SAFIA

Manual for SAFIA Mycotoxin Measurement

This manual describes how to perform a performance check with SAFIA Check particles and how to measure the SAFIA assay for mycotoxins using the CyFlow® Cube 6 V2m flow cytometer and the CyFlow® Robby autoloading station, as well as how to analyse the data with SAFIA Score 1.1.

Version 2.2

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Contents

1 General information on mycotoxin measurement with SAFIA

1.1 Mycotoxins

Mycotoxins are secondary metabolites produced by moulds or ergot fungi, mainly belonging to the species *Aspergillus*, *Alternaria*, *Fusarium*, *Penicillium* and *Claviceps*. When consumed, they can cause acute poisoning, chronic diseases and even cancer. Mycotoxins pose one of the greatest contamination risks to the food industry and are therefore heavily regulated by the EU (see Regulation (EC) No. 915/2023). The regulated mycotoxins that can be measured with the SAFIA kits are ochratoxin A (OTA), fumonisins (FUM, isomers FB1, FB2), deoxynivalenol (vomitoxin, DON), zearalenone (ZEN) and aflatoxins (AFL, isomers AFB1, AFB2, AFG1, AFG2) and T2 toxin (T2). The maximum permissible levels depend on the type of food.

1.2 Available kits

Table 1. Overview of available SAFIA kits with the parameters they contain

Order number	Kit	Parameter
FIE1L012	Field Kit	FUM, DON, ZEN, T-2 + Control
STO1L001	Storage Kit	OTA, AFL + Control
SCR1L013	Screening Kit	OTA, AFL, FUM, DON, ZEN, T-2 + Control

1.3 Principle of the SAFIA assay

The **s**uspension **a**rray **f**luorescence **i**mmuno**a**ssay, or SAFIA for short, is a particle-based multiplexing rapid test. Coded microparticles are used for multiplexing. The coding is based on different amounts of a red fluorescent dye incorporated into the microparticles. Each code, represented by a specific dye concentration, is used to encode a corresponding measured analyte. The entire measurement principle for the detection of mycotoxins is based on indirect competitive immunoassays. The mycotoxins are chemically immobilised on the surface of the particles. The sample or standard, a mixture of mycotoxin-specific antibodies and fluorophore-labelled antibodies are added to the particles. The specific antibodies competitively bind either the respective immobilised mycotoxin or the mycotoxin present in the sample. Bound antibodies are stained with dye (green fluorescent) labelled antibodies to produce a measurable signal. Due to the competitive reaction, the concentration of the mycotoxin is inversely proportional to the signal and can be determined using a calibration curve.

The red fluorescence used for coding and the green fluorescence used for quantification are read out using a flow cytometer. Inside the flow cytometer, the SAFIA microparticles are separated hydrodynamically and the fluorescence is measured independently for each particle using a blue and a red laser/detector system.

Compared to classic immunoassays such as ELISA, SAFIA is a mix-and-read immunoassay. Washing steps, which are used to avoid high signal backgrounds, matrix interference or to stop signal increase, are not necessary.

In addition to the mycotoxins, a control measurement is also performed in SAFIA. This indicates whether matrix effects interfere with the test during measurement or whether it was performed correctly. The interpretation takes place automatically in SAFIA Score, see section 7.4.

1.4 Performing the SAFIA assay

This manual describes how to perform measurements in microtitre plate (MTP) format using the *CyFlow® Cube 6 V2m* with *CyFlow® Robby Autoloading Station* (hereinafter referred to as "Cube 6").

Additional information on operating the Cube 6 can be found in the device instructions. The device may only be used by persons who have been instructed and trained in its use. **Please observe the Safety instructions.**

The assay is carried out in the following steps:

1. Sample preparation (extraction, decolourisation if necessary, dilution)
2. Performing the SAFIA assay (mixing the reagents)
3. Readout with the Cyflow® Cube 6 flow cytometer
4. Evaluation of the assay using SAFIA Score 1.1

Figure 1. Analysis procedure

2 Product information

2.1 Kit contents

Table 2. Kit contents

Component	Quantity and contents	Status	Information
96-well microtiter plate	1	Ready to use	
Calibration standards	8 x 0.5 mL	Ready to use	Labelled "Kal-1" to "Kal-8"
Sample buffer	1 x 15 mL	Concentrate	10-fold concentrate
Primary antibodies	1 x 5 mL	Ready to use	Labelled with "AK 1"
Secondary antibodies	2 x 5 mL	Ready to use	Labelled with "AK 2"
Particle stock solution	1 x 45 µL	Concentrate	In vial with insert
Particle buffer	1 x 1.5 mL	Ready to use	
Fixation solution	1 x 10 mL	Ready to use	
SAFIA PVPP adsorber		Ready to use	Optionally available Order number: SPVA-007
SAFIA PA adsorber		Ready to use	Optionally available. Order number: SPAA-008

Table 3. Concentrations of the individual calibration standards

Standard	c(OTA) µg L^{-1}	c(DON) µg L^{-1}	c(ZEN) µg L^{-1}	c(FUM) µg L^{-1}	c(AFL) µg L^{-1}	c(T2) µg L^{-1}	c(KON) µg L^{-1}
KAL-1	1,000	10,000	1,000	10,000	1,500	5,000	1,000
KAL-2	100	1,000	100	1,000	150	500	100
KAL-3	10	100	10	100	15	50	10
KAL-4	1	10	1	10	1.5	5	1
KAL-5	0.3	3	0.3	3	0.45	1.5	0.3
KAL-6	0.1	1	0.1	1	0.15	0.5	0.1
KAL-7	0.01	0.1	0.01	0.1	0.015	0.05	0.01
KAL-8	0.001	0.01	0.001	0.01	0.0015	0.005	0.001

2.2 Additional reagents and materials required

Specifications for the materials can be found in the Checklist in the appendix.

2.2.1 Equipment

- Analytical balance
- *CyFlow® Cube 6* flow cytometer in the single-laser version (488 nm, blue) *with CyFlow®*
- Single-channel and multi-channel micropipettes
- Glass vessel, sealable
- Microtiter plate shaker
- Multi-channel reservoir
- *Robby autoloading station*
- Tubes for sample weighing and extraction
- Shaker for vessels
- Centrifuge

2.2.2 Reagents

- Ethanol 99% denatured with MEK, IPA and Bitrex® (min. 99.8%), dilute to 70% (vol/vol) for analysis
- Deionised water

2.2.3 Materials for operating the Cube 6

- *Sheath fluid*, order number: 04-4007_R
- *Cleaning Solution*, order number: 04-4009_R
- *Decontamination Solution*, order number: 04-4010_R
- *Hypochlorite Solution*, order number: 04-4012_R

- 96-well microtiter plate, order number 04-2020
- Test tubes for Sysmex Cube 6 flow cytometer, order number: 04-2000

Optional: Material for the SAFIA Performance Check:

- *SAFIA Check Particles*, order number: SCP-1L-010
- *SAFIA Check software*, order number: SCSO-009
  Alternatively
- *SAFIA Check Starter Kit* (particles and software), order number: SCSK-1L-011

2.3 Storage and use of the kit

The kit must be stored in a refrigerator at 2–8 °C and must not be frozen under any circumstances (e.g. at –20 °C). Before use, all components must be brought to room temperature and direct exposure to light must be avoided. No guarantee can be given after the expiry date. Individual reagents in the kit must not be exchanged with reagents from other kits, even if they have the same batch number printed on them. The kit may only be used by trained personnel, and the instructions for use must be strictly followed. Once opened, we recommend using the kit within one month. The diluted sample buffer should be stored at room temperature.

2.4 Safety instructions

The kit components *Calibration Standards* and *Fixation Solution* contain small amounts of mycotoxins and must be handled with care.
The kits contain substances that are hazardous to health. Please refer to the safety data sheets (SDS) for safety instructions and precautions regarding the components contained in the kits.

All reagents and materials must be disposed of properly and responsibly after use. When disposing of these materials, observe the applicable national regulations and consult the safety data sheets if necessary.
We recommend decontaminating glassware or other equipment that has come into contact with toxic solutions using either an alkaline quick cleaner or a 10% hypochlorite solution.

2.5 Data storage

Several files and folders are created when the system is installed. Do not change these, as this will interfere with the automatic processing of the data. Table 4 provides an overview of the files and where they are stored. Only the files marked with * are those that you must select manually in CyView or SAFIA Score. For convenience, pin the *Export Files* subfolders to Quick Access.

Table 4. Overview of file paths and data storage in the SAFIA system

C:\ProgramData\PartecGmbH\Cube_18\config\Mycotoxins			
File(s)			**File names**
Configuration file for performing the performance check and measurements			Mycotoxins-SCR_A_Robby.cv85 [1] *
Configuration file for performing cleaning			Cleaning-MTP.cv85*
C:\ProgramData\PartecGmbH\Cube_18\templates\Quality Control			
File(s)			**File names**
FCS Express Layout Template (automatically loaded into FCS Express for data processing)			Calc_Mycotoxins-SCR_A
C:\ProgramData\PartecGmbH\Cube_18\data\cyflow			
File(s)			**File names**
.fcs files of the measurements (**automatically** saved here with date and ID)			Date_Time_Tray ID_Last Well ID.fcs
C:\User\cyflow\Documents\			
Folder	**Subfolder**	**File(s)**	**File format**
SAFIA check	Export files	Raw data for SAFIA score (**automatically** stored here via FCS Express)	.csv*
	Reports	Performance check reports (stored here via SAFIA Check)	.pdf
SAFIA Mycotoxins	Export files	Raw data for SAFIA Score (**automatically** stored here via FCS Express)	.csv*
	Optional:		
	SAFIA files	SAFIA Score files	.sdf
	FCS Express layouts	Layouts of individual measurements	.fey

[1] Please note that each device contains the original configuration file ("SCR_A_Robby.cv85") and a configuration file adapted to your device. Always use the adapted configuration file for measurement, as it contains the correctly set gain values for the detectors in your device.

3 Starting the Cube 6

1. Before starting, the *waste* bottle should be completely emptied.
2. The *sheath* bottle should be filled to approximately 80% with sheath fluid.
3. Switch on the device (small black button on the device).
4. The CyView™ measurement software opens automatically. Wait until it has loaded completely and the device is ready for use.
5. Log in to the software with your account.
6. Open the configuration file (Mycotoxins-SCR_A_Robby.cv85 [2]) for measurement via *CFG upload* 

.
7. To do this, click on Prime 

in the main bar and then on Start  (see Figure 2) and follow the instructions through the Prime programme (see Figure 3).

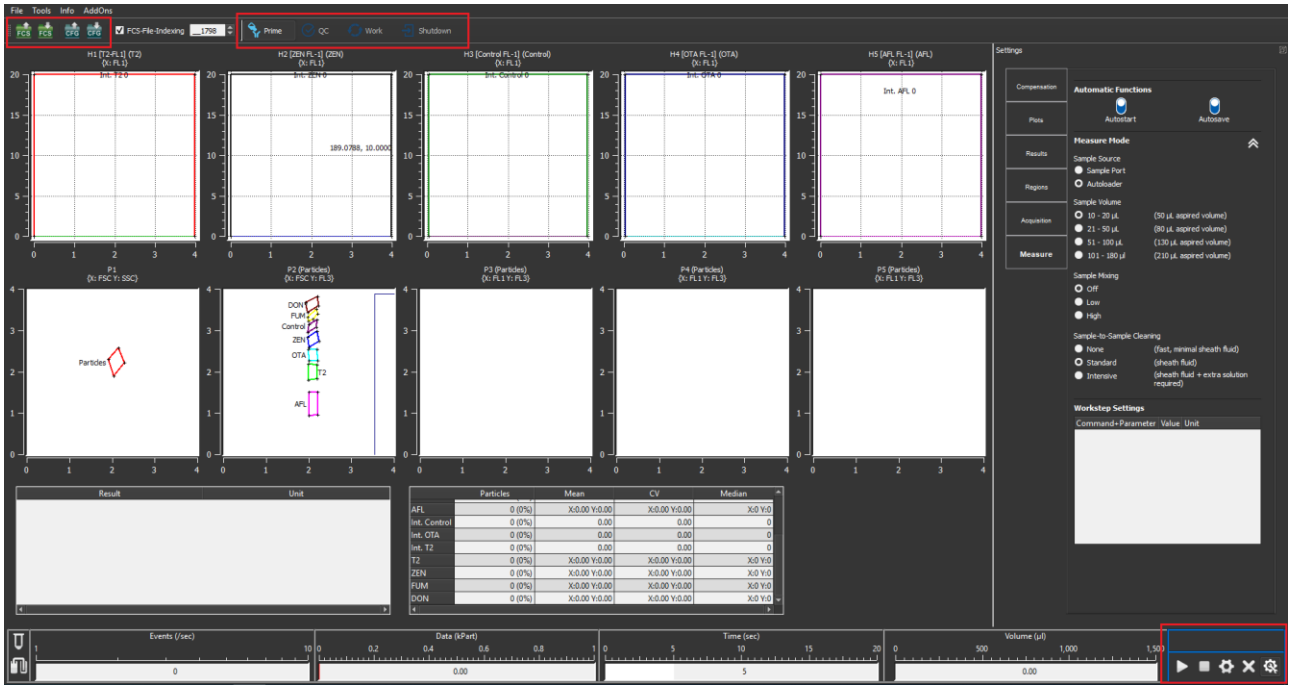
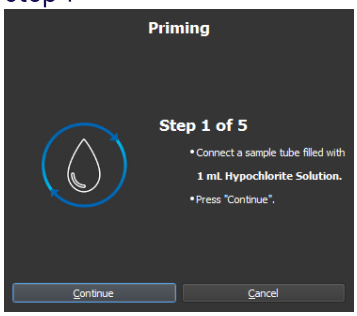


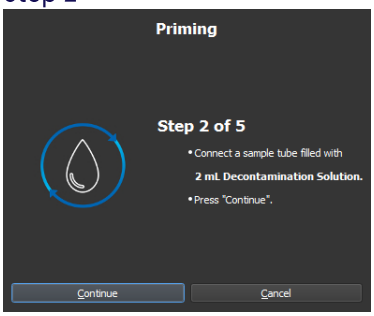
Figure 2. Location of the *Prime* programme, *settings* and *Start* button

[2] Please note that each device contains both the original configuration file ("SCR_A_Robby.cv85") and a configuration file tailored to your device. Always use the tailored configuration file for measurements, as it contains the correct gain settings for your device's detectors.

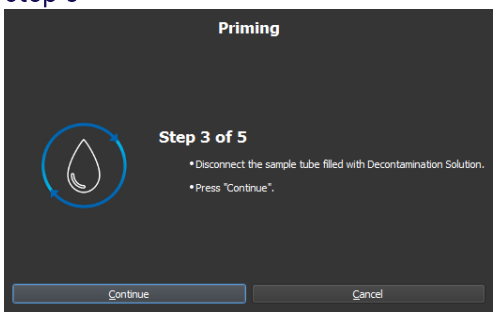
Step 1



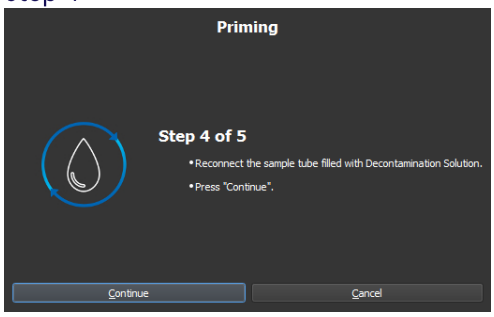
Step 2



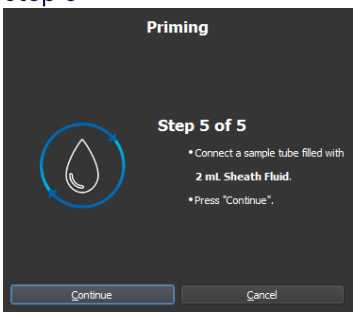
Step 3



Step 4



Step 5



Step 6

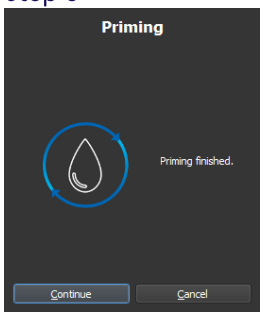


Figure 3. Steps of the Prime Programme

4 Conducting a *performance check*

4.1 Explanation of the *performance check*

We recommend checking the performance of the Cube 6 device daily using the *SAFIA Performance Check particles* and evaluating the results using *SAFIA Check*. If the performance check has already been carried out today, continue with section 5.

The *SAFIA Performance Check* is used to verify that the Cube 6 is functioning correctly for measuring SAFIA assays. To do this, a mixture of *SAFIA Performance Check particles* is measured. The measurement must have predefined intensity, particle count rates, coefficients of variation (%CV) and signal-to-noise (S/N) ratios in the corresponding FSC, SSC, FL-1 and FL-3 detectors in order to pass the *SAFIA Performance Check*. If the measurement is successful, 20,000 particles are measured, which appear in the FSC/SSC scatter plot as a population in the "Particle" gate (see Figure 4 A). Five gates are plotted in the FSC/FL-3 scatter plot, in each of which 1 or 2 populations are visible (see Figure 4 B). The populations from these 5 gates are separated into 5 histograms in FL-1, so that two peaks clearly represent two populations (see Figure 4 C).

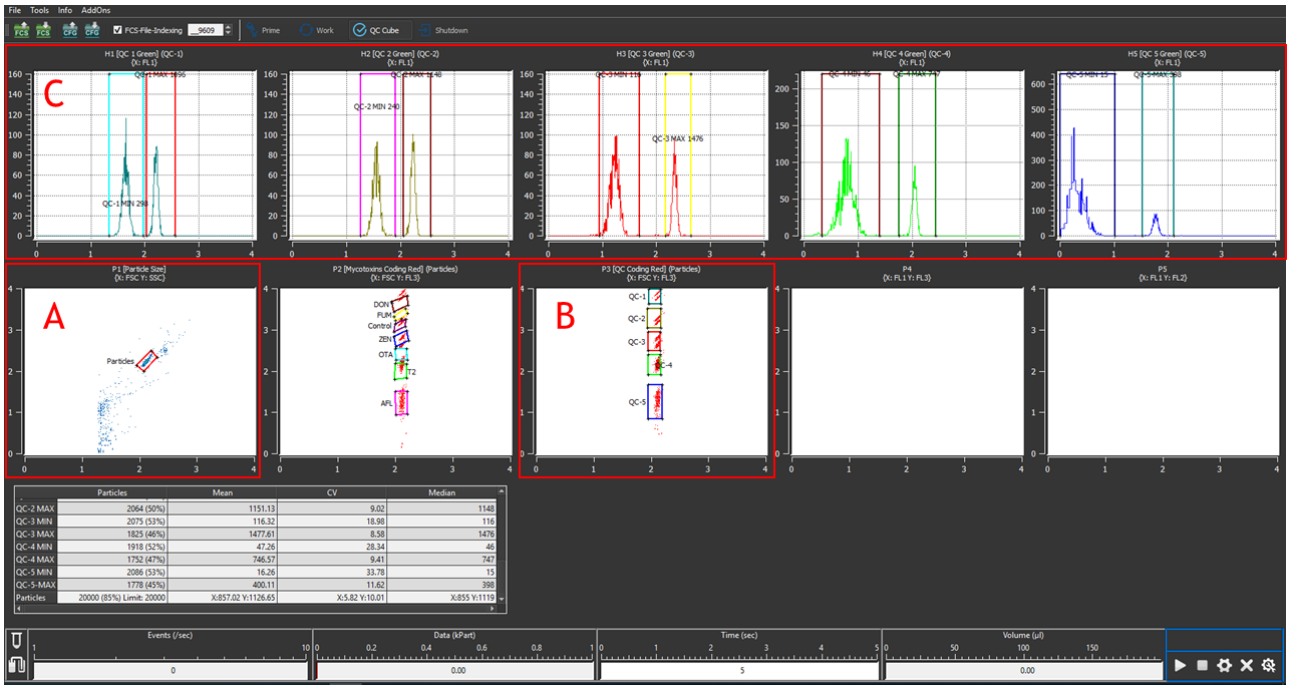


Figure 4. SAFIA Check overview. FSC/SSC scatter plot in A, FSC/FL-3 scatter plot in B and FL-1 histogram in C

The S/N ratio is calculated in *SAFIA Check* from the quotient of the intensity in FL-1 (MAX) and FL-1 (MIN) for each population in FL-1. The *SAFIA Performance Check* can therefore be used to check:

- the correct functioning of the fluidic system
- the status of the laser
- the correct functioning of the FL-3 detector (coding of the SAFIA assay particles)
- the correct functioning of the FL-1 detector (dynamic range of the SAFIA assays)

4.2 Preparation

The SAFIA Check particles must be diluted before performing the measurement.

1. Shake the bottle containing the *SAFIA check particles* vigorously for at least 15 seconds.

2. Remove 10 µL of the particle suspension and add it to 10 mL *of sheath fluid*.

3. Shake the particles again for at least 15 seconds.

4. The particles are now ready for measurement. The prepared solution can be used for a further 5 days, after which it must be discarded. **It should be stored in a refrigerator at 2–8 °C.**

4.3 Procedure

1. Click on QC Cube 

in the main bar and check under Settings  → Measure whether the Sample Port is selected as the Sample Source and that the settings match those in Figure 5.

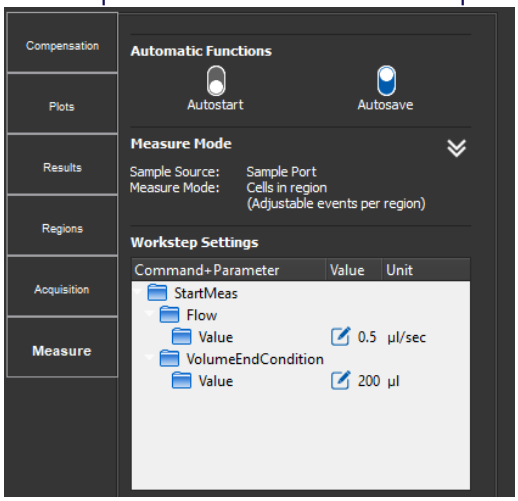


Figure 5. Measurement settings

2. Click *Start*  and follow the instructions in the QC programme (see Figure 6). If no particles are detected, follow the instructions in section 10.1.

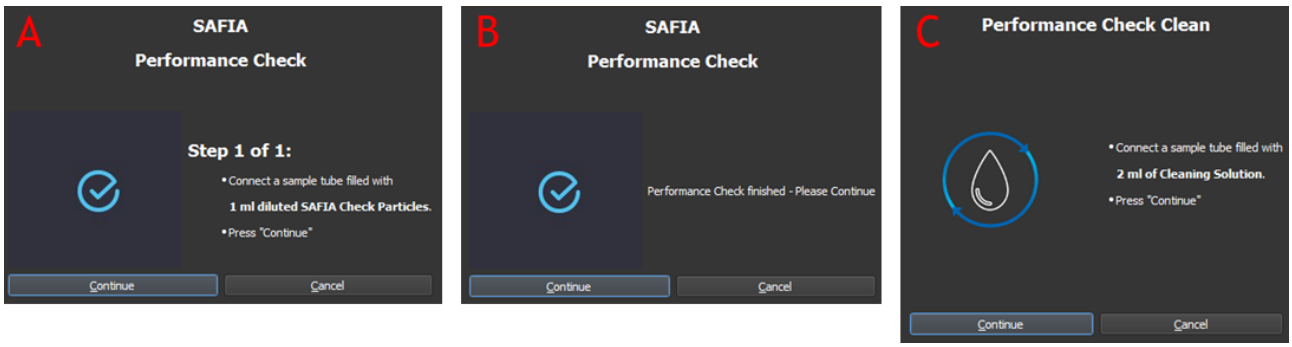


Figure 6. View of the instructions for the QC programme sequence

3. When the measurement is complete, the *FCS Express* programme opens automatically. The plots and histograms described above are displayed on the left-hand side, while the .fcs file used is visible on the right-hand side in *Datalist* (see Figure 7). If the correct data is not loaded automatically, please refer to the instructions in section 10.4.

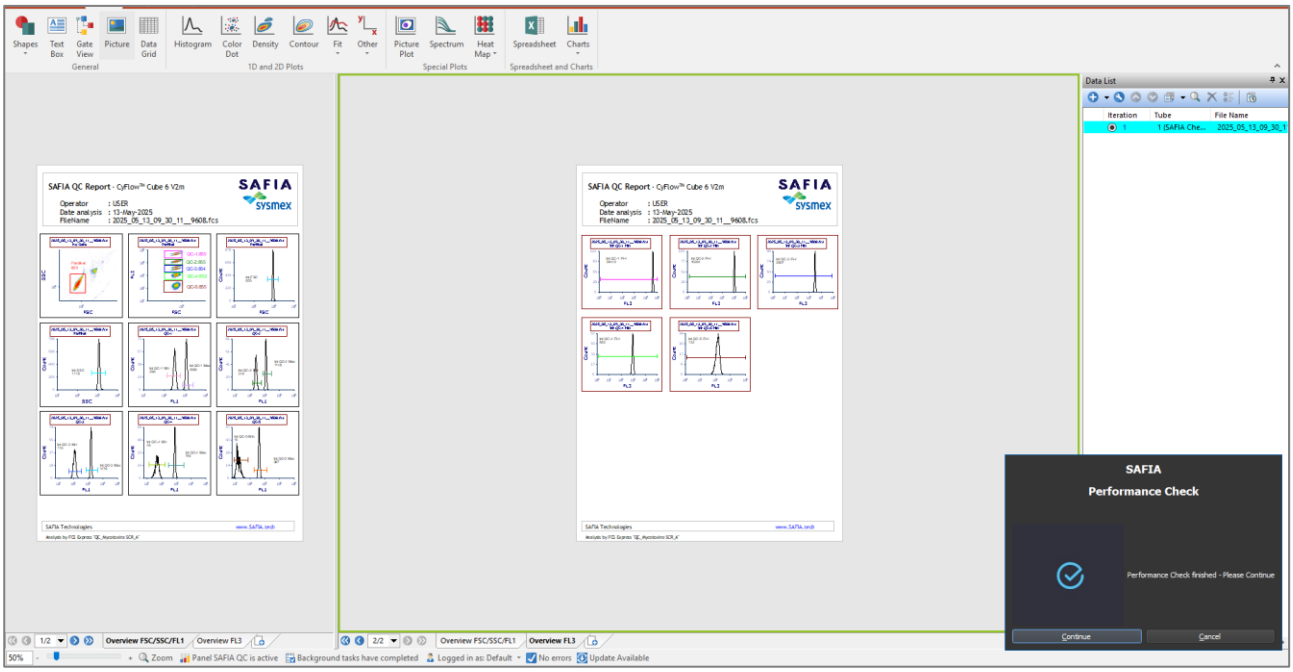


Figure 7. Display of the .fcs data in *FCS Express*

4. Check in *FCS Express* whether the populations are within the designated gates and adjust them slightly if necessary. To do this, click on the corresponding gate and move it to the desired position. Under the *Batch & Export* tab, click on *Run* (see Figure 8).
5. A window will open and close briefly, which you can ignore. The .fcs file will now be converted into a .csv file, which can be evaluated with SAFIA Check. The .csv file is automatically saved in the *SAFIA Check → Export-Files* folder.

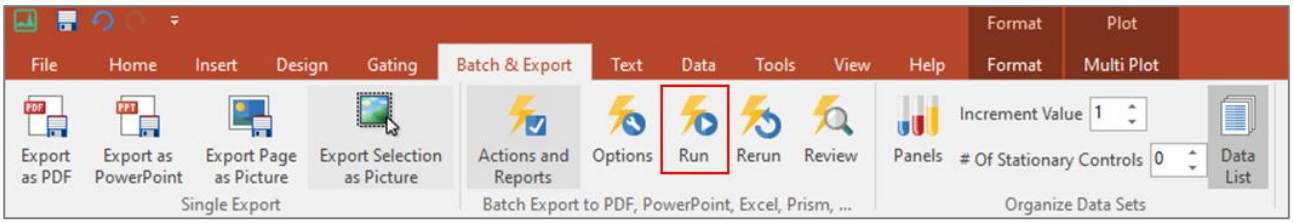


Figure 8. View of the *Batch & Export* tab in *FCS Express* and location of *Run*

6. Run the Performance Check Clean program in CyView. Once the measurement is complete, a window will open automatically to guide you through the process (see Figure 6 C). The cleaning process starts automatically and removes any remaining particles from the device.

4.4 Evaluation of a performance check with SAFIA Check

1. Open *SAFIA Check* and click on the button Add New Performence Check .

2. To add the .csv file, click *Load CSV File* in the window that opens. Select the appropriate file from the *SAFIA Check → Export Files* folder.

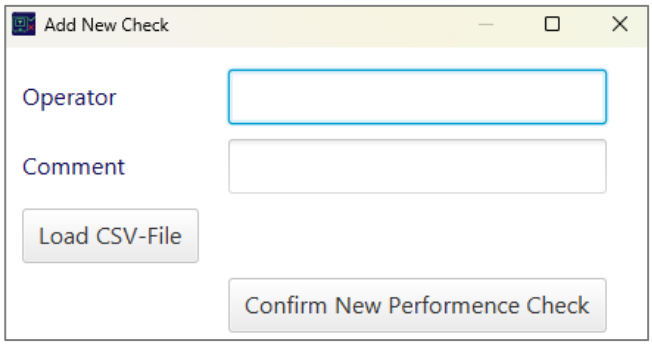


Figure 9. View of the *Add New Check* window with the *Load CSV File* and *Confirm New Performance Check* buttons and the *Operator* and *Comment* free text fields

You have the option of entering the name of the person performing the check and saving a comment that may be helpful for interpretation, e.g. if maintenance has been carried out on the device.

3. Clicking the *Confirm New Performance Check* button loads the .csv file into SAFIA Check and runs the performance check.

   It is then no longer possible to change or delete the performance check! Each .fcs file can only be evaluated once with the SAFIA Performance Check.

4. The result of the performance check is automatically saved as a .pdf file in the *SAFIA Check folder→ Reports.*

   Close *FCS Express* after completion without saving so that the template is not overwritten. If you want to save an adjustment to the gate positions, you must first remove the .fcs file from the data list.

4.5 Creating a new measurement series with a new LOT number

If you have received a new batch of *SAFIA Check particles* or want to start a new measurement series, e.g. after maintenance, you must create a new *LOT file*.

1. To do this, click on the button New LOT . In the window that opens, enter the *LOT ID* and the listed parameters (see Figure 10). The parameters are supplied with the *SAFIA Check particles*. They serve as benchmarks for each *performance check*.

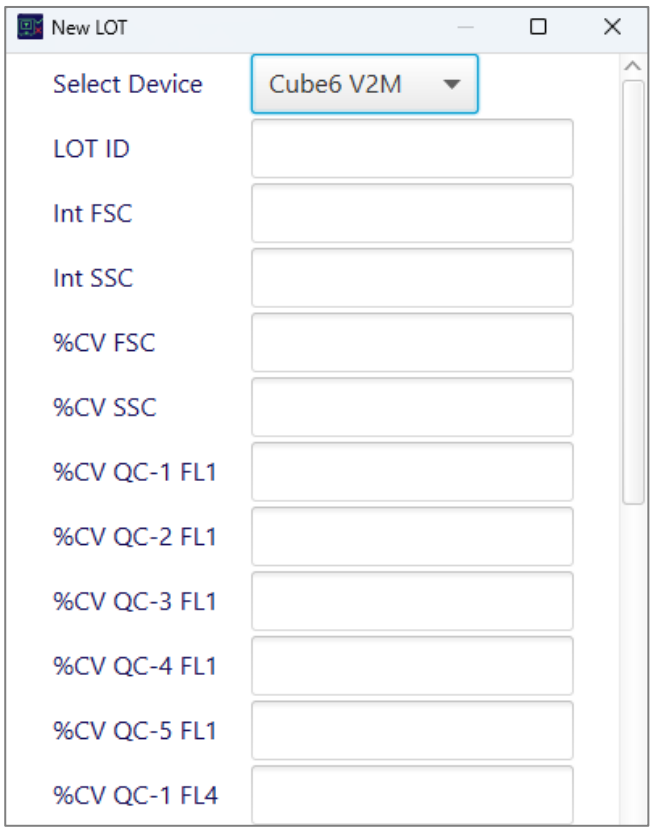


Figure 10. View of the *New LOT* window

2. Click on the *Add LOT File* button.

If the values measured during the first measurement of your SAFIA Check particles deviate from the benchmark values, the gain values of the detectors may need to be readjusted. This may also be the case after device maintenance. Please contact Sysmex Support for assistance.

5 Planning the SAFIA assay in SAFIA Score

Ensure that each plate (wells A1 to H12) contains at least one eight-point calibration curve! This should be performed in duplicate. Otherwise, it will not be possible to evaluate the test with SAFIA Score.

Only use the supplied black 96-well microtitre plate to perform the assay.

1. Open *SAFIA Score*.

In the menu bar, you will find the tabs *File* (loading, saving, editing files and exiting the software), *Export* (creating a report) and *Raw Data* (displaying the data from the .csv file). The software is divided into the workspaces *Start, Samples and Calibrations, Plate Layout, Calibration Curves, Results* and, if applicable, *Raw Data*. These tabs can be accessed by clicking on them. The workspaces *Calibration Curves, Results, References* and *Raw Data* are only activated after the .csv file has been imported and the *Calculate* button in the *Plate Layout* workspace has been clicked.

2. Start a new analysis in the *Start* tab by clicking on the green *+ New Analysis* button (see Figure 11).

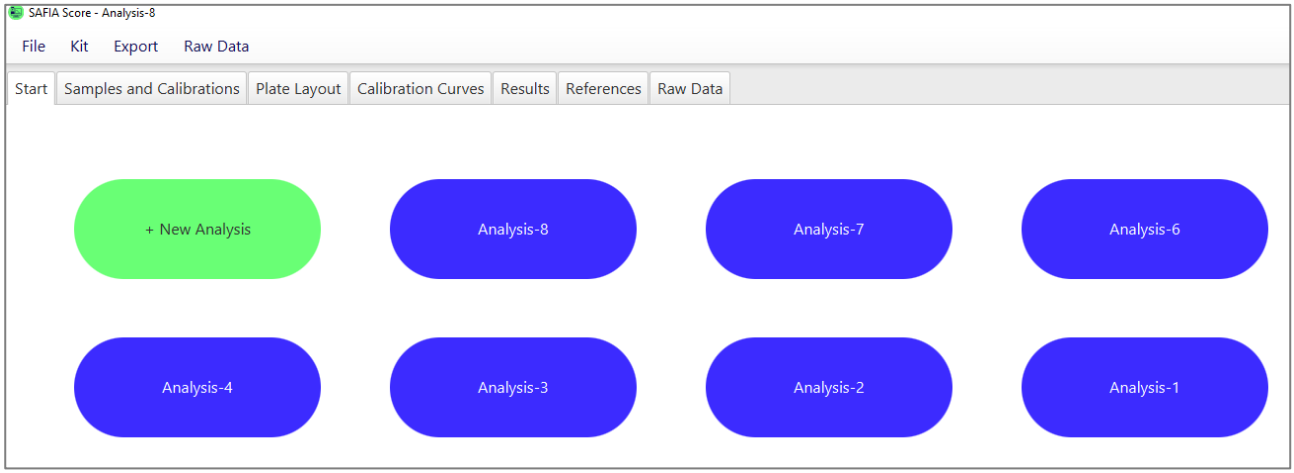


Figure 11. View of the SAFIA Score start area

You can also open an older analysis. To do this, click on the blue button of an older analysis in the start area. Alternatively, you can open a *SAFIA data file* (.sdf file) by double-clicking on it or by selecting *File → Load*.

3. In the window that opens, select the appropriate kit from the drop-down menu. Enter an analysis ID and click Start Analysis (see Figure 12).

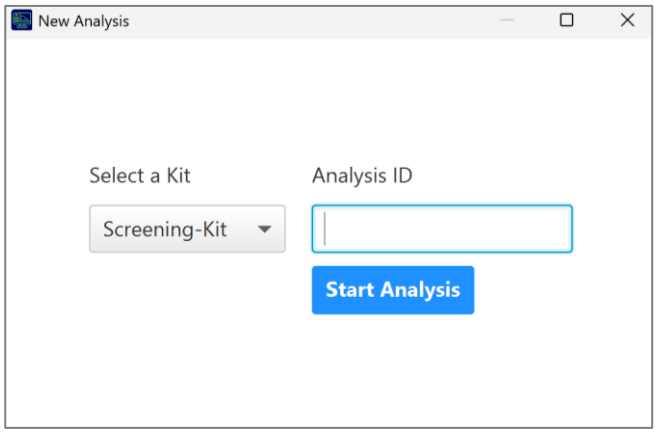


Figure 12. View of the *New Analysis* window

Assigning an *analysis ID* makes it easier to link .fcs, .csv and .sdf data. It can be changed in the menu under *File/Edit Analysis ID*.

4. Go to the *Sample and Calibration* workspace. Here you will find three tables that can be shown or hidden using the corresponding buttons. The *Calibration Table* contains the pre-filled concentrations of the individual standards supplied with the kit. You can edit these as required, e.g. change the concentration, delete and add a standard. In addition, the *Replicates* column can be used to specify how often a standard should be measured, see Figure 13.

Do not change this table if you plan to use all standards as supplied by us. Simply specify the number of repeat measurements.

We recommend measuring each standard or sample at least twice; inexperienced persons should measure each standard/sample three times.

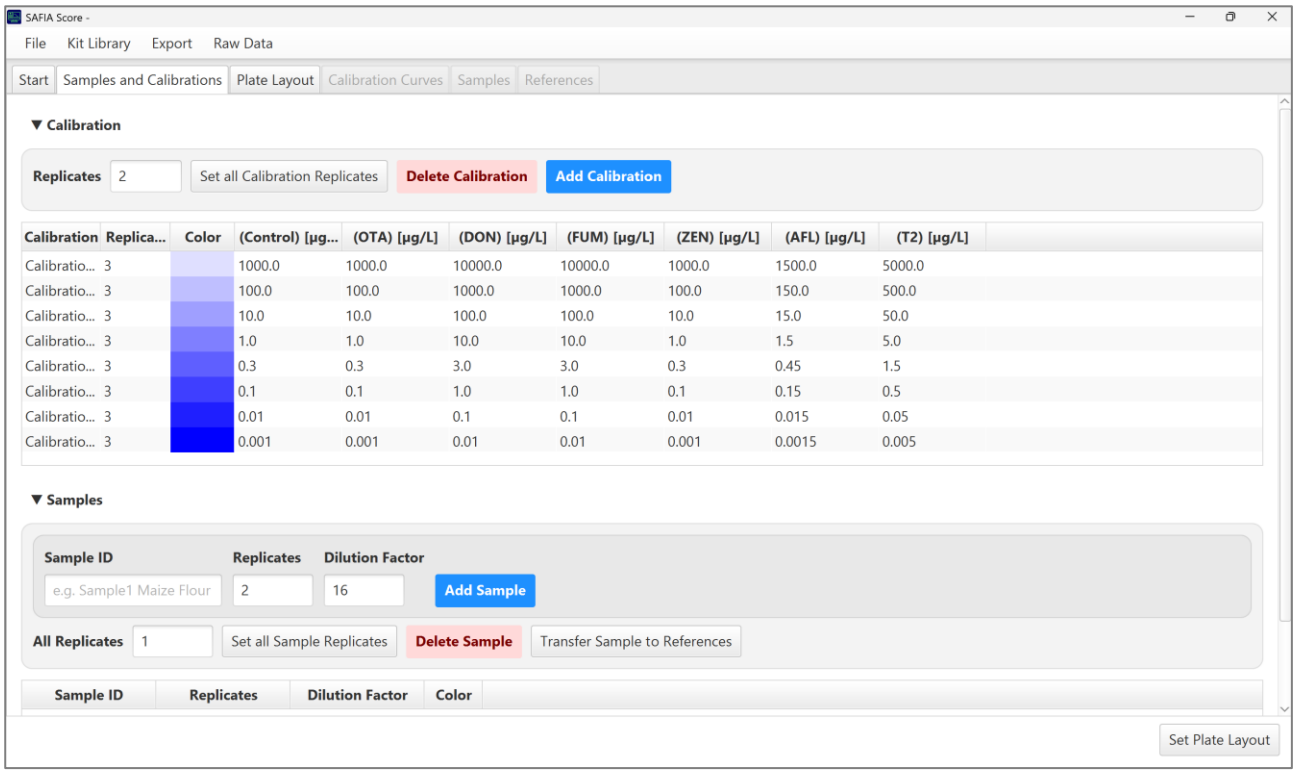


Figure 13. Overview of the functions of the Samples and Calibration workspace

5. Now enter your samples in the *Sample Table*. Assign a unique *sample ID* (sample name) to each sample and use the *Replicates* window to specify how often a sample should be measured. The sample ID can be changed by double-clicking in the corresponding cell, but it must never be assigned twice.

The sample ID can either be copied to the clipboard using a barcode scanner and pasted into SAFIA Score, or copied and pasted from a spreadsheet program. Make sure that only the relevant row is highlighted in blue (single click) when pasting. By double-clicking on a cell in the *Sample ID* column, you can enter or change the name of an individual sample ID.

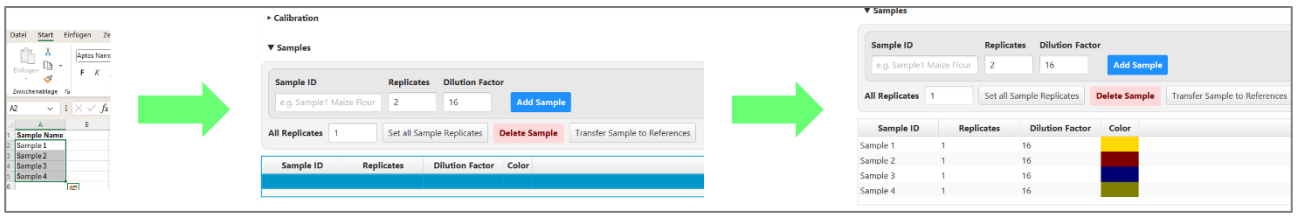


Figure 14. Pasting the sample IDs from a spreadsheet programme

6. Enter the dilution factor for the sample under *Dilution*. Then click *Add Sample* to add the sample to the table. The values in the *Replicates* and *Dilution* columns can also be edited later. The colour assigned to the sample will help in the next step when creating the plate layout.

The dilution factor can be found in section 6. For the SAFIA Mycotoxin Kit, it is "16" according to the standard procedure, "8" for liquid samples and "32" for herbs and spices. If you want to measure samples in several dilution steps, you must specify a separate *Sample ID* for each differently diluted sample.

7. Optional: You can enter samples with reference concentrations in the *Reference Table*, analogous to *the Sample Table* (e.g. reference materials or blank measurements).
   After the analysis is complete, *SAFIA Score* automatically determines the recovery rate of the reference samples. You can find this in the *References* workspace. Using the *Transfer Sample to References* button, you can also add reference samples that were accidentally entered in the Sample Table to *the References Table*.

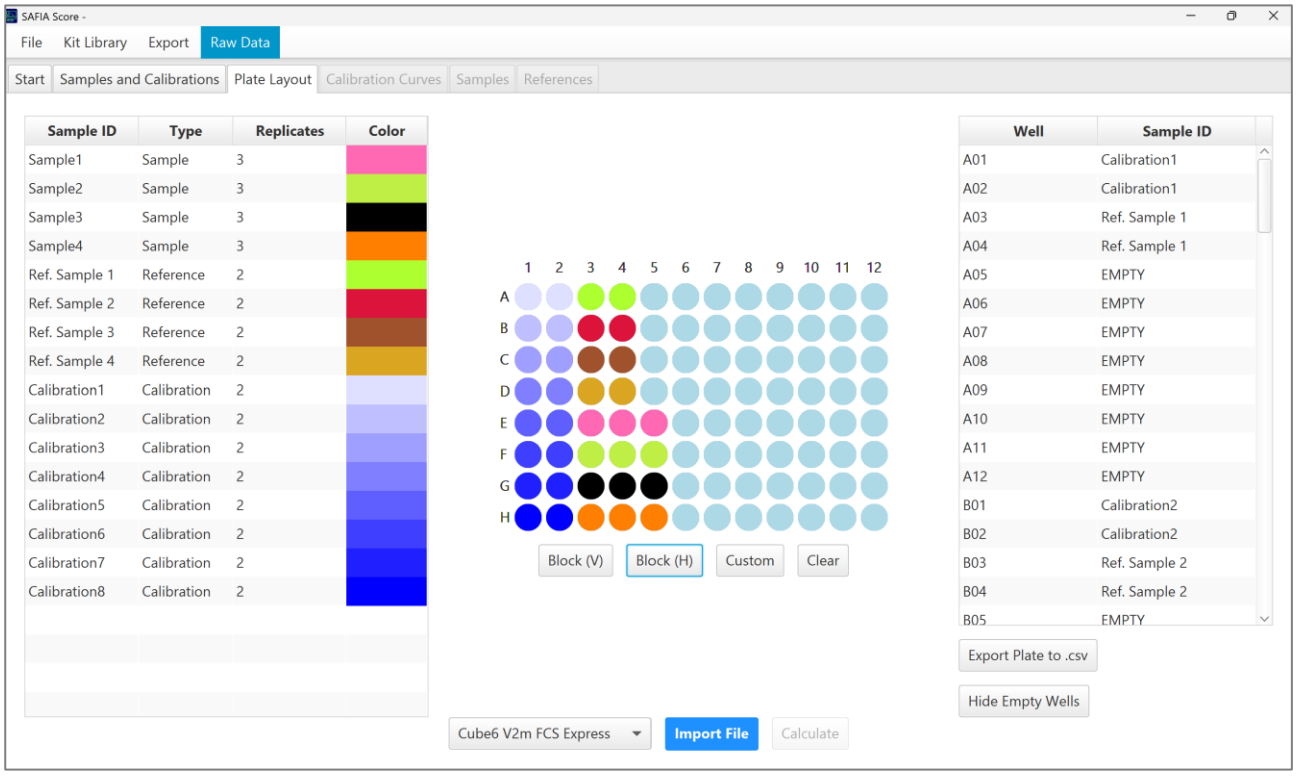


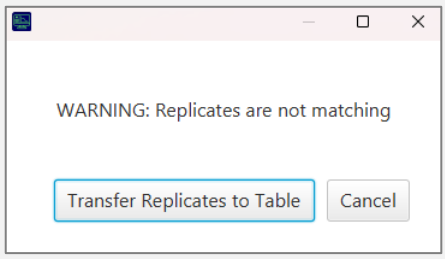
Figure 15. Overview of functions in the Plate Layout workspace

8. Go to the *Plate Layout* workspace to assign the samples and standards to the wells of the microtitre plate. The wells have an alphanumeric code (A1, B1, C1, etc. up to H12) (see Figure 15). The assignment can be done automatically using the *Block (V)* or *Block (H)* buttons. The replicates of the samples and reference samples are arranged either vertically (V) or horizontally (H). The replicates of the calibration standards are always placed horizontally on the microtitre plate. You can delete all entries using the *Clear* button.

9. The plate layout can also be created manually using the *Custom* button. This opens a separate window (see Figure 16). In the table on the left, select the desired sample and assign a single well to it by clicking, or multiple wells by clicking and dragging. Incorrectly assigned samples can be removed in the same way by activating the *EMPTY* button. Alternatively, all replicates of a sample can be dragged and dropped onto the

plate; these are then automatically arranged horizontally. The *Remaining* column shows the number of replicates that have not yet been placed on the plate. If more cavities are assigned to a sample than originally specified under *Replicates*, a negative number will appear there.

It is essential that the specified repeat measurements of the standards and samples from the *Samples and Calibration* workspace match the replicates in the *Plate Layout* workspace. If this is not the case after creating the *plate layout* using the *Custom* function, the following warning will automatically be displayed: "*WARNING: Replicates are not matching*".



You now have the option of either cancelling the process and changing the *plate layout* accordingly (*Cancel* button) or entering the replicate measurements directly into the corresponding table under *Sample and Calibration* (*Transfer Replicates to Table* button).

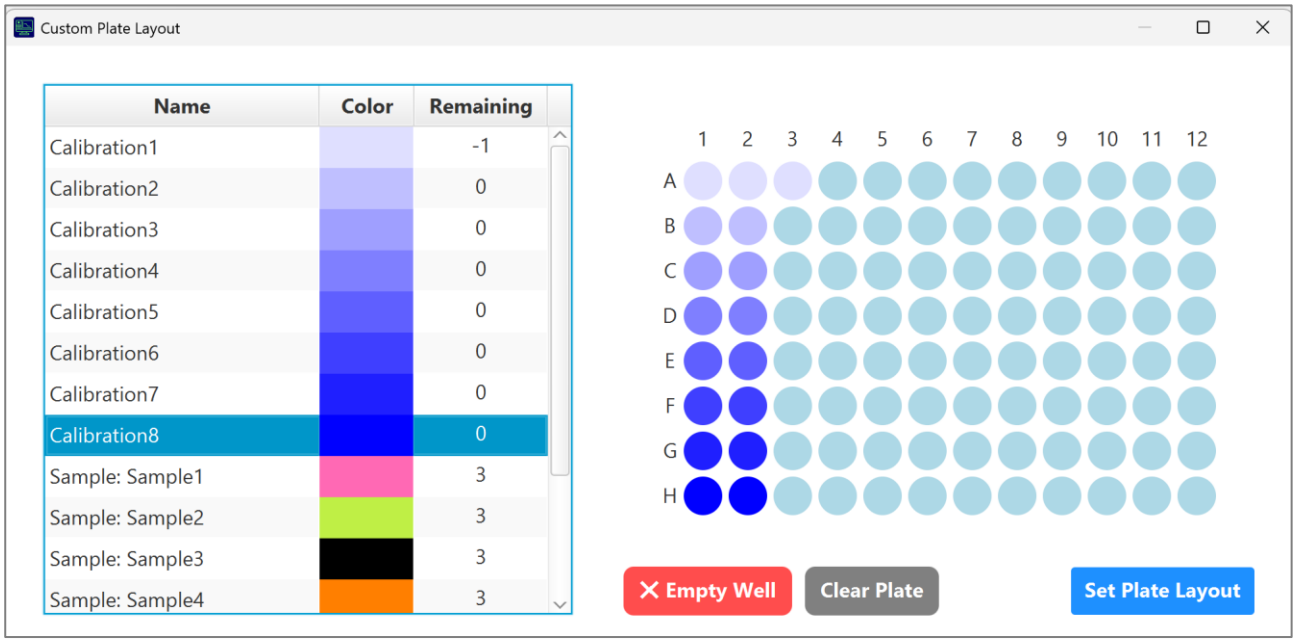


Figure 16. Menu for creating a custom plate layout

Use the *Export Plate Table* button to export the plate layout to a .csv file (.xlsx), which you can open with a spreadsheet programme, for example.

10. Save the *SAFIA data file* (.sdf) to continue the analysis later. This can be done under the *File→ Save As* menu.

11. The preparation of the assay in *SAFIA Score* is now complete. You can now use the created *plate layout* in the assay and perform the measurement in Cube 6.

6 Sample preparation

The sample preparation method to be selected depends on the type of food (see Table 5). The samples must be homogenised prior to extraction, e.g. by grinding the sample material with a mill. In doing so, attention must be paid to uniform grain sizes and the applicable regulations. The specified sample quantities should not be exceeded, but may be increased if necessary. It should be noted that the ratios of sample material and extraction agent must not be changed. Sample preparation is divided into the following steps (see Figure 17):

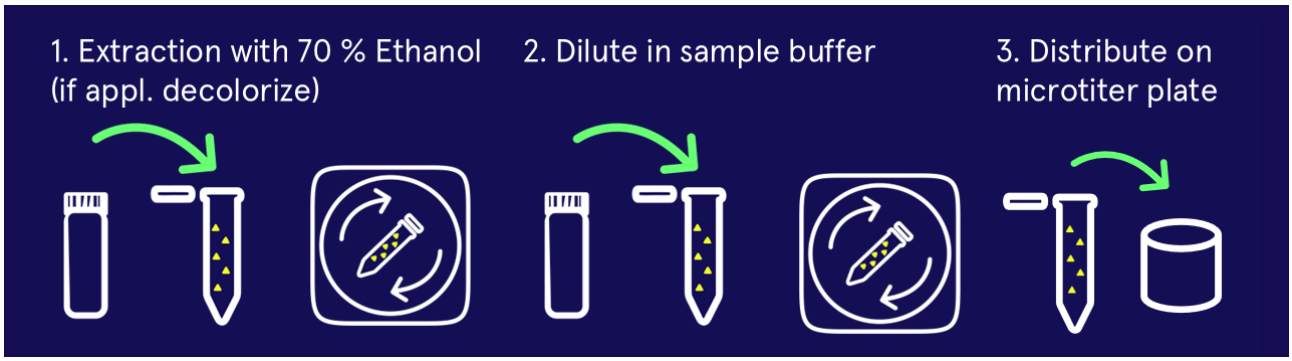


Figure 17. Overview of the sample preparation steps

6.1 Buffer preparation

Before processing the samples, the sample buffer concentrate must be diluted **1:10**:

- Pour **15 mL** of the sample buffer concentrate into a clean, sealable glass container.
- Add **135 mL of** deionised water.
- Store the diluted sample buffer in the refrigerator at 2–8 °C.
- Can be used until the expiry date of the kit.
- The sample buffer must be brought to room temperature before use.

When storing the sample buffer concentrate at 2–8 °C, a substance may precipitate. This will dissolve completely when diluted. **Please ensure that the entire contents of the bottle are dissolved. If necessary, rinse the bottle with the diluted sample buffer.**

6.2 Sample preparation instructions

To prepare samples, follow the protocol from Table 5 for the respective matrix. Sample preparation is matrix-specific, so for some matrices a slight adjustment of the standard protocol is required (solid foods with high protein content). Please note the information on special matrices in section 9.

Table 5. Sample preparation protocols for different matrices. Deviations from the standard protocol are highlighted in green

Sample type	Examples	Sample preparation	Factor
Solid foods with high protein content	Cereals, maize, legumes	1) Weigh **5 g** of sample material. 2) Add **20 mL** of 70% (vol/vol) ethanol and shake the samples for 15 minutes, e.g. in an overhead shaker. 3) Centrifuge **for 5 minutes** at **1,000 g**. 4) Dilute the supernatant **1:4** in sample buffer (e.g. 250 µL sample in 750 µL sample buffer) and shake briefly. 5) Centrifuge **for 10 minutes** at **12,000 g**.	16
Foods high in fat	Hazelnuts, peanuts, almonds, pistachios, Oils	1) Weigh **5 g** of sample material. 2) Add **20 mL** of 70% (vol/vol) ethanol and shake the samples for 15 minutes, e.g. in an overhead shaker. 3) Centrifuge **for 5 minutes** at **1,000 g**. 4) Dilute the supernatant **1:4** in sample buffer (e.g. 250 µL sample in 750 µL sample buffer) and shake briefly. 5) Add **3** spatula tips* of SAFIA PVPP adsorbent per 1 mL of diluted sample and shake **for 15 minutes**. 6) Centrifuge **for 10 minutes** at **12,000 g**.	16
Solid foods with high sugar content	Dried raisins, dates, figs	1) Weigh **5 g** of sample material. 2) Add **20 mL** of 70% (vol/vol) ethanol and shake the samples for 15 minutes, e.g. in an overhead shaker. 3) Centrifuge **for 5 minutes** at **1,000 g**. 4) Dilute the supernatant **1:4** in sample buffer (e.g. 250 µL sample in 750 µL sample buffer) and shake briefly. 5) Centrifuge **for 10 minutes** at **12,000 g**.	16

Sample type	Examples	Sample preparation	Factor
Speciality goods	Herbs and spices (paprika, chilli, ginger)	1) Weigh out **5 g** of sample material. 2) Add **40 mL** of 70% (vol/vol) ethanol and shake the samples for **15 minutes**, e.g. in an overhead shaker. 3) Centrifuge **for 5 minutes** at **1,000 g**. 4) Dilute the supernatant **1:4** in sample buffer (e.g. 250 µL sample in 750 µL sample buffer) and shake briefly. 5) Add **2 spatula tips*** of SAFIA PA adsorbent per **1 mL** of diluted sample and shake **for 15 minutes**. 6) Centrifuge for 10 minutes at 12,000 g.	32
	Weigh	1) Weigh **5 g** of sample material. 2) Add **40 mL** of 70% (vol/vol) ethanol and shake the samples for **15 min**, e.g. in an overhead shaker. 3) Centrifuge **for 5 minutes** at **1,000 g**. 4) Dilute the supernatant **1:4** in sample buffer (e.g. 250 µL sample in 750 µL sample buffer) and shake briefly. 5) Add **2 spatula tips*** of SAFIA PA adsorbent per **1 mL** of diluted sample and shake **for 15 minutes**. 6) Centrifuge for 10 minutes at 12,000 g.	32
	Cannabis	1) Weigh **5 g** of sample material. 2) Add **20 mL** of 70% (vol/vol) ethanol and shake the samples for **15 minutes**, e.g. in an overhead shaker. 3) Centrifuge **for 5 minutes** at **1,000 g**. 4) Dilute the supernatant **1:4** in sample buffer (e.g. 250 µL sample in 750 µL sample buffer) and shake briefly. 5) Add **3 spatula tips*** of SAFIA PVPP adsorbent per **1 mL** of diluted sample and shake **for 15 minutes**. 6) Centrifuge for 10 minutes at 12,000 g.	16

Sample type	Examples	Sample preparation	Factor
Heavily roasted products	Malt, molasses	1) Weigh **5 g** of sample material. 2) Add **20 mL** of 70% (vol/vol) ethanol and shake the samples for **15 min**, e.g. in an overhead shaker. 3) Centrifuge **for 5 minutes** at **1,000 g**. 4) Dilute the supernatant **1:4** in sample buffer (e.g. 250 µL sample in 750 µL sample buffer) and shake briefly. 5) Add **3 spatula tips*** of SAFIA PVPP adsorbent per **1 mL** of diluted sample and shake **for 15 minutes**. 6) Centrifuge for 10 minutes at 12,000 g.	16
Products with high chlorophyll content		1) Weigh **5 g** of sample material. 2) Add **20 mL** of 70% (vol/vol) ethanol and shake the samples for **15 minutes**, e.g. in an overhead shaker. 3) Centrifuge **for 5 minutes** at **1,000 g**. 4) Dilute the supernatant **1:4** in sample buffer (e.g. 250 µL sample in 750 µL sample buffer) and shake briefly. 5) Add **2 spatula tips*** of SAFIA PA adsorbent per **1 mL** of diluted sample and shake **for 15 minutes**. 6) Centrifuge for 10 minutes at 12,000 g.	16
Products rich in anthocyanins and polyphenols	Cranberries, raspberries	1) Weigh **5 g** of sample material. 2) Add **20 mL** of 70% (vol/vol) ethanol and shake the samples for **15 min**, e.g. in an overhead shaker. 3) Centrifuge **for 5 minutes** at **1,000 g**. 4) Dilute the supernatant **1:4** in sample buffer (e.g. 250 µL sample in 750 µL sample buffer) and shake briefly. 5) Add **3 spatula tips*** of SAFIA PVPP adsorbent per **1 mL** of diluted sample and shake **for 15 minutes**. 6) Centrifuge for 10 minutes at 12,000 g.	16

Sample type	Examples	Sample preparation	Factor
Liquid foods without many colourings	Juices, beverages, white wine	1) Measure out **5 mL** of sample. 2) Add **5 mL** of 100% (vol/vol) ethanol and shake the samples briefly. 3) Centrifuge **for 5 minutes** at **1,000 g** if there are a lot of suspended solids. 4) Dilute the supernatant **1:4** in sample buffer (e.g. 250 µL sample in 750 µL sample buffer) and shake briefly. 5) Centrifuge for 10 minutes at 12,000 g.	8
Liquid foods with a high concentration of red colourings	Cherry juice, berry juice, red grape juice, red wine	1) Measure out **5 mL** of sample. 2) Add **5 mL** of 100% (vol/vol) ethanol and shake the samples briefly. 3) Centrifuge **for 5 minutes** at **1,000 g** if there are many suspended solids. 4) Add **3 spatula tips* of** SAFIA PVPP adsorbent per **1 mL** of extract and shake **for 15 minutes.** 5) Centrifuge **for 5 minutes** at **1,000 g**. 6) Dilute the supernatant **1:4** in sample buffer (e.g. 250 µL sample in 750 µL sample buffer) and shake briefly. 7) Centrifuge **for 10 minutes** at **12,000 g**.	8

* 2 spatula tips of PA or 3 spatula tips of PVPP are approx. 15-25 mg. Decolourisation was tested in the range between 10 and 50 mg/mL PA/PVPP, and no influence of the amount of adsorbent on the analysis results was found.

Further protocols for specific matrices and applications, as well as updates to existing protocols, are available at www.safia.tech. Please check regularly for new updates.

7 Performing the SAFIA assay

Figure 18 shows a schematic representation of the individual steps involved in performing the SAFIA assay.

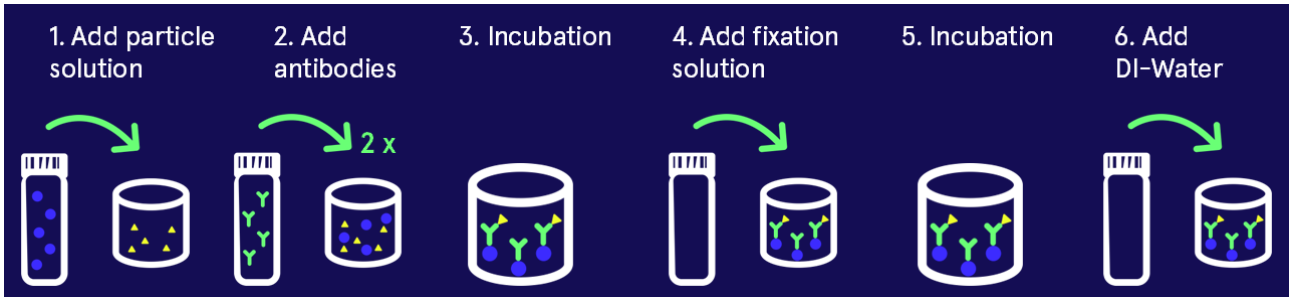


Figure 18. Performing the SAFIA assay

7.1 Preparation

1. If you have not already done so, plan your plate layout (assignment of samples and standards to the wells of the microtitre plate). This can be done using the SAFIA Score software (see section 5).
2. Bring all kit reagents to room temperature.
3. Shake the fixative solution before use, as storage at 2–8 °C may cause a component to precipitate, which will dissolve completely again at room temperature.
4. Prepare the required amount of particle working solution fresh:
   - Shake the particle stock solution vigorously for at least 20 seconds.
   - Dilute in particle buffer at a ratio of 1:33 (see Table 6).

Table 6. Pipetting scheme for 1, 3, 6, 9 and 12 strips of an MTP

	1 strip (8 wells)	**3 strips (24 wells)**	**6 strips (48 wells)**	**9 strips (72 wells)**	**1 whole MTP (96 wells)**
Particle stock solution	3.3	10 µl	20 µl	30 µl	45 µl (all)
Particle buffer	106.7 µl	320 µl	640 µl	960 µl	1,500 µl (all)
Total volume	110 µl	330 µl	660 µl	990 µl	1,545 µl

   - Shake the dilution well for at least **20 seconds**

The finished particle working solution must be used on the day of measurement and cannot be stored for longer!

All other reagents in the kit are ready for use.

7.2 Performing the assay

1. Add **25 µl** of diluted sample/standard to each well of the microtiter plate according to the selected plate layout.

2. Then add the following in succession:
   - **10 µL** particle working solution, shake well again before adding (at least **20 seconds**)
   - **25 µL** primary antibody (AK 1)
   - **50 µL** secondary antibody (AK 2).
3. Incubate for **20 minutes** at room temperature with shaking.
4. Add **50 µL** of fixation solution and incubate for **5 minutes**.
5. Add **140** µL DI water.

The reagents should be added using a multichannel pipette. If you are using a multichannel pipette with a dispensing function, you can use the following pipetting scheme:
Example
- You have filled 48 wells with standard/sample, in wells A1 to F8 (the first 6 columns)→ This means you have 6 strips with 8 samples each
- Use a multi-channel reservoir and pipette the volume required for one strip (see Table 7) 6 times in a row.
- Set the multi-channel pipette to the volume for 1 well (e.g. 10 µl for the particle solution) and 8 dispensing steps
- Now load 6 channels of the pipette with pipette tips and use each channel to draw up the prepared volume from the multi-channel reservoir.
- Now dispense the volume 8 times into the wells of rows 1-6.
- Work from bottom to top (H to A) to minimise errors due to cross-contamination.

Table 7. Required volumes of assay reagents for 1 well or one strip

Reagent	**1 wave**	**1 strip**
Particle solution	10 µl	110 µl
AK 1	25 µl	350 µl
AK 2	50 µl	700 µl
Fixation solution	50 µl	700 µl
DI water	140 µl	1,400 µl

A new pipette tip must be used for each different reagent/standard.

When pipetting, there should be no long intervals between the individual steps & pipetting should be carried out continuously so that there is no delay between the individual strips.

When adding the reagents to the individual wells, please note that the pipette tips must not touch the liquid if the same pipette tip is used for several wells!

6. The measurement in the flow cytometer can now be started, see section 7.3.

7.3 Readout with the Cyflow® Cube 6 flow cytometer

1. If you have not yet performed a performance check on the day of measurement, first follow the steps in section 4.
2. Pull out the movable tray of the CyFlow® Robby autoloading station and place the microtitre plate on the designated platform. Ensure that the plate is oriented correctly. Well A1 must be at the top right.
3. Click on *Work* Work in the main bar.
4. Under *Settings* → *Measure,* check that the parameters are set as shown in Figure 19.

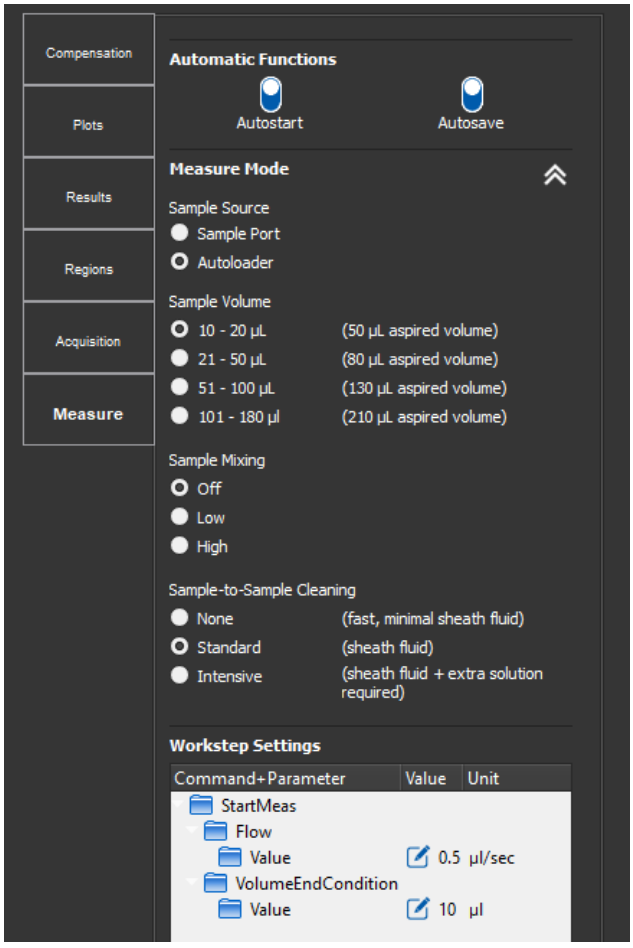


Figure 19. Cube 6 settings for measurement

5. Click on *Start* to start the measurement.

6. A window will now open (see Figure 20). Here you have the option of assigning a *tray ID* (batch number) (e.g. the analysis ID). Only use alphanumeric characters (A-Z, a-z, 0-9) and the underscore "_" for the tray ID (e.g. 20250213_TESTBATCH), as other characters and special characters are not recognised and will be deleted when the folder is created.

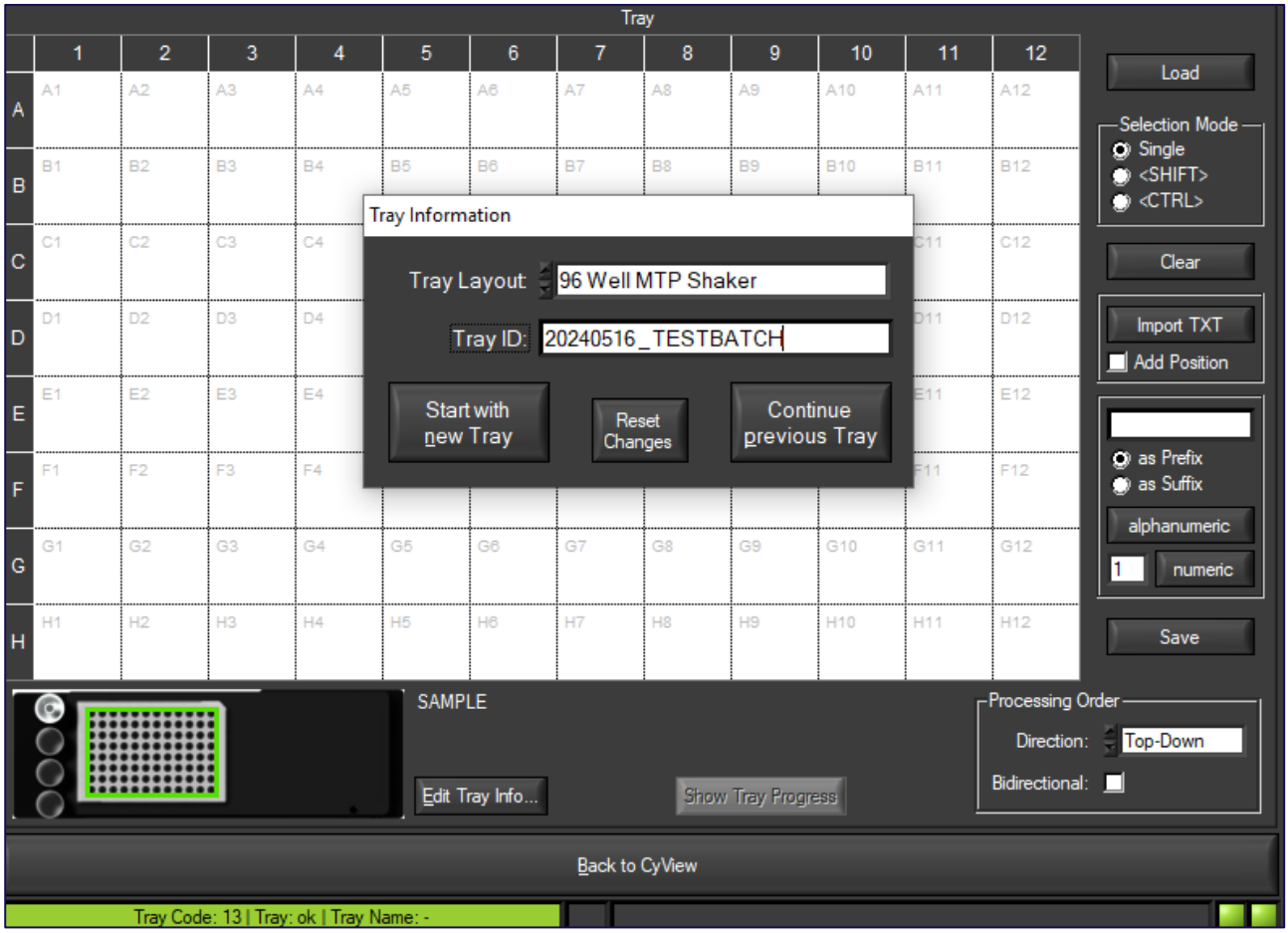


Figure 20. Selecting the disc layout

7. Click *Start with a new Tray* to create a new batch.

8. Now select the wells to be measured. This can be done individually or by dragging across the fields. Selected wells appear light blue (see Figure 21).

9. In order to evaluate the data using SAFIA Score, it is essential to enter the well ID. Always use the setting *as Prefix* for this. Press the *alphanumeric* button and the well ID will appear in the corresponding cavity. The well ID has been accepted when the values in the centre of the cavities are displayed in black.

10. Optionally, a sample name can be assigned to each sample. This is entered in the white field (see Figure 21, marked in red). Press the *alphanumeric* button to accept the sample name and display it in black in the corresponding cavity.

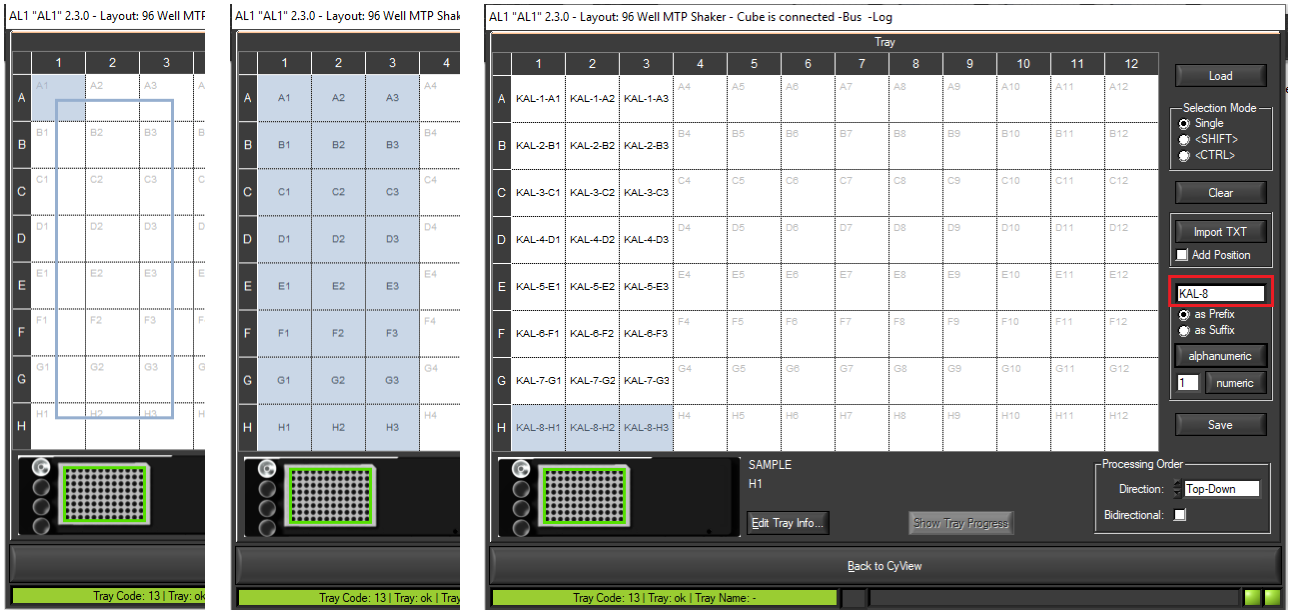


Figure 21. The menu for microtiter plate allocation

11. Now click on the *Back to CyView* button. This will take you to *CyView*, where you can start the measurement by *clicking Continue* ▶▶ .

12. Before and during the measurement, ensure that there is sufficient *sheath fluid*. Refill the bottle if a warning appears.

13. The measurement is now fully automatic. However, you should monitor the first 5 measurements to see whether, for example, air bubbles in the system are affecting the measurements.

If the measurement is successful, you will see a population appearing in the gate particles in the FSC/SSC scatter plot. Similarly, up to 7 populations form in the FSC/FL-3 scatter plot, see Figure 22. These do not have to be in the correct gates for the measurement; this can be corrected later in the evaluation if necessary. Depending on the kit used, different populations representing different mycotoxins can be seen in this plot.

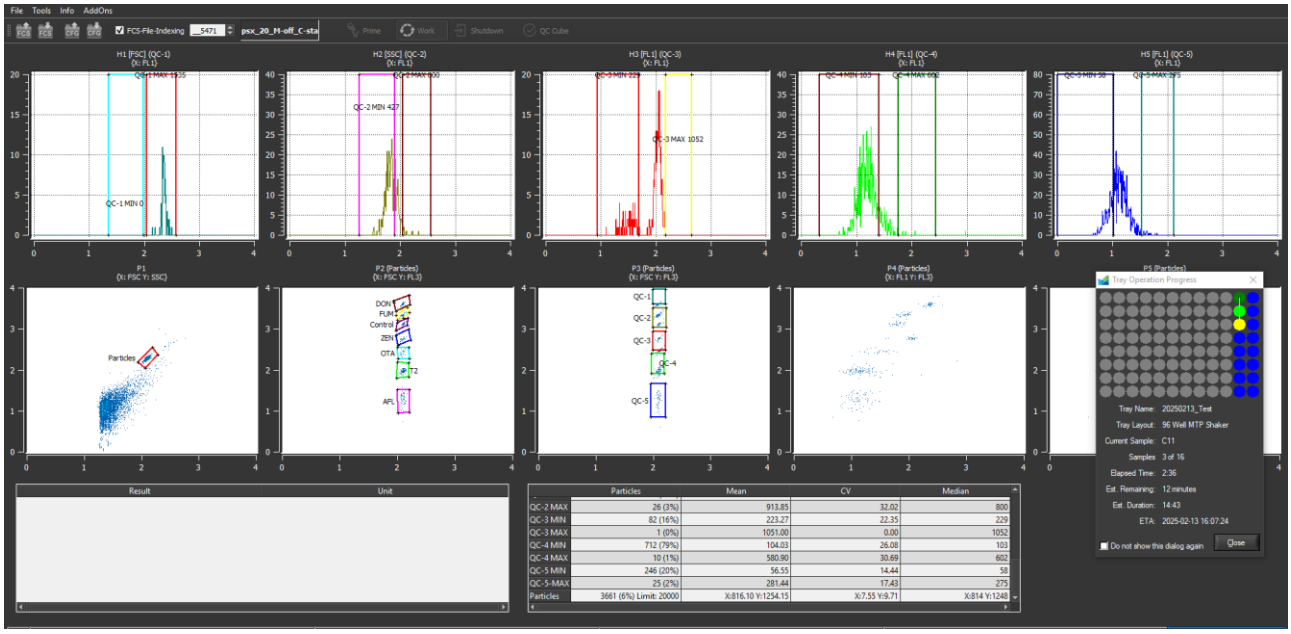


Figure 22. Typical view when measuring a SAFIA assay. Top: Histogram for the intensity in FL-1 of an analyte. Bottom: Scatter plot FSC/SSC and scatter plot FSC/FL-3

14. Optional after measurement: Perform a manual cleaning cycle, see section 10.1.

15. Once the measurement is complete, *FCS Express* opens automatically and displays the measured data (see Figure 23). If the correct data is not loaded automatically, follow the instructions in section 10.2.

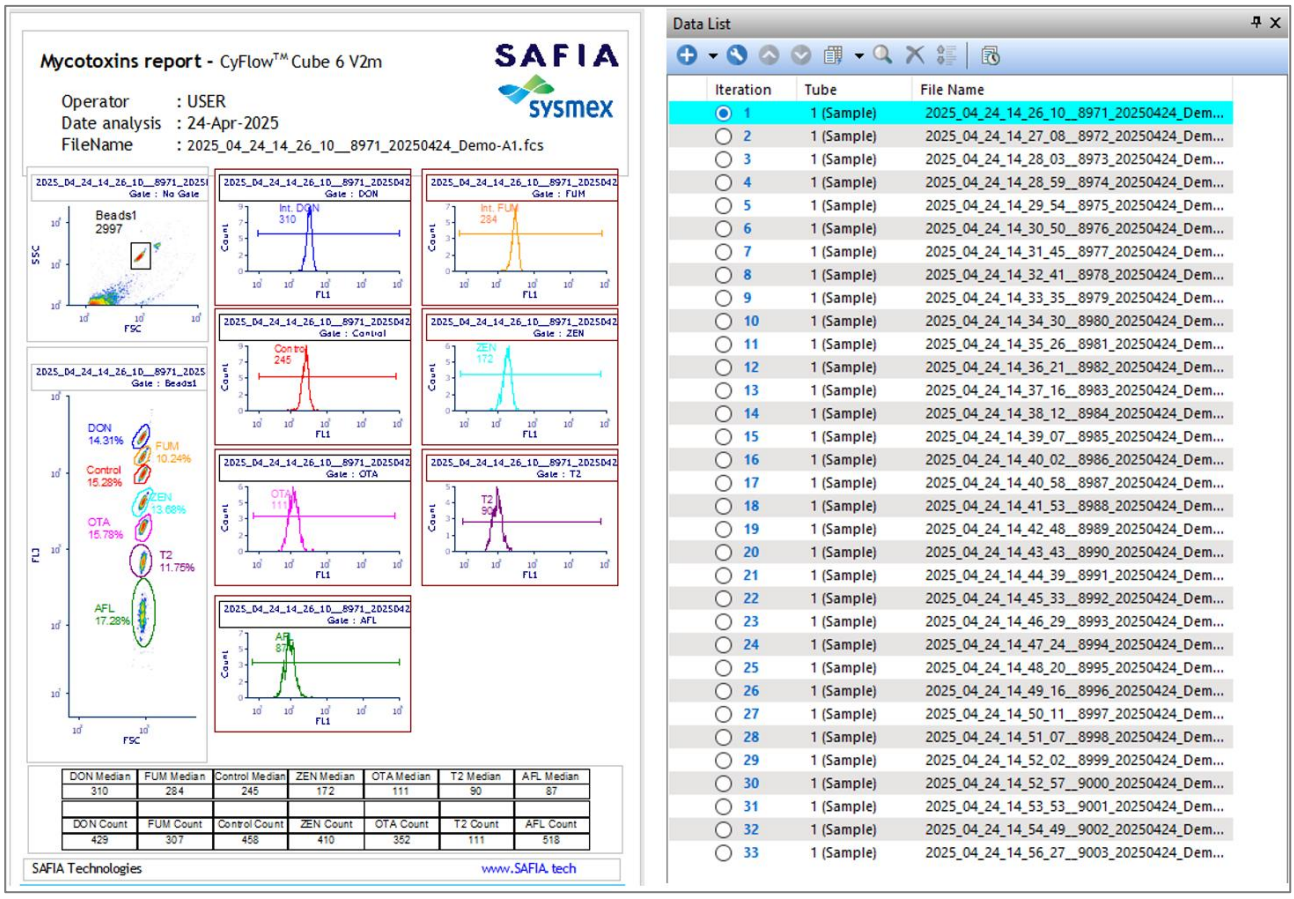


Figure 23. Display of .fcs data in FCS Express

16. In the Data List on the right-hand side, you will see that the first measurement is selected. The data from this measurement is displayed on the left-hand side. Check whether the populations are within the gates. If a population is not within the gate, it must be adjusted. To do this, click on the corresponding gate and move or enlarge/reduce it.

17. To perform this check for all measurements and convert the data, click *Run* in the *Batch & Export* tab. Before starting, make sure that the first .fcs file is selected (blue dot), as the iteration of the batch export always starts from the selected file.

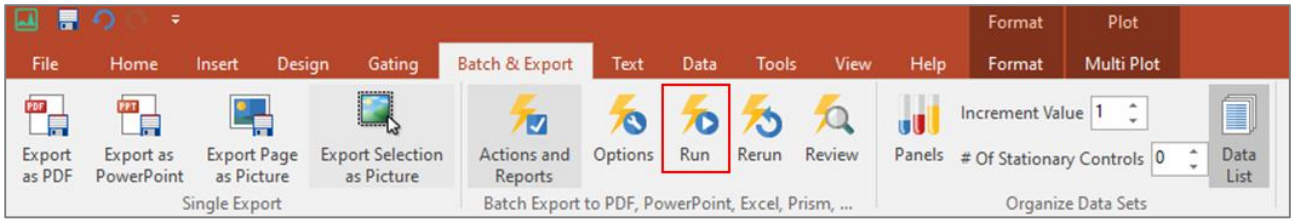


Figure 24. View of the Batch & Export tab in FCS Express and location of Run

18. Now check the gates of the second measurement and click *Continue* in the window that opens (see Figure 25) to proceed to the next measurement. If *Keep the changes* is selected, the adjustments will be applied to all measurements. If you do not want an adjustment to be applied to all measurements, select *Restore*

the state of the layout before the next iteration. With *Run to End*, you can jump to the last measurement, whereby the given gates are applied to all measurements and the conversion is performed.

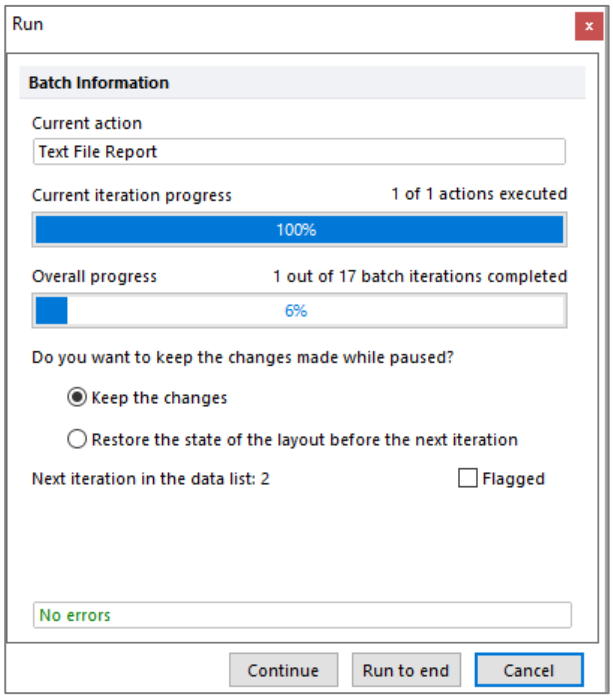


Figure 25. Iteration of measurements to convert .fcs files to .csv files

19. Once all measurements have been checked, the window closes and a .csv file containing the collected measurements is automatically created.

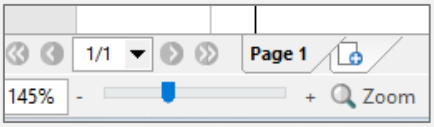
20. Close *FSC Express* without saving and continue with the evaluation in *SAFIA Score*.

Do not save the layout, as this will overwrite the template. If you want to save the individual layout, select the *Save As* option.

It is completely normal for the particle populations not always to lie completely within the gate, as the measurement is subject to daily fluctuations and can vary slightly depending on the batch.

Please note that the order of the gates and the names of the gates and regions must not be renamed. Swapping gates will result in incorrect decoding of the particles and thus incorrect evaluation! If you have any questions, please contact SAFIA Technologies Support .

If necessary, the plots can be enlarged using *the zoom function*.



7.4 Evaluation of the assay using SAFIA Score

1. If you have not yet planned your assay in SAFIA Score, follow the instructions in section 5.

2. In the *Plate Layout Cube6 V2m FCS Express* workspace, select *Import File* and then select the previously generated .csv files (see Figure 26).

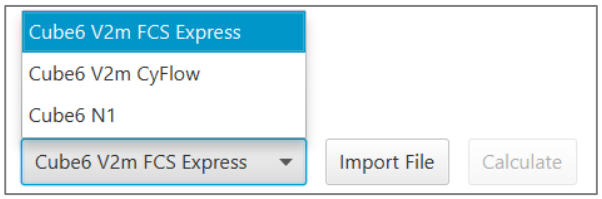


Figure 26. Excerpt from the *Plate Layout* workspace

3. Then click on the *Calculate* button. Sigmoid calibration curves are now created based on the plate layout and the measurement data of the calibration standards. These curves are used to automatically calculate the contents of the samples and reference samples (see Figure 27).

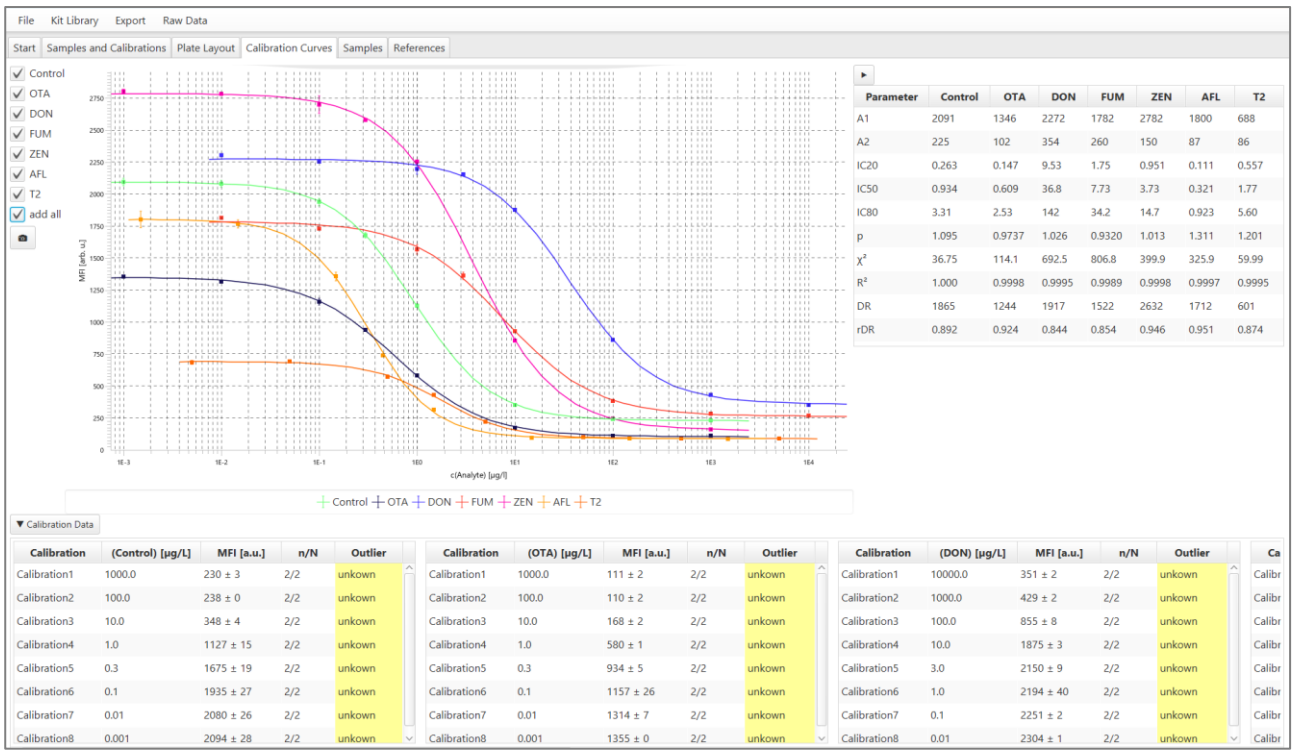


Figure 27. Overview of the functions in the *Calibration* workspace

4. The curve parameters calculated by the fit are displayed in the top right-hand corner. You can use these to assess whether the calibration of the assay was successful. A valid calibration curve should have the following parameters:

   - *Relative dynamic range* (rDR): at least 0.70, optimally > 0.80
   - Coefficient of determination (R^2): at least 0.990
   - Slope parameter of the curve at the test centre (p): approximately 0.5–2.0, optimally around 1
   - IC parameter

IC parameter	Control	OTA	DON	FUM	ZEN	AFL	T2
IC50 min [µg/L]	0.25	0.18	4.35	3.0	1.10	0.0650	0.360
IC50 Max [µg/L]	2.40	1.28	90.0	26.0	7.00	0.600	9.20

If a curve does not meet the reference values for these parameters, please refer to the information in section 10.7.

At the bottom of the tab, you will find a table summarising the results of the individual standards, sorted by analyte.

The calibration curve is calculated based on the mean fluorescence intensity (MFI, in a. u.) of the replicate measurement of each standard.

These and the corresponding standard deviation are shown in the table. If at least 3 replicates have been measured, a Grubbs outlier test (significance level α = 0.95) is automatically performed to identify any outlier measurements. Double-clicking on the individual rows of the table opens a detailed view (see Figure 28). The *Exclude Measurement* and *Exclude All Outliers* buttons can be used to exclude measurements either individually or based on the Grubbs test. This is taken into account in the calculation and saved. Previously excluded measurements can be re-included using Include Measurement. Click *Apply* to apply, or *Close* to discard.

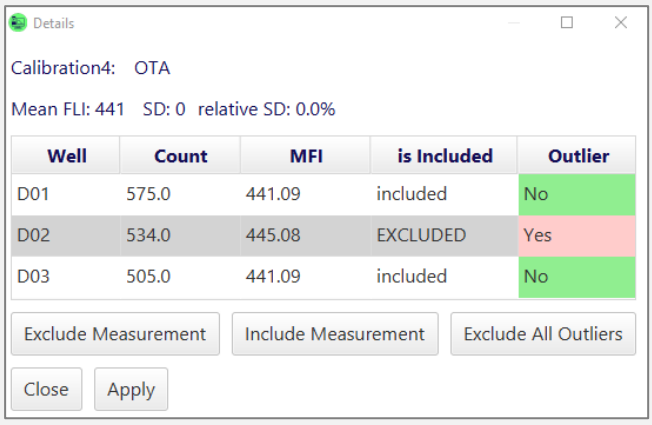


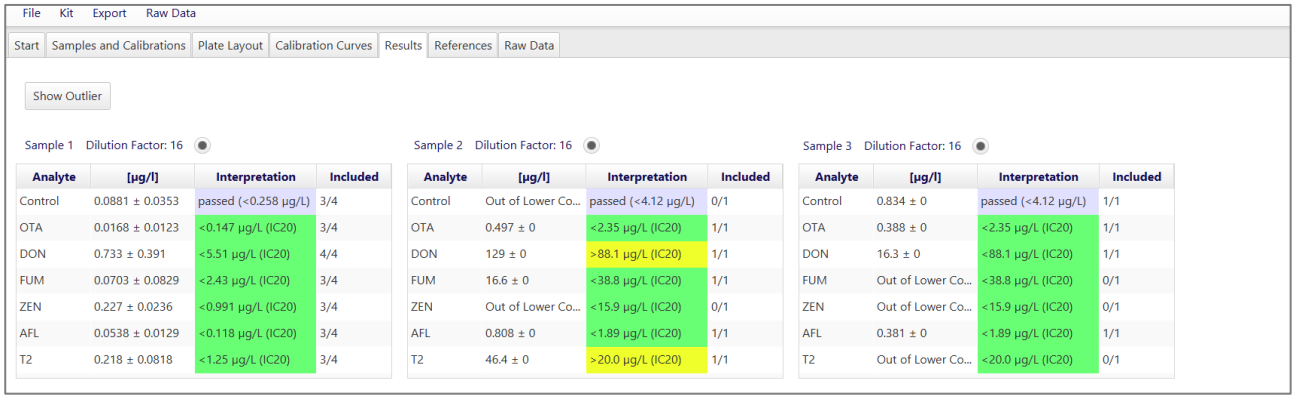
Figure 28. Detailed display of a single measurement in the *Calibration Curves* and *Results* workspaces. This can be opened by double-clicking on a table entry. The buttons can be used to include or exclude measurements from the calculation. In the *Outlier* field, you can see the results of the Grubbs outlier test

Please note that the Grubbs test is only intended as an aid in finding extreme measurement outliers and can generate unreliable results, especially with small sample sizes. For example, if two of the values in three replicate measurements match completely, the third value marked as an "outlier" should not be excluded.

In the *Calibration Curves* workspace, you can display the various calibration curves in the top left-hand corner and export them as a graphic using the screenshot function.

5. The *Results* and *References* work areas list the results of the individual samples and reference samples (see Figure 29).

6. You can switch between the interpretation view and the outlier view using the *Show Outlier* or *Show Interpretations* button. The outlier view is designed similarly to the outlier view in *Calibration Curves* and shows you the results of a Grubbs outlier test.



Analyte	[µg/l]	Interpretation	Included
Control	0.0881 ± 0.0353	passed (<0.258 µg/L)	3/4
OTA	0.0168 ± 0.0123	<0.147 µg/L (IC20)	3/4
DON	0.733 ± 0.391	<5.51 µg/L (IC20)	4/4
FUM	0.0703 ± 0.0829	<2.43 µg/L (IC20)	3/4
ZEN	0.227 ± 0.0236	<0.991 µg/L (IC20)	3/4
AFL	0.0538 ± 0.0129	<0.118 µg/L (IC20)	3/4
T2	0.218 ± 0.0818	<1.25 µg/L (IC20)	3/4

Analyte	[µg/l]	Interpretation	Included
Control	Out of Lower Co...	passed (<4.12 µg/L)	0/1
OTA	0.497 ± 0	<2.35 µg/L (IC20)	1/1
DON	129 ± 0	>88.1 µg/L (IC20)	1/1
FUM	16.6 ± 0	<38.8 µg/L (IC20)	1/1
ZEN	Out of Lower Co...	<15.9 µg/L (IC20)	0/1
AFL	0.808 ± 0	<1.89 µg/L (IC20)	1/1
T2	46.4 ± 0	>20.0 µg/L (IC20)	1/1

Analyte	[µg/l]	Interpretation	Included
Control	0.834 ± 0	passed (<4.12 µg/L)	1/1
OTA	0.388 ± 0	<2.35 µg/L (IC20)	1/1
DON	16.3 ± 0	<88.1 µg/L (IC20)	1/1
FUM	Out of Lower Co...	<38.8 µg/L (IC20)	0/1
ZEN	Out of Lower Co...	<15.9 µg/L (IC20)	0/1
AFL	0.381 ± 0	<1.89 µg/L (IC20)	1/1
T2	Out of Lower Co...	<20.0 µg/L (IC20)	0/1

Figure 29. Excerpt from the *Results* workspace with an overview of the results for the individual samples. This is identical to the view in the *References* workspace

The interpretation view provides you with a visual aid to show whether the control measurement has been passed (display: passed/failed) or whether the measured value for a toxin is above or below the IC-20 value. The IC-20 value can be regarded as an approximate limit of quantification for a non-validated matrix. **Please note that this is only an interpretation aid. The interpretation of whether a mycotoxin is above or below the detection or limit of quantification depends on the matrix tested and must be made by you based on the kit data or your own validation data for matrices not listed.**

The dilution factor for the calculation can be included or excluded with a click.

Similar to the tables in the *Calibration Curves* workspace, you can access a detailed view by double-clicking. Here, individual measurements can be included or excluded from the calculation. If the measured MFI is greater than the A1 value of the calibration curve, the value is displayed as *Out of Lower Concentration Range*, as quantification of the samples is mathematically impossible in this case. This means that the concentration of the analyte was lower than the assay's limit of quantification. The values are automatically excluded from the calculation. The same applies to values that are smaller than the A2 value of the calibration curve. A value below the A2 value means that the concentration of the analyte is very high. Results in this range should be questioned particularly critically; the probability of a measurement error is high. Also refer to the control measurement for interpretation and, if necessary, measure the sample again after strong dilution.

You can export the tables as a graphic using the *Screenshot Results Tables* button.

The concentrations determined are displayed in the lower area in the form of a bar chart, sorted by analyte. The chart can also be exported using the *Screenshot Results Graphs* button. In addition, it is possible to include the dilution factor in each graph and to display reference lines.

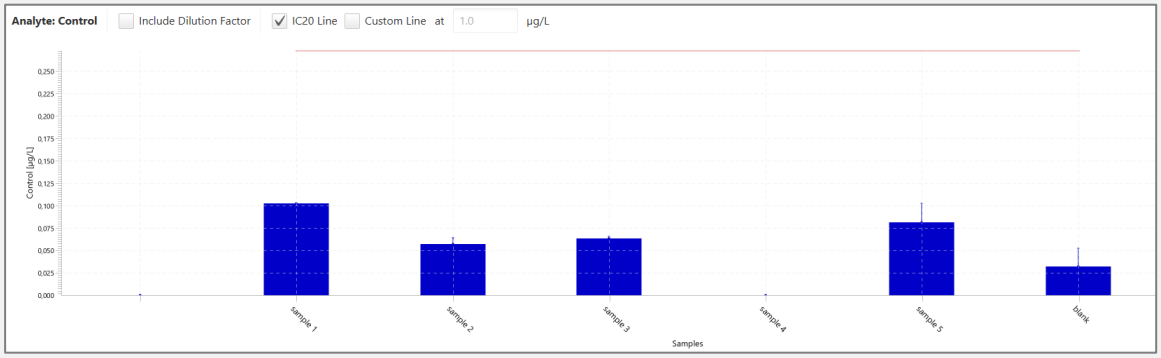


Figure 30. Excerpt from the *Results* workspace with the results of the individual samples as a bar chart. This is identical to the view in the *References* workspace

The analyte control is the control measurement. The control indicates whether a measurement result could be a false positive. The control is considered passed if the determined value of a sample is less than the IC20 value of the corresponding control calibration curve. This can be easily checked graphically. To do this, the *Include Dilution Factor* field must be deactivated and the *Set IC20 Reference Line* field must be activated (see Figure 30).

7. A table of the measured data can be displayed in the *Raw Data* menu.

This can be helpful when evaluating individual measurements. For example, if no or very few particles were detected, this can be seen in the *Count* column. The *Raw Data* workspace is only active if the *Show Raw Data* option has been activated in the *Raw Data* menu. This workspace can also be closed again if necessary.

8. Furthermore, the calculated values can be exported to a Microsoft® Excel® file (.xlsx). To do this, click on *Excel Export* in the *Export* menu and save the file. In the Microsoft® Excel® file, you will find the summarised results of the measurements of the samples and reference samples, as well as the parameters of the individual calibration curves, in the corresponding sheets.

8 Cleaning and shutting down the Cube 6

To ensure that the Cube 6 functions properly, we recommend cleaning the cytometer after completing the SAFIA measurements. This is only possible in MTP format. For the tube format, simply use the *Intermediate Cleaning function*.

1. Fill one strip at a time (8 cavities row A→ H) of a microtiter plate with 4 wells *of hypochlorite solution (1st column A→ D)*, 4 wells *of decontamination solution (1st column E→ H)*, 4 wells *of cleaning solution (2nd column A→ D)* and 4 wells *of sheath fluid (2nd column E→ H)*.

2. Pull out the movable tray of the CyFlow® Robby Autoloading Station and place the microtitre plate on the designated platform. Please note the correct orientation of the plate (well A1 at the top right).

3. Open the *configuration file* named *Cleaning-MTP*. To do this, click on the CFG button.

4. Now click on *Work* Work in the main bar.

5. Check that the following settings are configured under *Settings* → *Measure* (see Figure 31).

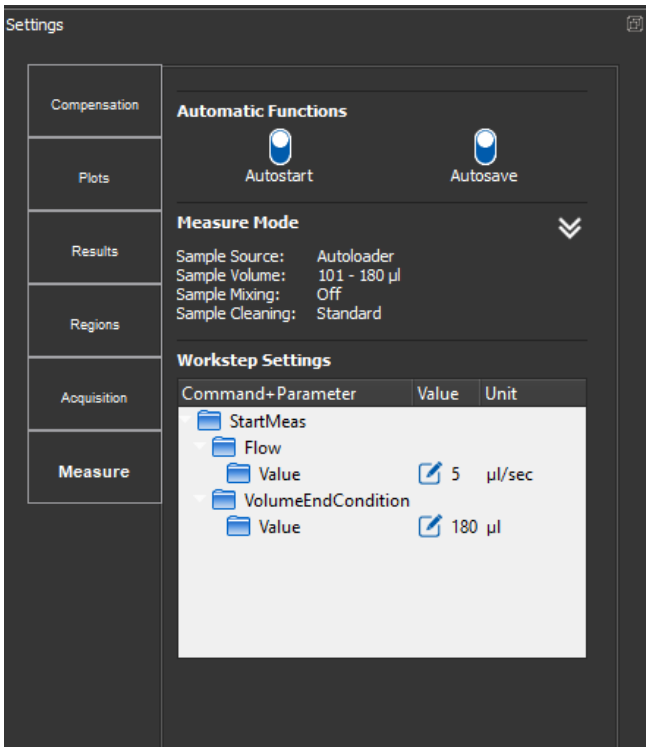


Figure 31. Settings for running the SAFIA cleaning programme (Cleaning-MTP)

6. Perform the measurement as described in the section 7.3.

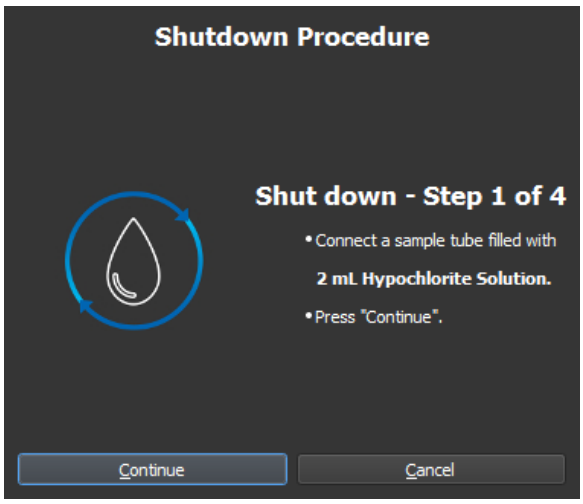
The *.fcs files* created by the cleaning programme do not contain any useful data, but are nevertheless automatically saved in the folder C:\Users\Public\Documents\Cyflow. You can delete these files for a better overview.

7. Now perform the shutdown by clicking *Shutdown* Shutdown .

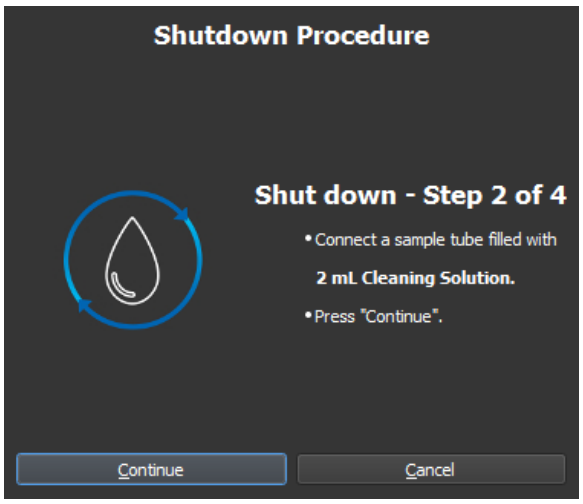
8. Start the programme by clicking *Start* ▶.

9. Follow the instructions in the shutdown programme, see Figure 32. Once the device has shut down, the CyView™ software closes automatically.

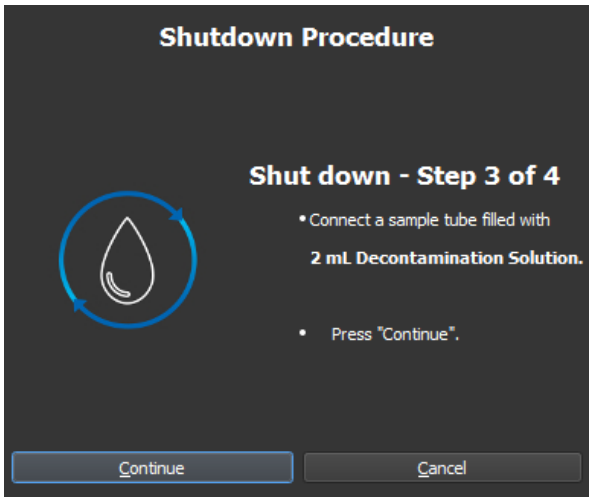
Step 1



Step 2



Step 3



Step 4

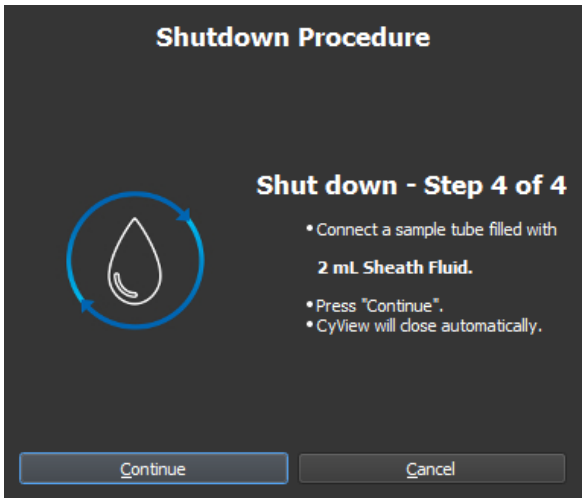


Figure 32. Figure 1. Steps of the Prime programme The software will shut down automatically once the process is complete. You can now shut down the PC

9 Notes for special matrices

Matrix	Note
Soy	High proportions of soy result in excessive OTA levels. Remedy: Soy products should be diluted a little more.
Cinnamon	Cinnamon cannot be analysed with SAFIA.
Coffee	Coffee cannot be analysed with SAFIA.
Mango	If the mango fruit content is approx. 30% or higher, an error may occur in the internal control. The internal control cannot be used for samples containing mango. The mycotoxin results are not affected by this.

10 Common errors and troubleshooting

10.1 Manual cleaning cycle

After completing a measurement, perform a cleaning cycle to completely remove residues from the flow cytometer and prevent carryover. This practice will help to prolong the life of your device.

> ! Please note that liquid is absorbed immediately when the red *intermediate cleaning button*  is pressed. Therefore, place the tube with the appropriate solution in the *sample port* before clicking the button.

1. To do this, fill a tube with approx. 2 ml of *cleaning solution* and connect it to the sample port.
2. Then click on the red *intermediate cleaning* button  .
3. Fill a tube with approx. 2 ml of *sheath fluid* and connect it to the *sample port*.
4. Click the *Intermediate Cleaning button* again  .

10.2 No particles are measured during the performance check or measurement

If no particles are detected, stop the measurement by clicking the *Stop* button  . Start a cleaning cycle by clicking the *Clean* button (see section 10.1) or run the Prime programme again (see section 3).

10.3 Particle populations are not in gates

The particle populations appear "elongated" and not in the gate (data looks "strange"): This can happen if an air bubble is stuck in the measuring cuvette or there is a problem with the device. In such cases, a cleaning cycle should first be performed and then the Prime programme restarted. If the problem persists, contact Sysmex Customer Service.

The *gain settings* and *thresholds* of the detectors were already set during installation of the system and must not be changed!

10.4 FCS Express does not open automatically with the correct data after the measurement is complete

If *FCS Express* does not open automatically with the data after your measurement, you can import it manually. To prevent this error in future, make sure that *FCS Express* is closed in CyView before starting the measurement.

1. Open *FCS Express* and click on *Open Layout*.

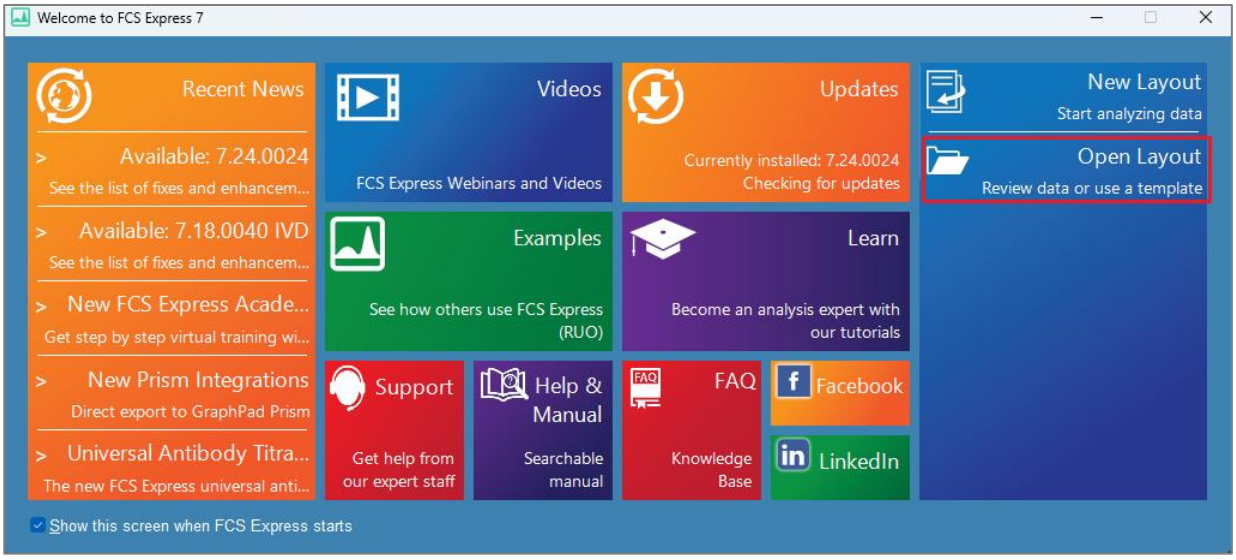


Figure2View of the *FCS Express* start page

2. Select the layout named Calc_Mycotoxins-SCR_A, located in C:\ProgramData\PartecGmbH\Cube_18\templates\Quality Control

   If you have already taken measurements, the most recently used layouts will appear below *Open Layout*, from which you can select the one you want.

3. A window will open with the empty layout on the left and the empty *Data List* on the right. You can *drag and* drop your .fcs files here. Your files are located in C:\ProgramData\PartecGmbH\Cube_18\data\cyflow\ in a folder with the name of the tray ID you assigned.

4. Now follow the instructions in section 7.3. If an error message appears when you click *Run*, follow the instructions in section 10.5.

10.5 FCS Express error message

1. If the error message (see Figure 33) appears when you click *Run,* click *Cancel* and perform the following steps.

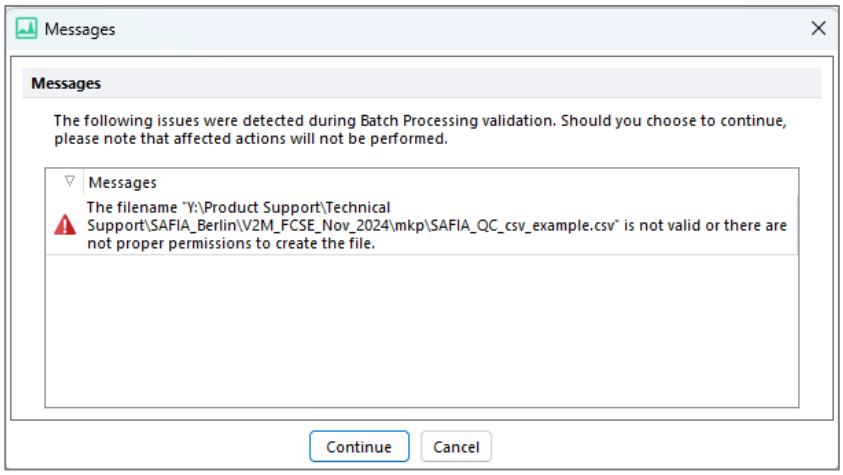


Figure 33. View of the error message in *FCS Express* when converting the .fcs file to a .csv file fails

2. Under Batch & Export, click Actions and Reports.

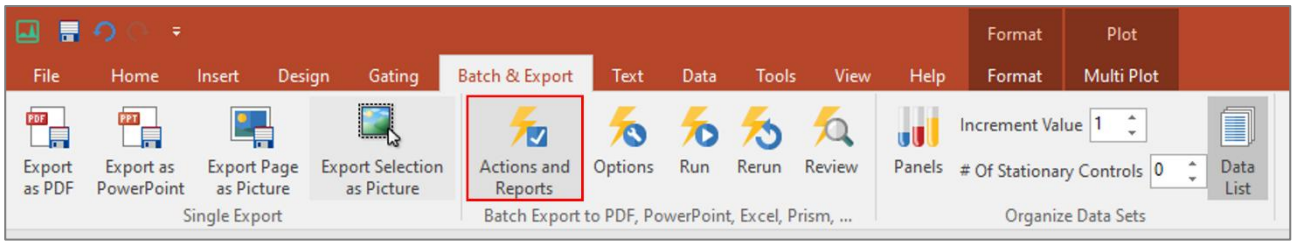


Figure 34. View of the *Batch & Export* tab in *FCS Express* and location of *Actions and Reports*

3. This window will then open:

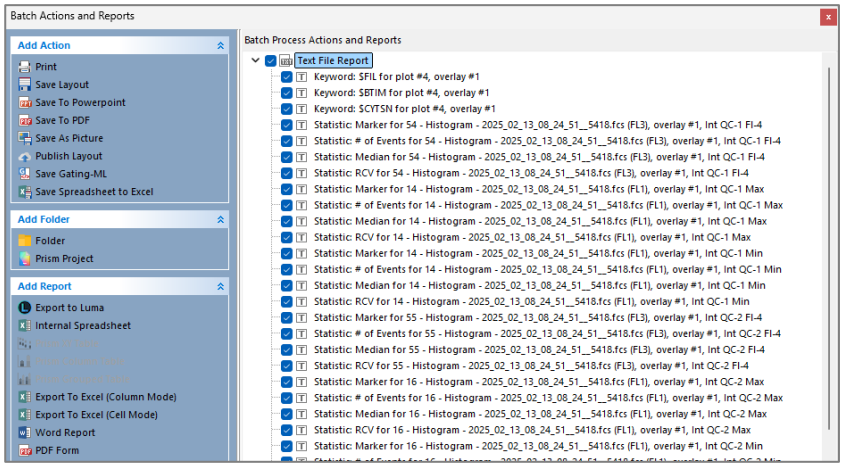


Figure 35. View of the *Batch Actions and Reports* window

4. Double-click on *Text File Report.*

5. A new window will open in the *File Options* tab:

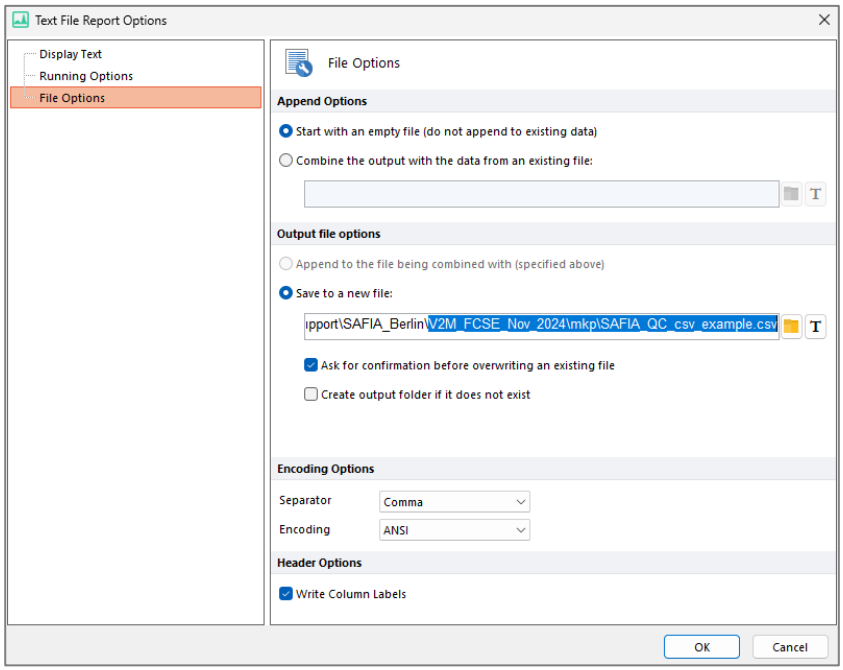


Figure 36. View of the *Text File Report Options* window

6. Check that *Start with an empty file []* is selected under *Append Options* and that *Comma* and *ANSI* are selected under *Encoding Options*.

7. Under Output file Options, select the storage location for Save to a new file by clicking on the folder icon. The storage location is in the SAFIA Check or SAFIA Mycotoxins folder→ Exportfiles. Enter the file name here.

8. Now click on the T (to the right of the folder icon) to open the *Insert a Token* window:

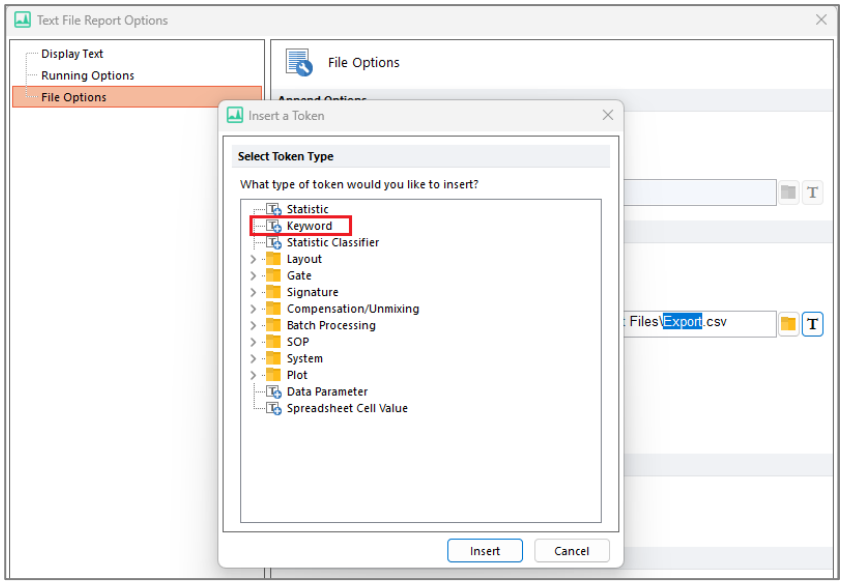


Figure 37. View of the *Insert a Token* window

9. Double-click *Keyword* to open the *Create Keyword* window. Select the *Keyword* tab and click on the three dots.

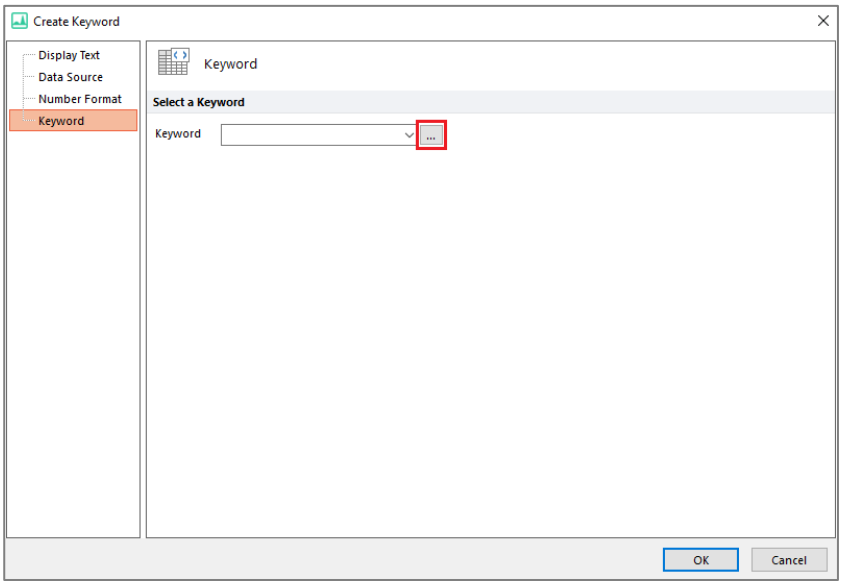


Figure 38. View of the *Create Keyword* window

10. The *Keyword List* window will now open. Select the keyword $FIL and confirm with OK. The Batch Actions and Reports window can now be closed.

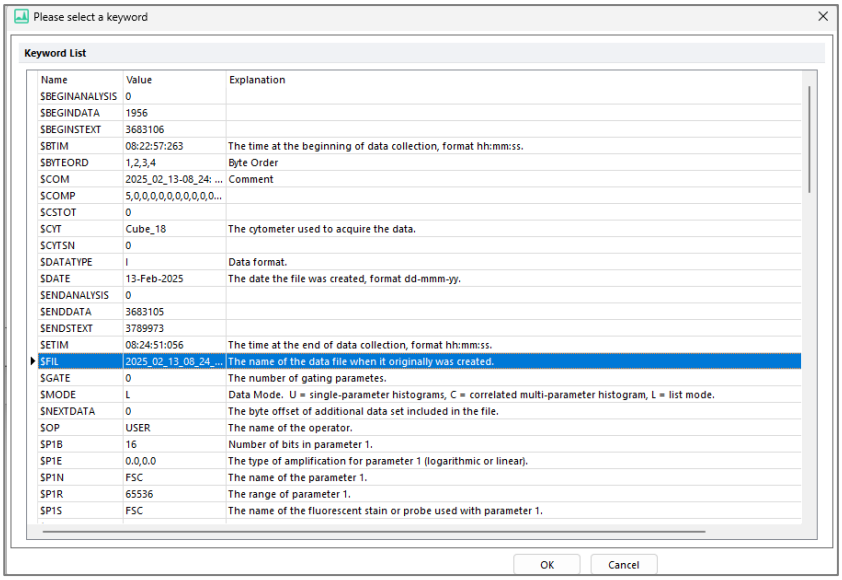


Figure 39. View of the *Please select a keyword* window

11. Click *Run* and follow the steps in section 7.3. Save the layout via File Save As with the name Calc_Mycotoxins-SCR_A.

10.6 Control is positive

If the control is positive, a substance in the matrix is interfering with the assay. The remaining results may therefore be incorrect. To remedy this, perform a purification step with PA or PVPP adsorbent to remove the interfering substances, see section 6. Also note the instructions for special matrices.

10.7 Calibration curve does not meet the reference values

10.7.1 Relative dynamic range, IC50, p-values

If the calibration curve is too flat, the relative dynamic range and/or other parameters of the calibration curve, such as the IC50 values and the slope parameter of the curve at the test centre (*p*), do not correspond to the reference values (see section 7.4). The samples cannot be quantified correctly. Check the best-before date of the kit and always ensure that the kit is stored correctly (see section 2.3). Repeat the measurement with a new kit that is still within its shelf life. Contact SAFIA Technologies Support at SAFIA Technologies Support if the curves do not meet the reference values for a kit within its shelf life.

10.7.2 R Square <0.990

If the coefficient of determination (R^2/R Square) is less than 0.990, check the individual measured values of the calibration curve in SAFIA Score. If at least 3 replicates have been measured, a Grubbs outlier test (significance level $\alpha = 0.95$) is automatically performed, which indicates possible outlier measurements. If fewer than 3 replicates were measured, you can identify obvious outliers by looking at the curve or by checking for a large standard deviation (in duplicates). Outliers can be easily excluded (see Figure 28), which increases the coefficient of determination. When pipetting the calibration standards, ensure clean pipetting so that the coefficient of determination is greater than 0.990.

10.8 SAFIA Score

If you do not get any results after loading your data into SAFIA Score, there may be several reasons for this. The following are common errors:

- You have not entered any values for one or more mycotoxins in a reference material→ Enter the values you know and 0.0 for unknown concentrations.
- The plate layout in SAFIA Score does not match the actual layout of your measurement plate→ Adjust the layout in SAFIA Score to match your actual layout and upload your measurement data again.

Contact SAFIA Technologies Support if you are unable to resolve your error.

11 Appendix

11.1 Checklist

Equipment	Specification	Example
Area		
Workstation	approx. 2 x 1 metre	
Equipment		
Centrifuge	15/50 mL: 1,000 g 2 mL: 12,000 g	
(Microtiter) plate shaker/orbital shaker		Titramax 101
Rotator/ overhead shaker		uniLOOPMIX 2
Analytical balance		Ohaus Pioneer PC
Mill for grinding sample material		A11 basic analytical mill (IKA)
Single channel pipette	10-100 µL	Research plus, 10-100 µL (Eppendorf)
	100-1000 µL	Research plus, 100-1000 µL (Eppendorf)
	1000-10000 µL	Research plus, 1000-10000 µL (Eppendorf)
8-channel pipette, manual		Research plus, 10-100 µL (Eppendorf)
Alternative to manual pipette / optimal		
8-channel pipette with dispensing function	5-100 µL	Xplorer 8-channel pipette 5-100 µL (Eppendorf)
Optional		
8-channel pipette with dispensing function	15-300 µL	Xplorer 8-channel pipette 15-300 µL (Eppendorf)
	50-1200 µL	Xplorer 8-channel pipette 50-1200 µL (Eppendorf)

Stand for centrifuge tubes	for 50 mL, 15 mL and 2 mL tubes	
PC screen		
Cutter knife		
Table waste bin		
Trays for multi-channel	1 chamber	
	8 or 12-chamber	Reagent reservoirs Trifill multi-channel (Carl ROTH)
Measuring cylinder	150 mL, 500 mL	
Laboratory bottle	250 mL, 500 mL	
Consumables		
Pipette tips for the respective pipettes		
Centrifuge tubes	2 mL	
	15 mL	
	50 mL	
96-well plate, flat bottom		
Table waste bags		

11.2 Glossary

Term	Explanation
$FIL	FCS key, displays the file name (sequential run number and date of measurement).
$WELLID	FCS key, returns the alphanumeric designation of a microtitre plate well.
.csv file	File exported from the *FCS Express* software that can be imported into SAFIA Score. This is a file in ASCII format. CSV stands for comma-*separated values*
.fcs file	A data format from flow cytometry. FCS stands for Flow Cytometry Standard (File). An .fcs file is a raw measurement data file generated by Cyflow® software. It is standardised and can be loaded into FCS Express, for example, to convert it into a .csv file.
.sdf file	*SAFIA Data File*. This is the storage file for all information generated in SAFIA Score, e.g. plate layouts, calibrations and measurement results. It can be opened in SAFIA Score.
Analysis ID	A unique analysis number assigned by the user. It should be assigned once for each microtitre plate measured.
Grubbs' outlier test	A statistical test used to detect and eliminate outliers in a given sample and to improve the remaining sample through iteration. It is implemented in SAFIA Score for calibration and results.
Batch	A batch refers to the collected measurements of a plate from a measurement run. Each measurement belongs to a batch.
Clean programme	Cleaning cycle of the Cube 6
Configuration file	A file containing specific settings for reading the assay with the Cyflow® Cube 6 flow cytometer.
Dilution (factor)	Dilution factor of a sample. The standard dilution factor in the SAFIA Mycotoxin (Cereal) Kit is 16.
FCS Express layout	Template for converting .fcs files to .csv files using FCS Express.
FL-1	Fluorescence detector 1 Detection channel for fluorescence of secondary antibodies in SAFIA
FL-3	Fluorescence detector 3 Detection channel for fluorescence of particle coding in SAFIA

FSC	*Forward scatter*. Detector for measuring scattered light at a small angle. FSC and SSC are used to distinguish SAFIA particles from other particles in the sample.
FSC key	A keyword that can be taken from the text part of an .fcs file and contains information about a measurement, for example.
Gate	Sorting window in flow cytometry. Used to separate relevant data from irrelevant data, e.g. SAFIA particles from other particles. Only particles located within a gate are included in subsequent calculations.
IC20 value	The IC20 value indicates the concentration of an analyte that causes a 20% reduction in the maximum signal (A1) in SAFIA. It can be used as a guideline value for the limit of quantification. In SAFIA, the IC20 value of the control measurement serves as a criterion for a false-positive sample.
MFI [a. u.]	Median fluorescence intensity in arbitrary units. Fluorescence intensity calculated from a histogram, e.g. for the FL-1 detector.
Plate layout	Assignment of the alphanumerically coded wells (A1 to H12) of a 96-well microtiter plate to a sample or standard.
Prime programme	Flushing programme that puts the Cube 6 into operating mode.
R Square (R^2)	Coefficient of determination
Recovery rate	Percentage ratio of the target value of a reference sample to the determined actual value.
Relative dynamic range (rDR)	The relative dynamic range is a measure of the signal-to-noise ratio of an immunoassay. It is calculated as the quotient of the difference between the upper and lower asymptotes (A1 and A2) and the lower asymptote (A2) of a calibration curve. A relative dynamic range of 0.80 corresponds to a signal-to-noise ratio of 5.
Replicates	Repeat measurement
Sample ID	Unique sample designation
Sheath fluid	Fluid used to operate a flow cytometer (hydrodynamic focusing).
SSC	*Side scatter*. Detector for measuring scattered light at a 90° angle. FSC and SSC are used to distinguish SAFIA particles from other particles in the sample.
MTP format	Microtiter plate format. The reading is performed in a 96-well plate using the CyFlow® Robby autoloading station.

12 Contact

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