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 1.5.

Version 3

www.SAFIA.tech

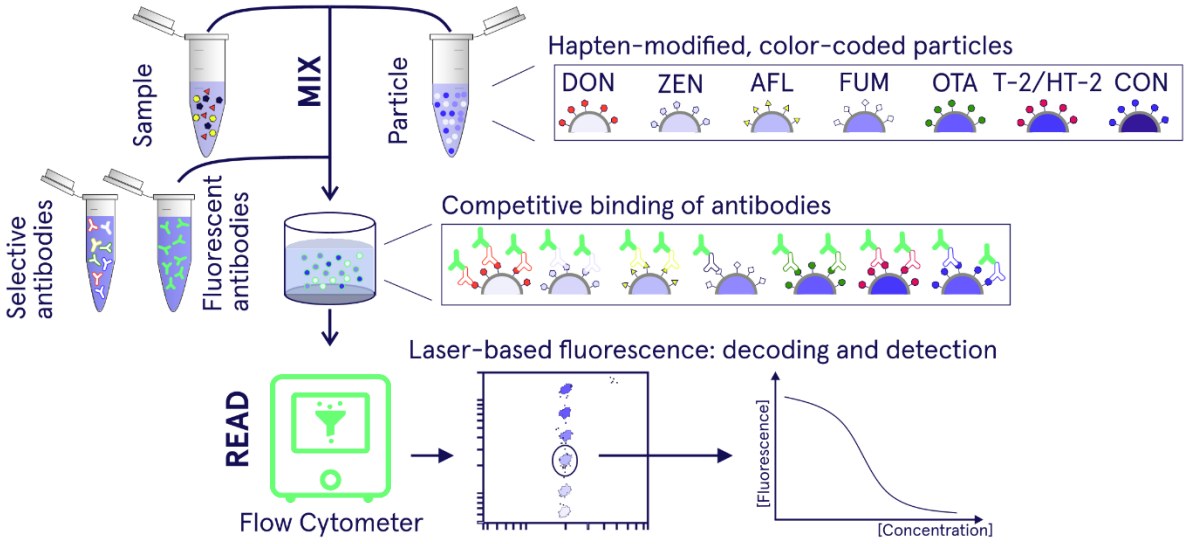
Contents

1 General information on mycotoxin measurement with SAFIA

1.1 Principle of the SAFIA assay

The suspension array fluorescence immunoassay, SAFIA for short, is a particle-based multiplex 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 the corresponding measured analyte. The overall measurement principle for detecting mycotoxins is based on indirect competitive immunoassays. The mycotoxins are chemically immobilised on the particle surface. Sample or standard, a mixture of mycotoxin-specific antibodies and fluorophore-labelled antibodies are added to the particles. The specific antibodies competitively bind either to the respective immobilised mycotoxin or to the mycotoxin present in the sample. Bound antibodies are stained with dye-labelled (green fluorescent) antibodies to generate a measurable signal. Due to the competitive reaction, the mycotoxin concentration 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 using a flow cytometer. Within the flow cytometer, the SAFIA microparticles are hydrodynamically separated and the fluorescence is measured independently for each particle using a blue laser/detector system.



Compared with classical immunoassays such as ELISA, SAFIA is a mix-and-read immunoassay. Washing steps used to avoid high signal backgrounds and matrix interference or to stop signal development are not required.

In addition to the mycotoxins, SAFIA includes a control measurement ("Control"). This indicates whether matrix effects interfere with the measurement or whether the assay was performed correctly. Interpretation is performed automatically in SAFIA Score; see section 7.5.

1.2 Performing the SAFIA assay

This manual describes how to perform measurements in microtiter plate (MTP) format with the CyFlow® Cube 6 V2m and CyFlow® Robby Autoloading Station (referred to below as "Cube 6").

Additional information on operating the Cube 6 can be found in the device instructions. The device may only be used by instructed and trained personnel. **Please observe the Safety instructions.**

The assay is performed 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. Evaluating the assay using SAFIA Score

Figure 1. Analysis workflow

2 Product information

2.1 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. 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 from the kit must not be exchanged with reagents from other kits, even if the same batch number is 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.

2.2 Safety instructions

Occupational safety

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

Disposal and decontamination

All reagents, reagent residues, contaminated consumables and sample residues must be disposed of after use in accordance with applicable local and national regulations. The current safety data sheets (SDS) must be observed. Reagents must not be disposed of via the sewer system or together with household waste. They must be collected as chemical laboratory waste and sent for proper disposal in accordance with applicable regulations. Assignment of a suitable waste code (e.g. according to AVV/EAK) is the responsibility of the waste producer or the responsible disposal organisation.

We recommend decontaminating glassware or other equipment that has come into contact with toxin-containing solutions using either an alkaline quick cleaner or a 10% hypochlorite solution.

2.3 Kit contents

Table 2. Overview of kit contents

Component	Quantity and content	Status	Information
96-well microtiter plate	1	Ready to use	
Calibration Standards (Calibration Standards)	8 x 0.5 mL	Ready to use	Labelled "Kal-1" to "Kal-8"
Sample Buffer (Sample Buffer)	1 x 15 mL	Concentrate	10-fold concentrate
Primary antibodies (Primary antibodies)	1 x 5 mL	Ready to use	Labelled "AK 1"
Secondary antibodies (Secondary antibodies)	2 x 5 mL	Ready to use	Labelled "AK 2"
Particle stock solution (Particle Stock solution)	1 x 45 µL	Concentrate	In vial with insert
Particle Buffer (Particle Buffer)	1 x 1.5 mL	Ready to use	
Fixation solution (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(T-2/HT-2) µg L^{-1}	c(KON) µg L^{-1}
KAL-1	1,000	10,000	1250	10,000	1,500	5,000	1,000
KAL-2	100	1,000	125	1,000	150	500	100
KAL-3	10	100	12.5	100	15	50	10
KAL-4	1	10	1.25	10	1.5	5	1
KAL-5	0.3	3	0.375	3	0.45	1.5	0.3
KAL-6	0.1	1	0.125	1	0.15	0.5	0.1
KAL-7	0.01	0.1	0.0125	0.1	0.015	0.05	0.01
KAL-8	0.001	0.01	0.00125	0.01	0.0015	0.005	0.001

2.4 Additional reagents and materials required

Contact us for recommendations and quotations.

Table 4. Overview of additional reagents and materials required, including specifications and examples.

Equipment		Specification	Example/recommendations
Workstation			
Area		approx. 2 x 1 m	no direct sunlight
Keyboard, mouse			Via Sysmex: order number: AV904041
Optional: PC monitor			Via Sysmex: order number: BY046024
Devices			
Centrifuges		2 mL: 12,000 g	uniCFUGE 5 (LLG)
		50 mL: 1,000 g	5810 R (Eppendorf)
(Microtiter) plate/orbital shaker			Titramax 101 (Heidolph)
Rotator/overhead shaker			uniLOOPMIX 2 (LLG)
Analytical balance			Pioneer PC (Ohaus)
Mill			Analytical mill A11 basic (IKA)
Single-channel pipettes		10–100 µL	Transferpette®, 10–100 µL (Brand)
		100–1000 µL	Transferpette®, 10–100 µL (Brand)
		1000–10000 µL	Transferpette, 1000–10000 µL (Eppendorf)
OPTION A	8-channel pipette, manual	10–100 µL	Transferpette® pro -8, 10–100 µL (Brand) Research® plus, 10–100 µL (Eppendorf)
	alternative to the 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)
	Reservoirs for multichannel pipettes	1 chamber	
		8/12 chamber	Trifill multichannel reagent reservoirs (Carl ROTH)
OPTION B	Electronic multistep pipette	Tips 1 mL, 5 mL	Brand HandyStep
Measuring cylinder		150 mL, 500 mL	
Laboratory bottle		250 mL, 500 mL	
Rack for centrifuge tubes		for 50 mL, 15 mL and 2 mL tubes	
Cutter knife			
Consumables			
Pipette tips for the respective pipettes			
Centrifuge tubes		2 mL	
		15 mL	

	50 mL	
96-well plate, flat-bottom		
Bench waste bags		
Reagents		
Ethanol 99%	denatured with MEK, IPA and Bitrex® (min. 99.8%), dilute to 70% (vol/vol) for analysis	
deionised water		
Materials for operating the Cube 6 (available from Sysmex)		
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
Sample tubes		Order number: 04-2000
Tube rack		Order number: 04-2000-02
Optional: material for the SAFIA Performance Check		
SAFIA Check Particles		Order number: SCP-1L-010
SAFIA Check Software		Order number: SCSOW-009
Alternative		
SAFIA Check Starter Kit (particles and software)		Order number: SCSK-1L-011

2.5 Data storage

Several files and folders are created when the system is installed. Do not change them, as this will interfere with automatic data processing. Table 5 provides an overview of the files and where they are stored. Only files marked with * are files that you must select manually in CyView or SAFIA Score. For convenient work, pin the cyflow subfolders to Quick Access.Table 5. Overview of file paths and data storage in the SAFIA system

Table 5. 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 a cleaning cycle	Cleaning-MTP.cv85*
C:\ProgramData\PartecGmbH\Cube_18\data\cyflow	
File(s)	**File names**
.fcs files from the measurements (automatically saved here with date and ID) are stored in a folder named after the tray ID	Date_Time_Tray-ID_Well ID.fcs
C:\User\cyflow\Documents\	

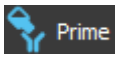
Folder	**Subfolder**	**File(s)**	**File format**
SAFIA-Check	Reports	Performance check reports (can be exported in SAFIA Check and stored here)	.pdf
SAFIA-Mycotoxins	SAFIA-Files	SAFIA Score files	.sdf

3 Starting the Cube 6

1. Before starting, the waste bottle should be completely emptied and the sheath bottle should be filled to approx. 80% with Sheath Fluid.
2. Switch on the device (small black button on the device).
3. The CyView™ measurement software opens automatically. Wait until it has fully loaded and the device is ready for use.
4. Log in to the software with your account (user name: USER, password: Cube1).

[1]Please note that each device has one original configuration file ("Mycotoxins-SCR_A_Robby.cv85") and one 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 of your device.

5. Open the configuration file (Mycotoxins-SCR_A_Robby.cv85 [2]) via CFG Upload 

.
6. Click Prime 

in the main bar, then click Start  and follow the instructions through the Prime program.

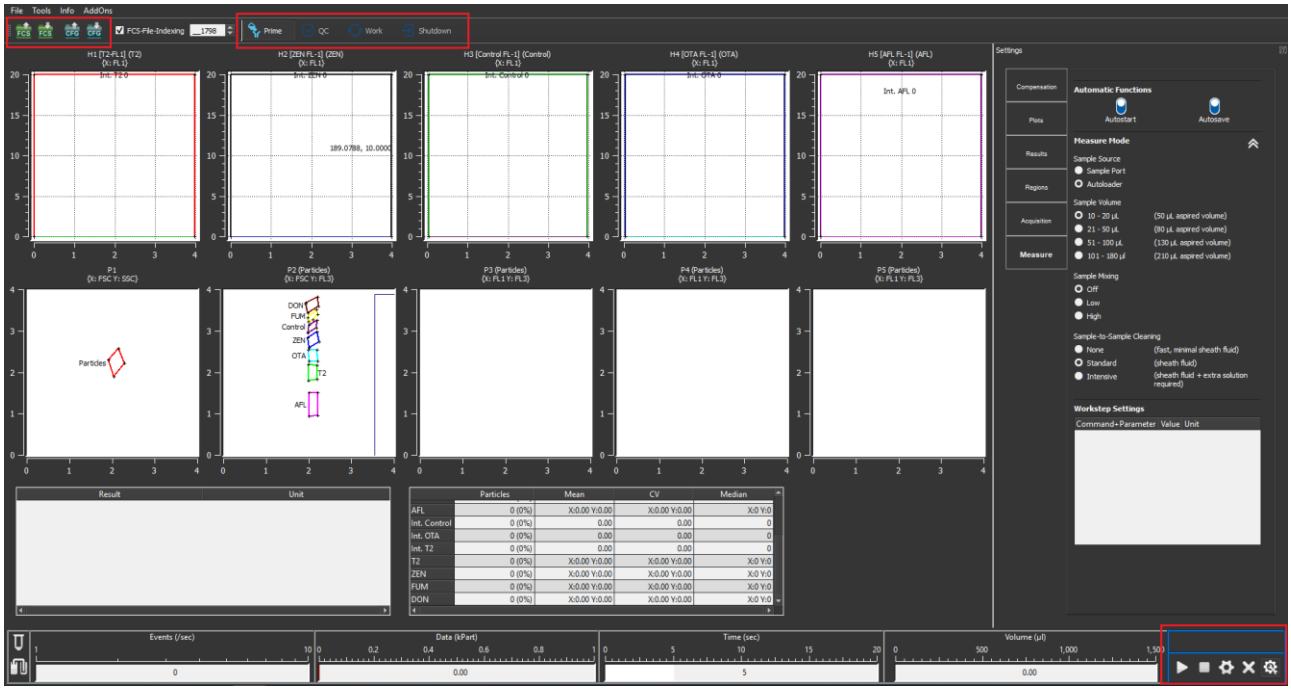
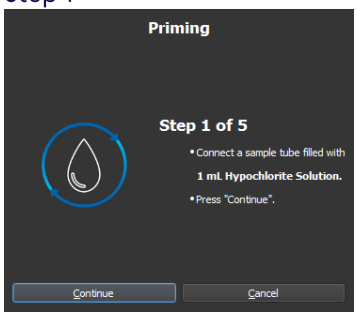
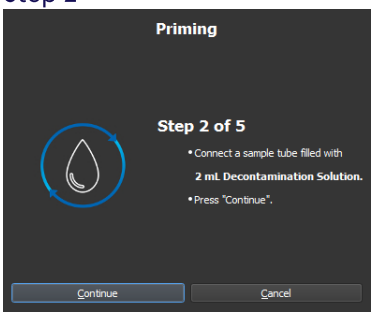


Figure 2. Location of the Prime program, settings and Start button

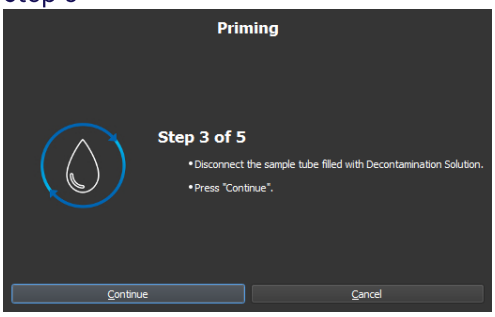
Step 1



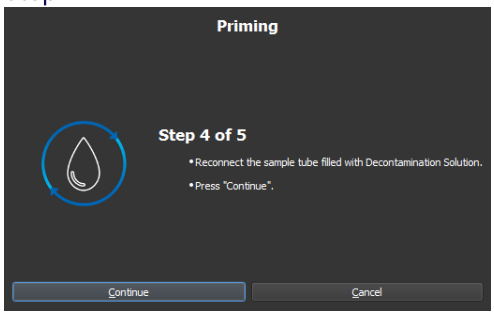
Step 2



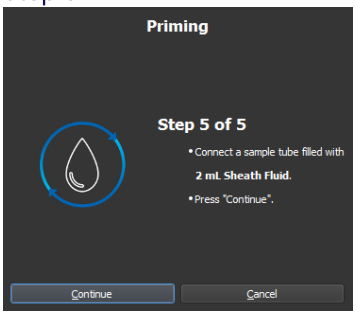
Step 3



Step 4



Step 5



Step 6

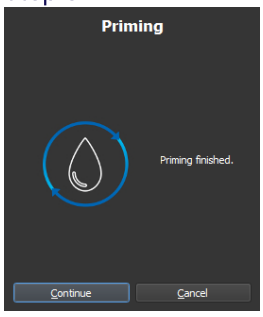


Figure 3. Steps of the Prime program

[2]Please note that each device has one original configuration file ("Mycotoxins-SCR_A_Robby.cv85") and one 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 of your device.

4 Performing a performance check

4.1 Explanation of the performance check

We recommend checking the Cube 6 device performance on each measurement day using the SAFIA Performance Check particles and evaluating it with SAFIA Check. If the performance check has already been performed on that day, continue with section 5.

The SAFIA Performance Check verifies that the Cube 6 is functioning correctly for measuring SAFIA assays. For this purpose, a mixture of SAFIA Performance Check particles is measured. To pass the SAFIA Performance Check, the measurement must meet predefined intensity values, particle count rates (count), coefficients of variation (%CV) and signal-to-noise (S/N) ratios in the corresponding FSC, SSC, FL-1 and FL-3 detectors. In a successful measurement, 20,000 particles are measured; these appear as a population in the "Particles" gate in the FSC/SSC scatter plot (see Figure 4 A). Five gates are drawn in the FSC/FL-3 scatter plot, with one or two populations visible in each gate (see Figure 4 B). The populations from these five gates are separated in five FL-1 histograms so that two peaks clearly represent two populations in each case (see Figure 4 C).

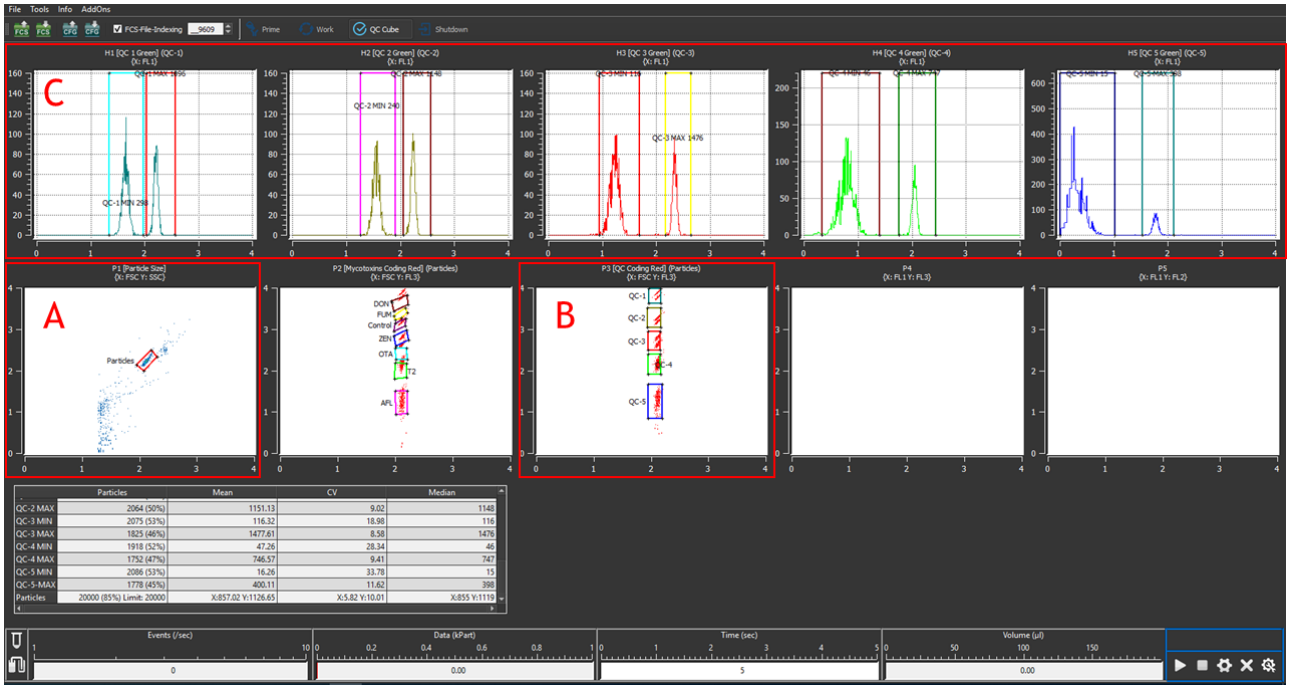


Figure 4. SAFIA Check overview. FSC/SSC scatter plot in A, FSC/FL-3 scatter plot in B and FL-1 histogram in C

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

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

4.2 Preparation

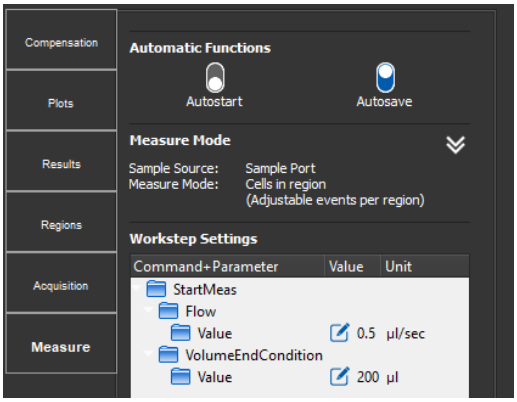
The SAFIA Check particles must be diluted before the measurement is performed.

1. Shake the bottle with the SAFIA Check particles vigorously for at least 15 seconds.
2. Take 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 that it must be discarded. Store it in a refrigerator at 2–8 °C.

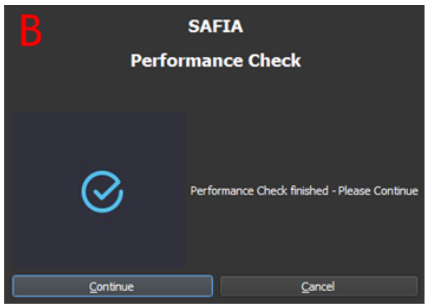
4.3 Procedure

1. Click QC Cube 

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



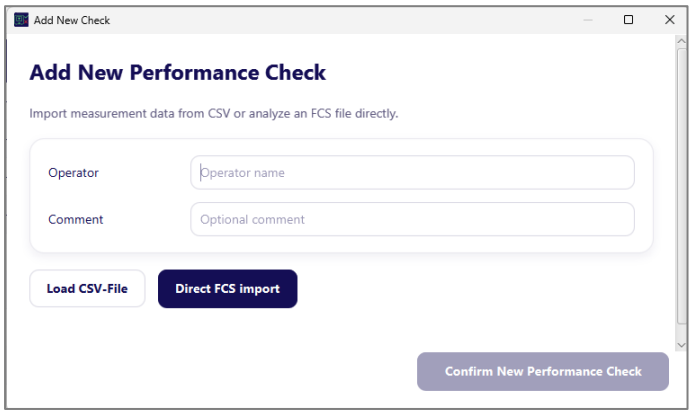
2. Click Start  and follow the instructions through the QC program. If no particles are detected, follow the instructions in section 9.2.
3. The measurement file for the performance check is automatically saved in the "cyflow" folder with the date and time of the measurement as the file name and an irrelevant number (e.g. 2024_08_01_08_59_53__1957.fcs).



4.4 Evaluating a performance check with SAFIA Check 

1. Open SAFIA Check and click the button at the top left:

2. To add the .fcs file, click Direct FCS Import in the window that opens. Select the corresponding file from the folder SAFIA Check → Export-Files.



3. The Check Autogating and Add Performance Check window opens. Click the Add Performance Check button at the bottom right.

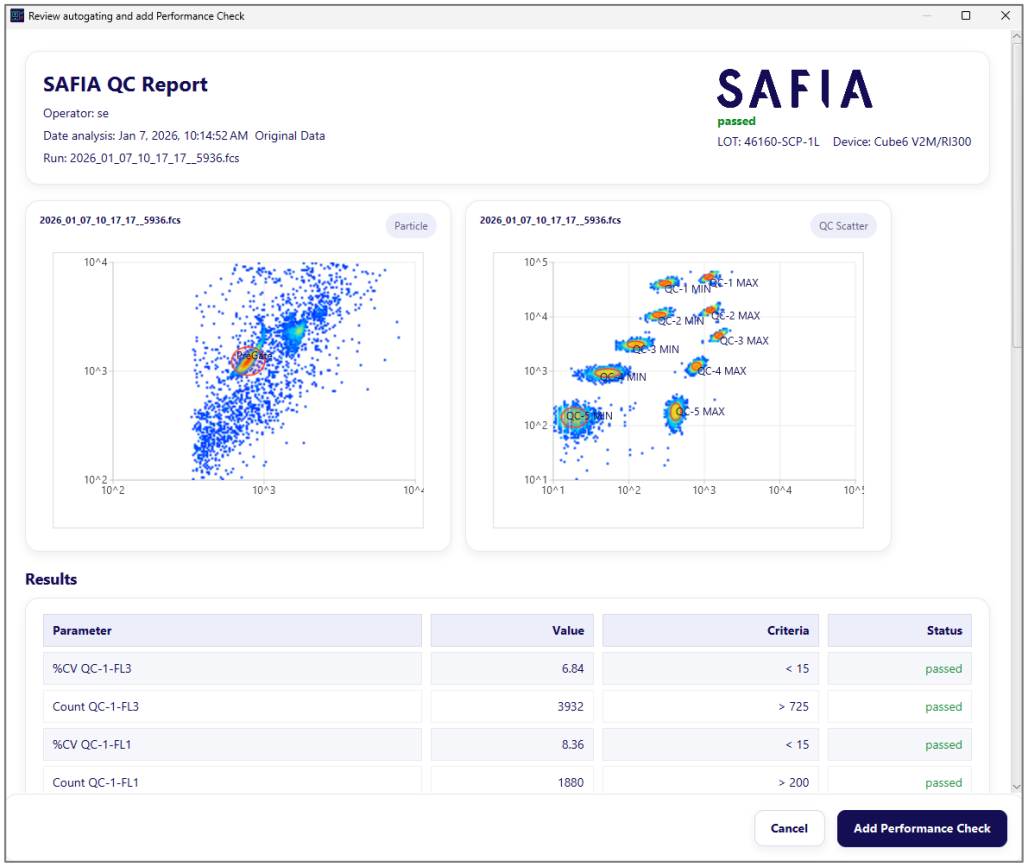
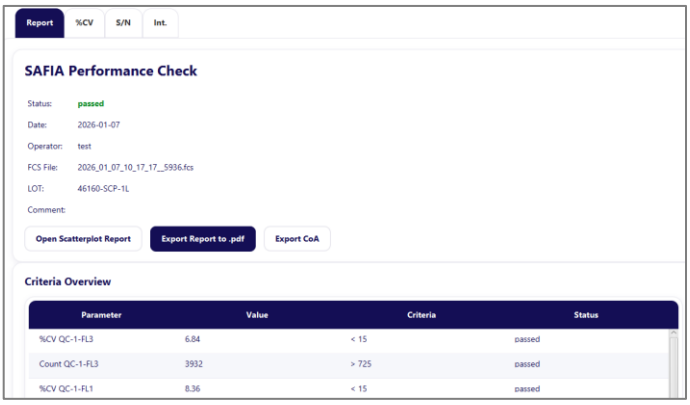


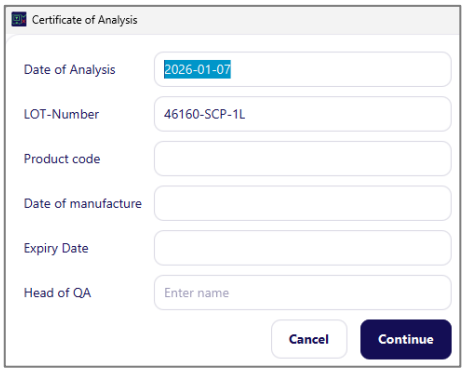
Figure 5. Display of the .fcs data in SAFIA Check

> ! The performance check can no longer be changed or deleted afterwards. Each .fcs file can only be evaluated once with the SAFIA Performance Check.

4. The saved performance check now appears on the right side of the window. The result of the performance check is saved automatically. Previous performance checks are listed on the left side of the window and can be opened. The window shown in Figure 5 can be reopened using the Open Scatterplot Report button.

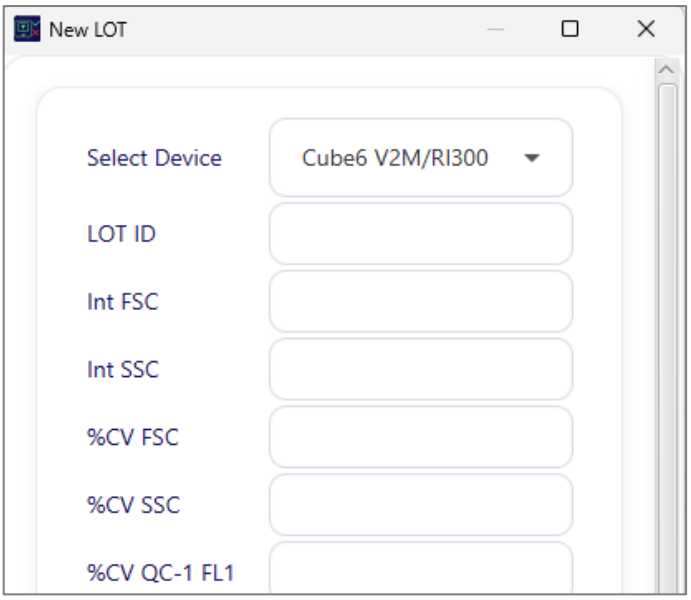


5. You can also export the report as a .pdf or export a CoA (certificate of analysis). To do this, fill in the corresponding fields that should be included on the CoA.



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, for example after maintenance, you must create a new LOT file.



1. To do this, click the button **New LOT** . Enter the LOT ID and the listed parameters in the window that opens. The parameters are supplied with the SAFIA Check particles. They serve as benchmark values for each performance check.
2. Click the button **Add LOT File** .

If the measured values from the first measurement of your SAFIA Check particles differ from the benchmark values, the gain values of the detectors may need to be readjusted. This can also be the case after device maintenance. Contact Sysmex Support for this.

5 Working with SAFIA Score

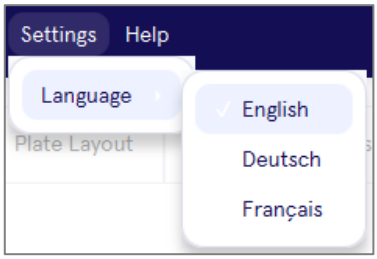
5.1 General information about SAFIA Score

SAFIA Score is installed by default on the computer of your flow cytometer. The software can also be installed on any number of additional devices. Send a request to support@safia.tech for this.

The software is divided into the workspaces **Start**, **Samples and Calibrations**, **Plate Layout**, **Calibration Curves**, **Samples** and **References**. These tabs can be opened by clicking them. The workspaces **Calibration Curves**, **Samples** and **References** are only enabled after the measurement data have been read in.

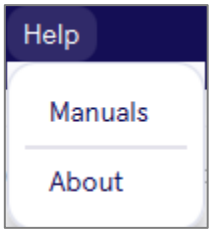
5.1.1 Changing the language

The user interface language can be changed at any time via Settings. German, English and French are available. After changing the language, close the application and restart it so that the change is applied.



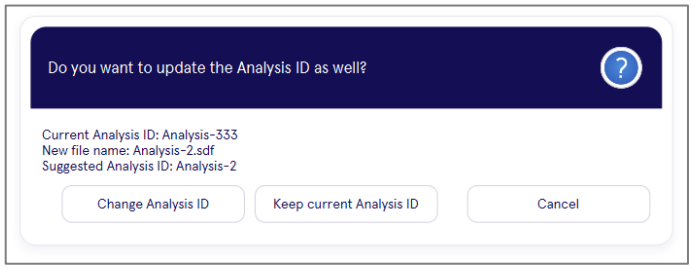
5.1.2 Manual and software version

The current version of this manual can be opened via the Help menu item. An internet connection is required for this. The currently installed software version can be viewed via the About menu item.



5.1.3 Saving

When creating an evaluation, you must assign an Analysis ID. By default, this Analysis ID is also used as the file name. If the Save As function is used, you will be asked whether the Analysis ID should also be changed. If you later change the file name in File Explorer, the Analysis ID of the evaluation remains unchanged.

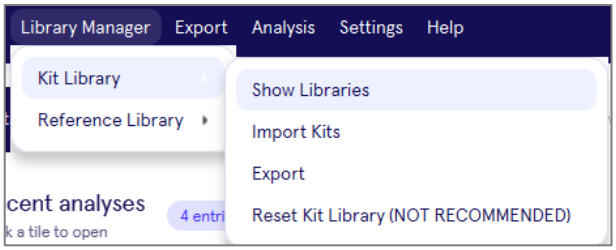


5.1.4 Opening files

You can also open an older analysis. To do this, click the blue button for an older analysis in the Start area. You will see the Analysis ID, the kit used and the storage location of the last 20 entries. Alternatively, you can open a SAFIA Data File (.sdf file) via File → Open or directly in File Explorer by double-clicking it.

5.1.5 Kit library

The Kit Library is located in the Library Manager menu. All kits available by default are preset there. These entries must not be changed.



5.1.6 Reference library

The Library Manager menu also contains the Reference Library, where you can manage and save the reference values of your reference materials. To create a new reference entry, click **Add Reference**, enter the corresponding values and save the entry. The Reference Library can be exported and imported again on another device, making it easy to transfer reference data between multiple devices.

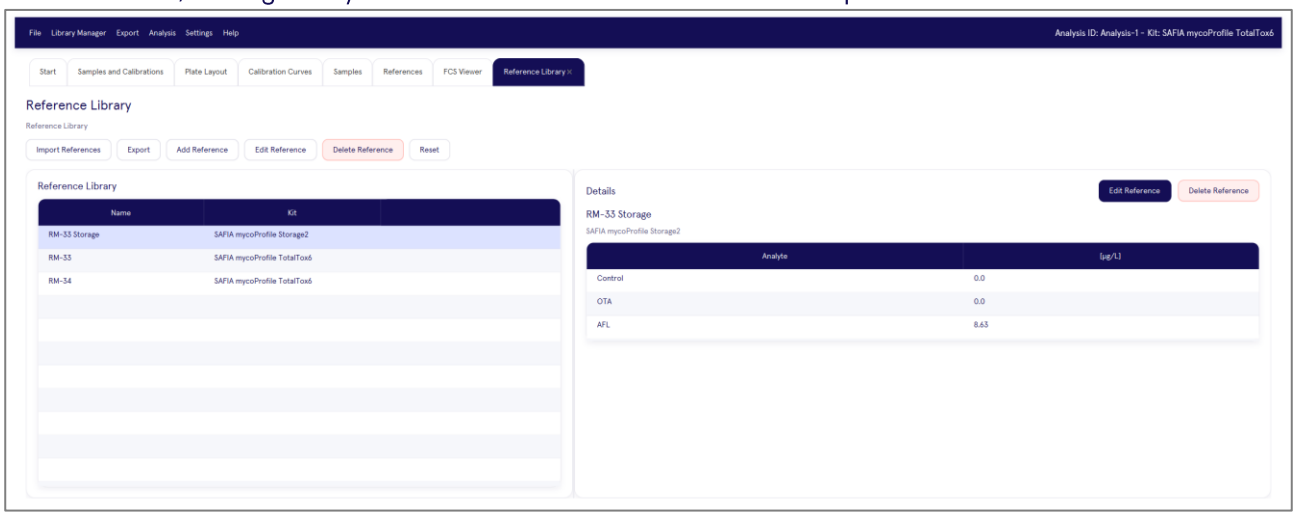


Figure 6. View of the Reference Library

5.2 Planning the SAFIA assay in SAFIA Score

1. Open SAFIA Score.
2. Start a new analysis in the Start tab using the green + New Analysis button.

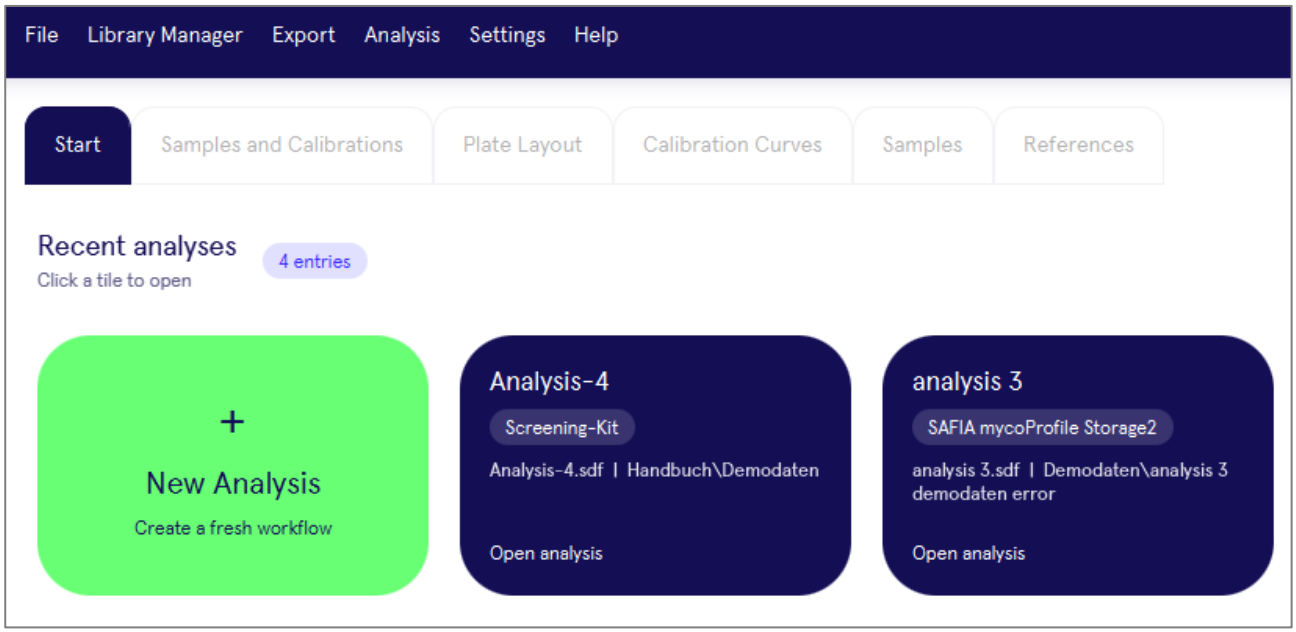
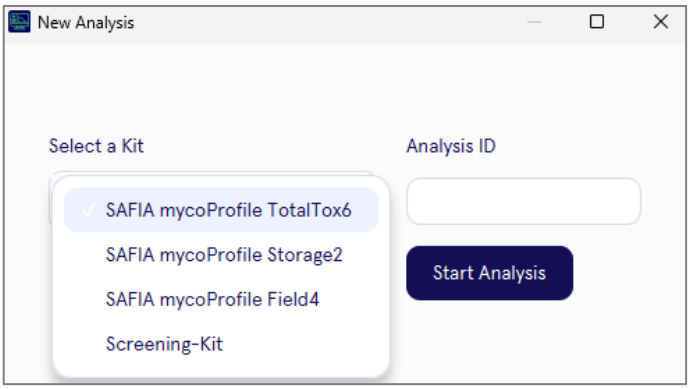
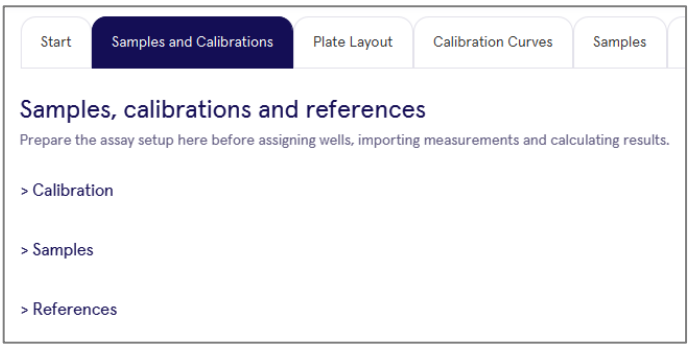


Figure 7. View of the SAFIA Score Start workspace

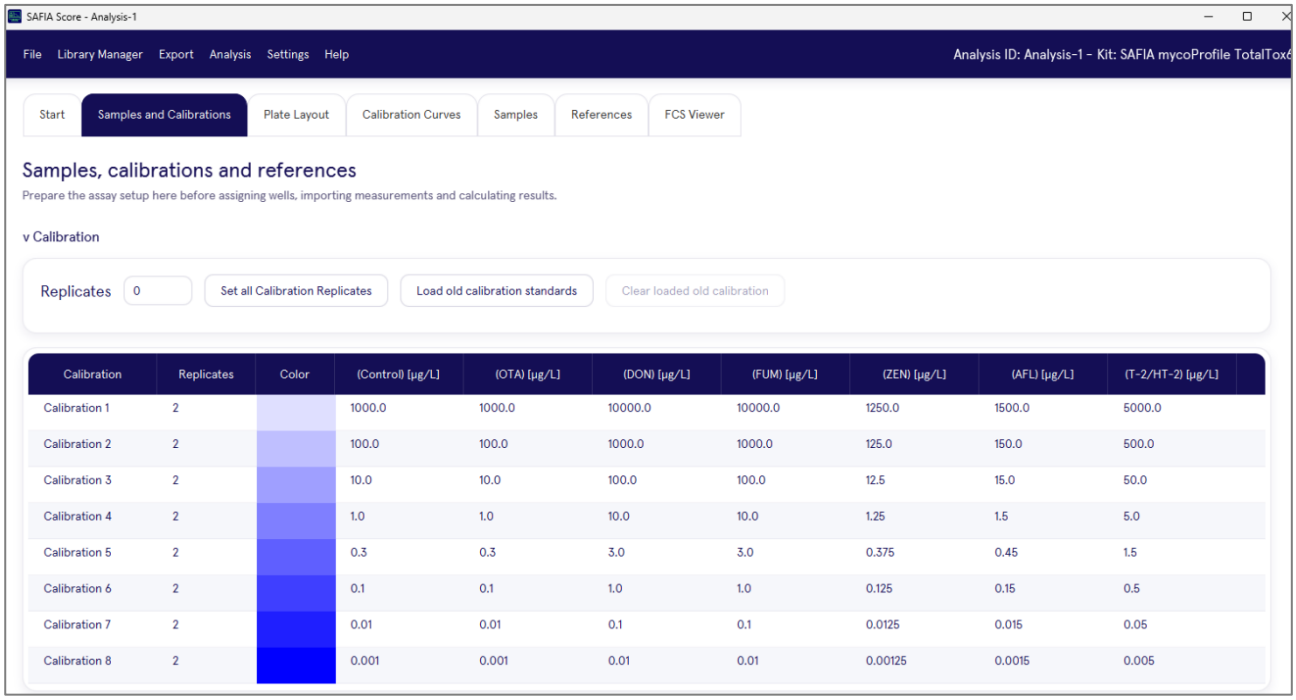
3. In the window that opens, select the corresponding kit from the drop-down menu. Enter an Analysis ID and click **Start Analysis**.



4. In the next step, you enter the **Samples and Calibrations** workspace. It contains three tables below one another, which can be expanded and collapsed by clicking the heading.



5. In the **Calibration** table, the concentrations of the individual standards supplied with the kit are prefilled. Use the Replicates column to define how often a standard should be measured.



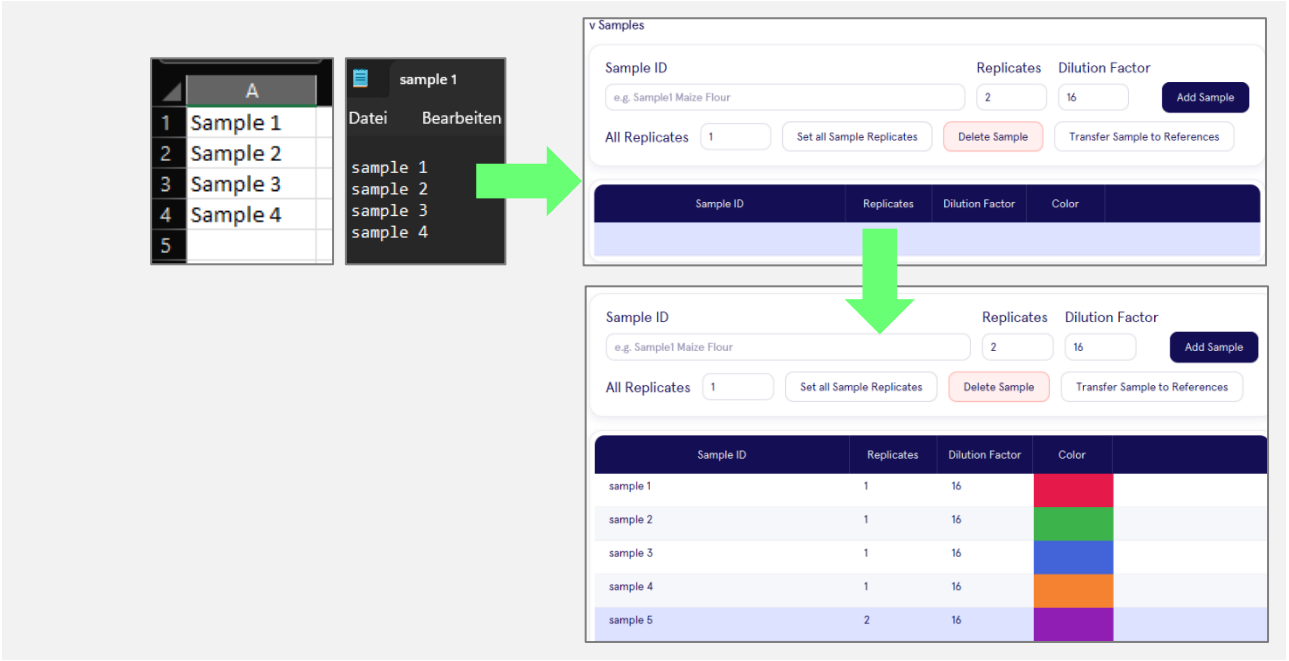
Calibration	Replicates	Color	(Control) [µg/L]	(OTA) [µg/L]	(DON) [µg/L]	(FUM) [µg/L]	(ZEN) [µg/L]	(AFL) [µg/L]	(T-2/HT-2) [µg/L]
Calibration 1	2		1000.0	1000.0	10000.0	10000.0	1250.0	1500.0	5000.0
Calibration 2	2		100.0	100.0	1000.0	1000.0	125.0	150.0	500.0
Calibration 3	2		10.0	10.0	100.0	100.0	12.5	15.0	50.0
Calibration 4	2		1.0	1.0	10.0	10.0	1.25	1.5	5.0
Calibration 5	2		0.3	0.3	3.0	3.0	0.375	0.45	1.5
Calibration 6	2		0.1	0.1	1.0	1.0	0.125	0.15	0.5
Calibration 7	2		0.01	0.01	0.1	0.1	0.0125	0.015	0.05
Calibration 8	2		0.001	0.001	0.01	0.01	0.00125	0.0015	0.005

Figure 8. Overview of the functions in the Samples and Calibrations workspace

6. Now enter your samples in the **Samples** table. Assign a unique Sample ID (sample designation) to each sample and define the number of replicates and the dilution factor. Both can also be edited directly in the table by clicking. The Sample ID can be changed by double-clicking the corresponding cell, but it must never be assigned twice.

The Sample ID can be typed directly, transferred to the clipboard with a barcode scanner and pasted into SAFIA Score, or copied and pasted from a spreadsheet program (e.g. Excel). By double-clicking a cell in the Sample ID column, you can enter or change the name of an individual sample.

1. Create and copy sample names as a list in Excel or a text program.
2. Select an empty light-blue row in the sample table and paste with Ctrl + V.
3. The sample names are automatically transferred to the sample table.

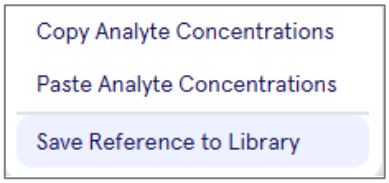


7. The values in the Replicates and Dilution Factor columns can be changed later at any time. Right-clicking a value allows it to be applied to all other samples.

The dilution factor can be found in section 6. In the high-throughput protocol it is 16; in the high-sensitivity protocol it is 8. If the same sample is measured at several dilution levels, a separate Sample ID must be assigned for each dilution level.

8. In the **References** table, you can enter samples with reference concentrations in the same way as in the Samples table. After the analysis is complete, SAFIA Score automatically determines the recovery rate of the reference samples. Using the button Transfer Sample to References , you can also move reference samples that were accidentally entered in the **Samples** table to the **References** table.

9. By right-clicking, you can copy and paste the analyte concentrations, for example if you want to measure a reference sample at two different dilution levels. You can also save the reference sample in the Reference Library.



10. Using the button Select from Reference Library , you can add a reference sample from your library. More information on the **Reference Library** can be found in section 5.1.6

11. Once you have entered all samples, click the button at the bottom Set Plate Layout right to assign the samples and standards to the wells of the microtiter plate. The wells have an alphanumeric code (A1, B1, C1, etc. through H12) (see Figure 9). Assignment can be performed 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 microtiter plate. The Clear button can be used to delete all entries.

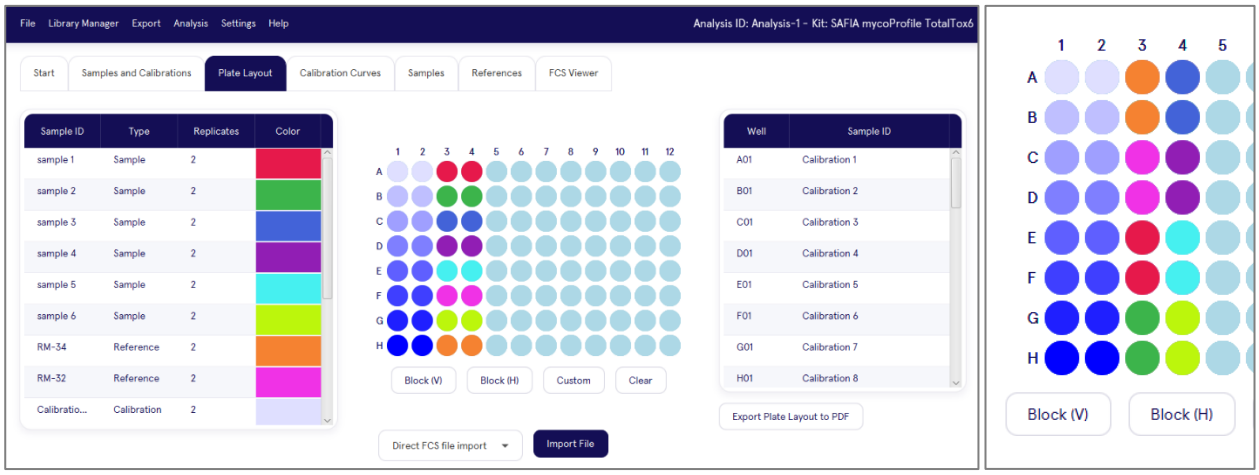
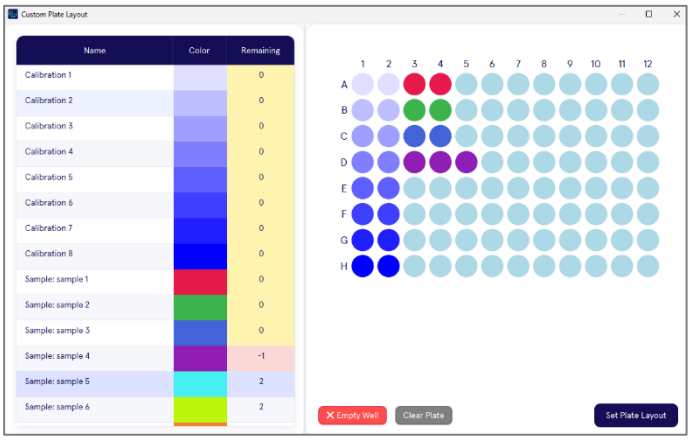


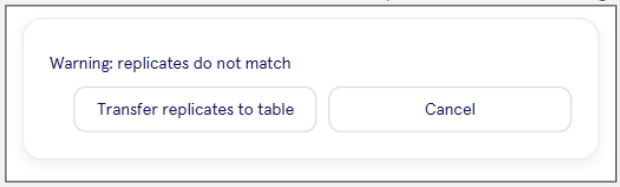
Figure 9. Overview of the functions in the Plate Layout workspace

The plate layout can also be created manually using the Custom button. This opens a separate window. In the table on the left, select the desired sample and assign it to a single well by clicking or to several wells by clicking and dragging. Incorrectly assigned samples can be removed in the same way by activating the Empty Well button. Alternatively, all replicates of a sample can be dragged and dropped onto the plate; they are then arranged horizontally automatically. The Remaining column shows the number of replicates that have not yet been placed on the plate. If more wells are assigned to a sample than originally specified under Replicates, a negative number is displayed there.



It is essential that the specified replicate measurements for standards and samples from the Samples and Calibrations workspace match the replicates in the Plate Layout workspace. If this is not the case after the plate layout has been created using the "Custom" function, a warning is displayed automatically.

You can now either cancel the operation and change the plate layout accordingly (Cancel button), or have the replicate measurements entered directly in the corresponding table under **Samples and Calibrations** (Transfer replicates to table button).



You can export the plate layout as a PDF using the button Export Plate Layout to PDF.

If you want to change the sample names after creating the plate layout, you must first delete the current plate layout.

12. Save the SAFIA Score file (.sdf) so that you can continue the analysis later. This can be done via the File → Save As.
13. The assay preparation in SAFIA Score is now complete. You can now use the created plate layout in the assay and perform the measurement on the Cube 6.

6 Sample preparation

The sample preparation method to be selected depends on the type of food (see Table 6). Samples must be homogenised before extraction, e.g. by grinding the sample material with a mill. Uniform grain sizes and the applicable regulations must be observed. The specified sample quantities should not be reduced, but may be increased if necessary. The ratio of sample material to extraction solvent must not be changed. Sample preparation is divided into the following steps:

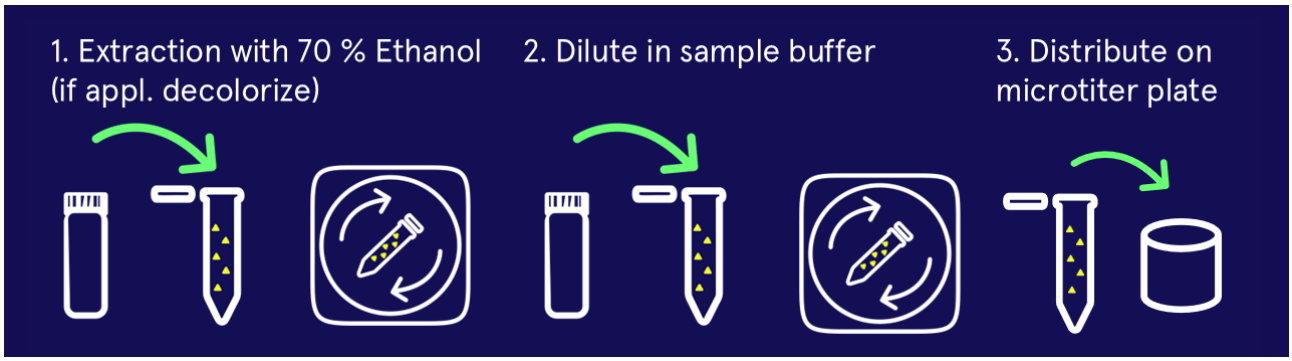


Figure 10. Overview of the sample preparation steps

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 a refrigerator at 2–8 °C.
- Can be used until the kit expiry date.
- Before use, the sample buffer must be brought to room temperature.

A substance may precipitate when the sample buffer concentrate is stored at 2–8 °C. It dissolves completely during dilution. Make sure that the entire contents of the bottle are dissolved. If necessary, rinse the bottle with the diluted sample buffer.

6.2 Sample preparation instructions

For sample preparation, follow the protocol specified in Table 6 for the respective matrix. Because sample preparation is matrix-specific, some matrices require minor adjustments to the standard protocol (e.g. solid foods with high protein content). Also observe the notes for special matrices in section 9.
The dilution factor is 16 in the high-throughput protocol because a larger amount of extraction solvent is used. In the high-sensitivity protocol it is 8 because extraction is performed with a smaller amount of extraction solvent. For some matrices, such as hay, herbs or spices, however, the extraction solvent cannot be reduced because otherwise the sample can no longer be extracted homogeneously.
Additional 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.

Table 6. 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, pulses	1) **Weigh out 5 g of sample material.** 2) **Add 20 mL (10 mL) of 70% (vol/vol) ethanol and shake the samples for 15 min, e.g. in an overhead shaker.** 3) **Centrifuge for 5 min 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 min at 12,000 g.**	16 (8)
Solid foods with high sugar content	Dried raisins, dates, figs		
Foods with high fat content	Hazelnuts, peanuts, almonds, pistachios, oils	1) **Weigh out 5 g of sample material.** 2) **Add 20 mL (10 mL) of 70% (vol/vol) ethanol and shake the samples for 15 min, e.g. in an overhead shaker.** 3) **Centrifuge for 5 min 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 measuring spoons* of SAFIA PVPP Adsorber per 1 mL of diluted sample and shake for 15 min.** 6) **Centrifuge for 10 min at 12,000 g.**	16 (8)

* 3 level measuring spoons of PA or PVPP are approx. 20 – 25 mg. Decolourisation was tested in the range of 10 – 50 mg/mL PA/PVPP; no influence of the adsorber quantity on the analytical results was observed.

Sample type	Examples	Sample preparation	Factor
Specialty products	Hay	1) **Weigh out 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 min 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 measuring spoons* of SAFIA PA Adsorber per 1 mL of diluted sample and shake for 15 min.** 6) Centrifuge for 10 min at 12,000 g.	32
Products with high anthocyanin and polyphenol content	Cranberries, raspberries	1) **Weigh out 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 min 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 measuring spoons* of SAFIA PVPP Adsorber per 1 mL of diluted sample and shake for 15 min.** 6) Centrifuge for 10 min at 12,000 g.	16
Liquid foods without many colourants	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 min at 1,000 g if many suspended solids are present.** 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 min at 12,000 g.	8

* 3 level measuring spoons of PA or PVPP are approx. 20 – 25 mg. Decolourisation was tested in the range of 10 – 50 mg/mL PA/PVPP; no influence of the adsorber quantity on the analytical results was observed.

Liquid foods with a high concentration of red colourants	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 min at 1,000 g if many suspended solids are present. 4) Add 3 measuring spoons of SAFIA PVPP Adsorber per 1 mL of extract and shake for 15 min. 5) Centrifuge for 5 min 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 min at 12,000 g.	8

* 3 level measuring spoons of PA or PVPP are approx. 20 – 25 mg. Decolourisation was tested in the range of 10 – 50 mg/mL PA/PVPP; no influence of the adsorber quantity on the analytical results was observed.

7 Performing the SAFIA assay

The SAFIA assay is performed in the following working steps.

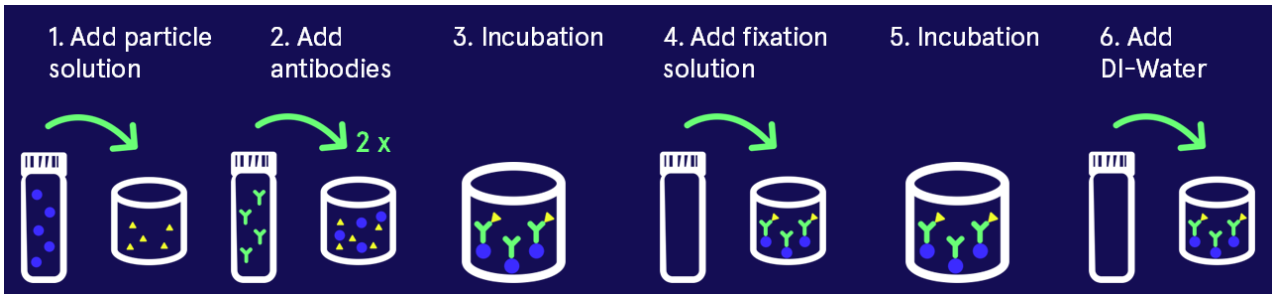


Figure 11. Performing the SAFIA assay

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 microtiter plate). This can be done using SAFIA Score software (see section 5).
2. Bring all kit reagents to room temperature.
3. Shake the fixation solution before use, as storage at 2-8 °C may cause one component to precipitate; it dissolves completely again at room temperature.
4. Prepare the required amount of particle working solution fresh:
5. Shake the particle stock solution vigorously for at least 20 s.
6. Dilute 1:33 in particle buffer (see Tables 7 and 8).

Table 7. 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 full 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	

Table 8. Pipetting scheme for a specific number of wells of an MTP

	1 well	30 wells				
Particle stock solution	0.4 µL	12 µL				
Particle buffer	13.3 µL	399 µL				
Total volume	13.7 µL	411 µL				

- Also shake the dilution well for at least 20 s.

The prepared 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 to use.

7.2 Performing the assay

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

When pipetting, avoid long intervals between the individual steps and pipette continuously so that no delay occurs between the individual strips.

When adding reagents to the individual wells, make sure that the pipette tips never touch the liquid if the same pipette tip is used for several wells.

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 sequence:
   - 10 µL particle working solution; shake it well again before addition (at least 20 s)
   - 25 µL primary antibody (AK 1)
   - 50 µL secondary antibody (AK 2).
3. Incubate for 20 min at room temperature while shaking.
4. Add 50 µL fixation solution and incubate for 5 min.
5. Add 140 µL DI water.
6. The measurement on the flow cytometer can now be started; see section 7.4.

7.3 Tips and notes on pipetting

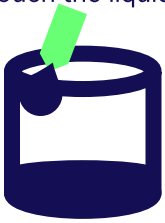
7.3.1 Important notes

- Select a slow aspiration and dispensing speed because many reagents contain surfactants.
- When using pipettes without dispensing mode, pre-wet the tip with liquid before aspirating.
- Use a new pipette tip for each reagent/standard/sample.
- Never touch the liquid in the well when pipetting.
- Because there are no washing steps, take particular care to work cleanly to avoid cross-contamination.
- For hand stability, it is helpful to work while seated so that the elbow can be supported.

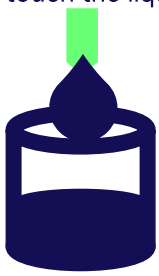
7.3.2 Suggested pipetting technique

- **Add sample/standard vertically**
- lightly touch the bottom

- **Add particle and AK-1 solution at a slight angle**
- touch the side wall
- Do not touch the liquid.



- **Add AK-2/fixation solution/water over the well**
- Do not touch the liquid.



7.3.3 Pipette selection

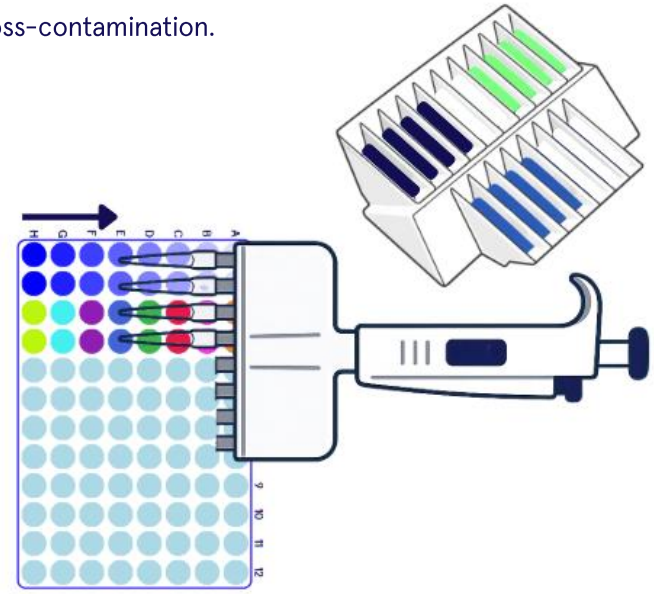
For adding the reagents, a multichannel pipette, ideally with a dispensing function, or an electronic multidispenser/multistepper can be used. The optimal pipetting steps with each pipette are described below.

Multichannel pipette:

- Particularly suitable for a high number of samples and for working in 8-well strips (up to the full plate).
- Prepare reagent quantities in the multichannel reservoir (e.g. for 6 strips, place 6 times the required amount per reagent per strip (see Table 7) next to one another in the reservoir).
- Set the multichannel pipette to the volume for one well (e.g. 10 µL particle solution). If possible, set 8 dispensing steps. Depending on the number of strips, fit tips to the required pipette channels and aspirate the reagent volume from the reservoir.
- Pipette or dispense the volume 8 times into the wells without touching samples or standards.
- Work from bottom to top (H -> A) to minimise cross-contamination.

Table 9. Required volumes of assay reagents for 1 well or one strip

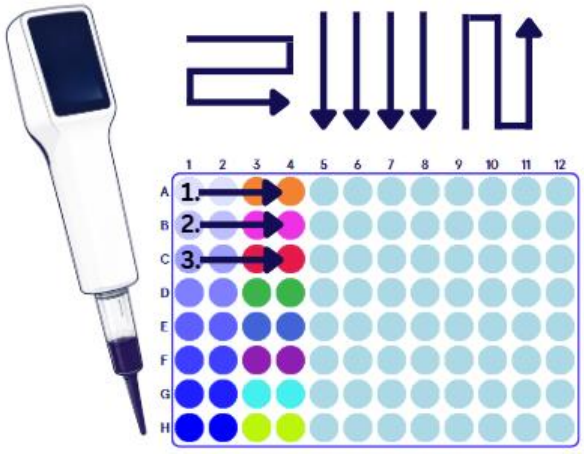
Reagent	1 well	1 strip
Particle solution	10 µL	110 µL
AK 1	25 µL	350 µL
AK 2	50 µL	700 µL
Fixation solution	50 µL	700 µL
DI water	140 µL	1.400 µL



- If you want to pipette more than 8 strips, perform the steps described above twice. Make sure to fill the wells in the same order each time.

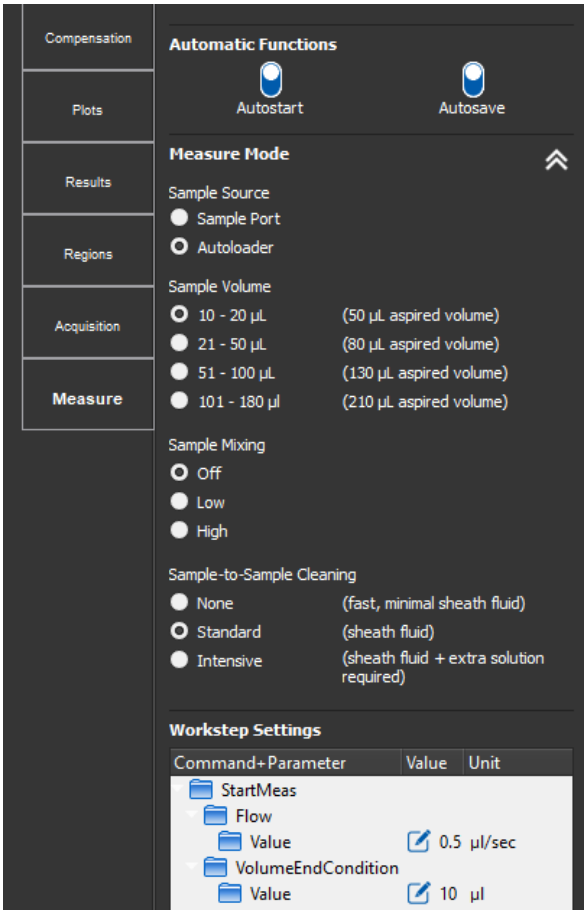
Multidispenser:

- Particularly suitable for a small number of samples because the layout is not bound to complete strips. Individual wells (e.g. A–D) of a strip can also be used.
- For 10 µL particle solution, use a 1 mL tip and set the number of steps according to the number of wells to be filled.
- A 1 mL tip can also be used for 25 µL. A maximum of 40 steps per filling is possible. Use a 2.5 mL tip for more than 40 samples.
- Use a 5 mL tip for 50 µL AK2 and fixation solution.
- Use a 5 mL tip for 140 µL water.
- The filling pattern of the plate can be chosen freely. It is important to use the same filling pattern for each reagent. The filling order must be maintained so that identical incubation times are ensured for all wells.

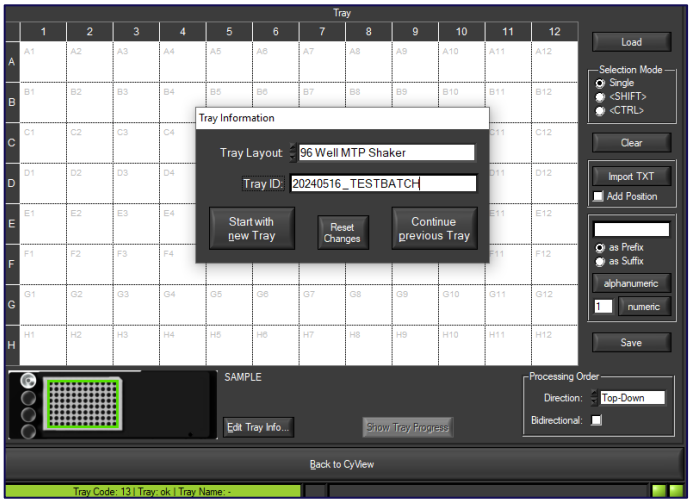


7.4 Readout with the CyFlow® Cube 6 flow cytometer

1. If you have not yet performed a performance check on the day of measurement, first follow the steps in section 4.4.
2. Pull out the movable tray of the CyFlow® Robby Autoloading Station and place the microtiter plate on the designated platform. Ensure the correct plate orientation. Well A1 must be at the top right.
3. Click the Work button in the main bar.
4. Under Settings → Measure, check whether the parameters are set as shown in the screenshot.
5. Click Start to begin the measurement.



6. A window now opens in which you assign a tray ID (batch number) (e.g. the Analysis ID). Use only alphanumeric characters (A-Z, a-z, 0-9) and the underscore "_" for the tray ID (e.g. 20250213_TESTBATCH), because other characters and special characters are not recognised and are deleted when the folder is created.
7. Click Start with a new Tray to create a new measurement.



8. Now select the wells to be measured. This can be done individually or by dragging over the fields. Selected wells appear light blue (see Figure 12).
9. To evaluate the data using SAFIA Score, it is essential to insert the well ID. Always use the setting as Prefix for this. Press the alphanumeric button; the well ID then appears in the corresponding well. The well ID has been accepted when the values in the centre of the wells are displayed in black.

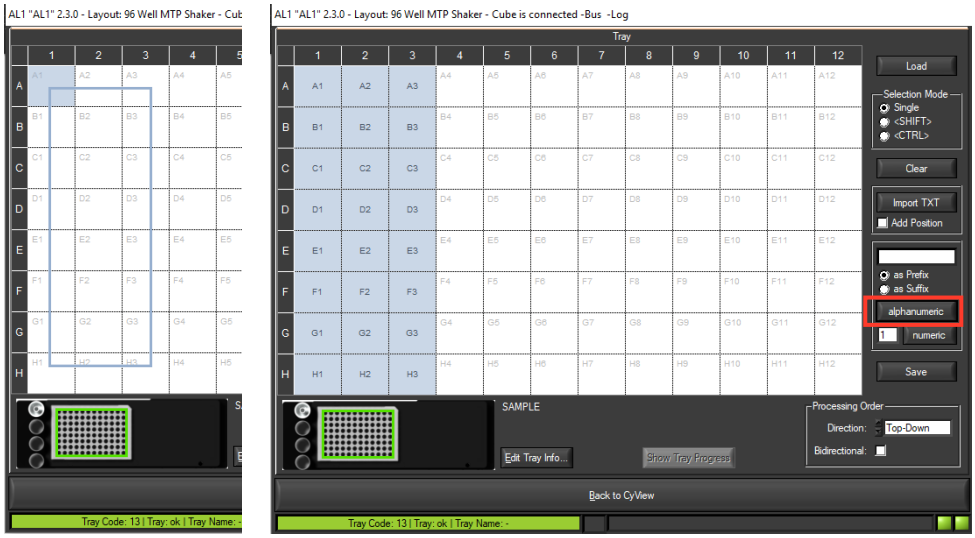


Figure 12. The menu for microtiter plate assignment

10. Now click the Back to CyView button. This takes you to CyView and the measurement can be started by clicking Continue  .

11. Before and during the measurement, make sure that sufficient Sheath Fluid is available. Refill the bottle if a warning appears.

12. The measurement now runs fully automatically. Nevertheless, you should monitor the first 3 measurements to check whether, for example, air bubbles in the system affect the measurements.

In a successful measurement, you see a population in the FSC/SSC scatter plot that appears in the Particles gate. Up to 7 populations also appear in the FSC/FL-3 scatter plot; see Figure 13. These do not need to be in the correct gates for the measurement, as this is corrected later during evaluation if necessary. Depending on the kit used, different populations representing the different mycotoxins can be seen in this plot.

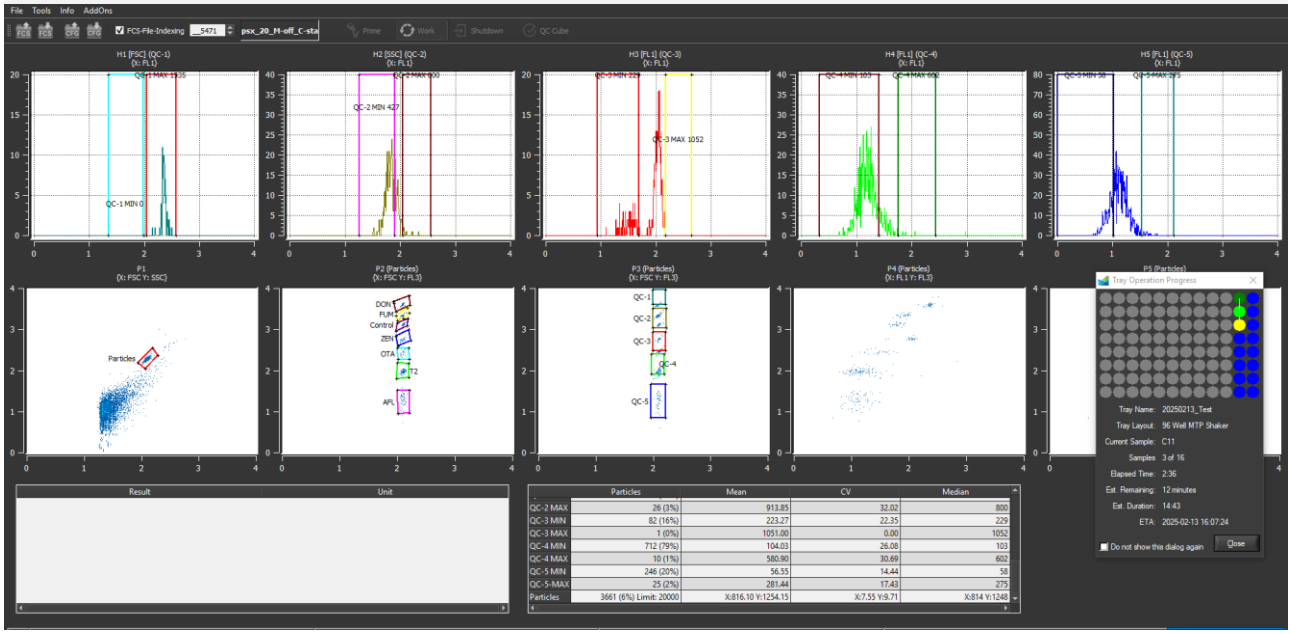


Figure 13. Typical view during measurement of a SAFIA assay. Top: histogram for the FL-1 intensity of an analyte. Bottom: FSC/SSC scatter plot and FSC/FL-3 scatter plot

13. Optional after the measurement: perform a manual cleaning cycle; see section 9.1

7.5 Evaluating 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 workspace, select Direct FCS File Import, then click Import File and select all previously generated .fcs files that were saved in the folder named after your tray ID:
   C:\ProgramData\PartecGmbH\Cube_18\data\cyflow\

3. Sigmoidal calibration curves are now generated based on the plate layout and the measurement data of the calibration standards (see Figure 14). The mycotoxin concentrations in the samples are calculated automatically on this basis.

4. The data are evaluated automatically. If deviations occur during the measurements, either the message "Review FCS" or "FCS file excluded" appears. Observe the corresponding instructions and carry out the recommended actions. Further information can be found in sections 10.5 and 10.6. If you want to view the measurement data in detail, click the **FCS Viewer** tab.

Figure 14. Overview of the functions in the Calibration Curves workspace

5. The curve parameters calculated by the fit are displayed at the top right. These can be used to assess whether assay calibration was successful. Move the mouse pointer over a parameter to display a brief explanation of its meaning and function. A valid calibration curve should have the following parameters:

   - *relative dynamic range (rDR): at least 0.60, optimal > 0.80*
   - coefficient of determination (R^2): at least 0.990
   - slope parameter of the curve at the test midpoint (p): approx. 0.5–2.0, optimal around 1
   - IC parameter | Control | OTA | DON | FUM | ZEN | AFL | T-2

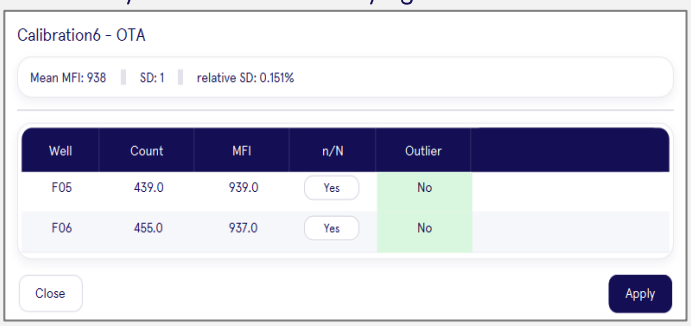
IC parameter	Control	OTA	DON	FUM	ZEN	AFL	T-2
IC50 min [µg/L]	0.250	0.180	4.35	3.00	1.10	0.0650	0.360
IC50 Max [µg/L]	2.40	1.28	90.0	26.0	7.00	0.600	9.20

   If a curve does not meet the guide values for these parameters, observe the notes in section 9.9

At the bottom of the window, the results of the individual calibration standards are summarised in a table, sorted by analyte. The calibration curve is calculated based on the mean fluorescence intensity (MFI, in a.u.) of the replicate measurements for each standard. These values 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 performed automatically and indicates possible outlier measurements. Double-clicking the individual rows of the table opens a detail view. In the n/N column, measurements included in the evaluation are marked "Yes". Clicking "Yes" changes the entry to "No" and excludes the corresponding measurement from the evaluation. Clicking again includes the measurement in the evaluation again. Click "Apply" to confirm your selection. Click "Close" to discard the changes.

Please note that the Grubbs test is intended only as an aid for identifying extreme measurement outliers and may produce unreliable conclusions, especially with a small sample size. For example, if 2 of the values from three replicate measurements are completely identical, the third value marked as an "outlier" should not be excluded.

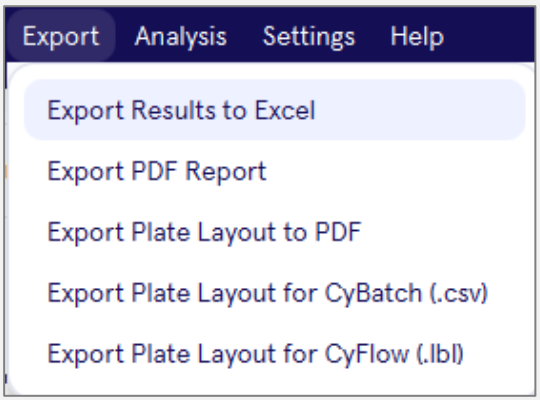


6. The results of the individual samples and reference samples are listed in the Samples and References workspaces (see Figure 15). Click Save to save your evaluation.
7. You can switch between Show Interpretation and Show Outlier using the corresponding buttons. The outlier view is designed analogously to the outlier view of the calibration curve and shows the results of a Grubbs outlier test.

Figure 15. Excerpt from the Samples workspace with an overview of the results of the individual samples. This is identical to the view in the References workspace.

Via Export, you have the option to export your results to Excel or as a PDF report. In the file, the corresponding sheets contain the summarised results of the measurements of the samples and reference samples, as well as the parameters of the individual calibration curves.

You can export the tables as an image using the Screenshot button.



7.5.1 Notes on data interpretation

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

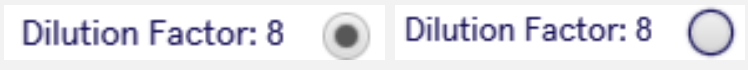
Colour coding of measurement results:

- **Green:** measurement result below the IC-20 value
- **Yellow:** measurement result above the IC-20 value
- **Orange:** measurement result above the IC-80 value
- **Red:** the internal control failed

Analyte	[µg/l]	Interpretation	n/N
Control	3.97 ± 0.437	failed (>3.3 µg/L)	3/3

If the measured MFI is greater than the A1 value (upper plateau of the curve), the measurement lies outside the calibration and mathematical quantification of the samples is not possible in this case. The result is therefore displayed as being below the concentration of A1. The values are automatically excluded from the calculation. The same applies to values that are lower than the A2 value (lower plateau of the curve). A value below A2 means that the analyte concentration is very high. Results in this range should be examined particularly critically; the probability of a measurement error is high. Also use the control measurement for interpretation and, if necessary, measure the sample again after strong dilution.

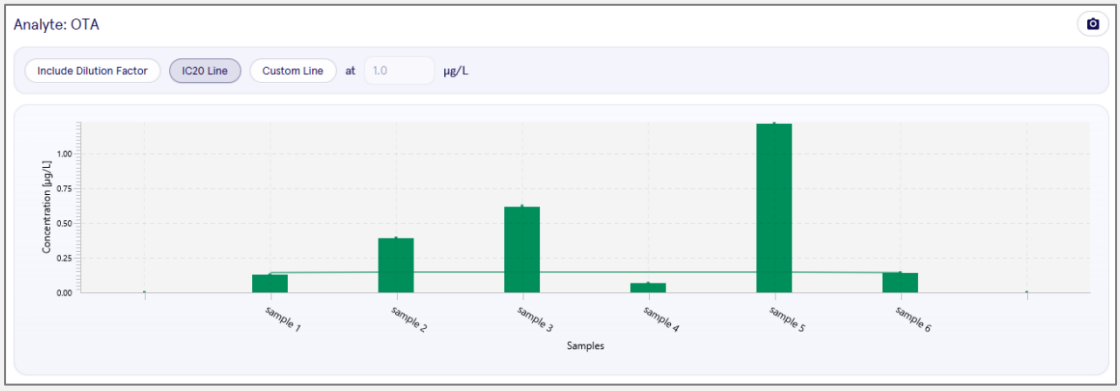
The dilution factor can be included in or excluded from the calculation by clicking.



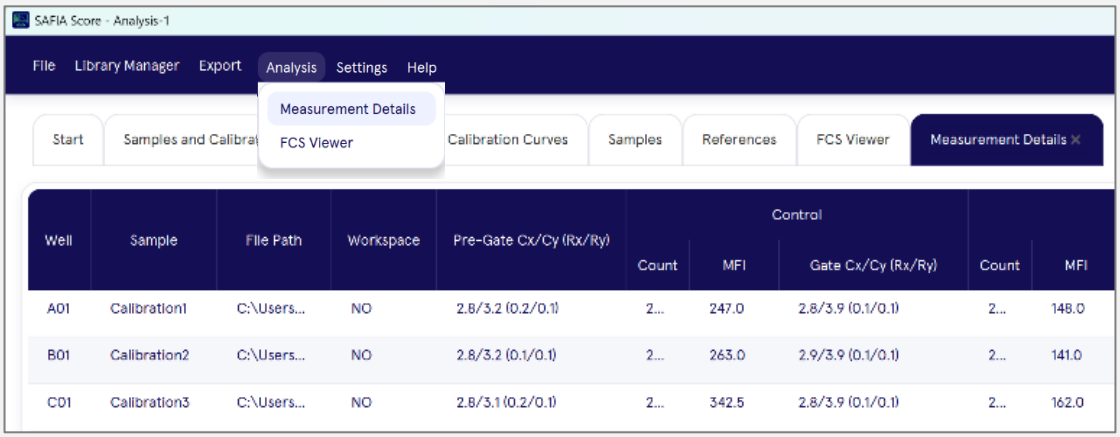
As with the tables in the **Calibration Curves** workspace, double-clicking opens a detail view. Here, individual measurements can be included in or excluded from the calculation (n/N "Yes" or "No").

In the lower area, the determined concentrations are displayed as a bar chart, sorted by analyte. The chart can also be copied using the Screenshot button. It is also possible to include the dilution factor in each chart and to display reference lines. If the IC-20 is displayed, the dilution factor must not be included.

The analyte Control is the control measurement. The control indicates whether a measurement result could be false positive. The control is considered passed if the determined value of a sample is lower than the IC20 value of the corresponding control calibration curve. This can be checked easily graphically. To do so, deactivate the Include dilution factor field and activate the IC-20 line field.



A table of the imported measurement data can be displayed in the **Analysis** menu.



This can be helpful when assessing individual measurements. For example, if no particles or only very few particles were detected, this can be seen in the Count column. The **Measurement Details** workspace is only active if the option has been enabled in the **Analysis** menu. This workspace can also be closed again if required.

7.5.2 Data view in the FCS Viewer

- In the **FCS Viewer**, measurement data are displayed analogously to the CyFlow view.
- On the left, the table shows the well ID, sample name and measurement file used. Under Status, you can see whether a measurement was included in or excluded from the evaluation; the reason for exclusion is shown in the Reason column if applicable. Events shows the total number of events, and Gates shows the number of detected gates.
- Two scatter plots are located on the right: in the FSC/SSC plot, all events are displayed according to size and granularity; SAFIA particles are automatically captured in the pre-gate (including event count). In the FSC/FL-3 plot, the measurement of the FL-3 detector is shown. It records the colour code of the SAFIA particles, which separates the particles previously captured in the pre-gate by analyte. Depending on the kit, up to seven populations with automatic gates and event counts are detected. Histograms for each analyte are shown below (FL1 vs. events). The peak centre corresponds to the MFI, from which the concentration is calculated via the calibration curve.

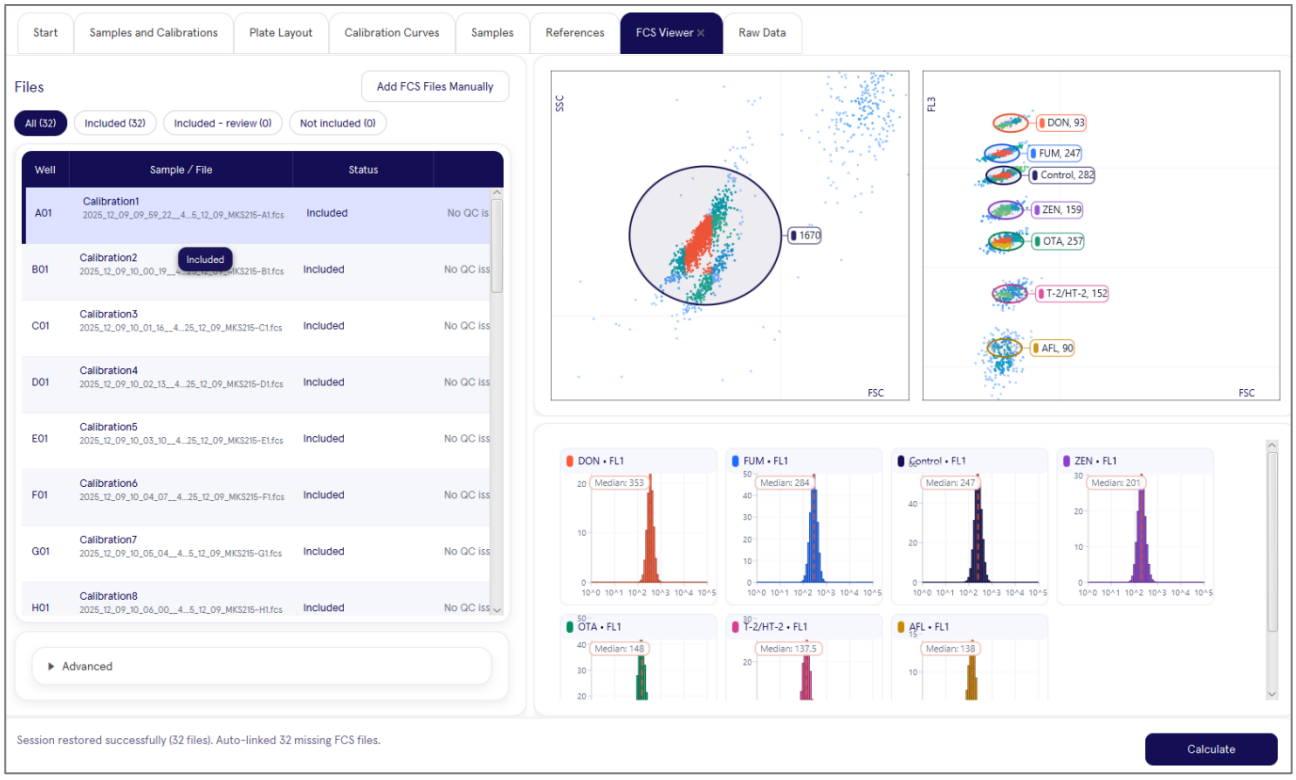
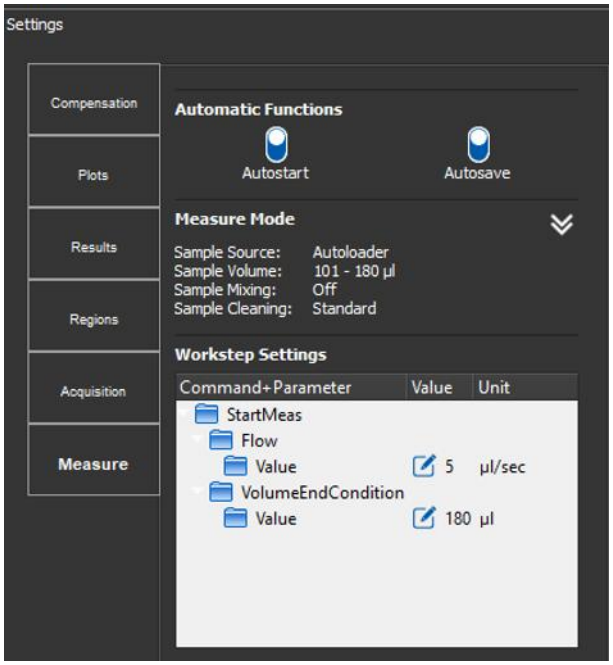


Figure 16. View of measurement data in the FCS Viewer in SAFIA Score

8 Cleaning and shutting down the Cube 6

To ensure proper function of the Cube 6, we recommend performing the following cytometer cleaning after completing the SAFIA measurements.

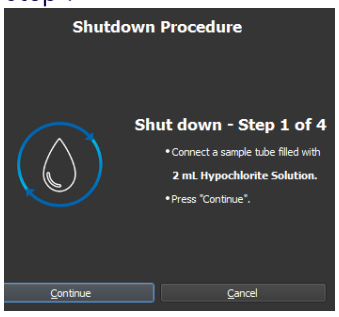
1. In two columns, fill 4 wells each with Hypochlorite Solution, 4 wells with Decontamination Solution, 4 wells with Cleaning Solution and 4 wells with Sheath Fluid.
2. Pull out the movable tray of the CyFlow® Robby Autoloading Station and place the microtiter plate on the designated platform. Please 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 the CFG button CFG .
4. Now click Work in the main bar.
5. Check whether the following settings are selected under Settings → Measure.
6. Perform the measurement analogously to section 7.4



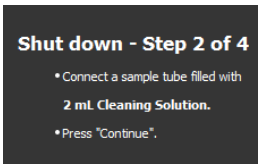
The .fcs files generated by the cleaning program do not contain meaningful data, but are nevertheless automatically saved in the folder C:\Users\Public\Documents\Cyflow. You can delete these files for better clarity.

7. Now perform the shutdown by clicking Shutdown Shutdown .
8. Start the program by clicking Start ▶ .
9. Follow the instructions through the Shutdown program; see Figure 17. After the device has finished, the CyView™ software closes automatically.

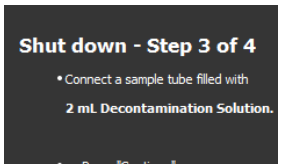
Step 1



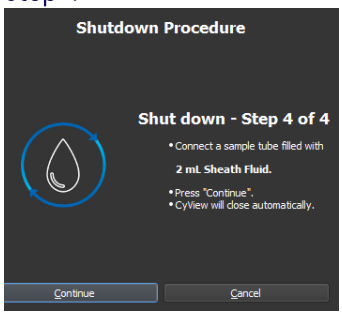
Step 2



Step 3



Step 4



Notes for special matrices

Matrix	Note
Soy	High proportions of soy result in OTA values that are too high. Remedy: soy products should be diluted somewhat more strongly.
Cinnamon	Cinnamon cannot be analysed with SAFIA.
Coffee	Coffee cannot be analysed with SAFIA.
Mango	At a mango fruit content of approx. 30% or more, an internal control error may occur. The internal control cannot be used for samples containing mango. The mycotoxin results are not affected.

9 Common errors and troubleshooting

9.1 Manual cleaning cycle

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

> ! Note that liquid is aspirated immediately as soon as the red Intermediate Cleaning button is pressed. Therefore, place the tube with the corresponding 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 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 .

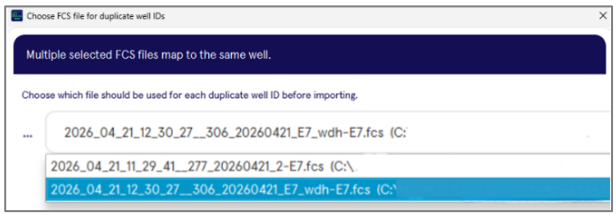
9.2 No particles are measured during the performance check or measurement

If no particles are detected, stop the measurement using the Stop button . Start a cleaning cycle using the Clean button (see section 9.1) or run the Prime program again (see section 3). Then repeat the measurement.

9.3 No particles are measured in individual wells

If you notice after a measurement that no particles were detected in individual wells, this may indicate that an air bubble in the Sysmex affected the measurement. In this case, perform another measurement with the same tray ID and specifically select the wells that you want to measure again.

When the same tray ID is used, the newly acquired measurement data are saved in the same folder as the previous data. When importing the .fcs files into SAFIA Score, you will then be prompted to select which of the two existing data sets should be used. Select the file with the later timestamp.

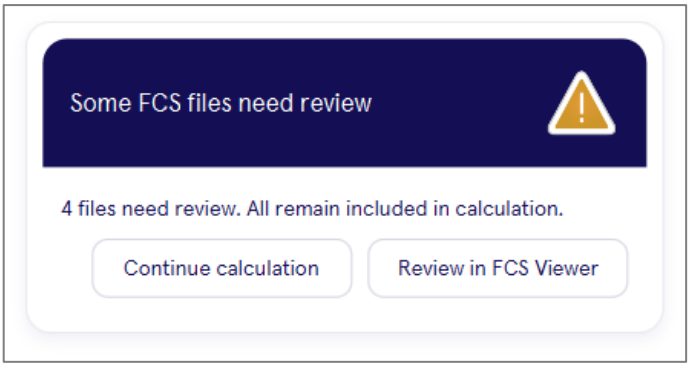


9.4 Particle populations are not in the gates

The particle populations appear "elongated" and are not in the gate (data look "strange"). This can happen if an air bubble is stuck in the measuring cuvette or if there is a device problem. In such cases, first perform a cleaning cycle and then restart the Prime program. If the problem persists, contact Sysmex customer service.

9.5 FCS review "Some FCS files need review"

If this message appears after loading your measurement results in SAFIA Score, one or more files are technically valid but have subject-specific abnormalities and should therefore be reviewed. The affected data are generally included in the evaluation.
Click "Continue calculation" to continue the evaluation without further review. Click "Review in FCS Viewer" to review the affected files.



Typical causes of this message are:

Cause	Recommended check/solution
Shifted populations	Check gates and, if necessary, regate or reset to average gates.
Estimated (not robust) gates	Check whether the average gates used are suitable for the measurement. Adjust gates if necessary.
Low event counts	Very low event counts significantly increase the uncertainty of the evaluation. However, a measurement is only excluded automatically if required gates are missing or if at least one required particle gate contains no events. Possible causes include insufficient mixing of the particle stock solution or particle working solution, as well as flow cytometer problems.
Unusual distributions within the expected structures	Check whether the gates are set correctly. If necessary, regate and reassess the distributions.

When you click "Review in FCS Viewer", the "FCS Viewer" tab opens. The table is filtered to show the measurements classified with the status "Used - Review". These measurements are highlighted in yellow.

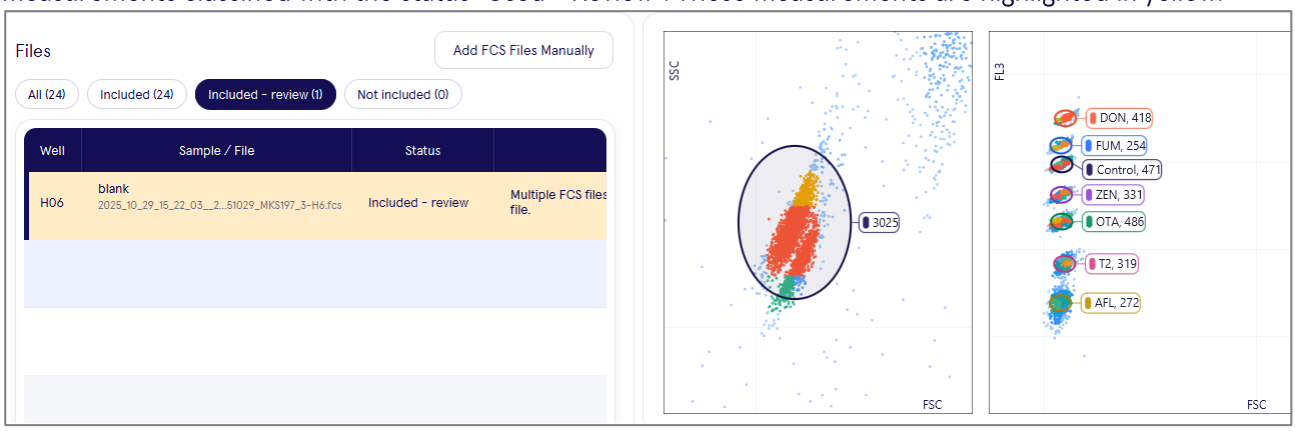


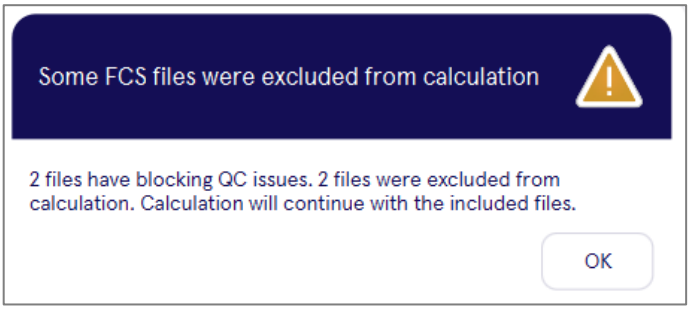
Figure 18. Overview view "Review in FCS Viewer"

When you click a highlighted measurement, a detail window opens. Under "QC Details", the reason for the classification as "Used – Review" is displayed. Check the data and, if necessary, perform manual regating. If the data are considered suitable after review, click "Apply and recalculate". If the measurement should not be included in the evaluation, click "Do not use measurement".

Figure 19. Detail view of a "Used – Review" file

9.6 FCS files excluded "Some FCS files were excluded from calculation"

If this message appears after loading your measurement results in SAFIA Score, one or more files have blocking technical or gate-related QC problems. By default, the affected data are not included in the calculation.



Typical causes of this message are:

Cause	Recommended check/solution
Missing or defective files	Check whether the plate layout stored in SAFIA Score matches the plate layout actually measured. If necessary, export and import missing or damaged FCS files again.
Incomplete gate structures	Check in the FCS Viewer whether the pre-gate and all expected particle gates are present. If gates were recognised incorrectly, use "Set to Average" or regate manually. Then click "Apply and recalculate". If the expected particle populations are actually not present, the measurement remains excluded.

Empty or invalid measurement data	Check whether the required particle gates contain events. If a required particle gate contains no events, reliable quantification is not possible for this measurement. Causes may include missing particles, insufficient mixing, measurement problems on the flow cytometer or strong background signal. If the gate was only set incorrectly, correct the gate and recalculate. The causes and solution steps are described in sections 10.2 and 10.3. Check the affected files for completeness and validity.
Import error	Ensure that "Direct FCS File Import" was selected during data import.

In the "FCS Viewer" tab, you can view the excluded files. The "Excluded" filter in the table filters and displays all excluded measurements. In the "Reason" column, you will find the respective QC note indicating the cause of exclusion. Click it to open the detail view in a separate window.

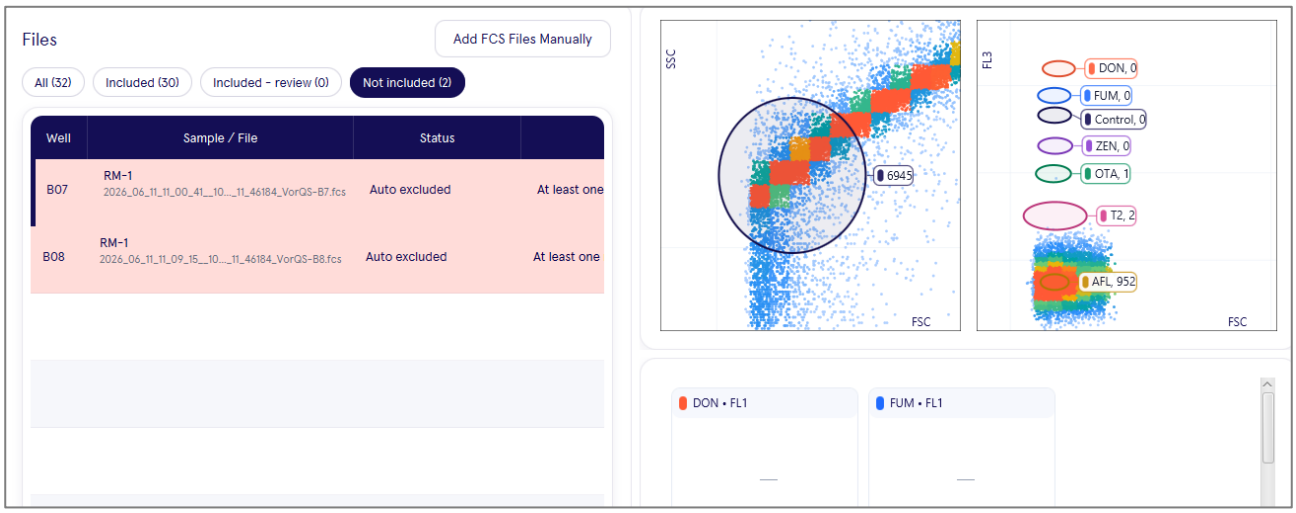


Figure 20. Overview view "FCS files excluded"

9.6.1 Example 1

Only very few SAFIA particles were measured, so no particles could be detected in some gates. Valid quantification is not possible under these conditions. The measurement therefore remains excluded.

Figure 21. Detail view example 1

9.6.2 Example 2

Due to strong background events, a local maximum was detected there instead of the particles. Click "Set to Average" for the pre-gate and particle gate. Based on the average gate positions of your other measurements, the gate is then shifted. You can zoom in or out further by scrolling with the mouse wheel in the plot.

Figure 22. Detail view example 2

You can now see that the average gate exactly includes the SAFIA particles. Now also click "Set to Average" for the particle gates.

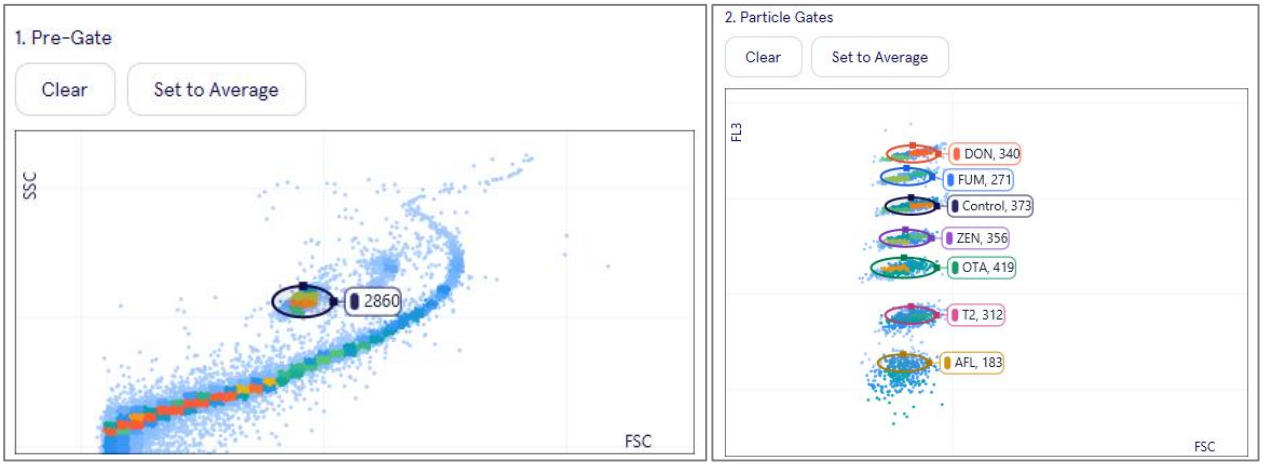


Figure 23. Detail view example 2 after "Set to Average" was applied

If you now click "Apply and recalculate", these data are included. They no longer appear under Excluded and are no longer highlighted in red.

9.7 Automatically set gates do not include all events in the population

During automatic gating (auto-gating), the software searches for local maxima. At this point, the MFI of many events is the same; it does not matter that not all events are included.

9.8 The 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. As a remedy, perform a cleanup step with PA or PVPP Adsorber to remove the interfering substances; see section 6. Also observe the notes for special matrices.

9.9 The calibration curve does not meet the guide values

9.9.1 Relative dynamic range, IC50, p values

If the calibration curve is too flat, the relative dynamic range and/or other calibration curve parameters such as the IC50 values and the slope parameter of the curve at the test midpoint (p) do not meet the guide values (see section 7.5). The samples cannot be quantified correctly. Check the kit's best-before date and always ensure correct kit storage (see section 2.1). Repeat the measurement with a new kit that is still within its shelf life. Contact SAFIA Technologies Support if the curves of a kit within its shelf life do not meet the guide values.

9.9.2 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 performed automatically and 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 (for duplicates). Outliers can be excluded easily, which increases the coefficient of

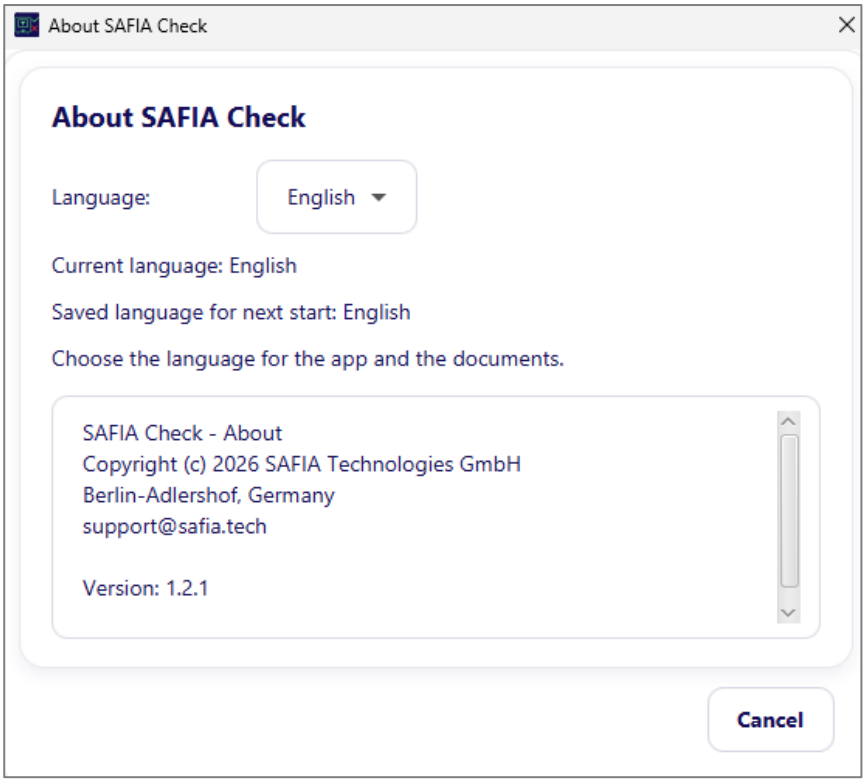
determination. When pipetting the calibration standards, ensure clean pipetting so that the coefficient of determination is greater than 0.990.

9.10 No evaluation in SAFIA Score

A common cause is that 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 cannot resolve the error.

9.11 Language setting in SAFIA Check

You can change the language in SAFIA Check under About.



9.12 Internet connection

The device does not have Wi-Fi functionality, but it can be operated completely without an internet connection. For individual functions, such as opening the current manual, and for support requests, an internet connection may nevertheless be useful. For internet access, the device can be connected via a LAN cable, a USB Wi-Fi adapter or a mobile phone connected by USB cable with USB tethering enabled.

10 Glossary

Term	Explanation
.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 the CyFlow® software.
.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 measured microtiter plate.
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.
Clean program	Cleaning cycle of the Cube 6
Configuration file	A file that contains specific settings for reading out the assay with the CyFlow® Cube 6 flow cytometer.
Dilution (Factor)	Dilution factor of a sample.
Event	An individual measurement event triggered when an object passes through the measurement area of the device and is detected. For each event, the signals for forward scatter (FSC), side scatter (SSC) and fluorescence intensities are recorded and stored.
FL-1	Fluorescence detector 1; detection channel for the fluorescence of the secondary antibodies in SAFIA.
FL-3	Fluorescence detector 3; detection channel for the fluorescence of the particle coding in SAFIA.
FSC	Forward scatter. Detector for measuring light scattered at a small angle. FSC and SSC are used to distinguish SAFIA particles from other particles in the sample.
Gate	Sorting window in flow cytometry. Used to separate relevant data from non-relevant 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 of the maximum signal (A1) in SAFIA. It can be

	used as an orientation 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 program	Rinsing program that brings the Cube 6 into operating condition.
R Square (R^2)	Coefficient of determination
Recovery rate	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	Replicate measurement
Sample ID	Unique sample designation
Sheath Fluid	Liquid used to operate a flow cytometer (hydrodynamic focusing).
SSC	*Side scatter. Detector for measuring light scattered at a 90° angle. FSC and SSC are used to distinguish SAFIA particles from other particles in the sample.*
MTP format	Microtiter plate format. Readout is performed in a 96-well plate via the CyFlow® Robby Autoloading Station.

11 Contact

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