
-
- **Copy Number Variation (CNV)**: The Copy Number Variation (CNV) is the ratio of the concentration of the targeted gene (Ctarget) versus the concentration of the reference gene (Cref) multiplied by the copy number of the reference species in the genome (CNref).



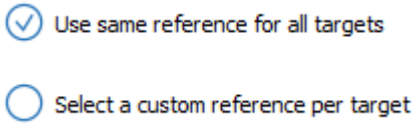
On the right- side of the screen, the “Settings” tab allows the user to select populations one after the other, each time by selecting the population in the left column and by clicking on “Add population to processing”



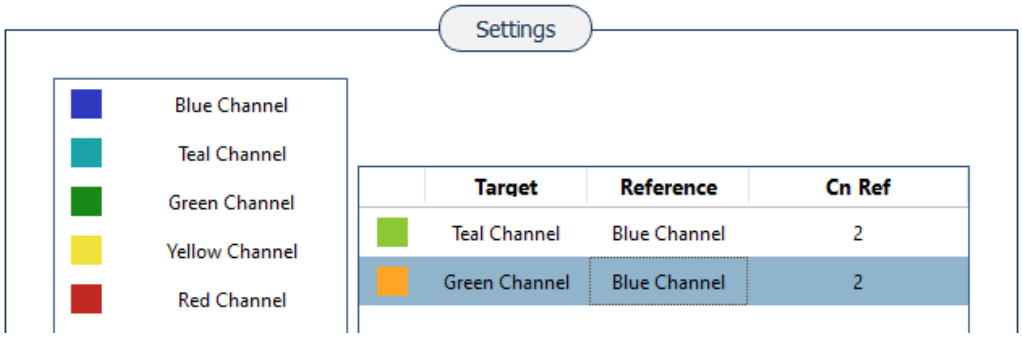
In the table on the right, the color in which the data will be displayed in the “Results” tab can be chosen by clicking on the colored square (orange arrow on the screenshot below).



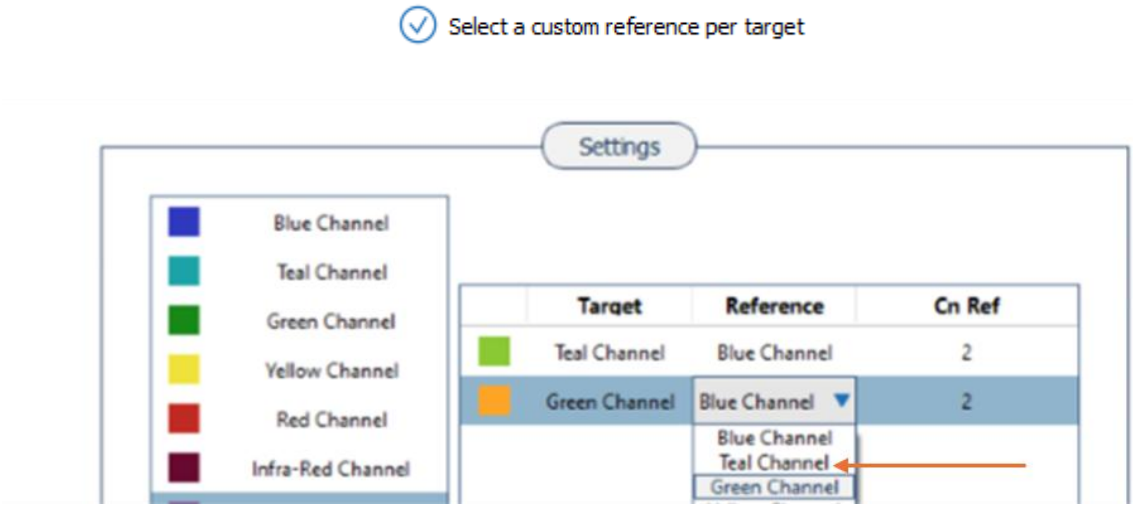
- By default, the “blue channel” is displayed in the “Reference” column. This can be modified by double-clicking on “blue channel” and selecting the desired reference displayed in the drop-down menu. If the “Use same reference for all targets” option is selected, then the reference chosen first will be applied automatically to the other populations.



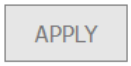
- Specifying the Cn ref value, which remains at 1 by default, can be done by double-clicking on each line in the relevant column “Cn Ref”, typing the value and pressing enter



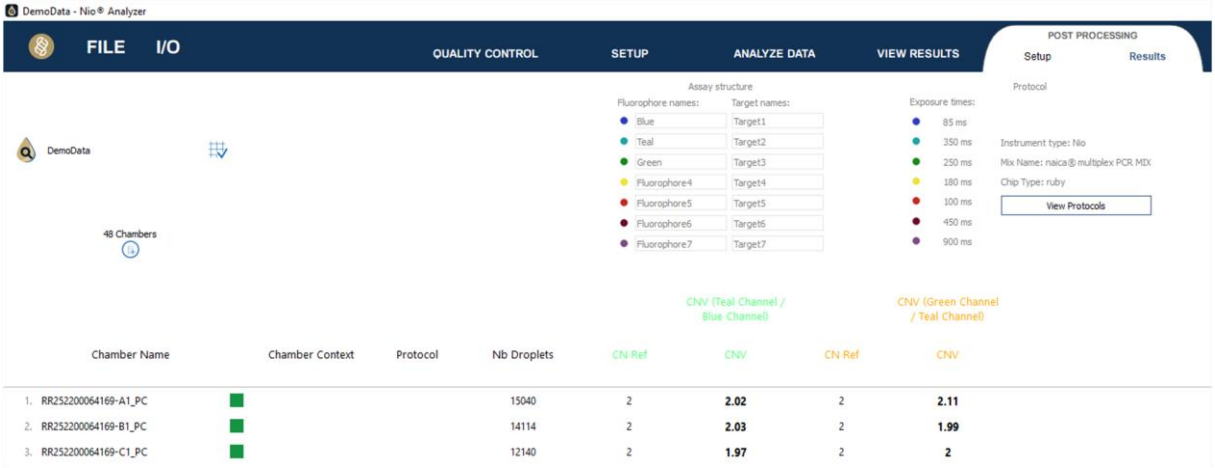
Selecting “Select a custom reference per target” will allow to define different reference gene for each target.



Clicking on apply triggers computation of the post-processing results.

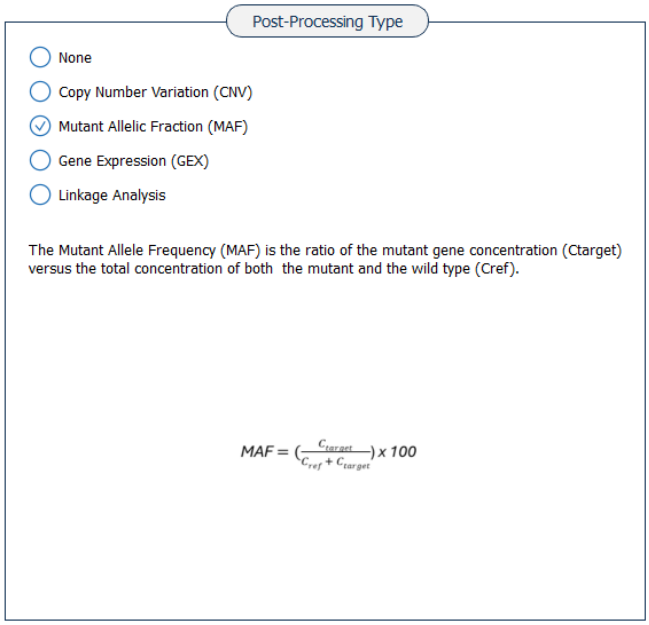


The values are displayed and can be viewed in the “Results” tab. By default, only the Cn ref and CNV values are displayed, in addition to the experiment parameters.

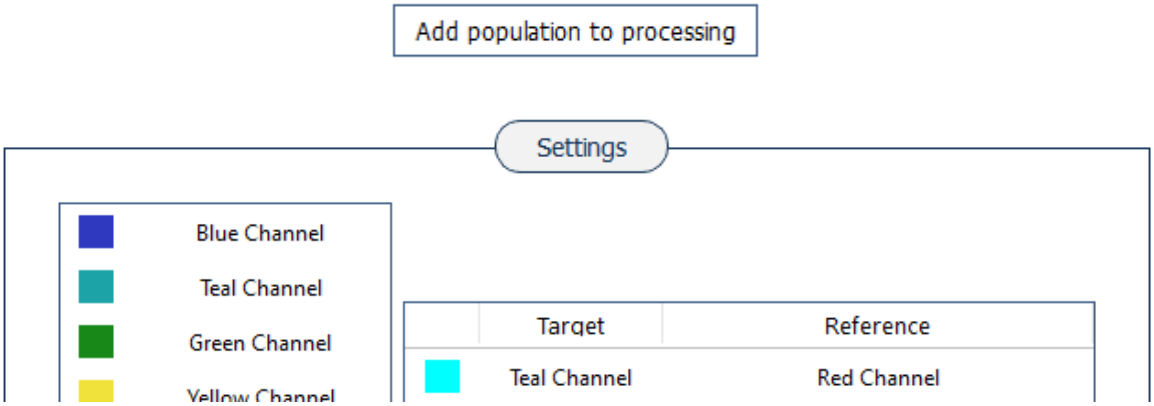


For more details on the statistics, click on the display additional columns icon (orange arrow). Additional columns are displayed, showing the confidence intervals and 95% uncertainty associated with the CNV value.

- **Mutant Allelic Fraction (MAF)**: The Mutant Allele Frequency (MAF) is the ratio of the concentration of the mutant gene concentration (Ctarget) versus the total concentration of both the mutant and the wild type (Cref) expressed in percentage.

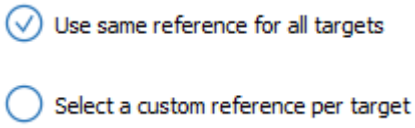


On the right- side of the screen, the “Settings” tab allows the user to select populations one after the other, each time by selecting the population in the left column and by clicking on “Add population to processing”

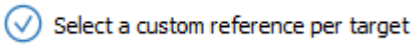


In the table, the color in which the data will be displayed in the “Results” tab can be chosen by clicking on the colored square as described in the previous section for CNV analysis.

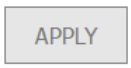
By default, the “blue channel” is displayed in the “Reference” column. This can be modified by double-clicking on “blue channel” and selecting the desired reference displayed in the drop-down menu. If the “Use same reference for all targets” option is selected, then the reference chosen first will be applied automatically to the other populations.



Selecting “Select a custom reference per target” will allow to define different reference gene per target.

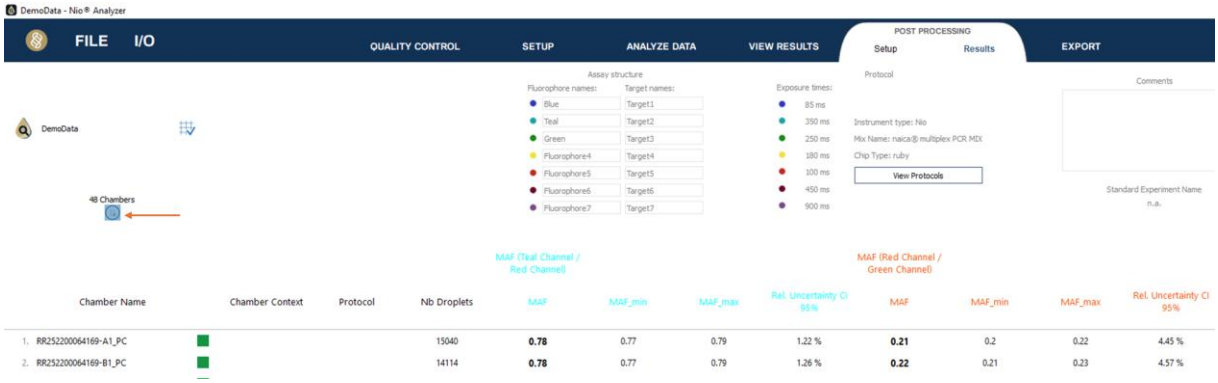


Clicking on apply will launch the calculation.

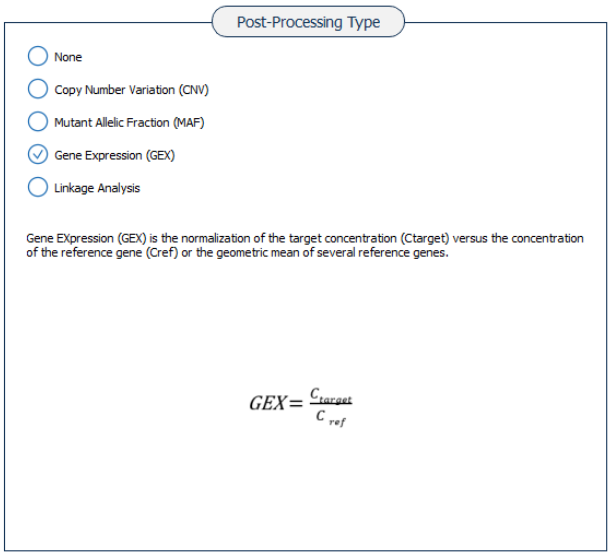


The values will be displayed in the “Results” tab. By default, only the MAF values are displayed, in addition to the experiment parameters.

For more details on the statistics, click on the display additional columns icon (orange arrow). Additional columns are displayed, showing the confidence intervals and 95% uncertainty associated with the MAF value.

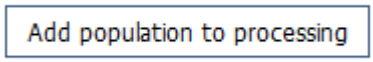


- **Gene Expression (GEX):** Gene EXpression (GEX) is the normalization of the target concentration (Ctarget) versus the concentration of the reference gene (Cref) or the geometric mean of several reference genes

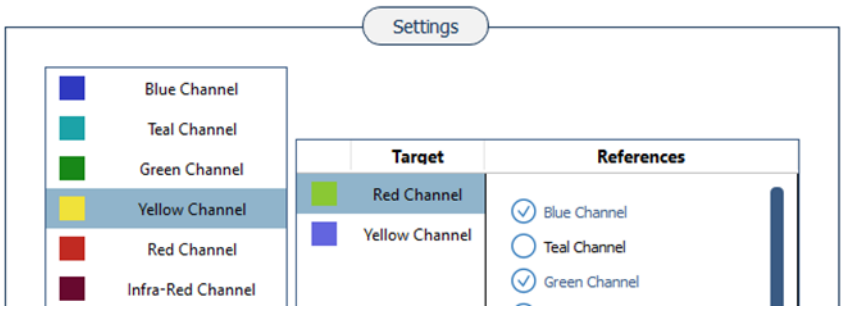


.

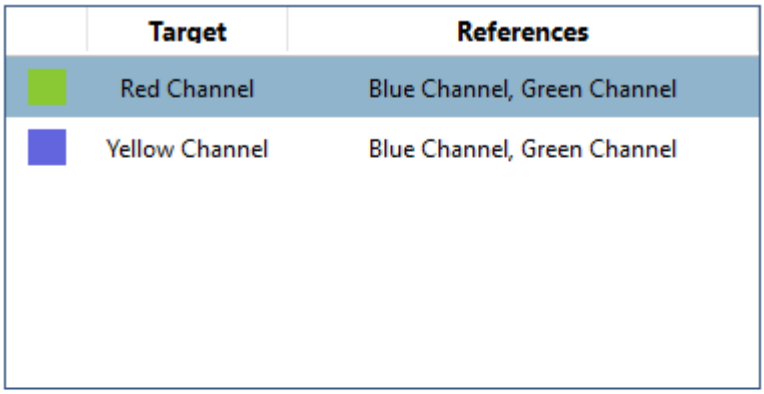
On the right- side of the screen, the “Settings” tab allows the user to select populations one after the other, each time by selecting the population in the left column and by clicking on “Add population to processing”

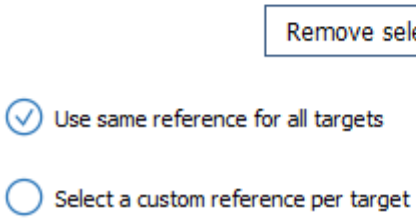


GEX analysis can be used to select one or more reference genes for the same target gene by double clicking in the column “References” where “none” is displayed. If several reference genes are selected, the GEX ratio is calculated using the geometric mean of the selected reference genes.

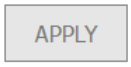


The user can choose “Use same reference for all targets” or “select a custom reference per target” by selecting the corresponding tab.



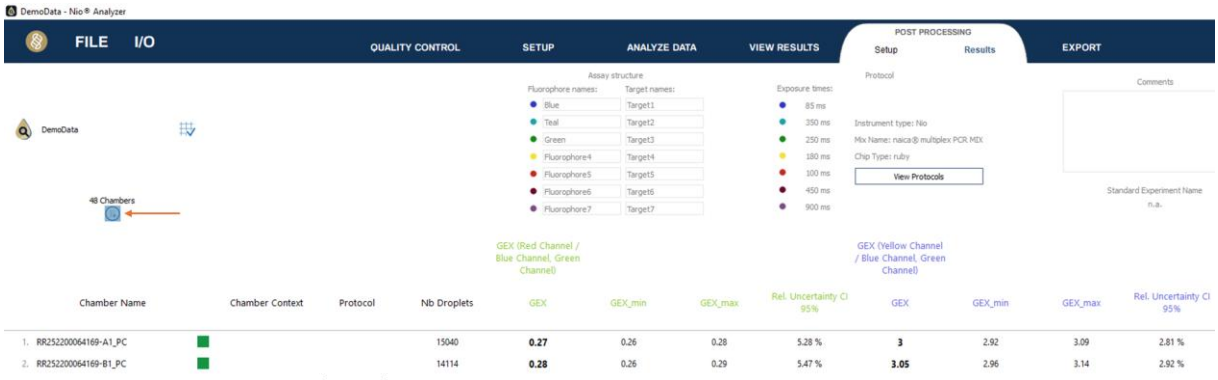


Clicking on apply will launch the calculation.

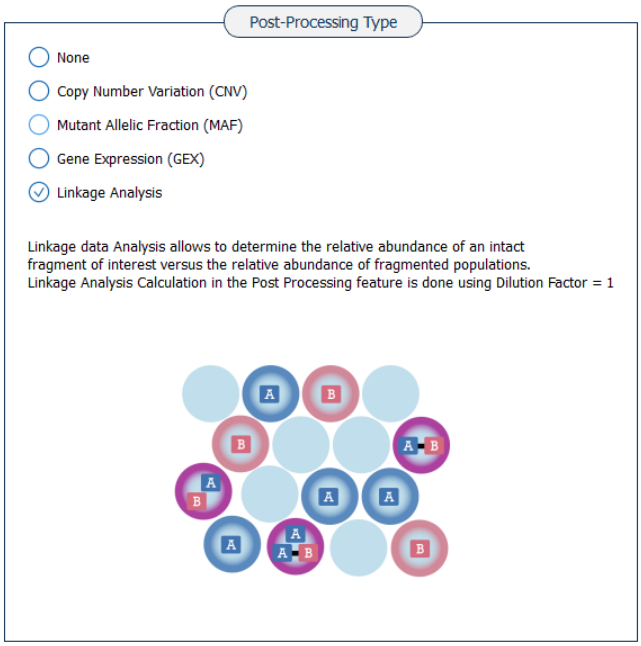


The values will be displayed in the “Results” tab. By default, only the GEX value is displayed, in addition to the experiment parameters.

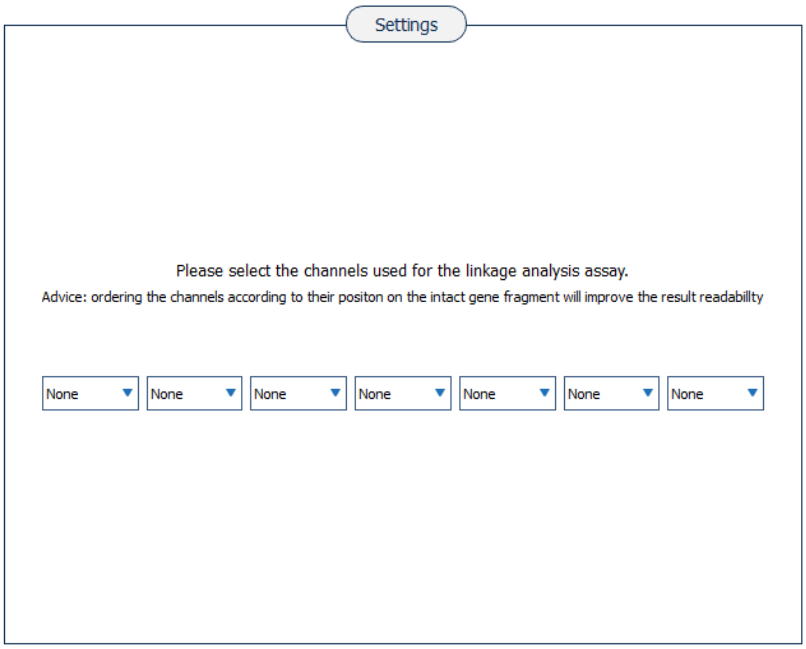
For more details on the statistics, click on the display additional columns icon (orange arrow). Additional columns are displayed, showing the confidence intervals and 95% uncertainty associated with the GEX value.



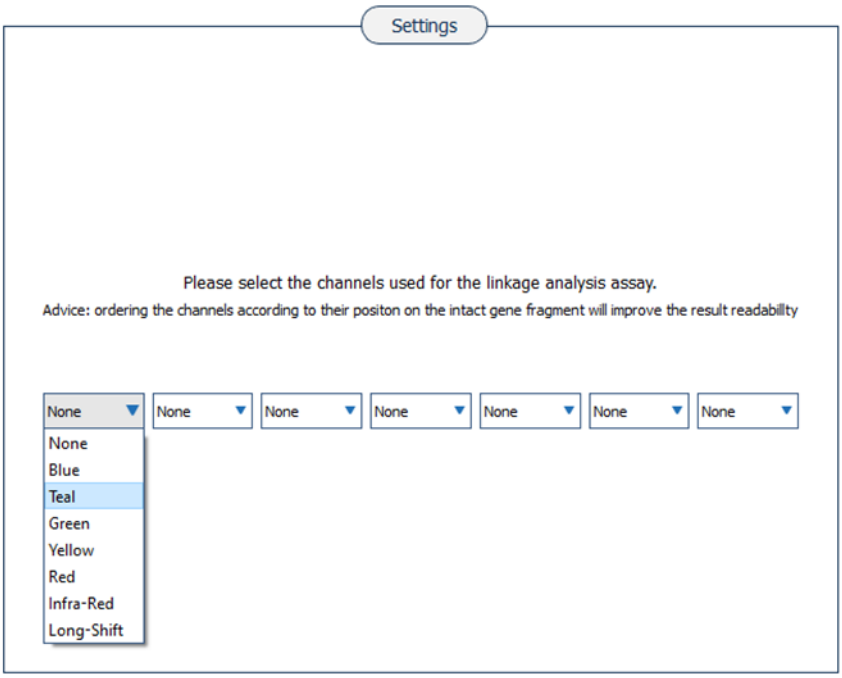
Linkage Analysis: This post-processing allows to **determine the estimated concentration and relative abundance of an intact fragment of interest (containing all the targets chosen for the analysis on the same nucleic acid molecule) versus the estimated concentration and relative abundance of each potential shorter fragment (containing only a subset of the targets on the same molecule).**



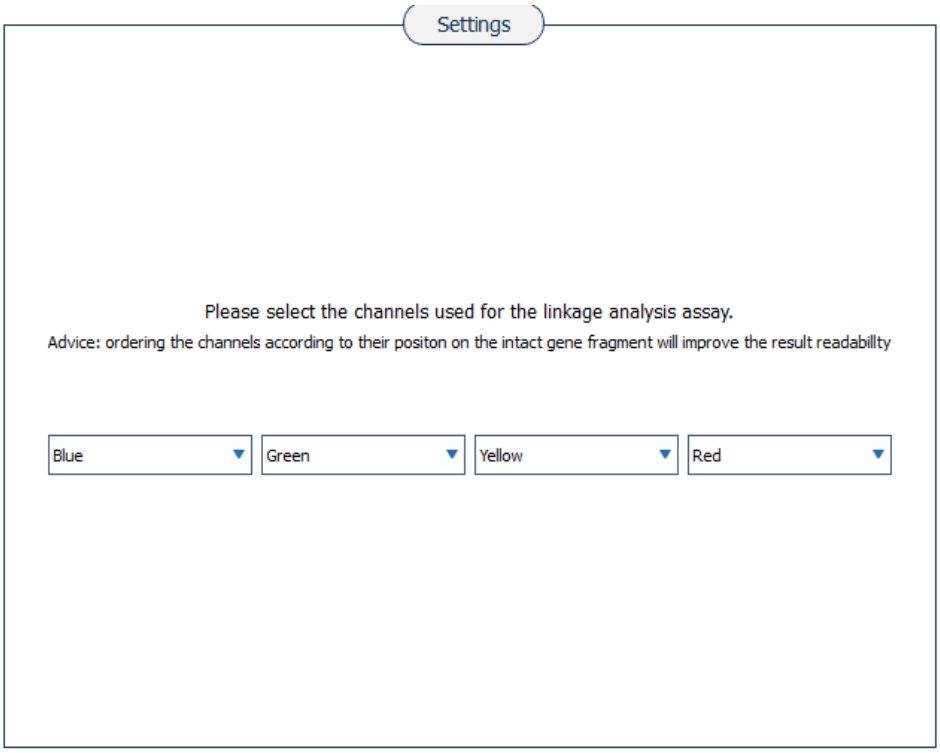
On the right- side of the screen, the “Settings” tab allows the user to select the channels used for the linkage analysis assay.



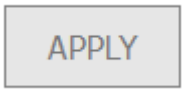
By clicking on each box, a drop-down menu is displayed, allowing the user to select the different channel used for the experiment.



To improve results readability, it is recommended to respect the order of the **different amplicons targeted on the intact fragment of interest.**

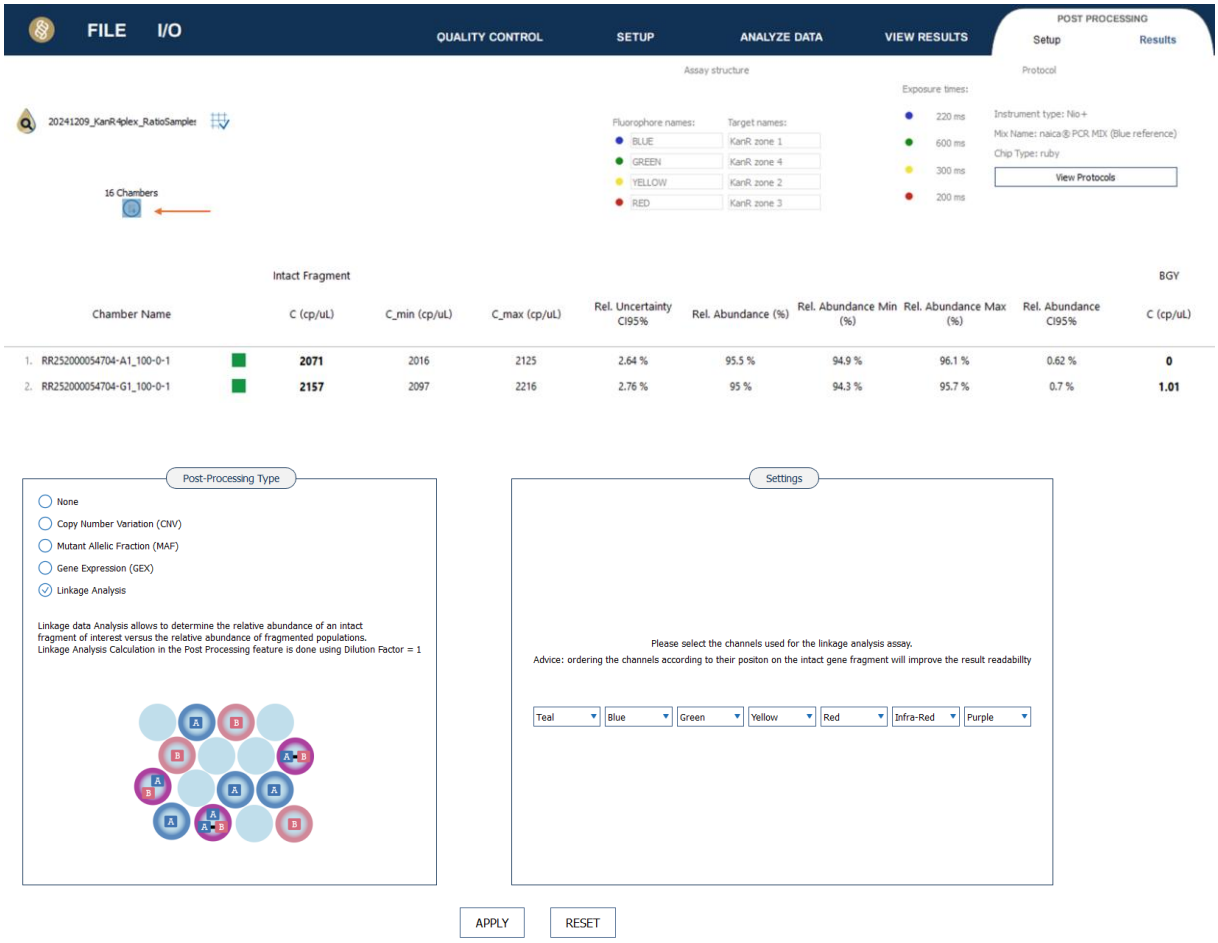


Clicking on apply triggers computation of the results.



The values are displayed in the “Results” tab. By default, only the concentration and values of relative abundance in % are displayed, in addition to the experiment parameters.

For more details on the statistics, click on the display additional columns icon (orange arrow).). Additional columns are displayed, showing the confidence intervals and 95% uncertainty associated with the concentrations and relative abundance values.



Important note for linkage analysis: Once the "linkage analysis" post processing is applied, the software automatically creates all the droplet populations with a positive or negative signal in each channel selected for the linkage analysis. For instance, when the Blue (B), Green (G), and Red (R) channels are selected, the populations B+G+R+ (positive in the three channels Blue, Green and Red), B+G+R- (positive in the Blue and Green channels, negative in the Red channel), B+G-R+, B-G+R+, B+G-R-, B-G+R-, B-G-R+, and B-G-R- are created. These populations will appear in the "Result Table" tab of the "VIEW RESULTS" tab with estimated concentrations, and they will appear in the drop-down menu for population selection in the "Advanced Graph" tab of the "VIEW RESULTS" tab.

By focusing on the B+G+R- population, it appears that it contains all the droplets that are positive in the Blue and the Green channels, but negative in the Red channel. This does not mean that these droplets all contain the nucleic acid fragment with both the target detected in the Blue channel and the target detected in the green channel.

Therefore, this population (and all the others mentioned above) do not correspond to the droplets containing each fragment analyzed, and the estimated concentrations displayed in the "VIEW RESULTS" tab are not the concentrations of each fragment. The concentration of each analyzed fragment is only available in the "POST PROCESSING" tab.

Step (6) - Export the data:

To export the data, click on the "EXPORT" menu, then select the desired data, choose the output directory, and click on the "Export" button.

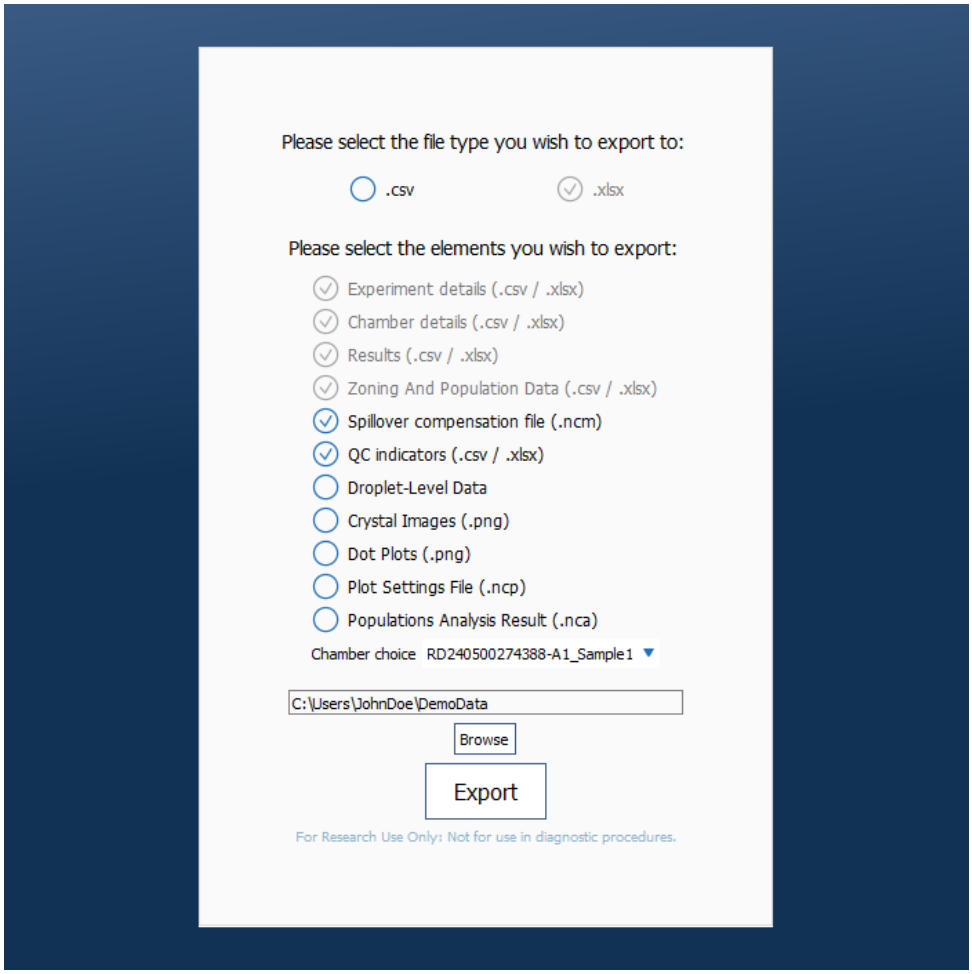


***Figure 55**: Export the data.*

Upon data export completion, the output folder will be automatically opened in a pop-up window.

Exporting data consists of obligatory and optional extra steps.

The following are always exported:

- experimental details
- chamber details
- results

- PP_setup
- PP_results
- zone details
- population details

Optional included data includes:

- QC indicators,
- droplet data files
- spillover
- crystal images
- dot plots
- analysis configuration
- plots configurations
- analysis templates

Always exported:

- Experiment details (".csv" file/"Experiment_Details" sheet of ".xlsx" file), including:
  - Fluorophore and target names.
  - User comments.
  - Scanning parameters: exposure times (in ms) in each channel.
  - Spillover compensation status (compensated or not).
  - The Nio Analyzer software (Standard or Regulatory) version used to export.
  - Timestamp of data export.
  - Thresholding type ("Line" vs. "Polygon" zoning) and definition scope ("common for all chambers" vs. "individual per chamber") for the experiment (see the section "How to define *custom populations?*" in the Advanced Functionalities).
  - Chip type.
  - Mixname.
  - Kit name.
  - Instrument configuration.
  - Standard experiment name if applicable.
- Chamber details (".csv" file/"Chamber_Details" sheet of ".xlsx" file), including for each chamber:
  - Chip barcode, chamber position ("A", "B", "C", etc.), sample name.
  - Pooling ID (empty if not applicable).
  - Protocol tag.
  - Protocol name.

- For each detection channel: sample type (“U”, “N”, “P”, or “S”); reference concentration (in copies/µL, applicable only if sample type = “S”).
- Original experiment name.
- Nio Reader software (Standard or Regulatory) version used to export.
- Scanning timestamp.
- The serial number of the Nio Digital PCR used to scan.
- Index of chip position (slot 1, 2, 3 from left to right on the chip plate) and index of chip plate at the time of the scan.
- Model of the chip (chip type), and droplet diameter (estimated average droplet diameter in microns).
- The number of manually removed droplets (see the sections "*How to explore the crystal populations?*" and “*How to perform quality control?*” in the Advanced Functionalities).
- Droplet volume (µl)

Note: if samples from different experiments (.niodata or .nioresult) are merged, the output details may differ.

- Results (“.csv” file/“Results” sheet of “.xlsx” file), including, for each chamber:
  - Chip barcode, chamber position, sample name
  - Total number of analyzed droplets
  - For each population:
    - sample type (only in “Line” thresholding mode)
    - dilution factor (N/A if not applicable)
    - estimated stock concentration (in copies/µL) with the following rounding rules:
      - If c > 1000 cp/µL, the estimate is truncated
      - If c > 10 cp/µL, the estimate is rounded to one digit
      - If c < or = 10 cp/µL, the estimate is rounded to two digits
    - number of positive droplets (i.e., belonging to the population)
    - number of negative droplets (i.e., not belonging to the population, if not specifically defined as “negatives” with respect to the population)
    - the separability score (see How to quantify fluorescence separability for the evaluation of amplification efficiency? for more details)
    - minimum and maximum limits of the 95% confidence interval for the estimated stock concentration (in copies/µL)
- 95% relative uncertainty for the estimated stock concentration (equal to the length of 95% confidence interval divided by 2 times the estimated concentration)

- PP_setup (“.csv” file/“PP_setup” sheet of “.xlsx” file), including for the experiment:
- The Type of post-processing analysis
- The target population
- The reference population

- The Cn ref value in case of CNV analysis
- The different channels used in case of linkage analysis
- PP_results (".csv" file/"PP_setup" sheet of ".xlsx" file), for each chamber:
- Chamber name, Pooling ID, Protocol name
- Total number of analyzed droplets
- For each CNV calculation: CN ref value, CNV value, minimum and maximum limits of the 95% confidence interval for the CNV value, 95% relative uncertainty for the estimated CNV value
- For each MAF calculation: MAF value, minimum and maximum limits of the 95% confidence interval for the MAF value, 95% relative uncertainty for the estimated MAF value
- For each GEX calculation: GEX value, minimum and maximum limits of the 95% confidence interval for the GEX value, 95% relative uncertainty for the estimated GEX value
- Linkage analysis:
  - for the total fragment: estimated stock concentration (in copies/µL), minimum and maximum limits of the 95% confidence interval for the estimated stock concentration (in copies/µL), 95% relative uncertainty for the estimated stock concentration (equal to the length of 95% confidence interval divided by 2 times the estimated concentration)
  - for the the intact fragrment or all the other combinations of channels, estimated stock concentration (in copies/µL), minimum and maximum limits of the 95% confidence interval for the estimated stock concentration (in copies/µL), 95% relative uncertainty for the estimated stock concentration, Relative abundance (%), minimum and maximum limits of the 95% confidence interval for the relative abundance and 95% relative uncertainty for the relative abundance

- Zone Details (".csv" file/"Zone_Details" sheet of ".xlsx" file), including details for each fluorescence zone (see the section "How to define *custom populations?*" in the Advanced Functionalities):
  - Chip barcode and chamber position, sample name
  - Zone ID (note that in "Line" thresholding mode there are always 2^N fluorescence zones, where N is the number of detection channels, while in "Polygon" thresholding mode there is one zone per user-defined polygon)
  - Zone type (quadrant in "Line" mode, polygon in "Polygon" mode)
  - In 3-color experiments only:
    - The x and y channels are used to define the zone, and the z channel (where "Chan1" stands for "Blue", "Chan 2" for Green, "Chan 3" for "Red", etc.).
    - List of x-y coordinates of the 2D points defining the zone (note that in "Line" thresholding mode there are always 5 points defining a square)
    - Coordinates of the z boundaries of the zone (note that in "Polygon" thresholding mode the z-values for the boundaries are always 0-65535 RFU)
- Population Details (".csv" file/"Population_Details" sheet of ".xlsx" file) including details for each population (see the section "How to define custom populations?" in the Advanced Functionalities):

- Population name
- Population color (RGB values)
- List of the ID(s) of the zone(s) from which the populations have been built
- *If applicable:* list of the ID(s) of the zone(s) specifically defined as “negatives” for the population.

Optional data exports

- QC Indicators (".csv" file / "QC_Indicators" sheet of .xlsx file), including for each chamber:
  - Chip barcode, chamber position and sample name
  - Quality flags
- Advanced QC Indicators (".csv" file / "QC_Advanced" sheet of .xlsx file), including for each chamber:
  - Chip barcode, chamber position and sample name
  - In "Line" thresholding mode only:
    - Automated threshold values for each channel
    - Manual threshold values for each channel
  - In "Line" and "Polygon" thresholding modes, for each droplet population
    - dimensions: channel ID to define the zone (where 1 stands for "Blue", 2 for Green, 3 for "Red", etc.)
    - separability score: please refer to the specific section on this
    - μ pos: mean of the fluorescence values of the positive droplet population computed in 1D, 2D or 3D, depending on the number of channels used to define the population
    - μ neg: mean of the fluorescence values of the negative droplet population, computed in 1D, 2D or 3D, depending on the number of channels used to define the population
    - std-dev pos: for 1D-defined populations: L1 standard deviation of the positive droplet fluorescence values in 1D. For 2D- or 3D- defined populations: mean of the projected distances between the positive droplet fluorescence points and their center in 2D or 3D along the axis defined by the positive population center and the negative population center in 2D or 3D
    - std-dev neg: for 1D-defined populations: L1 standard deviation of the negative droplet fluorescence values in 1D. For 2D- or 3D- defined populations: mean of the projected distances between the negative droplet fluorescence points and their center in 2D or 3D along the axis defined by the positive population center and the negative population center in 2D or 3D
- Droplet-level data files (".csv" files), for each chamber, each population (by default the "Blue channel", "Green channel" and "Red channel", etc. populations), and each compensation status (compensated and not compensated):
  - Each file includes one line per droplet and for each droplet the file contains the following information:
    - the x and y coordinates of the droplet center in the chamber
    - the fluorescence values (in RFU) of the droplet in each channel
    - the numerical index of the droplet
  - For each chamber, there is:
    - one general file which contains the information of all the droplets analyzed in the chamber.

- for each population (named “XXX”), there are 2 population-specific files:
- the first file with a filename including “XXX_POS”, which contains the information of the subset of droplets belonging to the population of interest.
- the second file with a filename including “XXX_NEG”, which contains the information of the subset of droplets defined as negative for the population.

- Spillover compensation file (“.ncm” format), including:
  - the translation vector for the fluorescence background in each channel.
  - the excitation matrix for the degree of excitation of each fluorophore by each LED.
- Crystal images (“.png” format in 8 bits), including the images of each chamber in each channel.
- Dot Plots (“.png” format), including, in the relevant channels:
  - the 1D dot plots of each chamber
  - the 2D dot plots of each chamber
  - the concatenated 1D graphs with all the chambers in sequential view
  - the concentration graphs
  - the uncertainty curves
  - the droplet fluorescence histograms
- Plots configuration file (exported in “.ncp” format); including the Histogram, 1D, and 2D dot plot configurations.
- Analysis template file (exported in “.nca” format); including the following information as data analysis parameters):
  - manual or automated thresholding strategy
  - thresholding mode and scope
  - list of the populations (default or custom) with their name, color, and zones
  - coordinates defining each zone. Only the coordinates associated with the first chamber of the current experiment are saved. These coordinates will apply to each new chamber of any further experiments.
- If the zoning mode is set to ‘Individual per chamber’, then the user is invited to specify the chamber for which the zoning configuration will be exported in the “.nca” file.

Notes:

- *Exporting all the chamber images or graphs will make exporting data take longer*
- *The indicative size for the exported chamber images in PNG format is 3 MB/channel/chamber.*
- *From each menu of the application, it is also possible to save a given image view or graph view by activating one of the following states.*
  -  *Save as an image file (“.png”).*
  -  *Save as a vector file (“.svg”).*
  -  *Copy to clipboard.*

And then click on the image or graph of interest.

Finally, to save the experiment, simply click on the “FILE” menu and click on “Save” or “Save as” depending on what is needed.

5. Advanced functionalities

How to edit the experiment?

To edit the experiment, click on the “SETUP” > “Edit Experiment” submenu.

Figure 56: *Edit the experiment properties, scroll right using the bar at the bottom of the page to view results for all the fluorophores.*

The editable parameters regarding the context of the experiment are:

- The fluorophore names (for each fluorescence channel).
- The target names (for each fluorescence channel).
- The user comments.

The editable parameters regarding the context of each chamber are:

- The sample name.
- The sample type (U: unknown; N: negative; P: positive; S: standard).
- The dilution factor, where a value of 10 equals a 10-fold dilution.
- The standard concentration, which is the known stock concentration, in the case when the sample type is “S”.

Note: If the dilution factor is modified from “1” to “10”, then the estimated stock concentration will be multiplied by 10. . The updated dilution factor is considered in post-processing data calculations, except for Linkage Analysis, which is always calculated at a dilution factor of 1.

Some fields are read-only because their content is used to identify the chambers.

To modify the order of the chambers in the list, click on the “up” and “down” icons above the chamber list to sort them by name, or click on the title of any column to sort them according to the column value in ascending or descending order.

For example, the chambers can be sorted according to ascending stock concentration values in the “Blue” channel.

To pool (resp. unpool) a set of chambers in which the same sample has been loaded, simply select these chambers in the left panel using “Ctrl+Click” or “Shift+Click”, and then click on the “Pool” button (resp. “Unpool” button) above the chamber list.

For more details about chamber pooling, check out the next chapter “How to pool replicate chambers for better sensitivity and precision?”.

To remove one or more chambers from the current experiment, select the chamber(s) in the left panel list, then click on the “-” icon above the list.

To add the chambers of another experiment into the current experiment, click on the “+” icon above the chamber list, then select the experiment to be imported (in “.niodata” or “.nioresult” format) or drag & drop the file in the application window.

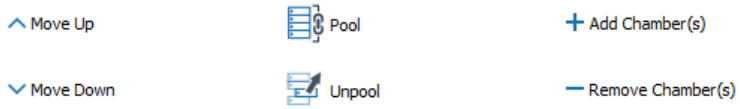


Figure 57: *The “EDIT EXPERIMENT” submenu contains icons to sort, pool/unpool, or add/remove experiment chambers.*

CAUTION!

To avoid compromising the results the experiment to be added should have identical experiment parameters as the current experiment, i.e.,

- Fluorophore names and target names should be the same, otherwise, they will be erased by those of the current experiment.
- Scanning parameters (exposure times) should be the same, otherwise, these too will be erased by those of the current experiment.
- Image analysis parameters should be the same, otherwise, a warning message will be displayed.

How to navigate in the 3D graph?

To visualize the 3D graph, click on the “ANALYZE DATA” > “Plots & Populations” submenu, then click on the “3D dot plot” tab. The 3D graph is displayed, where each axis represents the fluorescence value of the droplets in any given channel.

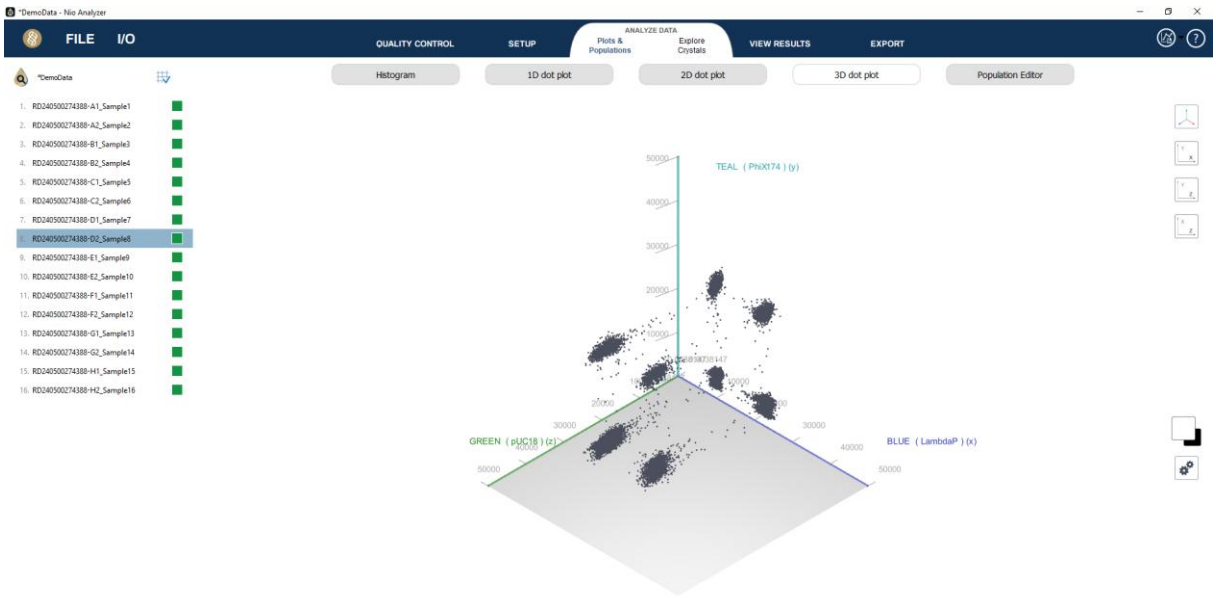


Figure 58: *Navigation in the 3D dot plot.*

To navigate in the 3D graph:

- Use mouse scroll to zoom into the graph
- Use right mouse click and drag to rotate the graph
- Use left mouse click and drag to move the graph and the rotation center
- Double-click with the left mouse button to reset the view

Icon description:

Reset the view.

Project the view on the first and second axis.

Project the view on the second and third axis

Project the view on the first and third axis

Switch between black and white backgrounds

Modify the point size, the line width, the grid step or the population axis

How to read the droplet fluorescence histograms?

To view the histograms of the droplet fluorescence values, click on the “ANALYZE DATA” > “Plots & Populations” submenu, then click on the “Histogram” tab.

The histograms of the currently selected chamber(s) are displayed for each channel, where the x-axis shows the fluorescence value (in RFU) and the y-axis shows the number of associated droplets:

***Figure 59**: Histograms of fluorescence.*

This histogram view is useful to appreciate the separability between the negative population (on the left side of the histogram) and the positive droplet population (on the right side of the histogram), and thus to check or adjust the fluorescence thresholds.

If multiple chambers are selected, then the number of droplets in each fluorescence bar are added together.

How to interpret the uncertainty curves?

To visualize the curve of relative uncertainty at a 95% confidence level, click on the “VIEW RESULTS” > “Advanced Graphs” submenu, then click on the “Uncertainty Curves” tab.

The 95% relative uncertainty curves are displayed for each channel, where:

- The x-axis shows all possible chamber concentrations (in copies/µL)
- The y-axis shows the 95% relative uncertainty (in %) of the chamber concentration (which is based on the total number of droplets analyzed in the currently selected chamber

- The colored curve corresponds to the relative uncertainty curve of the currently selected chamber (the higher the total number of droplets in the chamber, the lower the curve)
- The upper gray curve corresponds to the relative uncertainty curve of the chamber with the least droplets.
- The lower gray curve corresponds to the relative uncertainty curve of the chamber with the most droplets. Note, the two gray curves can be obscured under the colored curve of the selected chamber if they are the same.
- The filled point(s) located on the curve correspond to the predicted concentrations in the currently selected chamber(s) on the left panel. Be sure to distinguish between the chamber concentration and the stock concentration which is equal to the chamber concentration times the dilution factor.
- The white point located on the curve represents the chamber concentration which minimizes the relative uncertainty. This indicative value may help to estimate to optimal dilution for the experiment.

Note that whatever the number of droplets and the confidence level, the white point is reached where ~79.7% of the droplets are positive.

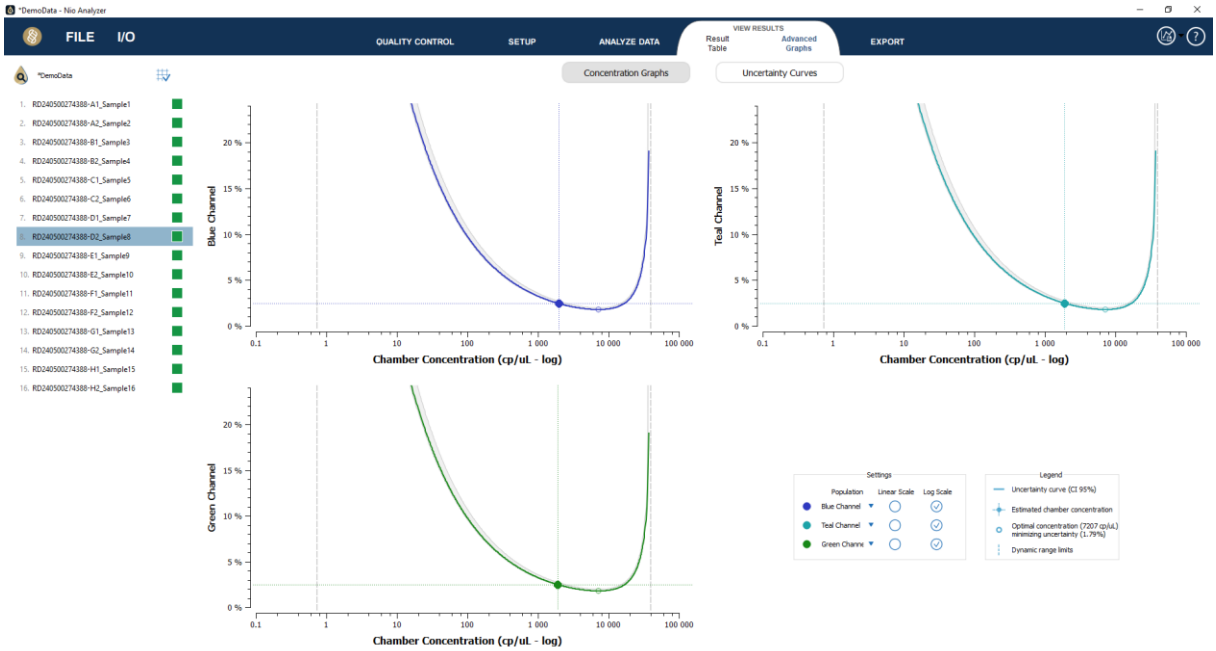


***Figure 60**: Relative Uncertainty Curve.*

How to pool replicate chambers for improved sensitivity and precision?

CAUTION!

The chamber pooling functionality should only be used under the assumption that pure replicates have been loaded in the pooled chambers (e.g., the same sample has been loaded).

Pooling a set of chambers in which the same sample has been loaded, increases both detection sensitivity and quantification precision. Indeed, by considering each set of pooled chambers as one larger chamber, this pooling strategy allows to increase the analyzed volume.

If the pooled chambers have not been previously defined with the Nio Reader software, it is still possible to define them with the Nio Analyzer software for experiment analysis:

- Go to the “SETUP” > “Edit Experiment” submenu.
- Select the chambers required to pool together in the list of the chambers displayed on the left, using “Ctrl” + “Left-Click” or “Shift” + “Left-Click”. The names of the selected chambers will be highlighted in blue in the list.
- Click on the “Pool” button above the list of chambers. A new row in the table representing the parent of the pooled chambers, and named with their “Sample name”, will be created above them in the list and an expand icon “>” will show the results lines of the children chambers on demand.
- All chambers pooled together will automatically share the same “Pool ID” (recorded in the “Chamber_Details” sheet of the exported “.xlsx” file), as well as the same “sample name”. For each detection channel, they will also share the same “sample type”, “reference concentration” and “dilution factor”.

To “Unpool” the chambers, click the “Unpool” button.

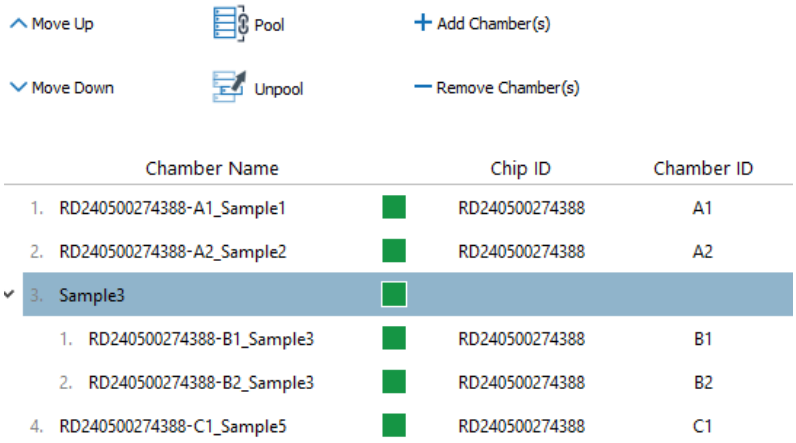


Figure 61: *Chamber pooling feature of the Nio Analyzer software (SETUP > Edit Experiment).*

Once the chambers are pooled, the software automatically computes the concentration of each target in the pooled chambers represented by their “parent”. The higher the number of pooled chambers, the higher the total number of analyzed droplets, so the lower the sampling error and the partitioning error, which means that the quantification certainty increases.

The predicted concentrations of the pooled chambers are displayed in the “Result Table” tab of the “VIEW RESULTS” menu:

Chamber Name	Chamber Context	Nb Droplets	Dilution	C (cp/uL)	Nb Pos	Nb Neg	Separability Score	C_min (cp/uL)	C_max (cp/uL)	Relative Uncertainty (CI 95%)
1. RD240500274388-A1_Sample1		15921	1	**1914**	5491	10430	11	1863	1966	2.66 %
2. RD240500274388-A2_Sample2		17031	1	**1973**	6017	11014	12	1923	2023	2.55 %
3. Sample3		34491	1	**1849**	11570	22921		1816	1884	1.83 %
1. RD240500274388-B1_Sample3		17378	1	**1816**	5745	11633	10	1769	1864	2.6 %
2. RD240500274388-B2_Sample3		17113	1	**1883**	5825	11288	10	1835	1932	2.59 %
4. RD240500274388-C1_Sample5		15908	1	**2024**	5736	10172	7	1971	2077	2.61 %

Figure 62: *Example of pooled results (VIEW RESULTS > Result Table), where the relative uncertainty of the parent pool is lower than those of the individual constituent chambers.*

In the “VIEW RESULTS” > “Result Table” submenu, one line is added for the result of the pooled “parent chamber” and the result lines of the non-pooled “children chambers” are kept below. These new lines are exported in the same order in the “Results” sheet of the exported “.xlsx” file.

In the “VIEW RESULTS” > “Advanced graphs” submenu, the user can visualize both the uncertainty curve and concentration graphs, either for the pooled “parent chamber” or for each “children chamber” composing the pool.

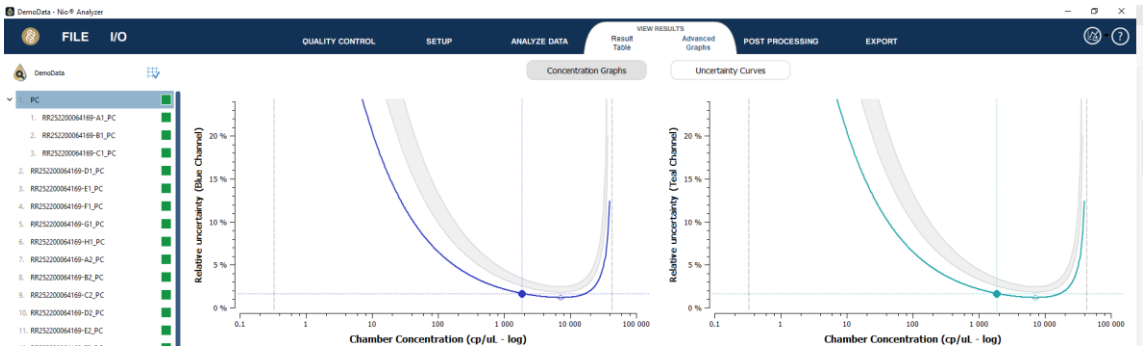


Figure XX: *Example of uncertainty curve (VIEW RESULTS > Advanced graphs for the pooled “parent chamber”*

Warning: When running Nio Analyzer in Regulatory mode, validating a pool of chambers will automatically validate each chamber of the pool individually. Stilla technologies recommends making sure that each individual chamber composing the pool is indeed valid. Please refer to section 0 for more details about the features specific to the Regulatory mode, such as sample validation.

How to concatenate or aggregate graphs?

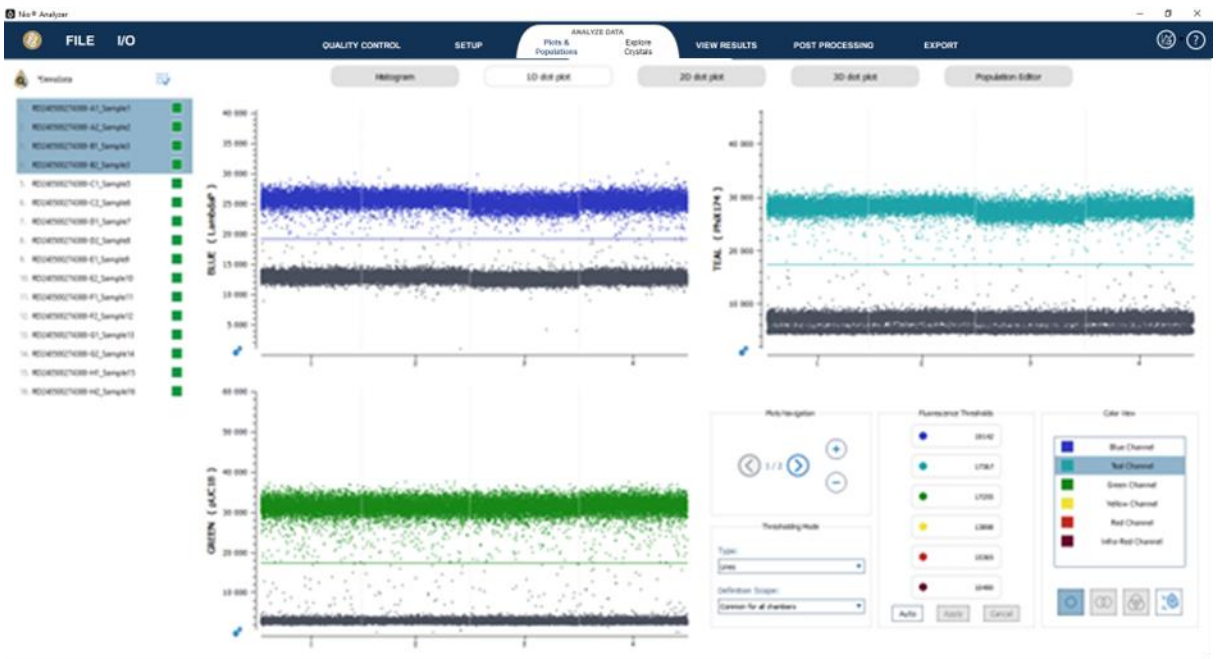


Figure 63: *Concatenated view of 1D dot plots.*

To concatenate 1D graphs, select multiple chambers in the left panel (using “Ctrl+Left-Click”). The 1D graphs of the selected chambers are then concatenated in a sequential view (in the order of the chamber list).
To aggregate 2D graphs, select multiple chambers in the left panel (using “Ctrl+Left-Click”). The 2D graphs of the selected chambers are then aggregated.

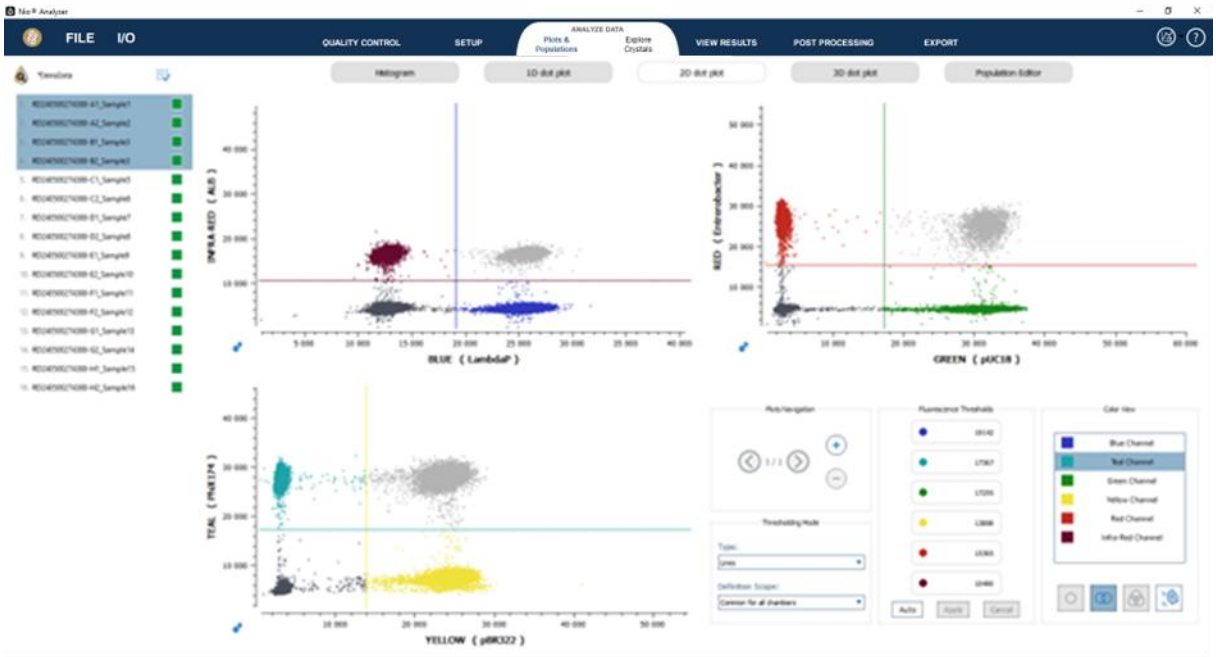


***Figure 64**: 2D dot plots of pooled chambers.*

To aggregate 3D graphs, select multiple chambers in the left panel (using “Ctrl+Left-Click”). The 3D graphs of the selected chambers are then aggregated.

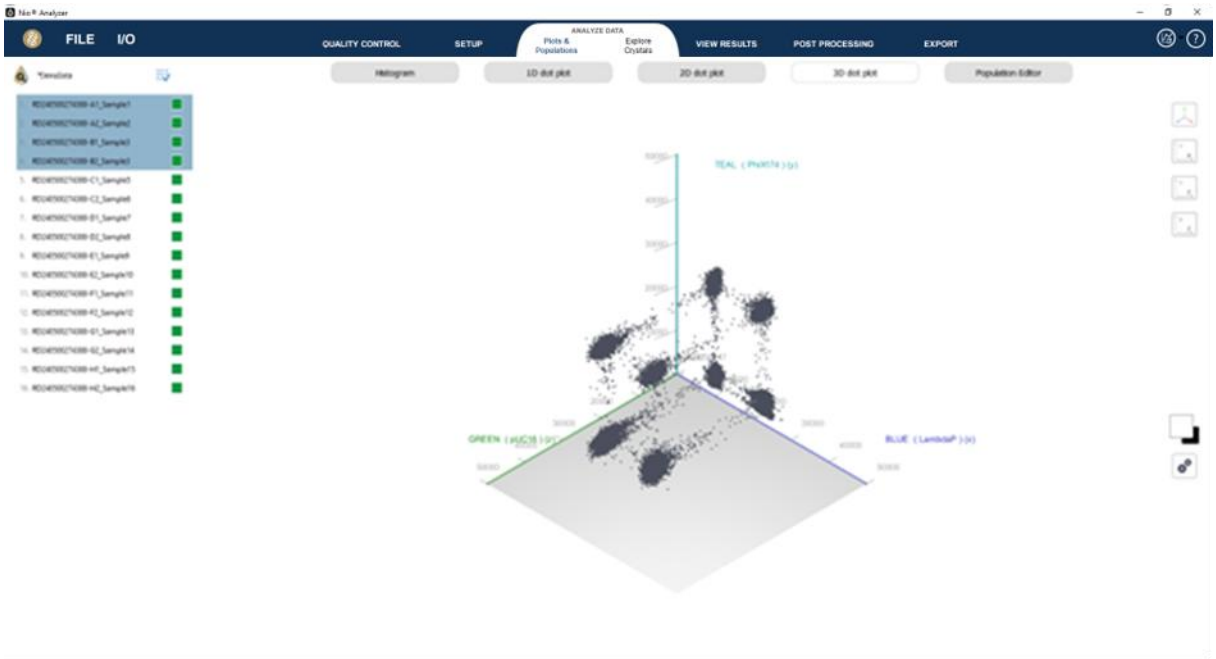


***Figure 65**: 3D dot plots of pooled chambers.*

Similarly, the histograms and uncertainty curves can display aggregated chambers.

***Figure 66**: Histograms of pools of chambers.*

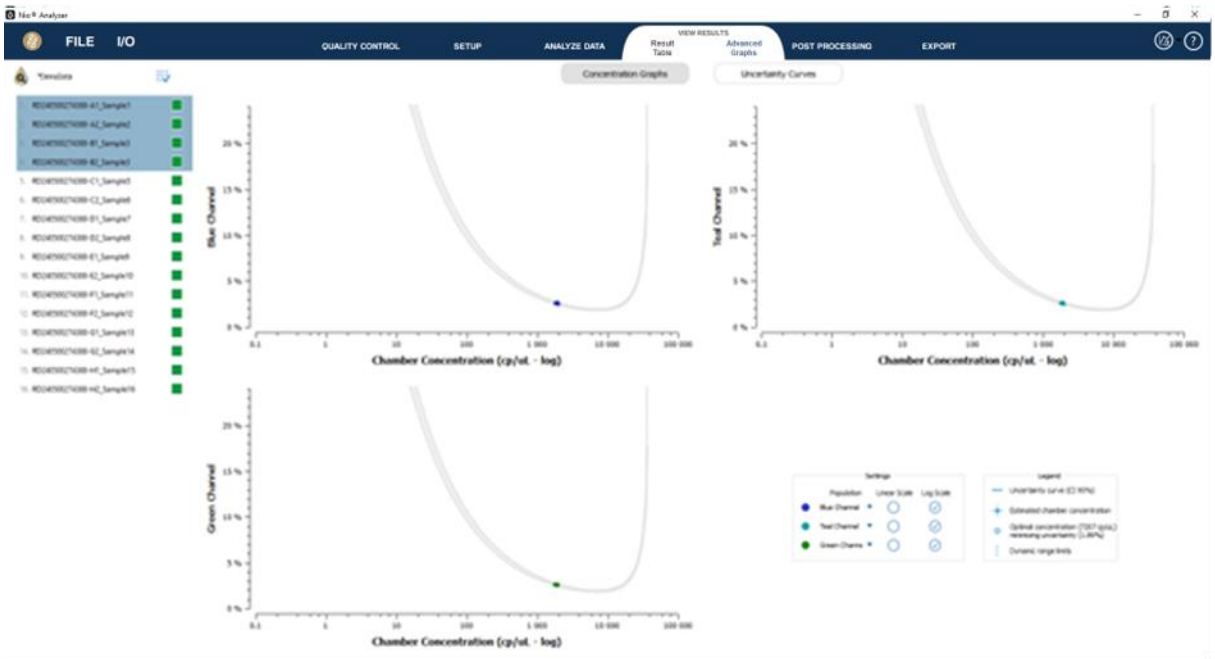


***Figure 67:** Uncertainty Curves of pooled chambers.*

How to explore the Droplet Crystal populations?

To explore the crystal populations, click on the “ANALYZE DATA” - “Explore Crystals” submenu, then select the chamber of interest from the list.

To navigate in the crystal image of a chamber:

- Use mouse scroll to zoom in and out in the image.
- Use left mouse click to drag the image.
- Double-click using the left mouse button to reset the view.

- Click on the LED icons in the “Channel selection” widget to change the detection channel (“Blue”, “Teal”, “Green”, “Yellow”, “Red”, “Infra-Red” and “Purple” LED).
- Click on the “Auto” button (resp. “Reset” button) in the “Contrast Adjustment” widget to automatically adjust image contrast (resp. reset image contrast).

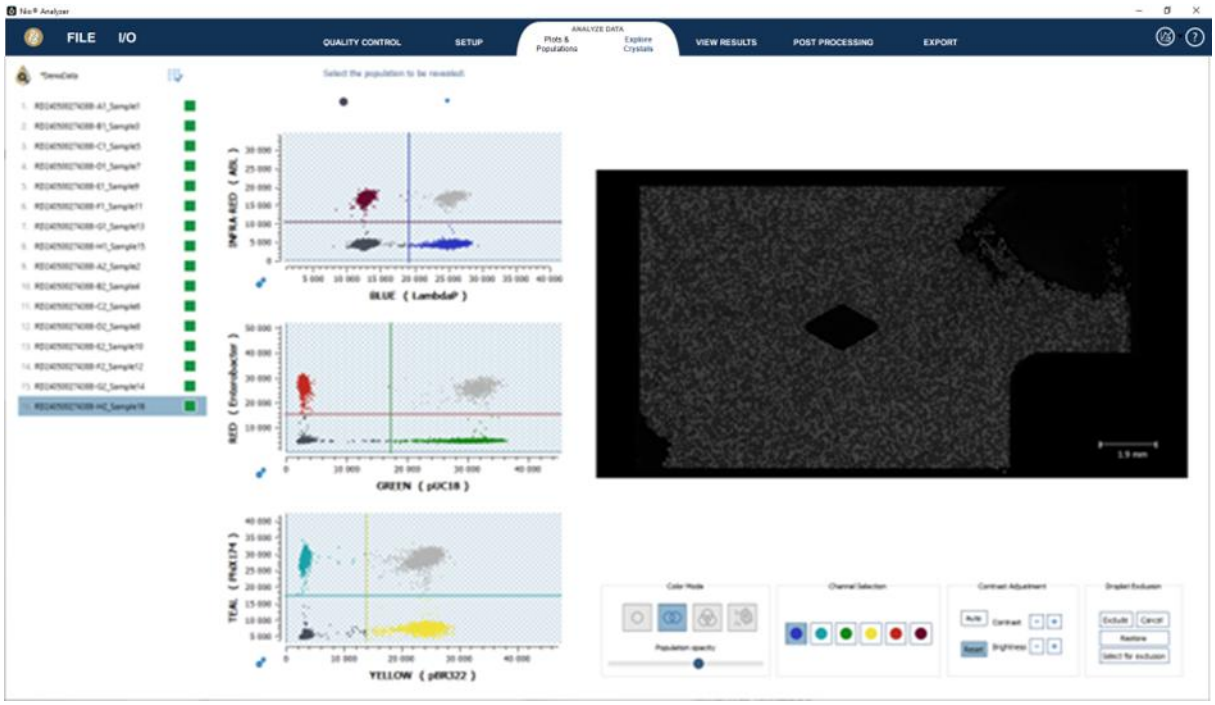


Figure 68: *Explore crystals in 2D simplified-color mode.*

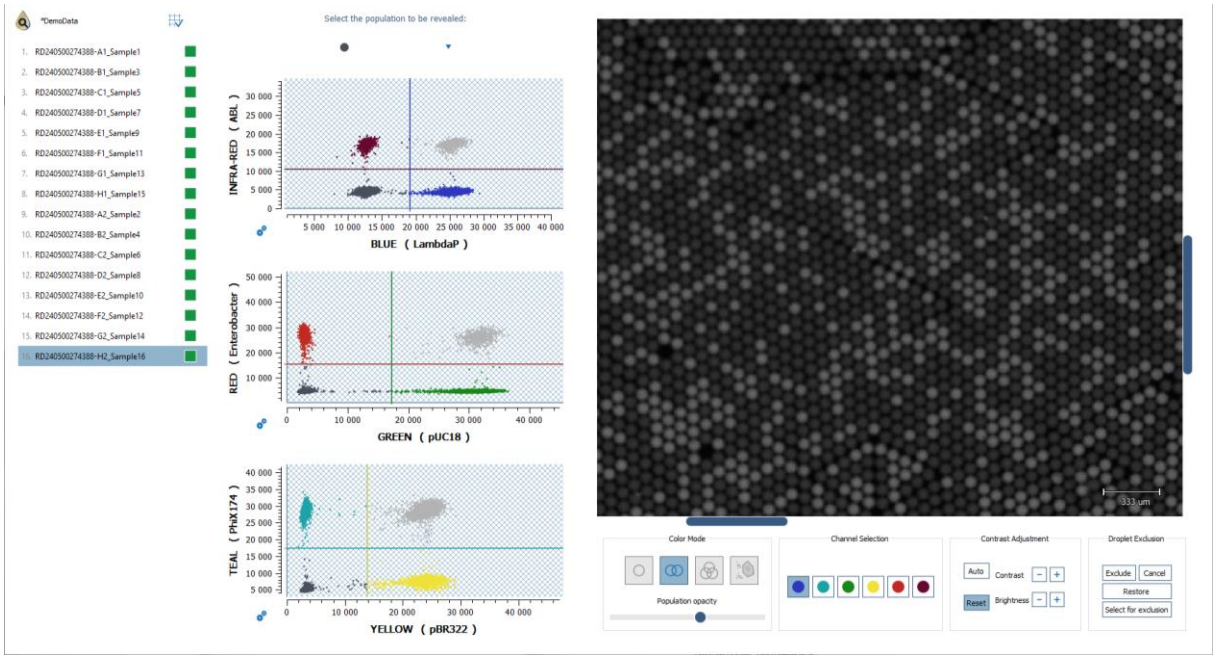


Figure 69*: Zoom in on the crystal.*

To highlight a specific droplet in the crystal image:

- To activate/deactivate the highlighted droplets included in one quadrant of a 2D graph, Left-Click on the quadrant of interest.

- The activated quadrant will be filled in transparent orange in the graph and the droplets included in this quadrant will be highlighted in their current color codes in the image. The “Population opacity” cursor can be used to adjust the opacity.
- Use “Shift + Left-Click” to select multiple quadrants in any given 2D graph and highlight multiple droplet populations.

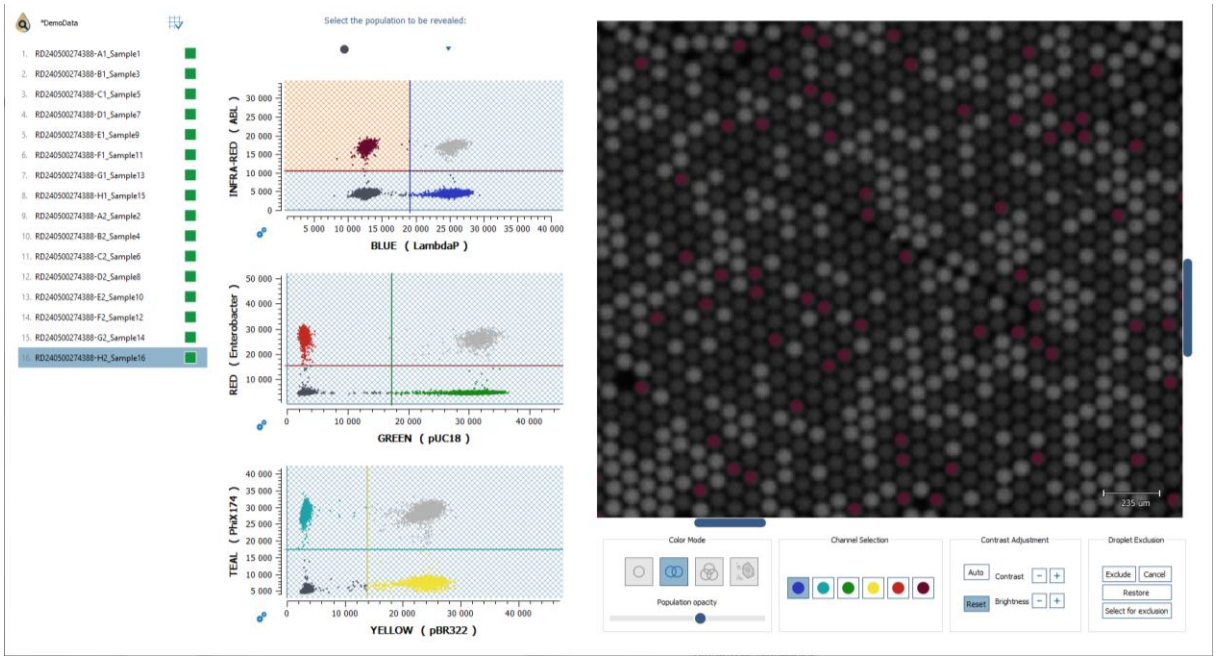


Figure 70: *Highlighted droplet populations in the selected graph quadrant are revealed in their color code on the right.*

To highlight the droplets belonging to a given population, select the population name in the drop-down menu above the 2D dot plots.

The associated zones (quadrants or polygons) will be filled in transparent orange in the graph and the droplets included in these zones will be highlighted with the population color in the image.

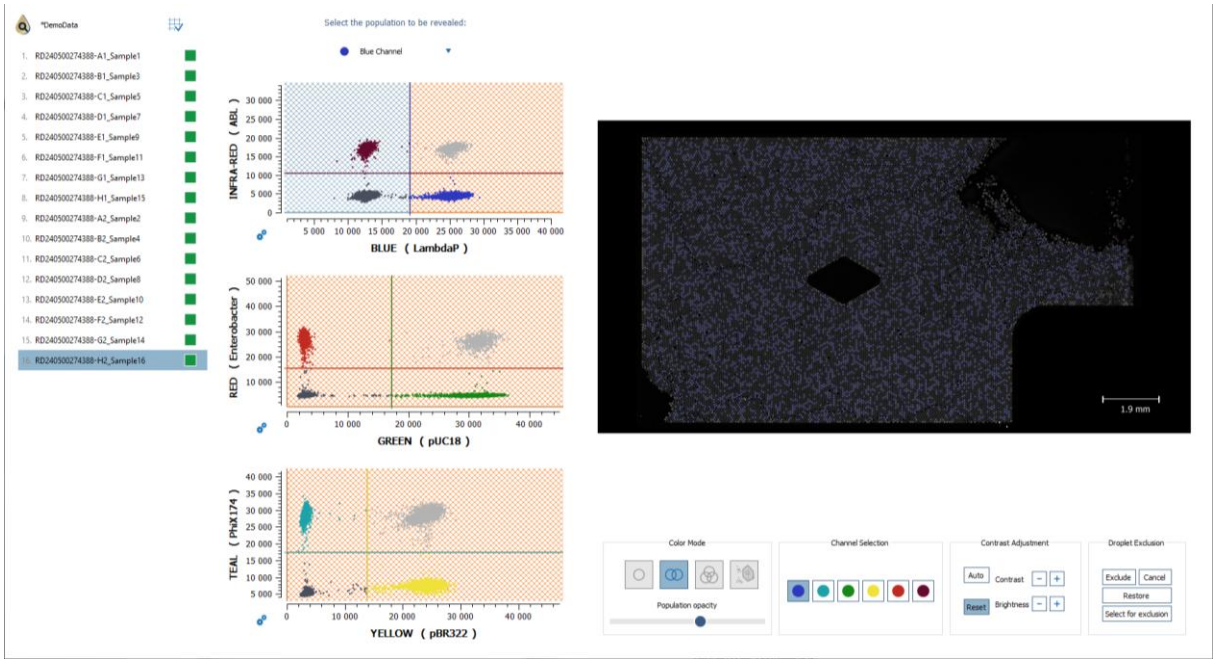


Figure 71: *Highlighted droplet populations using graph quadrants.*

To highlight droplets included in any user-defined polygonal region of a 2D graph, press the “Ctrl” key, then Right-Click several times to define the polygonal region to be drawn (one-click = one vertex), and leave the “Ctrl” to define the last polygon vertex.

The polygon will then be drawn with orange edges on the 2D graph and the droplets included in this polygon will be highlighted in orange color in the image.

To edit the polygon:

- Right-Click outside the polygon to remove it.
- Left-Click on an edge of the polygon then drag & drop to move the polygon. Note that left-clicking inside a polygon will not move the polygon but rather the 2D view.
- Left-Click on any vertex of the polygon to modify its position.
- Right-Click on a vertex of the polygon to remove this vertex.
- Left-Click on an edge of the polygon to add a new vertex in the middle of this edge.
- To define a basic diamond polygon in one click, hold the “Shift” key while right-clicking. Then edit it normally.

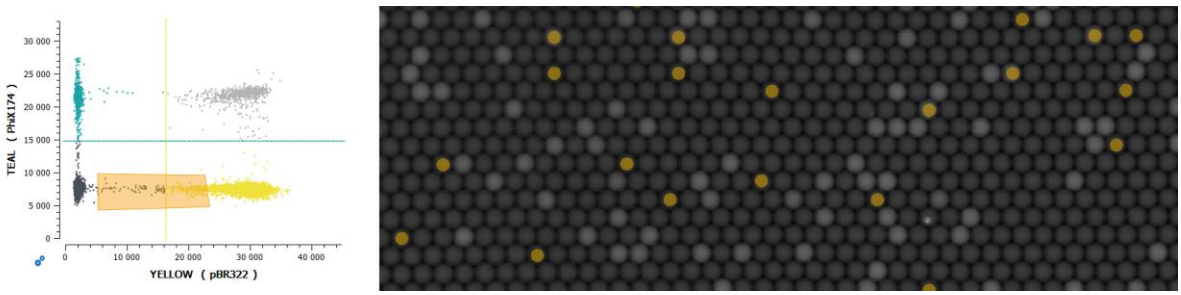


Figure 72: *Highlighted droplet population using polygon drawn in the 2D graph (left); included droplets are highlighted in orange color (right).*

To exclude highlighted droplets from the analysis, click on the “Select for exclusion” button in the “Droplet Exclusion” widget, and finally click on the “Exclude” button in the “Droplet Exclusion” widget to remove them from the analysis. If it is required to exclude some droplets from the highlighted polygon, after clicking on “Select for exclusion”, select/deselect the droplets by right-clicking on them in the crystal image and then click on “Exclude”.

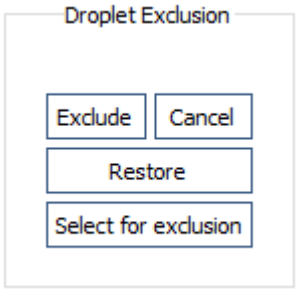


Figure 73: *Widget for manual droplet exclusion in “EXPLORE CRYSTAL” submenu.*

Manually excluded droplets will be marked with yellow hexagons in the crystal images and will be removed from any dot plot:

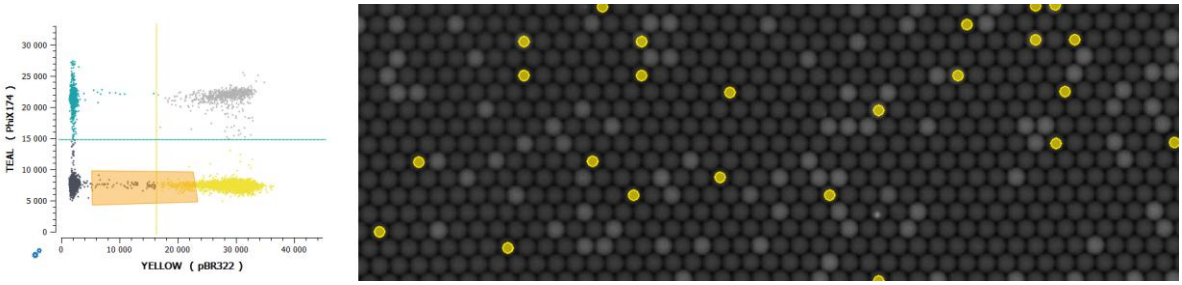


Figure 74: *Excluded droplets are removed from the 2D graph and marked with yellow hexagons in the image.*

To restore all the manually excluded droplets, click on the “Restore” button in the “Droplet Exclusion” widget.

How to perform quality control?

To perform quality control, click on the “Quality Control” menu.

Chamber Quality Flag

A chamber quality flag is displayed next to each chamber’s name on the top left side of the application window. This chamber quality flag provides visual feedback for chamber quality control. The color of the quality flag depends on quality indicators such as the presence of saturated objects and the number of analyzable droplets in the chamber:

- “Number of analyzable droplets”: the higher the number the better the confidence in predicted concentration results.
- “Number of saturated objects”: the lower the number the better the chamber quality. *Note: Saturated objects are either saturated droplets caused by a high exposure time or saturated artifacts (this can be corrected when using Nio Reader software by checking that exposure times are not too high, or if the chip should be cleaned using an appropriate wipe to remove dust or fluorescent elements on the foil -- Please check Nio Digital PCR User Manual for instructions).*

Each indicator can be either green, yellow or green with an eye icon inside.

A green flag indicates that all quality indicators are within expected specifications.

A green flag with an eye icon on it means that Stilla Technologies recommends inspecting visually the droplet crystals to decide whether the result should be taken into account. Refer to the Ruby Chip troubleshooting section of the Nio Digital PCR User Manual for more details.

A yellow flag indicates that there is either too many saturated artefacts on the picture or there are less than 1000 detected droplets in the chamber. When saturated artefacts are caused by dust particles, Stilla Technologies recommends cleaning the bottom of the Ruby Chip with a dust free tissue and read the chambers again (refer to the Nio Digital PCR User Manuel for detailed instructions).

Figure 75: *Annotated crystal for quality control.*

To inspect the images, click on the chamber in the chamber list. The chamber crystal is then displayed with the following annotations:

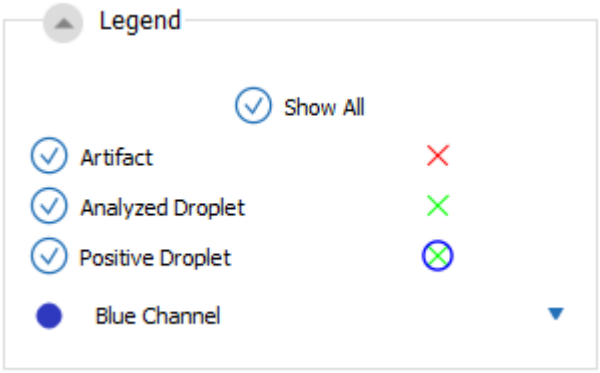


Figure 76*: Legend for image annotation in the Quality Control menu (checkboxes allow to show/hide specific annotations).*

- Red Cross: artifact (e.g., dust, undersize droplet, oversize droplet, irregularly shaped droplet, boundary droplet, etc.) not counted as an analyzable droplet.
- Green Cross only: negative droplet in the currently selected channel (counted as an analyzable droplet).
- Note that all negative droplets should be visible in the “Blue” channel image owing to the addition of the reference fluorophore (e.g., Fluorescein or FITC fluorophore).
- Blue circle around Green Cross: positive droplet in the currently selected population in the drop-down menu just below (counted as an analyzable droplet).

To navigate in a chamber crystal:

- Use mouse scroll to zoom in and out in the image.
- Use left mouse click to drag the image.

- Double-click with the left mouse button to reset the view.
- Click on the LED icons in the “Channel selection” widget to change the detection channel (“Blue”, “Teal”, “Green”, “Yellow”, “Red”, “Infra-Red” and “Purple” LED).
- Click on the “Auto” button .in the “Contrast Adjustment” widget to automatically adjust image contrast. Click on the “Reset" button in the “Contrast Adjustment” widget to reset image contrast.

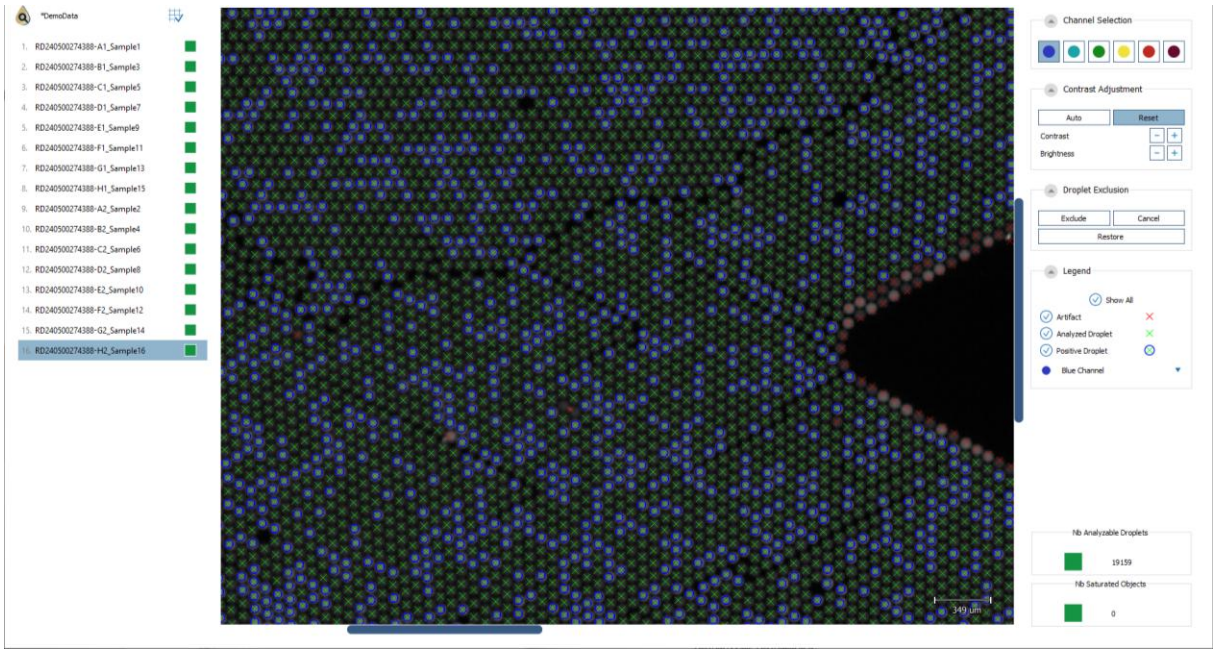


***Figure 77**: Zoom into the annotated crystal for quality control.*

To exclude one or several droplets considered as false positive(s) (i.e., artifacts not to be counted as analyzable droplets):

- Right-Click on each droplet to be excluded.
- Click on the “Exclude” button in the “Droplet Exclusion” widget.

Note: Excluded droplets are marked with yellow hexagons.

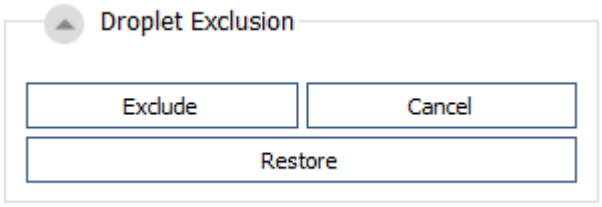


***Figure 78:** Widget for manual droplet exclusion in the Quality control menu.*

To exclude all the droplets belonging to an image region:

- Press the “Ctrl” key, then Right-Click several times to define the polygonal image region to be excluded (one click = one vertex), then leave the “Ctrl” to define the last polygon vertex.
- Click on the “Exclude” button in the “Droplet Exclusion” widget.

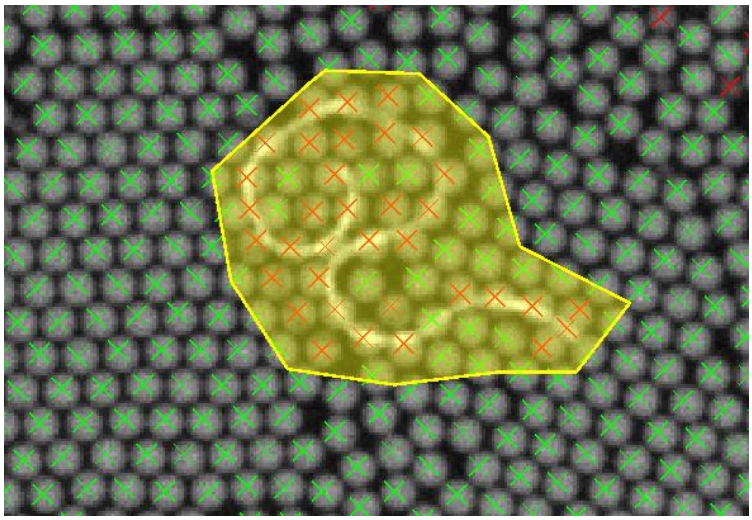


Figure 79: *Selection of droplet or polygonal image regions to be excluded.*
Note: Droplet annotations are still visible in the yellow shape before clicking on "Exclude".

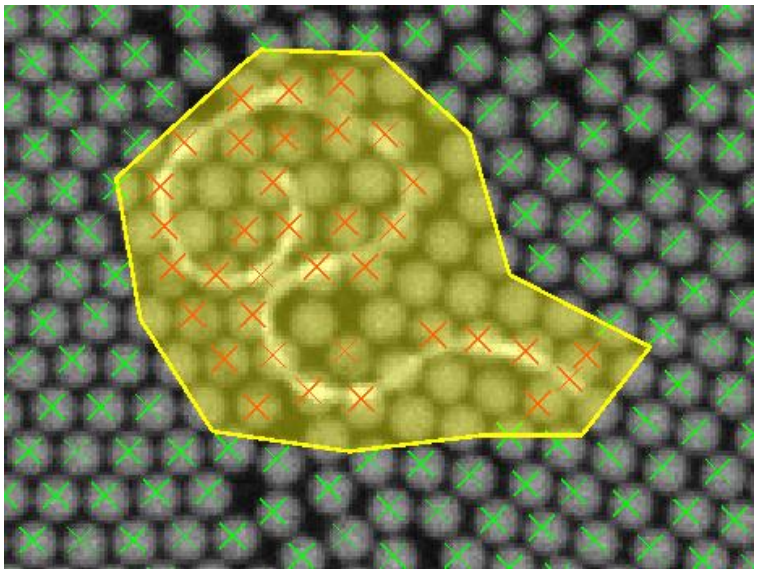


Figure 80: *All selected droplet or image regions are removed.*
Note: Droplet annotations are no longer visible in the yellow shape after clicking on "Exclude".

Note: All droplets belonging to the polygonal region will be removed from the analysis and marked with yellow color.

To cancel the current selection of droplets to be removed, click on the "Cancel" button in the "Droplet Exclusion" widget.

To restore all the manually excluded droplets, click on the "Restore" button in the "Droplet Exclusion" widget.

Note: It is possible there may be oversegmentation (two or more droplets (crosses) instead of one) or undersegmentation (one droplet instead of two or more) of the droplets which may lead to false positive or false negative results. Manually inspect the droplets. Where there are red crosses, the software automatically rejects the droplets, and no action is required. However, if aberrant crosses are green, they should be excluded manually.

Note: Artifacts are detected in the detection channel but - depending on the experiment/assay/chemistry used - some artefacts could exist and be visible only in other channels. It is recommended to check all the channels available in the experiment and ensure that no droplet need to be excluded manually.

How to quantify fluorescence separability

The Separability Score is an objective measure of the separation of the positive and negative clusters of any given amplified target in a sample. The Separability Score is based on the distance between the positive and negative clusters and the positive and negative cluster spreads. (**Figure 81**).

- the “positive” cluster of droplets includes the amplified target of interest and thus represents a high fluorescence intensity.
- the “negative” cluster of droplets does not include the target of interest and therefore has a lower fluorescence intensity compared to the positive cluster.

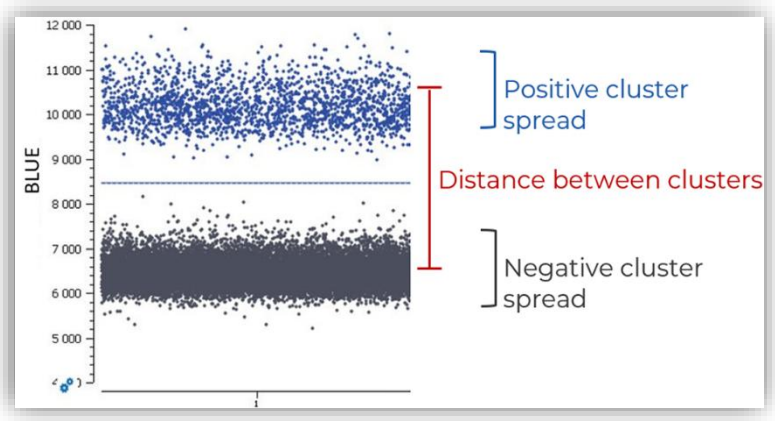


Figure 81: *Illustration of the parameters that define the Separability Score.*

The Separability Score is given for each detection channel and is automatically computed by the Nio Analyzer software. It can be viewed for each target population in the “VIEW RESULTS” menu under the “Result Table” submenu by clicking on the “display/hide additional columns” icon (Figures 82 and 83).

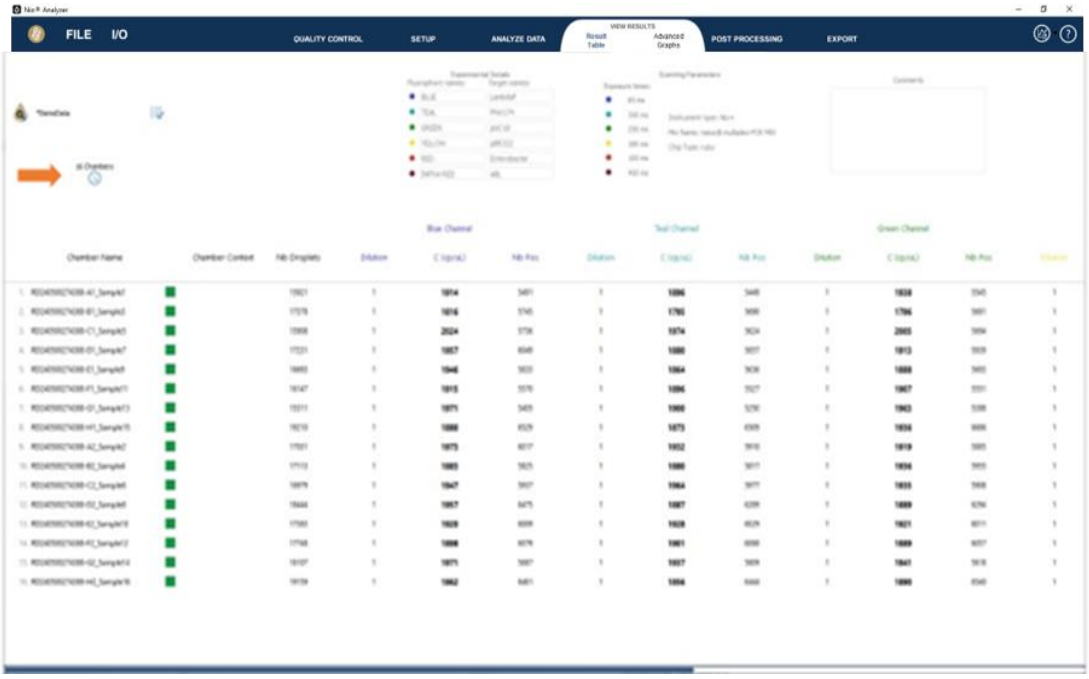


***Figure 82**: Unexpanded view of the Results Table.*

***Figure 83:** Separability Score displayed by clicking the "display/hide additional columns" icon under the "Results Table" submenu.*

The Separability Score is one of the relevant criteria for both quality control and assay optimization.

The higher the Separability Score for the droplet population, the better the amplification efficiency for the target of interest. A low Separability Score, or a decrease in a previously characterized Separability Score, can signal a quality issue impacting the PCR amplification efficiency (for example: use of an incorrect PCR program, sample degradation, incorrect probe/primer concentrations).

Note: The Separability Score is not the only parameter to be evaluated when optimizing assays. Proper quantification must be confirmed using known quantities of positive controls. For example, the Separability Score for any given channel could be high, but if the reaction is not specific to the desired

target, the target quantification could be incorrect (see below "Using the Separability Score for assay optimization"

Separability Score values:

- For the Blue channel, a Separability Score of 4 is the minimum acceptable value to distinguish clearly and set the threshold between the positive and negative clusters on a 1D dot-plot.
- The Separability Score is low for the Blue channel because of the addition of the reference dye, fluorescein, which allows detection of the droplets by the analysis software but increases the background signal (negative population fluorescence) in the Blue channel.
- For all other channels, a Separability Score above 8 is acceptable, depending on the assay and the fluorophore used.

Using the Separability Score for assay optimization:

The Separability Score can be used to determine the optimal elongation temperature common to all target amplifications. The Separability Score is critical for assay optimization because unoptimized probes/primers may cause non-specific amplification (**Figure**). This non-specific amplification can further lead to a distinct second population due to undesired probe/primer interactions.

The Separability Score can also be used to optimize assays and help determine the ideal PCR conditions common for all primers and probes used in an assay. Sub-optimal PCR conditions can result in 'rain', droplets of intermediate fluorescence due to inefficient amplification (**Figure 85**). Poor PCR can also lead to more than one distinct positive population, which can result in difficulty defining a threshold and/or inaccurate quantification of the target of interest.

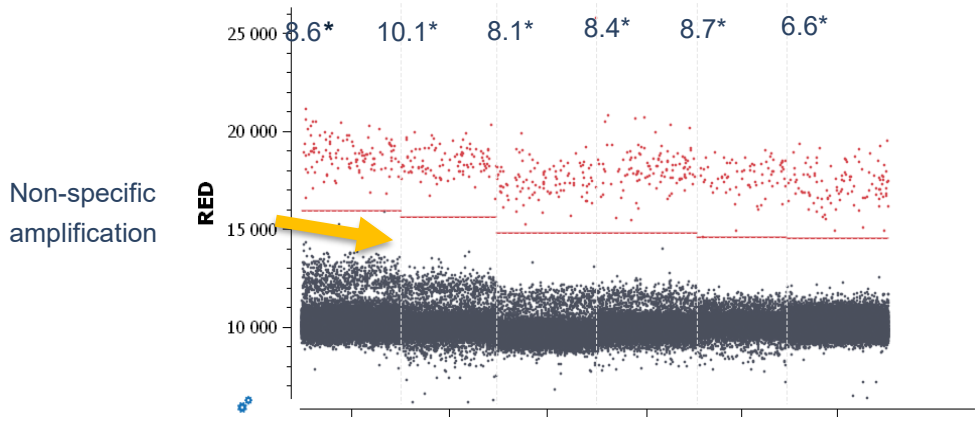


Figure 84: *Identification of non-specific amplification.*

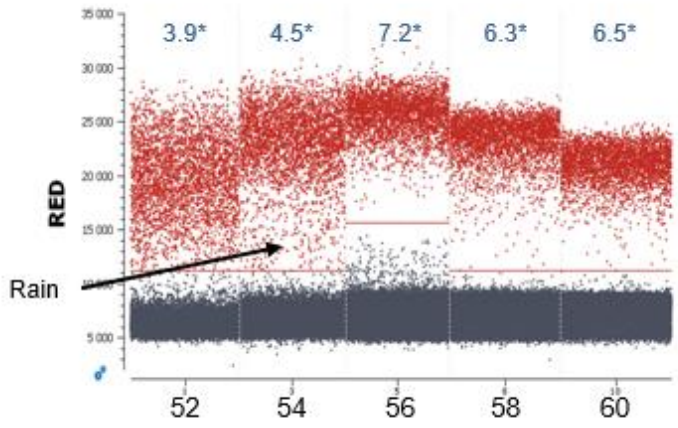


***Figure 85**: Example of rain.*

The Separability Score can also be used to determine the optimal probe/primer concentration at which there is good separability between the positive and negative clusters, and no performance loss. It can be especially useful to determine the lowest concentration of probes/primers needed for an assay to reduce assay complexity and the overall quantities of reagents required for the reaction.

Relative Separability Scores can be used to evaluate the assay performance. For example, if the Separability Score was 4 in the Blue channel, but after changing the elongation temp from 59 °C to 61 °C, the Separability Score became 6, this would indicate a potential improvement in the assay. A comparison of the Separability Score before and after making a reaction change should be done for all channels to evaluate the impact on assay performance.

How to define custom droplet populations?

Populations are entities for which Nio Analyzer provides results: number of positive and negative droplets, concentration, confidence interval etc.

The process of defining more refined populations than the default ones proposed for each experiment (i.e., Blue, Teal, Green, Yellow, Red, Infra-Red, Purple populations), is split into two main stages:

- the individual zones need to be defined
- then the population needs to be built from those zones.

Finally, negative zones can be defined if needed.

How to define a zone?

There are two ways to partition the fluorescence space in Nio Analyzer software:

- 1D Line thresholds
- 2D Polygons.

The default is to use a line to delimit a threshold, but polygons can also act as thresholds. To select the appropriate thresholding type to be applied to the whole experiment, go to “ANALYZE DATA” > “Plots

& Populations” > “2D dot plot”, then go to the “Thresholding Mode” widget and use the “Type” combo box to select “polygon” mode.

1D "Line" thresholds:

The 1D "Line" thresholds are the default zoning for any freshly scanned experiment.

Each 1D line splits each channel into 2 parts. See section IV. 1. f "*Data visualization and interpretation*" for a full explanation. See also section IV. 3. "*Check the fluorescence thresholds*" for more details on how to manipulate Line thresholds. The "Lines" can be manipulated in the following plots:

- Histograms
- 1D-dotplot
- 2D-dotplot

2D "Polygon" thresholds :

The 2D "Polygon" thresholds provide a flexible way to define zones in the fluorescence space when line thresholding is not possible. The polygons are only editable in 2D-dotplots.

By default, upon switching to the "Polygon" thresholding type, the experiment no longer contains the default zones and populations.

Polygon zones can be manually created based on the user's requirements. For this purpose, go to "ANALYZE DATA" > "Plots & Populations" > "2D dot plot" and select "Polygons" in the "Type" drop-down button of the "Thresholding Mode" widget:

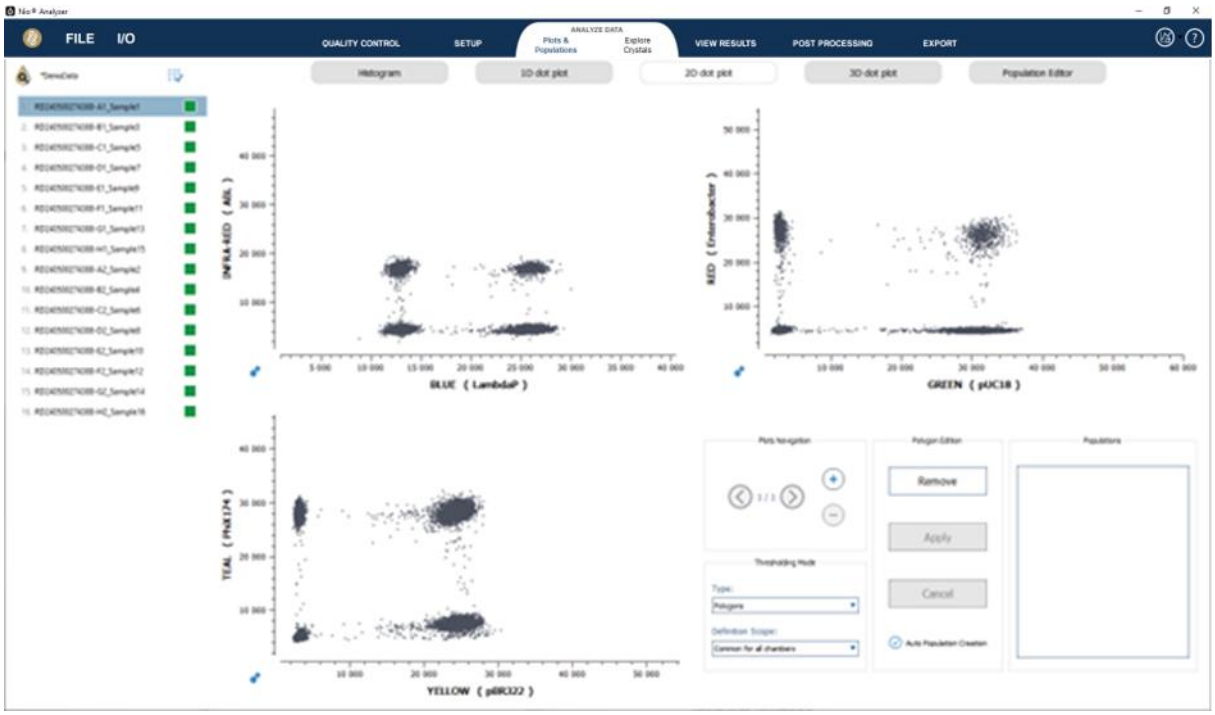


Figure 86: *An experiment that has been converted to "Polygon" thresholding mode.*

The polygons can be created and edited in the same way as in the "Explore Crystals" 2D-plots above:

- In the Polygon Edition widget, the checkbox "Auto Population Creation" is checked by default. This means that, each time a polygon is created, a pop-up is displayed asking the user to associate it to a new population. The latter is created by assigning a name and a color to it.

- Alternatively, unchecking the “Auto Population Creation” checkbox will prevent the pop-up display, allowing the user to first create all the polygons needed and associate them with the corresponding population in a second step.

Note: Starting a polygon too close to an existing polygon vertex may remove the existing vertex while creating the new polygon. To avoid it, create the polygon, away from any existing polygon vertex, and drag and drop the polygon or polygon vertex to its final position afterwards.

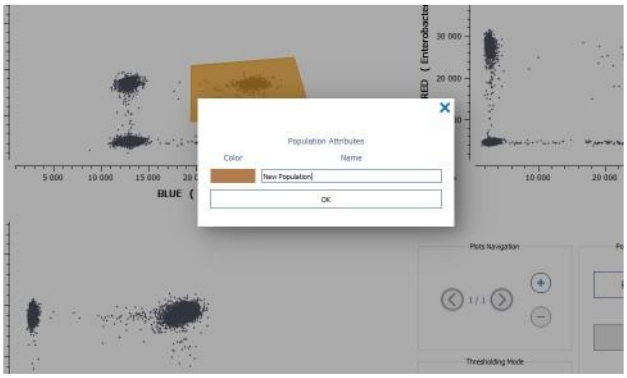


Figure 87: *Creating a new population upon polygon creation.*

If a polygon is associated with a single population, it will be displayed with the color of the population, otherwise, it will be orange by default.

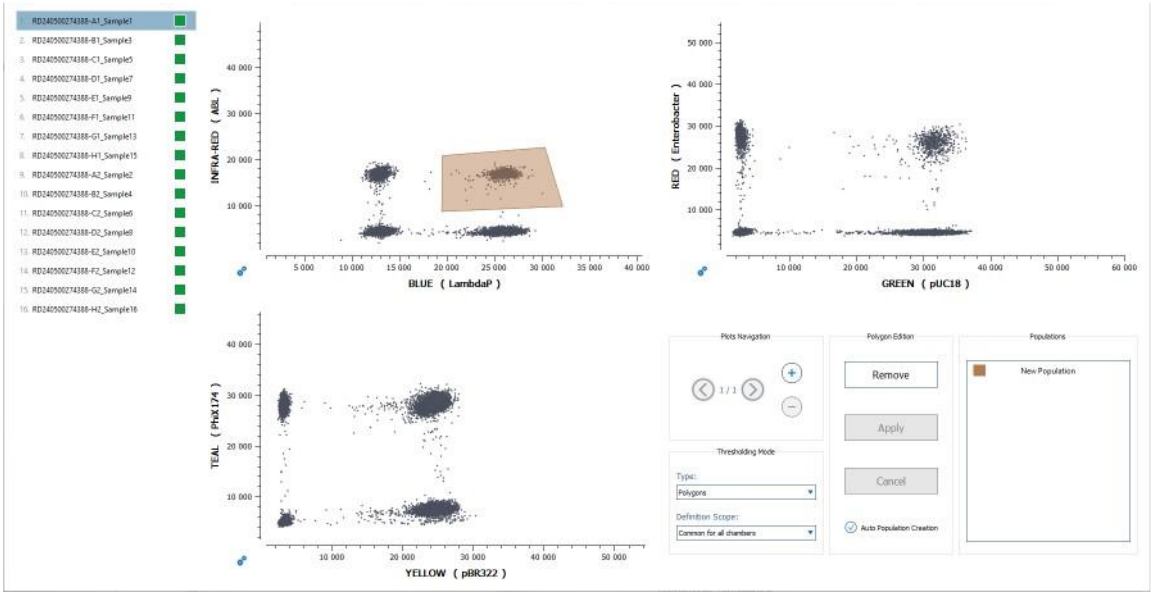


Figure 88: *A polygon is colored with the population color if it is associated with only one population.*

The edges of a polygon can be edited by dragging & dropping, adding or removing summits and edges. To apply (resp. “Cancel”) the changes made to a polygon, click on the “Apply” button (resp. “Cancel” button) of the Polygon Edition widget.

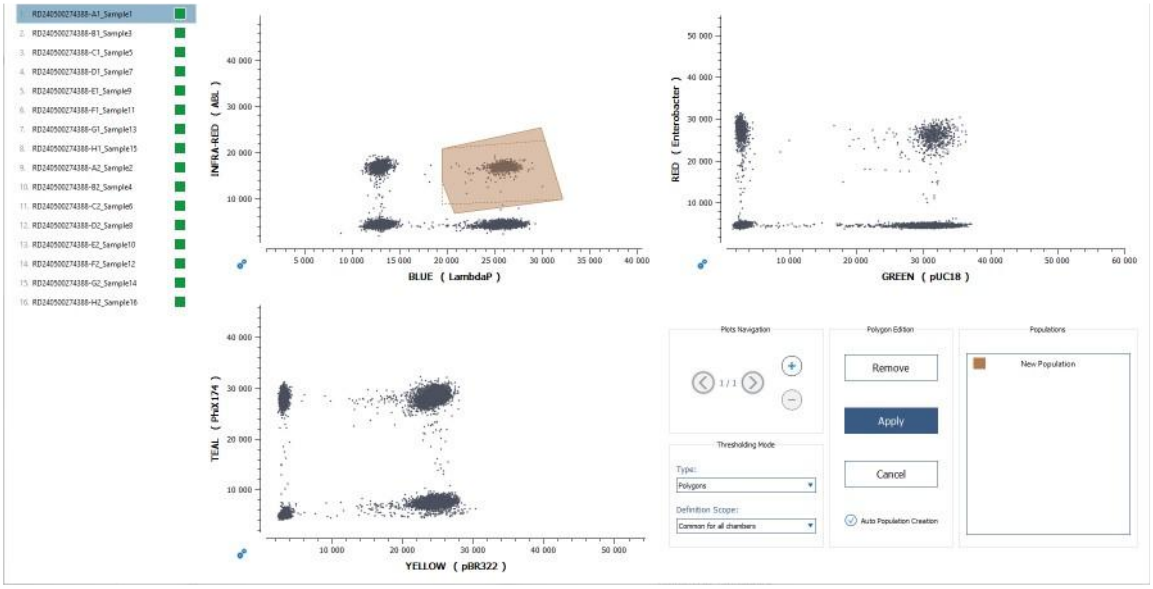


Figure 89: *If one polygon has been moved or edited, the change can be applied or cancelled.*

The user can choose to either set the polygon thresholds uniformly across the whole experiment or adjust each polygon zone, chamber by chamber. The polygon localization in the 2D dot plots can be either common to every chamber of an experiment or adjusted chamber by chamber. For the latter case, the checkbox “Individual per chamber” must be selected in the “Definition Scope” combo box of the “Thresholding Mode” widget.

Note: In the “Individual per chamber” mode, no polygon zones will be displayed in the 2D dot plots if more than one chamber is selected in the left panel.

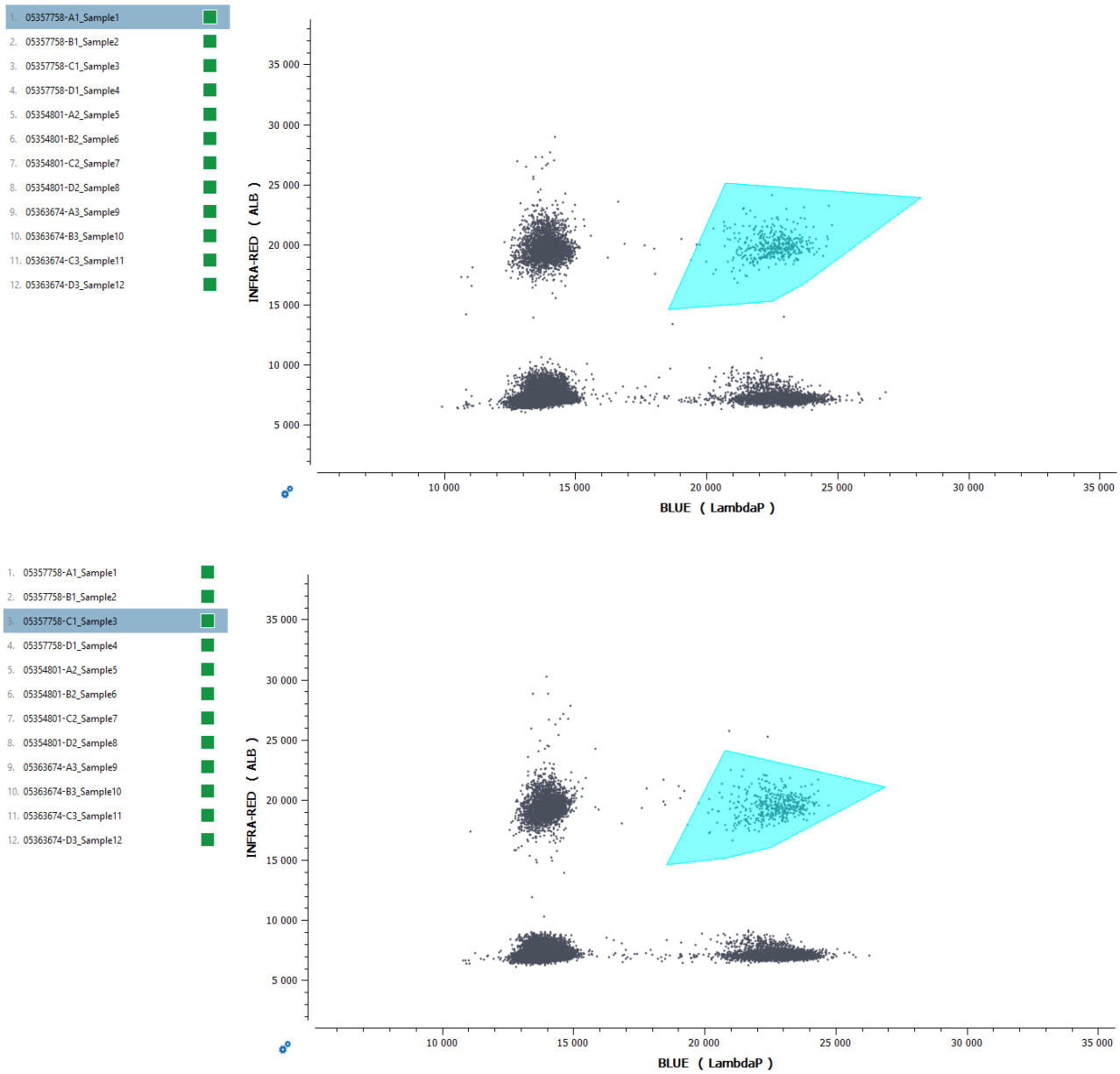


Figure 90 *Example of polygon zone that is adjusted differently in 2 different chambers.*

Heatmap visualization

The heatmap visualization is a convenient analysis tool to delimit 2D zones. In heatmap visualization mode, the colormap represents the different droplet density levels in the fluorescence populations. Droplet densities are visualized utilizing a color gradient displayed on the second y axis. Low droplet densities are represented by shades of colours on the lower axis (light grey) while increasing droplet densities are color coded in shades of blue, green, yellow and red representing the highest droplet density.

To activate the heatmap visualization in 2D dot plots, open plots settings and check the “Display heatmap” checkbox.

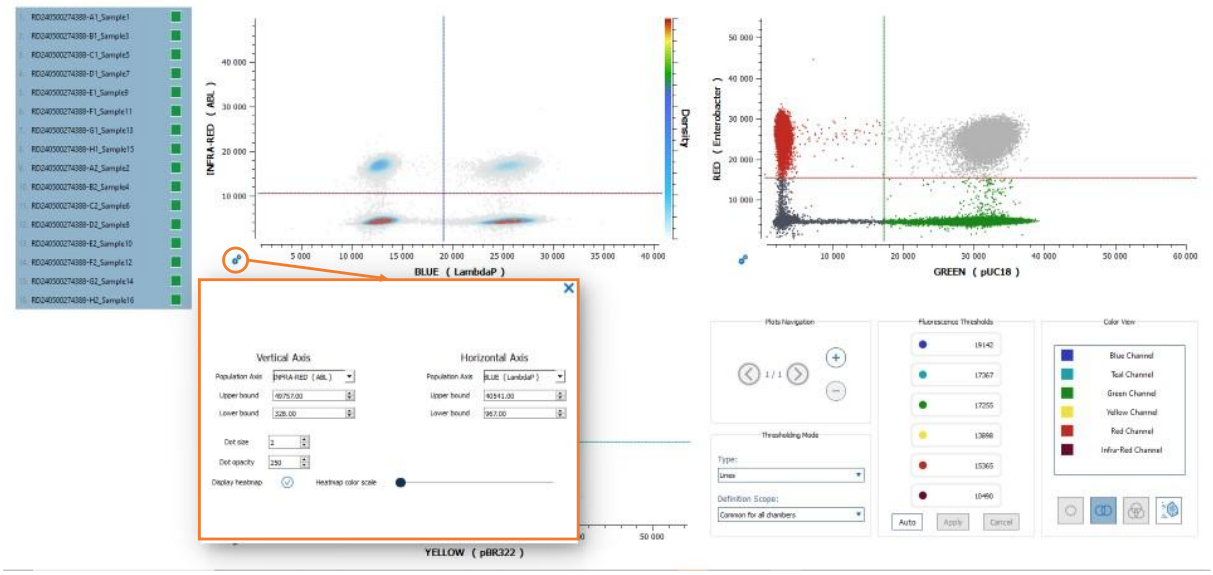


Figure 91: *Plots settings with the display heatmap option*

The color scale on the right of the graph gives the complete color range that is used to represent the different density levels.

Most of the time, the negative population is very dense, which has the effect of hiding smaller density variations between less dense areas. The Heatmap color scale parameter can be modified to highlight populations of lower density levels.

Example:

When droplet clusters are not well separated, the heatmap feature will allow the definition of distinctive droplet clusters.

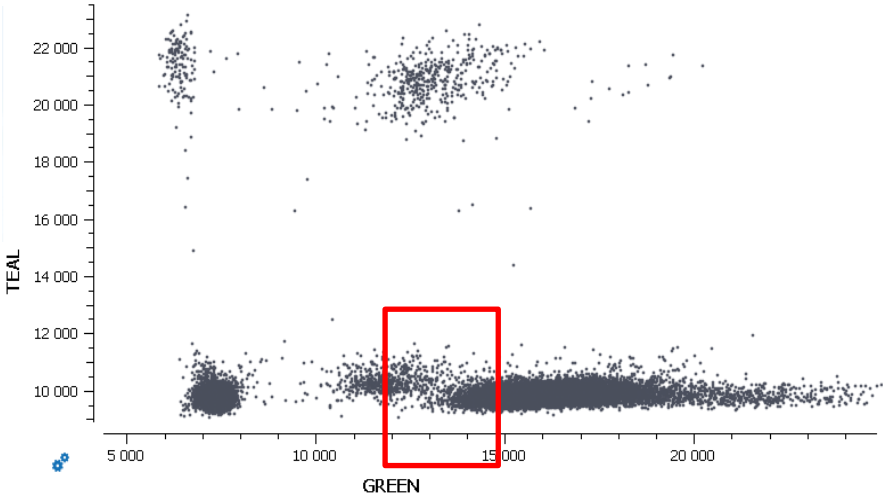


Figure 92: *Example of using heatmap to correctly position a polygon on a 2D dot-plot with 2 green positive populations that are not well separated*

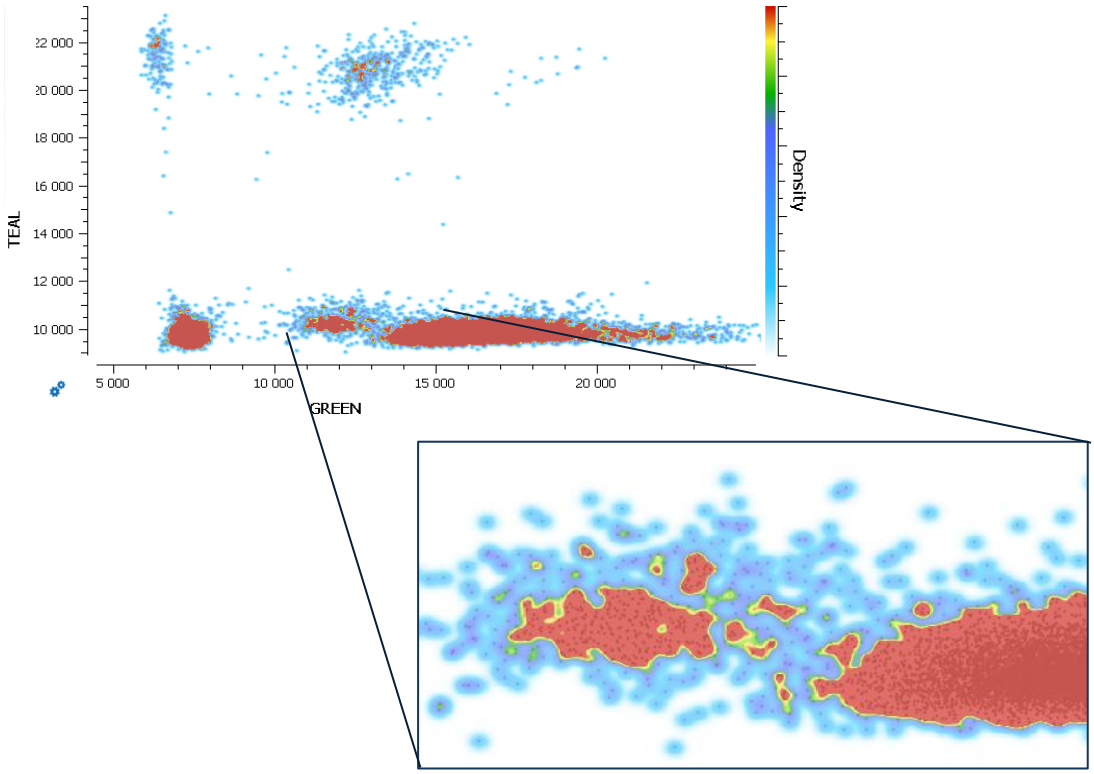


Figure 93: *Activation of heatmap feature*

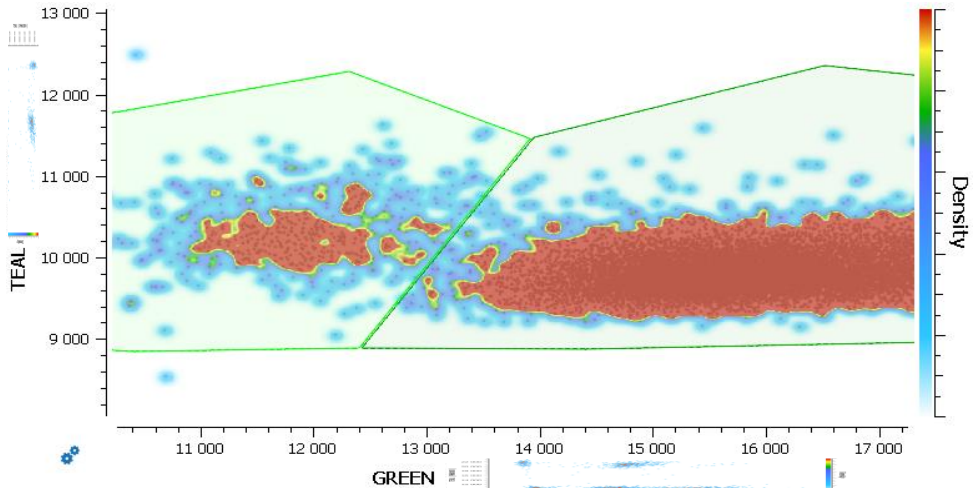
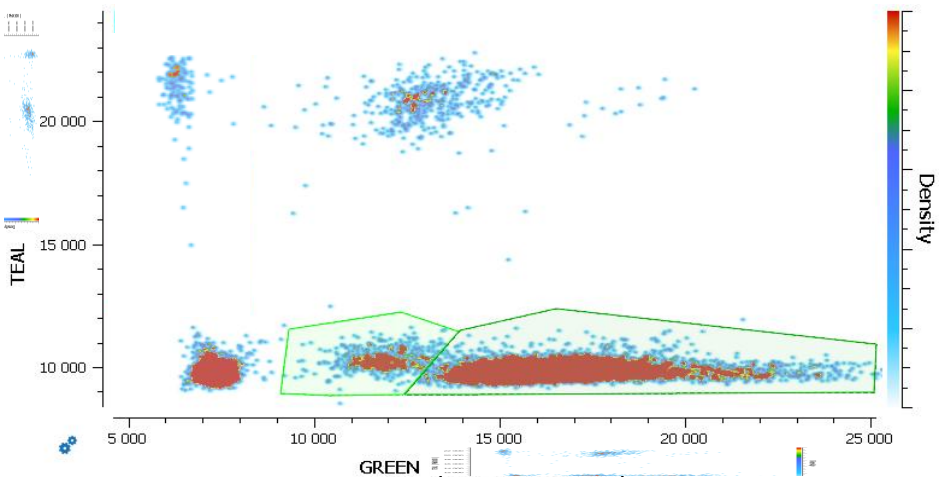


Figure 94: *Zoom in the 2D-dot plot and placement of polygons in the intersection of the 2 clusters guided by the heatmap color gradient (red indicating the most dense population)*



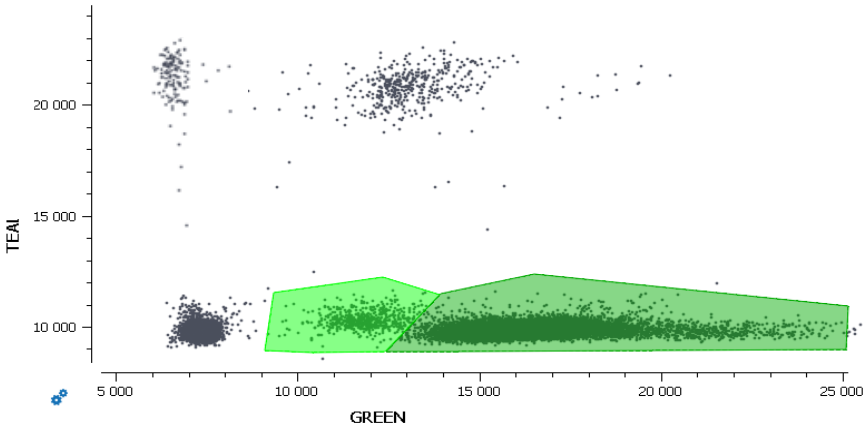


Figure 95: *Zoom out and deactivate the heatmap feature*

Population definition:

A population is an entity defined by a zone or several zones. The Population Editor widget is available to specify which zones make up a population.

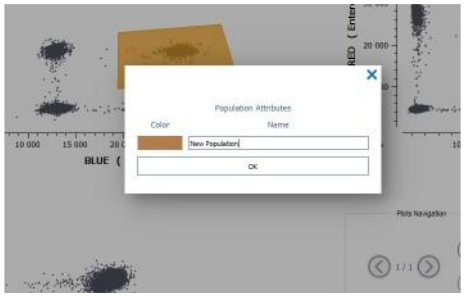


Figure 96: *Creating a new population upon polygon creation.*

Note: if you have the option "**Automatically create population**" checked, after drawing your polygon, a pop-up will be displayed asking for the new population details.

Note: you can discard the pop-up. Clicking on the cross button at the top right corner of the popup will perform the discard and no population will be automatically created.

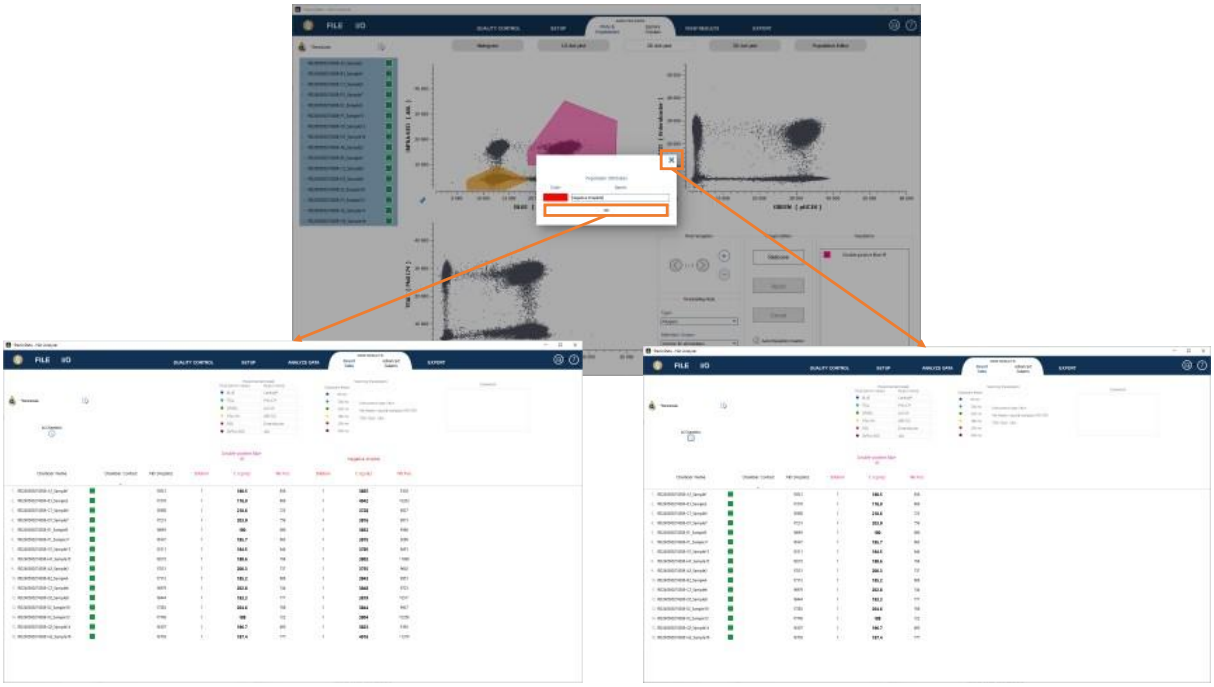


Figure 97: *Automatic creation of population upon polygon creation*

Go to "ANALYZE DATA" > "Plots & Populations" and then to the "Population Editor" widget.

In the “Population Editor” widget, for each population created, simply clicking on the population name will display the associated zones, both in the list and in the plots/Rosace view/Positivity Combination View.

Figure 98: *Population Editor widget.*

In the Zones panel, several tabs provide different way of viewing the zones:

- Rosace: providing an easy selection of simple, double- or triple-positive zones in a RGB 3-color experiment.
- 2D Dot plot: to view thresholds and polygons in a 2D space
- 3D Dot plot: to view the volumes that are related to the thresholds or polygons. In this view you can see the projection of a 2D polygon along the 3D axis of the 3D-dotplot.
- Positivity Combination: please see description below.

Some tabs are deactivated depending on your experiment settings, e.g.:

- The Rosace is only available for experiments using Red-Blue-Green channels
- The Positivity combination is only available in "Lines” thresholding mode.

By clicking on the “Edit” button, the color and the name of a population can be changed. It is also possible to update the zones that are included in the population by modifying the check box associated to a zone.

Note: when viewing the zones in the 2D-dotplot, it is possible to interact by:

- Selecting a zone: either by clicking in the zone in the plot or clicking in the row in the Zone List. The selected zone will both be selected in the table and also visually displayed in the dotplot.
- Checking the zone: using the checkbox to include it in the currently edited population. Checking a zone for a population is only possible if the “Edit” button under the population list has been clicked.

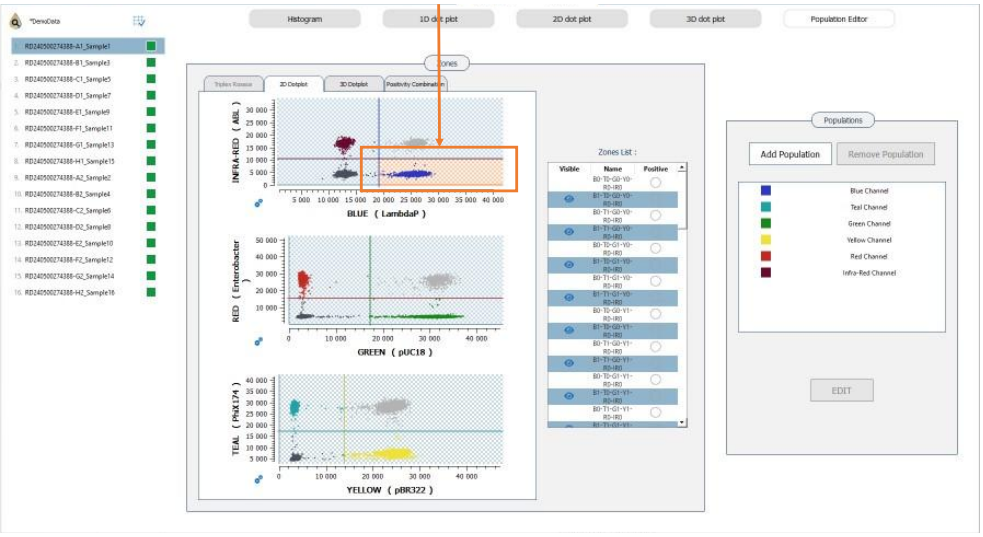


Figure 99: *Orange arrow : selected quadrant to display the desired population.*

“Positivity Combination” widget: This widget can be accessed under the “Population Editor” tab > “Zones”. Click on a quadrant to display the population under the “Zones list” widget.

Now, let’s consider a new 6-color experiment using “Lines” as thresholding mode.

Click on the “Positivity Combination” widget and the status of the populations will be displayed as either positive, negative or undefined. This widget is useful for visualization and creation of populations.

For example, the user can create a "Simple Blue Positive" population by setting the blue channel to "Positive" and the other channels to “Negative”

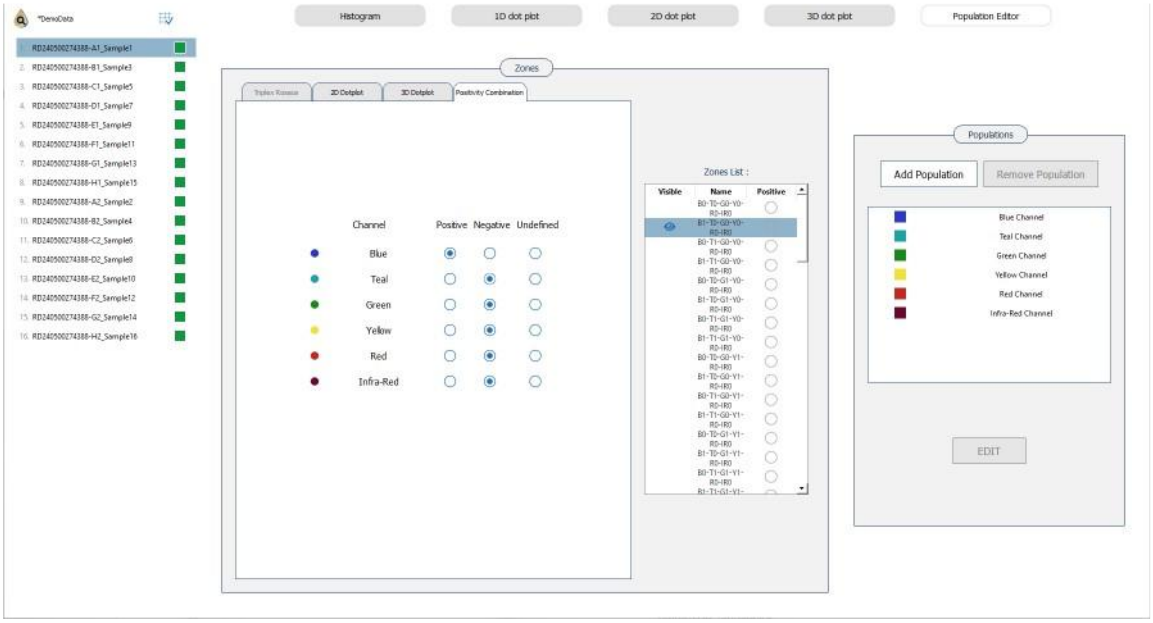


Figure 100: *The "Positivity Combination" widget for population visualization and creation.*

By definition, the droplets included in the population are counted as positive droplets.

By default, all the droplets that are not included in the population are counted as negative droplets for this population.

In the following paragraph we will see how to define negative zones in a population.

Negative zones:

For some applications, such as drop-off applications, it might be necessary to choose a specific negative droplet population by specifying the "negative droplets" in a zone to be considered for a given population:

To define a negative zone for a population, a specific zone has to be selected. Let's take polygons as example. If the polygon has not already been drawn, go to the "ANALYZE DATA" > "Plots & Populations" and then to the "2D dot plot" widget and create a new polygon as previously described.

Then to define the negative population in a given population, select the population of interest in the "Population Editor" and click on the "EDIT" button.

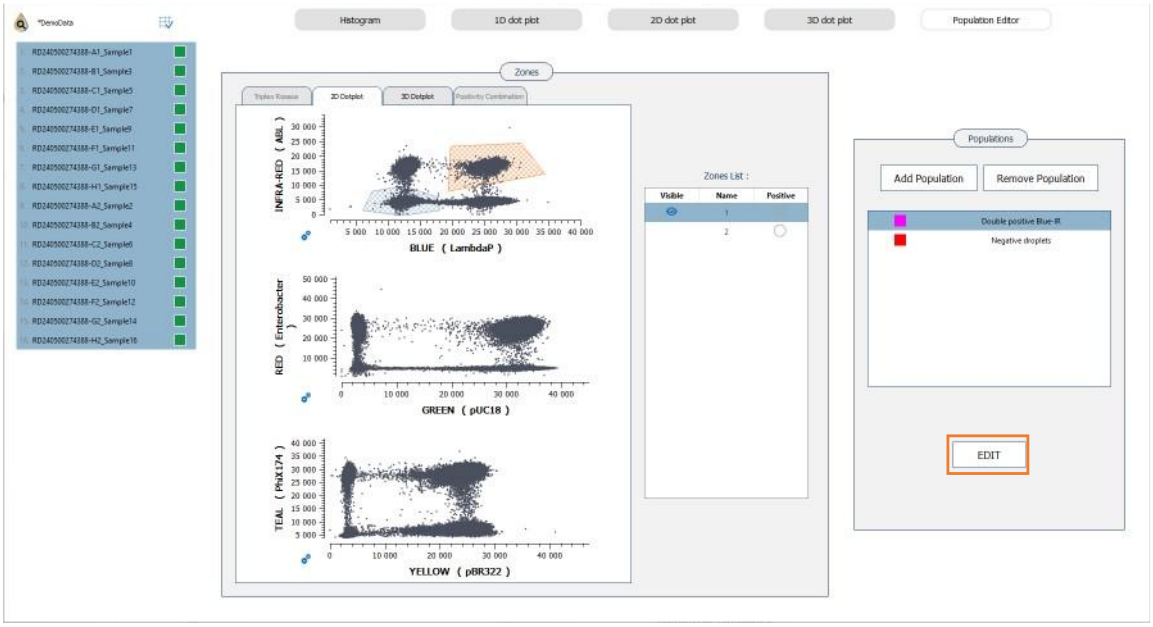
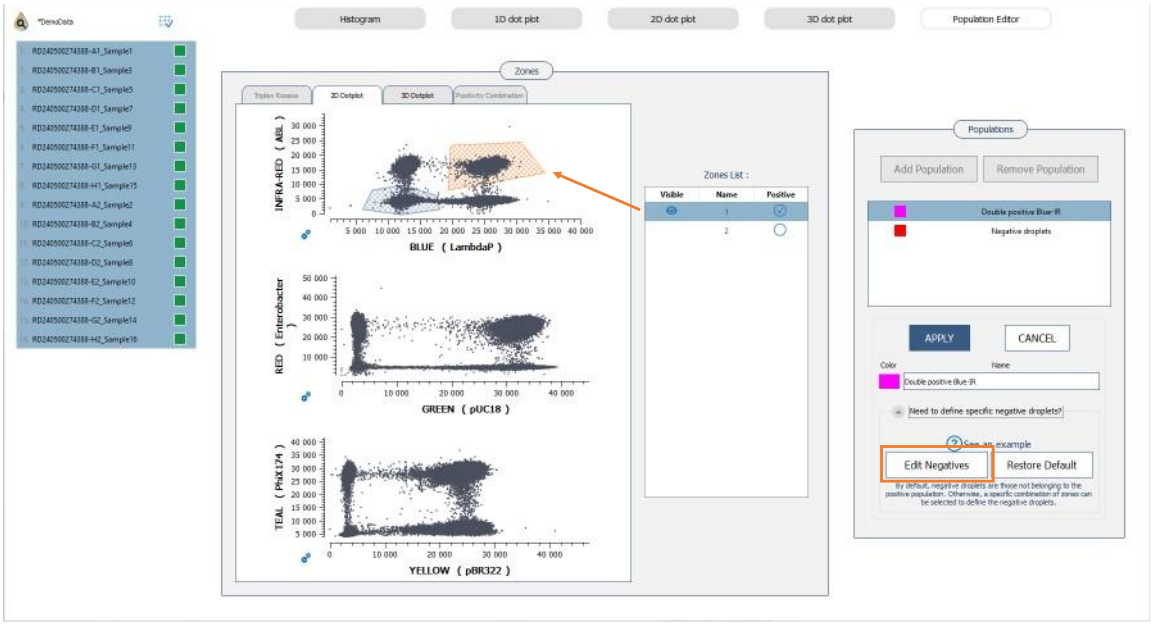


Figure 101: *Defining the negative population in the "Population Editor" widget.*

Upon expanding the panel "Need to define specific negative droplets?", and clicking on the button "Edit Negatives", a new column appears in the zone list, allowing the required negative zones to be defined. Once this is done, click on "Edit Negatives". After selecting the negative droplets polygon, check the box "Negative" and finally, click on "APPLY". Now the concentration calculation of the polygon of interest only considers the droplets detected in the negative droplet polygon.



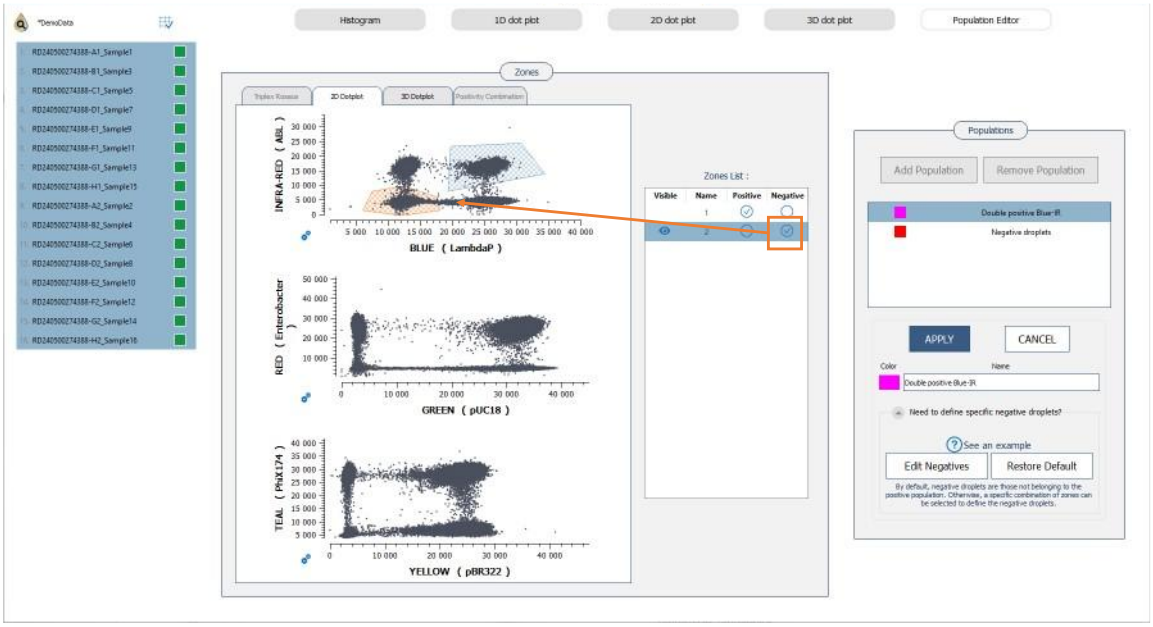
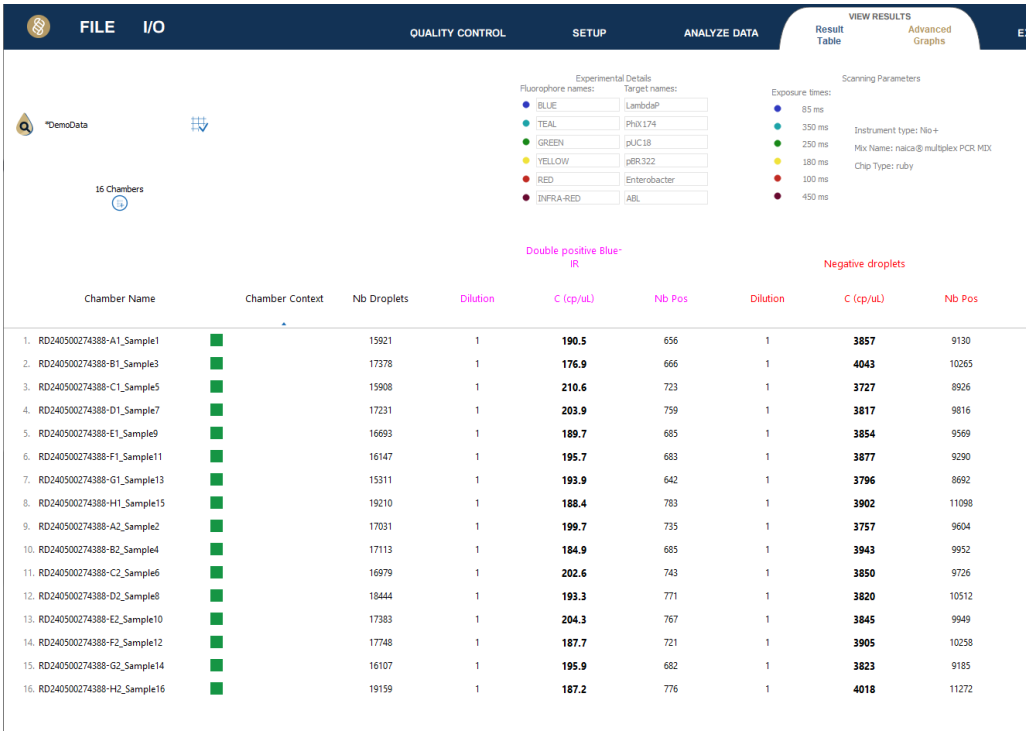


Figure 102: *Editing the negative zones of a population.*

Populations for which specific negative droplets have been defined are indicated with the symbol "*" suffixed to the population name (meaning that the sum of positive and negative droplets for such populations may not be equal to the total number of droplets).

The "*" suffix will be displayed throughout the Result Table information in Nio Analyzer software.



	Chamber Name	Chamber Context	Nb Droplets	Dilution	C (cp/uL)	Nb Pos	Dilution	C (cp/uL)	Nb Pos
1.	RD240500274388-A1_Sample1		15921	1	**190.5**	656	1	**3857**	9130
2.	RD240500274388-B1_Sample3		17378	1	**176.9**	666	1	**4043**	10265
3.	RD240500274388-C1_Sample5		15908	1	**210.6**	723	1	**3727**	8926
4.	RD240500274388-D1_Sample7		17231	1	**203.9**	759	1	**3817**	9816
5.	RD240500274388-E1_Sample9		16693	1	**189.7**	685	1	**3854**	9569
6.	RD240500274388-F1_Sample11		16147	1	**195.7**	683	1	**3877**	9290
7.	RD240500274388-G1_Sample13		15311	1	**193.9**	642	1	**3796**	8692
8.	RD240500274388-H1_Sample15		19210	1	**188.4**	783	1	**3902**	11098
9.	RD240500274388-A2_Sample2		17031	1	**199.7**	735	1	**3757**	9604
10.	RD240500274388-B2_Sample4		17113	1	**184.9**	685	1	**3943**	9952
11.	RD240500274388-C2_Sample6		16979	1	**202.6**	743	1	**3850**	9726
12.	RD240500274388-D2_Sample8		18444	1	**193.3**	771	1	**3820**	10512
13.	RD240500274388-E2_Sample10		17383	1	**204.3**	767	1	**3845**	9949
14.	RD240500274388-F2_Sample12		17748	1	**187.7**	721	1	**3905**	10258
15.	RD240500274388-G2_Sample14		16107	1	**195.9**	682	1	**3823**	9185
16.	RD240500274388-H2_Sample16		19159	1	**187.2**	776	1	**4018**	11272

Figure 103: *Result Table for the populations modified in Figure 102. Populations with the specific negative droplets have the "*" symbol suffixed to population name.*

6. Regulatory mode

In the following, “Nio software suite Regulatory” refers to either Nio Reader software or Nio Analyzer software or both in regulatory mode.

To ensure fulfilment of all 21 CFR Part 11 sections user´s organization responsibilities to establish respective Standard Operation Procedures (SOPs) are indicated in the table with “X”.

Section	Subject	User´s organization	Nio software suite Regulatory	Compliance management
11.10 (a)	Validation	X	X.	System validation as well as experiment result validation is supported by Nio Reader and Nio Analyzer software. SOPs of the user´s organization for validation is required.
11.10 (b)	Human readable records	n.a.	X	Electronic records in human readable form supported throughout Nio software and Nio Analyzer software.
11.10 (c)	Protection of records	X	X	All electronic records are kept within the Nio Reader and Analyzer software environment until the user transfers them to external electronic archives in line with user´s organization policies.
11.10 (d)	Limited system access	X	X	Control of access to Nio Reader and Nio Analyzer software through individual user authentication. Establishing and maintaining the appropriate list of user accounts is the responsibility of the user´s organization.
11.10 (e)	Audit trails	X	X	Nio Reader and Nio Analyzer software tracks changes in an audit trail which does not expire. The creation of backups is under the responsibility and control of the user´s organization.
11.10 (f)	Operating system checks	X	X	Nio Reader and Nio Analyzer software provides guidance and checks for setting up an experiment. Nio Reader and Nio Analyzer software offers the possibility to create custom standard experiments. Such experiments can be released for routine workflows following validation by the user.
11.10 (g)	Authority checks	X	X	Control of access to Nio Reader and Nio Analyzer software by individual authentication and User Role assignments. User cannot modify electronic records. Experiments modification require specified permissions and requires

Section	Subject	User´s organization	Nio software suite Regulatory	Compliance management
				validation prior to release for routine workflow.
11.10 (h)	Device checks	X	X	Experiment configuration and parameters are checked by Nio Reader and Nio Analyzer software. The sample ID input and standard experiments validation is under the responsibility and control of the user´s organization.
11.10 (i)	Determination of education	X	X	User Manuals and training documentation are provided by Stilla Technologies. Establishing and maintaining the appropriate training level is the responsibility of the user´s organization.
11.10 (j)	Written policies	X	n.a.	Establishing and maintaining SOP to comply with the regulation is the responsibility of the user´s organization.
11.10 (k)	System documentation	X	X	Nio Reader and Nio Analyzer software documentation cannot be changed by the user. The distribution of documentation to the users and version control of the documentation is the responsibility of the user´s organization.
11.100 (a)	Electronic Signature general requirements	X	X	Unique to individual users.
11.100 (b)		n.a.	X	Verification of identity
11.100 (c)		n.a.	X	Certification of equivalence.
11.200 (a/b)	Electronic Signature components and control	n.a.	X	Signature with username and password ensure genuine owner.
11.300 (a)	Controls for identification username and	X	X	Uniqueness of username and password.

Section	Subject	User´s organization	Nio software suite Regulatory	Compliance management
11.300 (b)	passwords	X	X	Periodical check of issuance.
11.300 (c)		X	X	Loss management.
11.300 (d)		X	X	Safeguards and detection of unauthorized attempts.

naica® Data Service Permissions

The naica® Data Service software introduces the notion of Permissions. Each User Role grants assigned users access to a set of predefined permissions for the Nio Reader software and Nio Analyzer software.

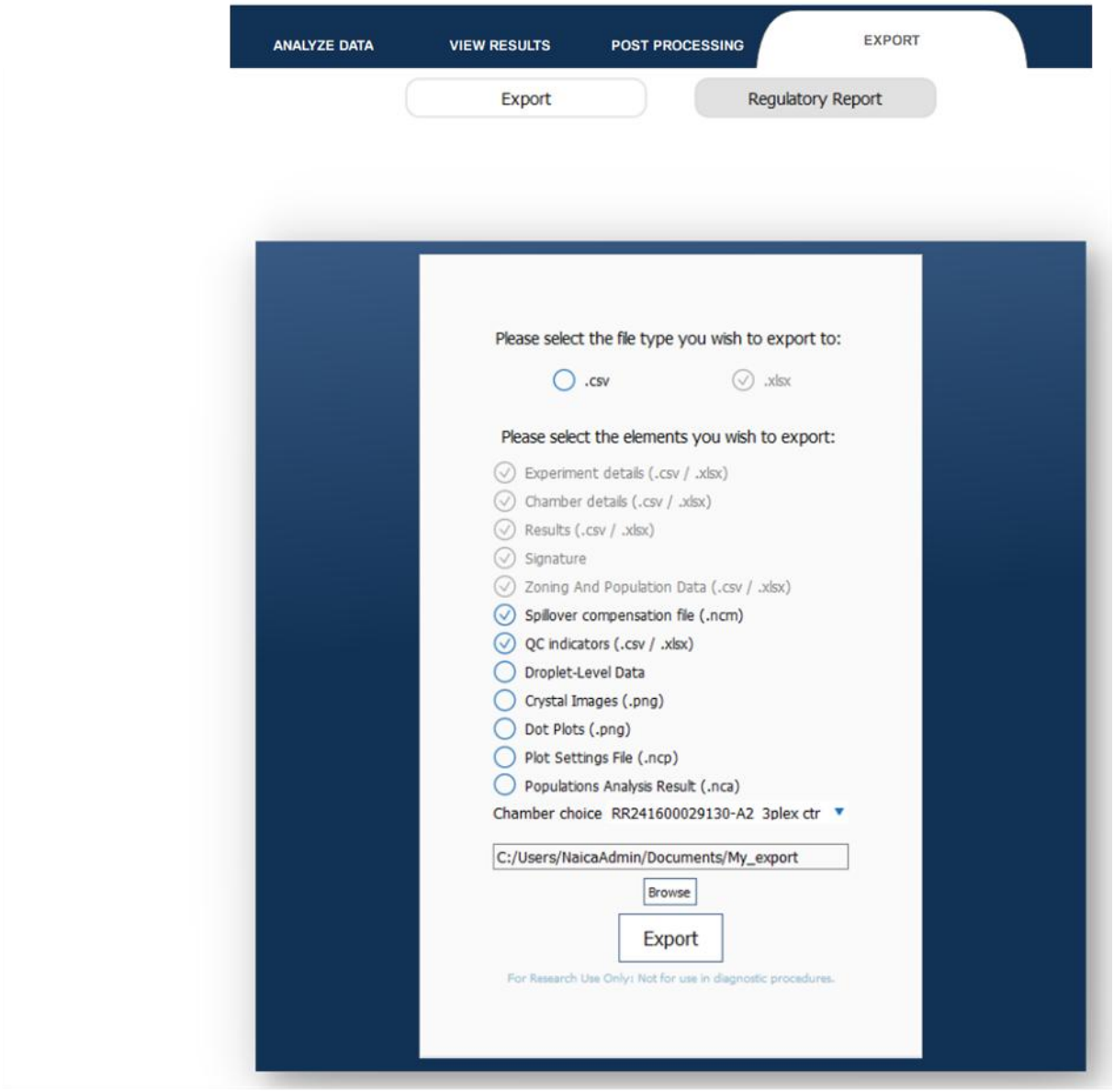
The permission settings can be administrated through the Roles / Permission tab on the naica® Data Service user interface. All available Permissions are displayed in a list format, where granted permissions are marked with an activated check-box symbol for a defined User Role. Permissions not granted for a User Role display an unchecked box.

The available permissions to select for Nio Analyzer are:

1. Assay edition

2. Export Data

In addition to analytical data included in the 21 CFR Part 11 Regulatory Report, the «Export Data » permission allows the User Role to export all the data files listed below with Nio Analysis software. The preselected data elements (greyed out) are always part of the data export. Additional data elements can be selected by activating the checkbox.



Warning: Once exported, the data in XLSX and CSV file formats can be edited / modified, outside the Nio Analyzer software environment, without Experiment Audit Trail recording. Therefore, only the generated PDF report is to be used for sample results reporting compliant with 21 CFR Part 11.

3. Generate PDF report

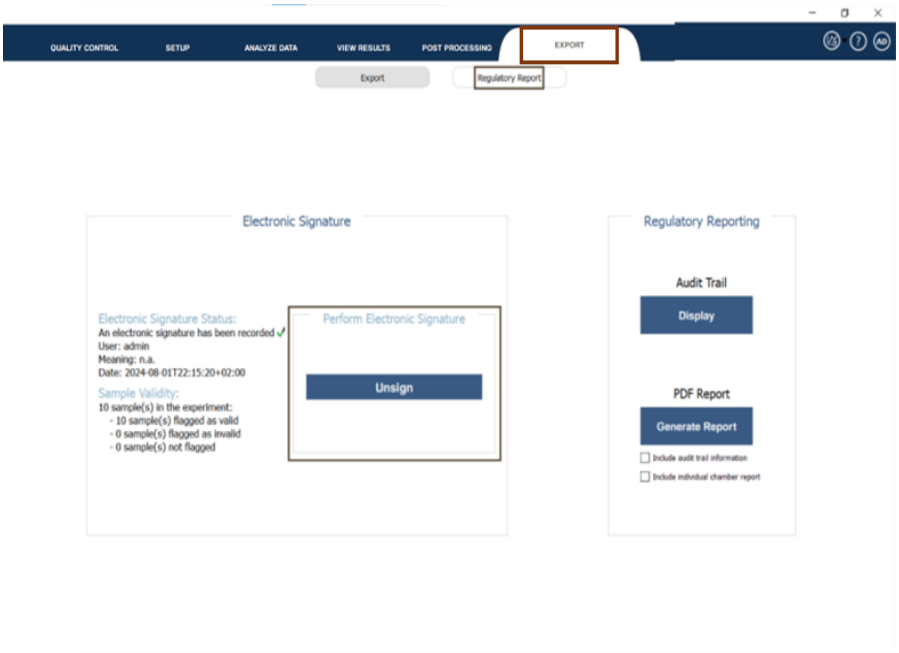
The «Generate PDF report» permission allows the user to create and export a 21 CFR Part 11-compliant PDF report of electronically signed experiments using Nio Analyzer software. The user has the option to include the experimental audit trail information as part of the PDF report. To electronically sign an experiment, User Role permission «Validate Experiments » is required.

4. Invalidate Experiments

The «Invalidate Experiments» permission allows the user removing an existing electronic signature executed by a different user. Once the signature is removed, the user can make further data edits or modifications.

In GMP / GLP environments organizations are often operating under « two-man rule / 4-eyes principle », where results must be reviewed by a second person as a control mechanism designed to achieve a high level of data security. Under this rule, data access and actions to modify data results, must always be available to more than one authorized person. The «Invalidate Experiments» permission supports the respective 4-eyes principle hierarchy within an organization with full compliance for 21 CFR Part 11.

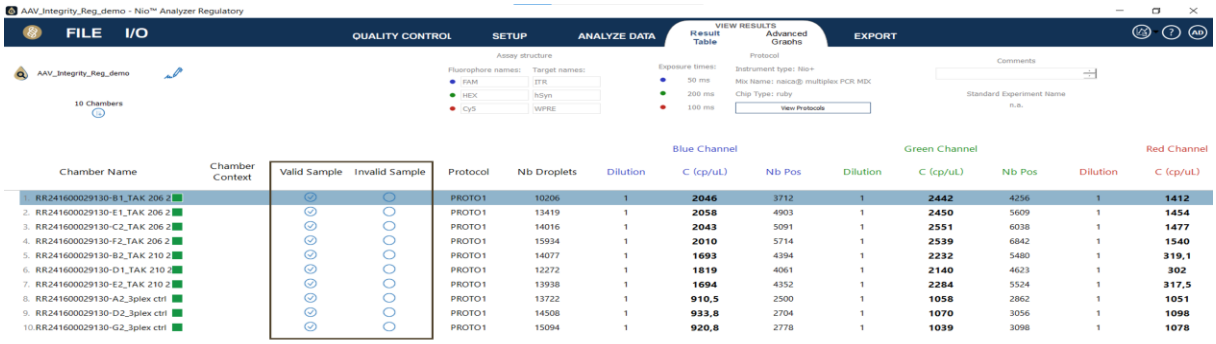
Note: For detailed instructions please see Section 0 - Electronic Signatures.



5. Validate Experiments

The «Validate Experiments » permission allows the user to conclude the result of the experiment for each individual sample in the experimental run in Nio Analyzer software Result Table.

The «Validate Experiments» permission further allows the user to electronically sign the validated result of the entire experiment in the “Regulatory Report” page of the “Export” menu of Nio Analyzer software.



Note: For detailed instructions please see Section 0 - Sample Validation and Electronic Signatures.

The naica® Data Service software introduces the notion of User Role which can be administrated through the Roles / Permission tab on the naica® Data Service user interface. For further explanation about User Role, please refer to Nio Reader software User Manual.

Individual User authentication

For 21 CFR Part 11 compliance it is obligatory to ensure that only authorized individuals have access to Nio Analyzer software. Therefore, all users are obliged to log in to Nio Analyzer software using individual user authentication credentials (username and password).

A failed login attempt can come from using incorrect login credentials.

Note: Following several incorrect entries for user login credentials the naica® Data Service account can be locked. Only an IT administrator can reset the user's Windows account to be activated again.

Failed log-in attempts can also be due to a connectivity issue to the naica® Data Service. Please contact the respective IT department to ensure that naica® Data Service connection is provided.

If the user's session expires, the Nio Analyzer software will request a new authentication. Any attempt to avoid user authentication will automatically exit the software, which may lead to unsaved changes.

Automatic log-out is managed through the individual organization's setup for the Microsoft Windows user account settings. However, it is recommended to always proactively close all Nio Analyzer software if the application is not in use.

Note: The login attempt can fail if the underlying Windows authentication is denied or if the software loses the connection to the naica® Data Service.

Sample validation

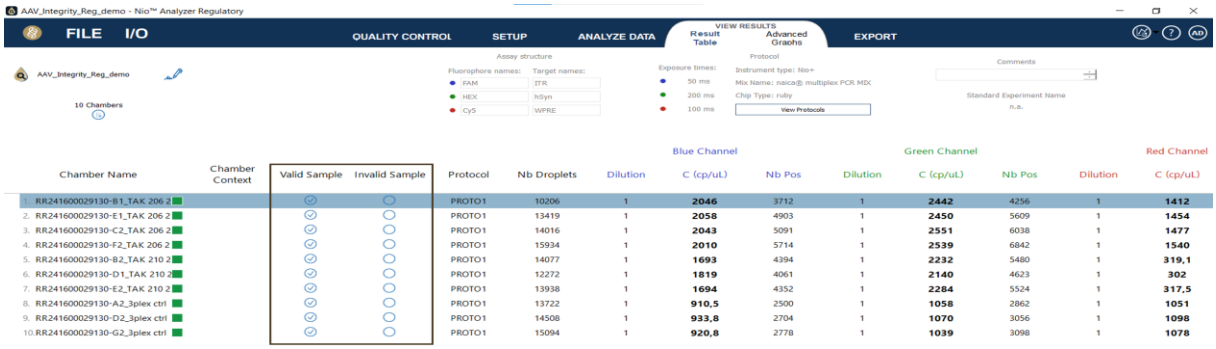
To be able to sign and export the experiment results, Nio Analyzer software requires each individual sample to be reviewed and flagged as "valid" or "invalid".

Sample acceptance criteria may vary depending on the specific SOP for an application or assay. As such, sample conformity criteria must be defined by the organization using the Nio Digital PCR within their application area.

The flagging of each individual sample as "valid" or "invalid" is performed in Nio Analyzer "View Result" → "Result Table".

The experiment result table displays two columns: "Valid Sample" and "Invalid Sample".

By default, the status of all samples is undefined. It is possible to apply the same status to a selection of samples; select the samples while pressing the "Shift" or "Alt" key and click on the desired validity status.



	Chamber Name	Chamber Context	Valid Sample	Invalid Sample	Protocol	Nb Droplets	Blue Channel Dilution	Blue Channel C (cp/uL)	Blue Channel Nb Pos	Green Channel Dilution	Green Channel C (cp/uL)	Green Channel Nb Pos	Red Channel Dilution	Red Channel C (cp/uL)
1.	RR241600029130-B1_TAK 206 2		✓	○	PROTO1	10206	1	**2046**	3712	1	**2442**	4256	1	**1412**
2.	RR241600029130-E1_TAK 206 2		✓	○	PROTO1	13419	1	**2058**	4903	1	**2450**	5609	1	**1454**
3.	RR241600029130-C2_TAK 206 2		✓	○	PROTO1	14016	1	**2043**	5091	1	**2551**	6038	1	**1477**
4.	RR241600029130-F2_TAK 206 2		✓	○	PROTO1	15934	1	**2010**	5714	1	**2539**	6842	1	**1540**
5.	RR241600029130-B2_TAK 210 2		✓	○	PROTO1	14077	1	**1693**	4394	1	**2232**	5480	1	**319,1**
6.	RR241600029130-D1_TAK 210 2		✓	○	PROTO1	12272	1	**1819**	4061	1	**2140**	4623	1	**302**
7.	RR241600029130-E2_TAK 210 2		✓	○	PROTO1	13938	1	**1694**	4352	1	**2284**	5524	1	**317,5**
8.	RR241600029130-A2_3plex ctrl		✓	○	PROTO1	13722	1	**910,5**	2500	1	**1058**	2862	1	**1051**
9.	RR241600029130-D2_3plex ctrl		✓	○	PROTO1	14508	1	**933,8**	2704	1	**1070**	3056	1	**1098**
10.	RR241600029130-G2_3plex ctrl		✓	○	PROTO1	15094	1	**920,8**	2778	1	**1039**	3098	1	**1078**

Sample validation for pooled chambers:

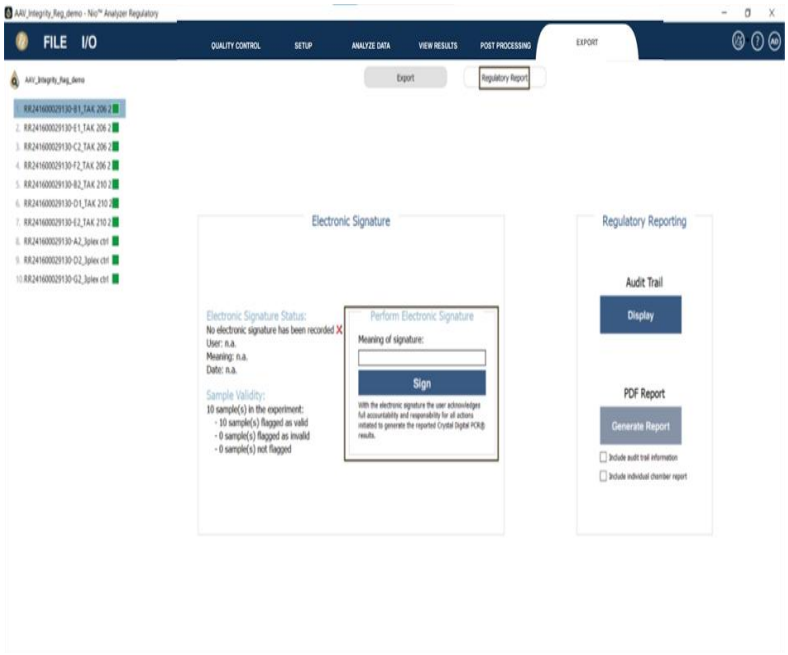
When using the “Pooled Chamber” feature, the experiment validity status of the individual sample chambers cannot differ from the pooled chamber sample result.

- If the validity status of all individual sample chambers that compose a pooled chamber sample result are consistent, the validity status of the pooled chamber sample is implicitly set to the same value.
- If the validity status of the individual sample chambers selected for pooling differ, the validity status of the pooled chamber sample remains undefined.

It is also possible to assign a validity status for the pooled chamber sample directly. By doing this, the validity status of the individual sample chambers will be overwritten to match the pooled chamber sample validation status.

Electronic signatures

Nio Analyzer software enables the electronic signature of all validated experiment results, provided that the respective User Role has signature permissions. “Electronic Signature” is available in the “Regulatory Report” page of the “EXPORT” menu.



The Electronic Signature section is structured as follows:

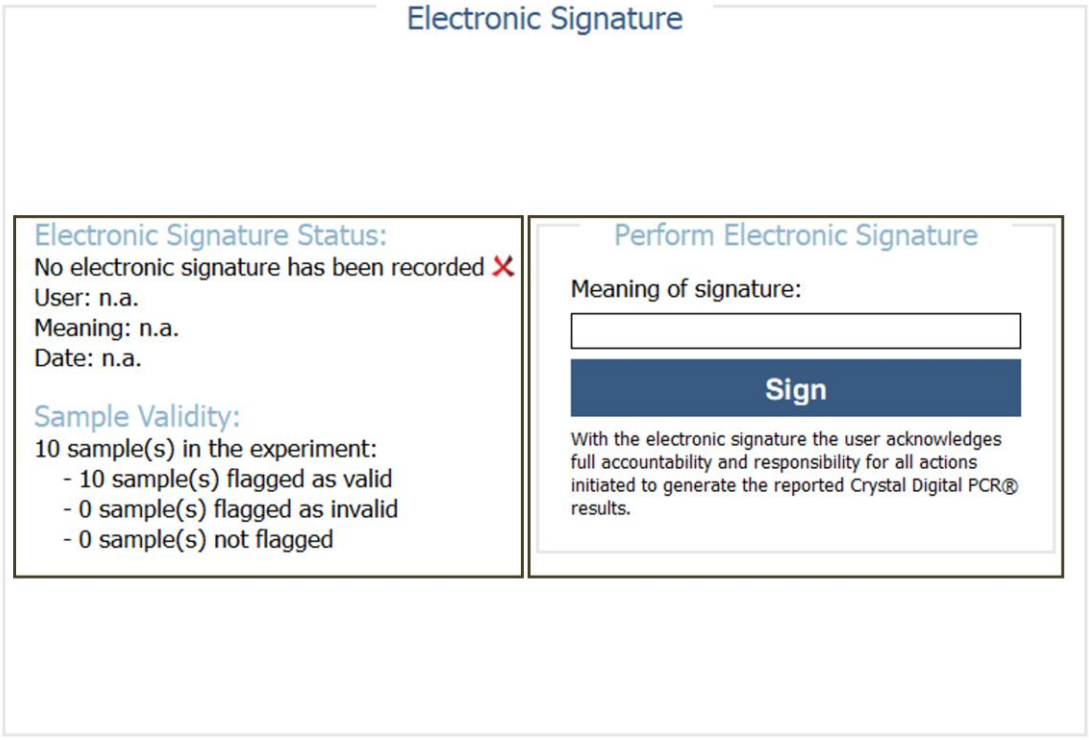
- Electronic Signature Status displays a summary of the electronic signature status for the individual experiment file:
  - Record of the file has already been signed or not
  - Record of signature user identification
  - Record of signature meaning
  - Record of signature date and time
- Sample Validity displays a summary of the concluded sample results for the experiments:
  - Record of number of samples in the experiment file flagged as valid
  - Record of number of samples in the experiment file flagged as invalid
  - Record of number of samples in the experiment file not flagged

Note: To electronically sign an experiment, it is mandatory to first flag the result of all samples in the experiment file as “valid” or “invalid” in the “Result Table” page of the “VIEW RESULTS” menu. If the samples have not all been flagged, a warning message is displayed in the “Perform Electronic Signature” section, and it will not be possible to proceed with electronic signature.

- Perform Electronic Signature
  - To execute the electronic signature for the experiment file it is optional to define the meaning of the electronic signature.
- Meaning of the electronic signature
  - The default value for the meaning of electronic signature is set to display “n.a.”

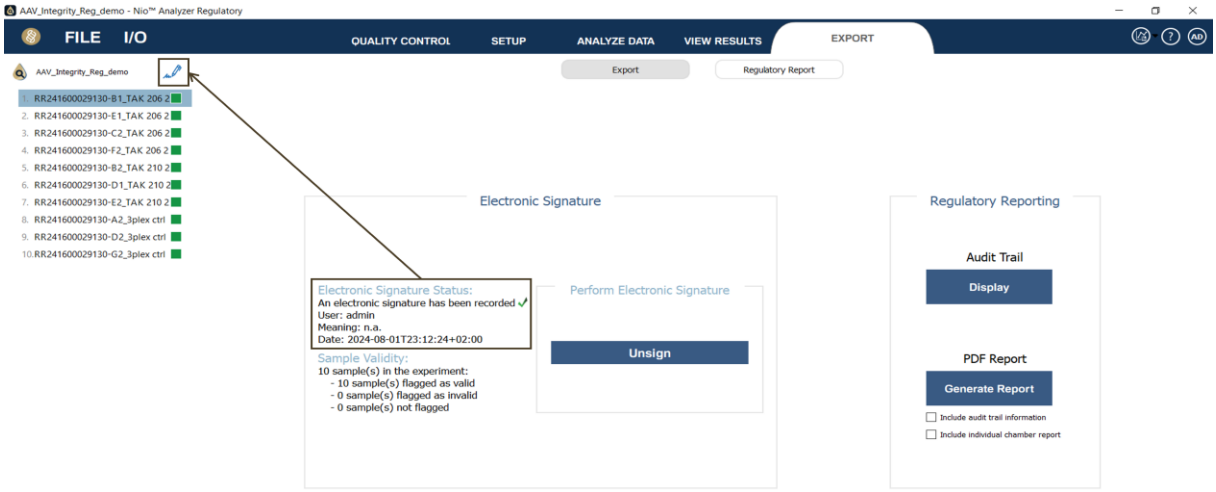
- In GMP / GLP environments, organizations are often operating under « two-man rule / 4-eyes principle », where results must be reviewed by a second person as a control mechanism designed to achieve a high level of data security. The «Meaning of electronic signature» field allows to establish an organization-tailored result-release hierarchy with full compliance for 21 CFR Part 11.
- The individual meanings of electronic signatures within an organization must be defined within the specific organization process.

- Sign
  - To execute the electronic signature for the experiment file, it is mandatory to confirm the electronic signature, by again performing a new authentication with login (username and password). With the electronic signature the user acknowledges full accountability and responsibility for all actions initiated to generate the reported Crystal Digital PCR® results.



Once the experiment is signed, a signature icon appears to the right of the experiment name in the top left corner of the experiment file that remains displayed in all Nio Analyzer software menus (see below).

The signature icon provides the visual indication that the experiment has been electronically signed and is therefore locked to prevent any further modifications. Subsequently, all Nio Analyzer software features allowing modifications to the experiment file are disabled throughout the software menu.



- Unsign

  The author of an electronic signature is always allowed to remove their electronic signature. To remove the electronic signature of an experiment, click on the “Unsign” button and confirm the user identity with login authentication (username and password) to finally remove the electronic signature.

To be able to edit the experiment file, previously electronically signed by a different Author, the user who attempts modifying the experiment file must have the User Role permission “Invalidate experiment”. If this “Invalidate experiment” permission is not held by the current user, a “Permission denied” message will be displayed.

Experiment Audit Trail

The Experiment Audit Trail records all edition events in Nio Reader and Nio Analyzer software for an experiment file. Events included in the Audit Trail record are:

- Scanning Template parameters
- Chip IDs
- Scanning parameters modifications
- Droplet recognition edition
- Sample edition
- Experiment loading
- Spill-over compensation edition
- Threshold or polygon position edition
- Post-processing setup
- Electronic signature record of the experiment file including user identification and date and timestamp.
- Experiment design (pooling chambers, importing/setting/removing assays and protocols, enabling/disabling chambers)

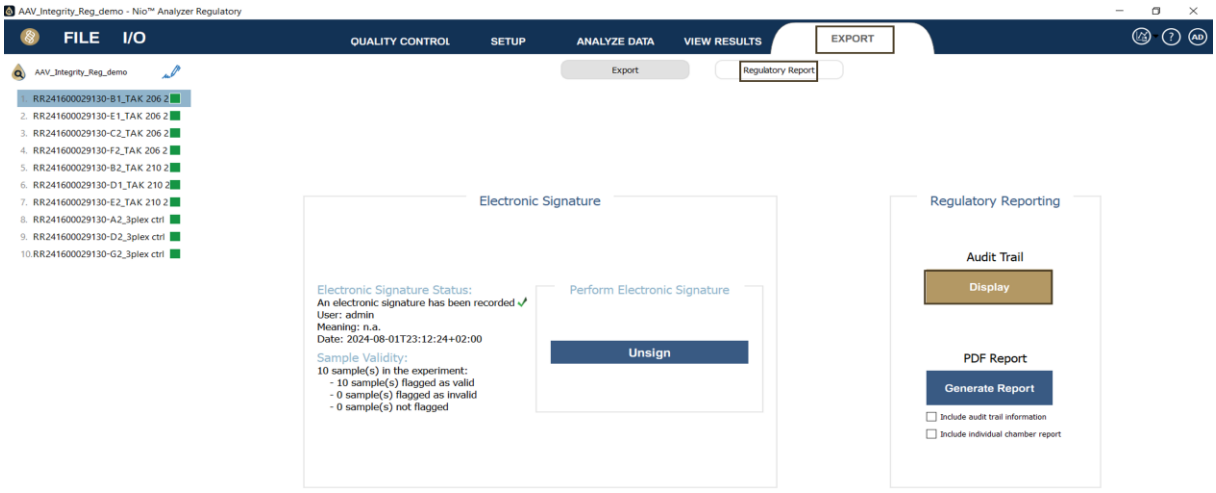
- Starting/Cancelling a run
- Re-reading chambers
- Changing a run priority

The Experiment Audit Trail does not track events related to the “Export” page in the “EXPORT” menu. Export events are not in the scope of the “Regulatory Report” page.

To produce a consistent Experiment Audit Trail, all computers running Nio software suite must be configured with the correct date and time.

The Experiment Audit Trail can be reviewed within Nio Analyzer software. To open the Experiment Audit Trail viewer:

- Click on the “EXPORT” menu
- Open the “Regulatory Report” tab
- In the “Regulatory Reporting” section click on “Display” underneath “Audit Trail”

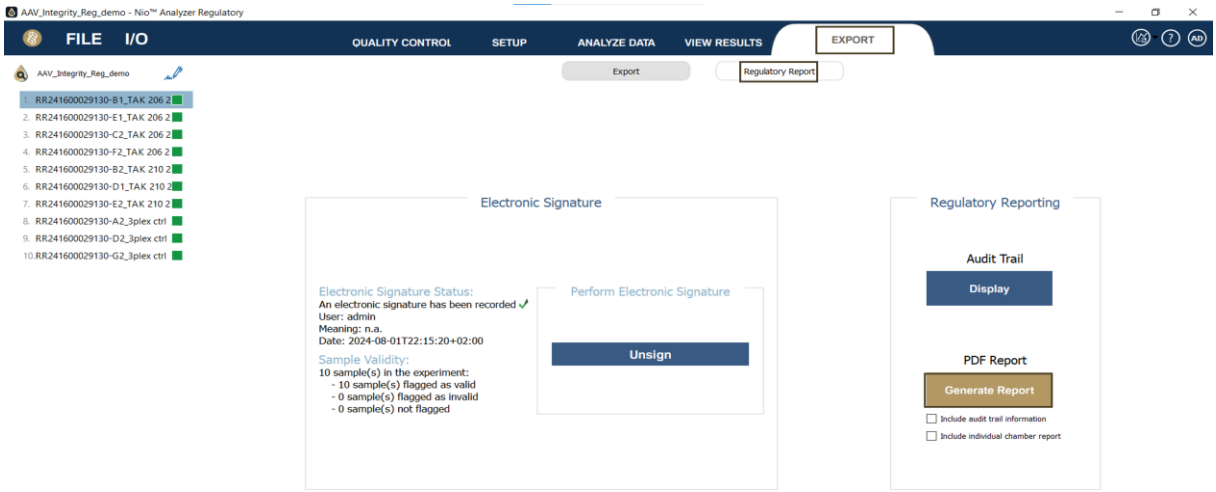


Experiment PDF Report creation

Note: The Experiment PDF report can only be created if the experiment file is signed electronically. All date and time stamps are displayed with the ISO 8601 format.

To export the Experiment PDF Report:

- Open the “Regulatory Report” tab
- In the “Regulatory Reporting” section, click on “Generate Report” underneath “PDF Report”. Optionally it is possible to include individual chamber report or the audit trail in the Experiment PDF Report:
    - To include the Experiment Audit Trail in the PDF Report, check the “Include audit trail information” box underneath “Generate Report”.
    - To include the individual chamber report in the PDF Report, check the “Include individual chamber report” box underneath “Generate Report”.
- Choose the file directory and confirm by clicking ok.
- Upon PDF Report export completion, the PDF file is automatically opened with the default PDF viewer.



The PDF report can be printed in US Letter or A4 format. The PDF report is not editable after edition.

The Experiment PDF Report includes:

- The experiment name
- A “User and software traceability information” section, including:
    - Electronic signature operator, date, and time
    - Electronic signature meaning
    - Scan operator, date, and time
    - Report generation operator, date, and time
    - Software name and version used to create the PDF Report
    - Software name and version used to scan the chambers

- An “Experiment and analysis setup information” section, including:
  - Instrument type identification
  - Instrument Serial Number
  - Chip type
  - Mix type
  - Standard Experiment status
  - Number of analyzed chambers
  - Number of analyzed samples (considering pooled chambers)
  - Spill-over compensation matrix
  - Thresholding scope (common to all chambers or individually per chamber)
  - Thresholding type (Lines or Polygons)
  - A description of the scanned channels, including:
    - Channel name
    - Fluorophore name
    - Target name
    - The time exposure
- A “Thermocycling program” section, including:
  - Protocol(s) tag(s)
  - Protocol(s) name(s)
  - Altitude profile(s)
  - PCR program(s)’ steps
  - The layout of the protocol(s) in the chips of the Chip Plate
- A “Chip Layout” section, including for each Chip Plate:
  - Chip ID
  - Chamber ID
  - Sample name
  - Pooling ID – if chambers are pooled, the pooling ID is written between brackets next to the Chamber ID.
  - Protocol tag
- A Post-processing set-up section, describing
  - The type of post-processing analysis
  - The target and reference populations
- A "QC Indicator" section, included for each individual chamber:
  - Chamber ID
  - Sample name
  - Chamber context
  - Image Sharpness quality for chamber
  - Number of analysable droplets for chamber
  - Number of saturated objects for chamber
  - General quality flag for chamber
    For the quality flags, High is equivalent to a “Green" flag, Low is equivalent to a “Yellow” flag and “Medium” is equivalent to the green eye flag.

- A “Result Table” section, included for each individual chamber and pooled chamber:
  - Chip ID
  - Chamber ID
  - Chamber context
  - Sample name
  - Sample validity status
  - Protocol tag
  - Population name
  - Dilution factor
  - Concentration
  - Number of positive droplets per population
  - Number of negative droplets per population
  - Separability score
  - Minimum concentration
  - Maximum concentration
  - Relative uncertainty
- Footer section including:
  - Report generation operator, date and time
  - Experiment name
  - Current page and total number of pages

The Experiment PDF Report can also include the following two optional sections, if selected by the user:

- Individual and pooled chamber level information.
  This includes, for each individual chamber and each pooled chamber:
  - The pooling/chamber name
  - A “Pooling/Chamber details” section, including:
    - Chamber ID
    - Sample name
    - Chamber/Pooling context
    - Pooling ID
    - General quality flag for pooling
    - Image sharpness quality for chamber
    - Number of analyzable droplets for chamber
    - Number of saturated objects for chamber
  - Per channel: The sample type
    - U = unknown
    - P = positive
    - N = negative
    - S = standard
    - Dilution factor
  - A “Chamber/Pooling result” section, including:
    - Sample validity status

        - Total number of droplets per chamber / pooling
        - Per channel:
    - Dilution factor
    - Concentration
    - Number of positive droplets
    - Number of negative droplets
    - Minimum concentration
    - Maximum concentration
    - Relative uncertainty
    - Separability score
  - The PDF Report displays the experiment 2D plots, as configured in the “ANALYZE DATA” menu, “Plots & Population” tab, “2D dot plot” section.
- The experiment audit trail.

Note: The chambers in the ‘QC indicators’ table and the ‘Results’ table will appear in the same order as they appear in the Nio Analyzer software when the Experiment PDF Report is generated. The “Populations” that appear in the “Results” table correspond to the same populations that are visible in Nio Analyzer software.

7. Maintenance and Technical Support

For technical support inquiries:

- **United States:** support@bio-rad.com **| 1-800-424-6723**[AA1]
- **Austria / Germany / Switzerland:** cts-ce@bio-rad.com | 00 800 00 24 67 23
- **France:** sp-lsg@bio-rad.com | 00 800 00 24 67 23
- **Denmark:** techsupport.nordic@bio-rad.com | 00 800 00 24 67 23
- **United Kingdom:** lsgtechsupport.uk@bio-rad.com | 00 800 00 24 67 23
- **Belgium / Netherlands:** cts.benelux@bio-rad.com | 00 800 00 24 67 23

To enable remote software maintenance on the Nio Digital PCR provided by Stilla Technologies, check that the PC is turned on and connected to the Internet. Install Team Viewer on the Nio Digital PCR. Then, double-click on the “Team Viewer” desktop icon and send both the user ID & password information by email to the Technical Support Team.

***Figure 104**: Desktop icon of the “Team Viewer” application for remote software maintenance.*

Please have the following information ready for remote maintenance:

- The software version, which is available in the “About” menu accessible via FILE > About.
- The log files which have been generated in the directory:
- “%USERPROFILE%\Public\Stilla\NioAnalyzer\logs”

8. Troubleshooting

OBSERVATION	RECOMMENDATION
The Nio Analyzer software application stops responding.	Go to “Control Panel” > “Task Manager” and end the Nio Analyzer software process, then restart it.
Data cannot be exported	Check that an exported file is not already open in the export directory.
The Nio Analyzer software application is too slow.	If the data needs to be analyzed offsite from the Nio Digital PCR, check that the PC/laptop used meets the minimum requirements specified in this manual. If you run the Nio Analyzer software application on a portable PC, the laptop should be plugged into a power source for optimal use. Opening several windows of the Nio Analyzer software on a PC/laptop can also slow down the application.
After the last action, the displayed frozen cursor does not disappear.	Go to “FILE” > “Save” or “FILE” > “Save as” to save the current experiment. This will make the frozen cursor disappear.

If these observations persist, please contact Technical Support.

9. Software License Information

How to view the software version?

To find which version of the software is installed, click on the “FILE” menu, then click on “About”. The software version is displayed in the pop-up window.

Figure 2: Nio Analyzer software Standard mode’s “About” menu

Nio Analyzer software Regulatory mode’s “About” menu.

Nio Analyzer software license

For Research Use Only. Not for use in diagnostic procedures.

Third-party licenses

The Nio Analyzer software uses the following third-party software components:

- Boost
- Catch
- CMake
- CPP REST SDK
- Cryptopp
- cuDNN
- Docopt.cpp
- Eigen
- GLC-Lib
- GSL (Guidelines Support Library)
- ITK
- JSON for Modern C++
- JWT CPP
- MagicEnum
- onnxruntime
- OpenCV
- Python
- Qt
- Quazip
- Qwt
- stlab
- tl-expected
- xlnt
- ".yaml"-cpp
- ZLib

To view the license information, please visit the third-party websites or check the Nio Analyzer software installation directory: "C:\Program Files\Stilla\NioAnalyzer\licenses".

Note: The license information of all the third-party software components is also accessible in the Nio Analyzer software application, by clicking on "About" in the "FILE" menu.