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Benchmarking of Two Real-Time PCR-Based Rapid Sterility Testing Kits

Enhancing ATMP QC With Microsart® ATMP Sterile Release: A Comparison With Alternative Solutions

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Abstract

Advanced Therapy Medicinal Products (ATMPs) are increasingly conquering the pharmaceutical market and are necessary for the targeted treatment of various diseases. Before ATMPs can be administered their sterility must be ensured by testing them for bacterial and fungal contaminations. Patient safety is enhanced by using PCR-based rapid sterility tests, since the turnaround time is drastically reduced to few hours compared to 14 days for classical growth-based sterility testing. PCR techniques provide sensitive and accurate detection of bacterial and fungal DNA. In this application note the Microsart® ATMP Sterile Release kit is compared with an alternative PCR-based rapid sterility solution regarding their sensitivity on CFU and GC (genomic copies) level, and their convenience.

Introduction

Sterility Testing

Sterility testing is crucial for the release of cell therapy products, as microbial contamination can be fatal for recipients. The current compendial sterility test requires 14 days for most microbes to ultimately rule out any contaminations.[1,2,3] However, for cell-based therapeutics with a short shelf-life, particularly autologous cell therapies for critically ill patients, the time to result is a significant factor. Consequently, there is a growing demand for rapid, growth-independent tests. To meet this demand, we have developed and comprehensively validated a highly sensitive and broad-range microbial detection system to allow a rapid release in a matter of hours instead of days. This system combines the efficient Microsart® ATMP Extraction protocol for DNA isolation with the robust Microsart® ATMP Bacteria & Fungi real-time PCR assay. To guarantee a proper quality assurance with PCR-based methods, it is of great importance to detect contaminating DNA in the product reliably, but at the same time avoid false positives due to contamination of the sample during the test procedure or background DNA contamination of the test components themselves. A high level of sensitivity to ensure product sterility upon release is just as essential. These requirements were addressed by developing the efficient Microsart® ATMP protocols with a low number of hands-on steps eliminating the risk to introduce environmental contamination, coupled to a well-designed PCR assay including internal controls and TaqMan® probes to rule out false positives and negatives.

Through comparative benchmarking, this study highlights how the Microsart® ATMP Sterile Release kit combines efficiency in sample preparation and reliability in bacterial and fungal DNA detection, demonstrating advantages over alternative methods.

The Tested Kits

Microsart® ATMP Sterile Release
Microsart® ATMP Sterile Release is validated according to EP 5.1.6., EP 2.6.27., USP 1223 to rapidly detect bacterial and fungal contamination in various samples such as cell cultures, cell culture-derived biologicals, and ATMPs.

The delivered kit components include DNA-free processing tubes, Lysis Buffer, Suspension Buffer, and all the needed PCR reagents like an Internal Control DNA, a Bacteria and Fungi Master mix, the Positive Control DNA and PCR-grade water as well as Rehydration Buffer. The mentioned kit components and their purposes are summarized in Table 1.

To ensure a contamination/DNA-free working procedure during DNA extraction a Negative Extraction Control (NEC) is processed in parallel to the unknown samples. NECs and all unknown samples are processed using a minimal number of extraction steps to avoid cross contamination. Each sample is processed in duplicate.

Table 1: Microsart® ATMP Sterile Release DNA Free Kit Components and Their Functions

Material Lysis	Texture	Function	Storage conditions
DNA-free processing tube	plastic	Ensure contamination free DNA extraction process	Room temperature
Lysis Buffer	liquid	Lysing potential bacterial and fungal contaminants	
Suspension Buffer	liquid	Resuspending the isolated DNA pellet for further use in the PCR reactions	
Material PCR	**Texture**	**Function**	**Storage conditions**
Internal Control	lyophilized	Monitor the extraction process and PCR inhibition	+ 2 °C to + 8 °C after rehydration ≤ - 18 °C
Positive Control	lyophilized	Monitor the PCR functionality	
PCR-grade water	liquid	Used to rehydrate Internal Control and Positive Control upon usage	
Fungi Mix	lyophilized	Master mix for the detection of fungal contaminants	
Bacteria Mix	lyophilized	Master mix for the detection of bacterial contaminants	
Rehydration Buffer	liquid	Used to rehydrate Fungi and Bacteria Mix upon usage	

Bacterial and fungal contamination detection are performed using two different Master mixes namely the Bacteria mix and the Fungi mix. Bacteria and fungi species are specifically detected by amplifying a highly conserved 16S or 18S rRNA coding region, respectively. The amplification is detected at 520 nm (FAM™ channel) by employing specific TaqMan® probes to avoid false positive signals. Additionally, the ready-to-use Master mix includes ROX™-labeled probes (610 nm) which visualize the amplification of an Internal Control DNA (IC, Table 2) to prevent false negative results. This duplex assay including sample target and Internal Control target enables functionality testing of every measured reaction. The IC can either be added to each sample before starting the extraction process, to monitor the whole process including DNA extraction, or serve as a PCR control when only being added to the Master mix. Summarized, the IC detects false-negative results due to PCR inhibition or improper DNA extraction. To prove the functionality of the Master mix a Positive Control (PC) is provided. Every PCR test run should be completed with a No Template Control (NTC) to ensure the cleanliness of all PCR reagents. All controls are summarized in Table 3. Additionally, to the PC and NTC customers can perform positive extraction controls using Microsart® Validation Standards if required.

Results of this kit do not leave any room for interpretation. As soon as a Ct-value ≤ 40 is generated a contamination is detected. Only undetected samples are considered as negative (Table 4).

Table 2: Required Fluorescence Channels for Internal Control and Target Detection Using the Duplex Microsart® ATMP Sterile Release Kit

Channel	Target
ROX™	Internal Control
FAM™	Bacteria | Fungi

Table 3: Recommended Controls to Perform Along With a Microsart® ATMP Sterile Release PCR Sample Test Run

Abbreviation	Name	What is in there?	Extraction needed (Yes | No)	Purpose
NTC	No Template Control	PCR-grade water | Suspension Buffer	No	Prove PCR reagents and PCR setup cleanliness; rule out false positives
PC	Positive Template Control	Positive Control DNA	No	Prove PCR reagents functionality
NEC	Negative Extraction Control	Known negative sample matrix e.g., pure medium	No	Prove cleanliness of extraction process

Table 4: Interpretation of Results for Controls and Unknown Samples Using Microsart® ATMP Sterile Release

Channel	Sample type	Ct value definitions
FAM™	NTC	Expected to be negative
	PC	Expected to be positive (QC release criteria is a Ct-value between 22 and 26)
	NEC	Expected to be negative
	Unknown sample	≤ 40: contamination with bacterial | fungal DNA
ROX™	NTC	Expected to be positive (QC release criteria is a Ct-value between 27 and 31)
	PC	Irrelevant, if FAM™ is positive
	NEC	If used as PCR control, IC of FAM™ negative samples must show Ct values of ± 2 cycles of the NTC. If used as process control, IC of FAM™ negative samples must show Ct values of ± 3 cycles of the NTC.
	Unknown sample	If used as PCR control, IC of FAM™ negative samples must show Ct values of ± 2 cycles of the NTC. If used as process control, IC of FAM™ negative samples must show Ct values of ± 3 cycles of the NTC.

All kit components are freeze-dried or room-temperature stable materials and only need to be rehydrated upon usage (Table 1). A list of suitable real-time PCR cyclers enabling the detection in FAM™ and ROX™ channel can be found in the product's user manual.

Alternative Solution

The alternative solution is also designed to detect bacterial and fungal contaminations in biological samples. According to the product information from the supplier the kit meets the sensitivity requirements of EP 2.6.27 and USP 1071.

There are no DNA extraction reagents or protocol included in the alternative solution. It is recommended to combine the PCR kit either with the DNA extraction kit MolYsis™ Complete5 from Molzym or DNeasy PowerSoil extraction kit manufactured by QIAGEN. Thus, the competitor solution does not offer a completely validated process. In contrast, the Sartorius solution comes with a comprehensive validation of the complete workflow, consisting of DNA extraction and PCR detection.

Unlike the Microsart® ATMP Sterile Release kit, in which fungi and bacteria are detected in separate reactions using two different Master mixes, the alternative kit detects bacteria and fungi in a single reaction. The alternative solution is a multiplex assay and uses 5 different fluorescence channels in total, which poses the risk of losing efficiency. The ROX™ channel is utilized for reference dye detection. Bacterial contamination is detected in the VIC™ channel whereas fungal contamination is detected in the FAM™ channel.

Additionally, to the actual target detection, one channel is used to detect the Positive Control (AlexaFlour™ 647) and one for the Internal Process Control (ABY™). A summary of all necessary fluorescent channel is displayed in Table 5.

Table 5: Channels for Control and Target Detection Using the Alternative Solution

Channel	Target
ROX™	Reference dye
FAM™	Fungi
VIC™	Bacteria
Alexa Flour 647™	Discriminatory Positive Control
ABY™	Internal Process Control

According to the instructions for use multiple extraction controls and triplicates of all sample extractions (can be adjusted when sample volume is limited) should be processed: A Negative Extraction Control (NEC), a Sample Negative Extraction Control (SNEC), a Positive Extraction Control (PEC) and a Sample Positive Extraction Control (SPEC). To spike the Positive Extraction Controls and additionally serving as a PCR Positive Template Control (PTC) the kit comes with a frozen Discriminatory Positive Control which needs to be diluted prior to use. The dilution buffer is provided with the kit. All dilutions and the stock solution of the Discriminatory Positive Control should be kept on ice until use and during the process, as the kit components are not lyophilized and require - 25 °C to - 15 °C storage and handling.

Table 6: Competitors Kit Components and Their Functions

Material Lysis	Texture	Function	Storage conditions
Not included Extraction kits MolYsis™ Complete5 from Molzym or DNeasy PowerSoil extraction kit manufactured by QIAGEN are recommended			
Material PCR	**Texture**	**Function**	**Storage conditions**
Assay Mix	liquid	Includes primer and probes for fungal and bacterial contaminant detection as well as for amplifying Internal Process Control DNA*	- 25 °C to - 15 °C
2x qPCR Master mix Plus	liquid	Includes general PCR components e.g dNTPS, DNA Polymerase, mixed with Assay Mix to create functional Master mix*	- 25 °C to - 15 °C, after first thawed store at 2 °C to 8 °C
Discriminatory Positive Control (1×10^5 copies/µL)	liquid	Used as a positive control template as well as being diluted to serve as a spike for PEC and SPEC	- 25 °C to - 15 °C
DNA Dilution Buffer	liquid	Used to dilute Discriminatory Positive Control	- 25 °C to - 15 °C, after first thawed store at room temperature

Note: *interpretation by Sartorius, no clear function statement in the manual of the alternative solution.

Table 7: Required Controls for the Alternative Solution

Abbreviation	Name	What is in there?	Extraction needed (Yes/No)	Purpose
NTC	No Template Control	SDDB dilution buffer	No	Detecting contaminations
NEC	Negative Extraction Control	SDDB dilution buffer	Yes	Monitor for contamination of the extraction reagents, equipment, and work areas; Determine background residual contamination level of the sample preparation kit.
SNEC	Sample Negative Extraction Control	Sample Matrix	Yes	Determine background residual contamination level of the sample matrix
PTC	Positive Template Control	Discriminatory Positive Control dilution D2		Prove PCR reagents functionality*
PEC	Positive Extraction Control	SDDB + Spike1,000 | Spike100	Yes	Verify reagent and system performance; Evaluate the efficiency of DNA extraction.
SPEC	Sample Positive Extraction control	Sample Matrix + Spike1,000 | Spike100	Yes	Evaluate the efficiency of DNA extraction; Evaluate presence of PCR inhibitors in matrix.

Note: *interpretation by Sartorius, no clear purpose statement in the manual of the alternative solution.

The delivery of frozen kit components requires a well-functioning cooling chain during the whole transport. For further details about the controls see Table 7.
The Internal Process Control is an integrated part of the provided Master mix and can hence not be used as an extraction control at the same time. Thus, the described control set up does not allow an extraction efficiency control for every single sample but only for the separately extracted PECs and SPECs.

For PCR Master mix set up two components are delivered. A universal 2x qPCR Master mix Plus and a specific Assay Mix. Both frozen components must be prepared according to the instruction for use after thawing on ice. An information on all delivered components and their function can be found in Table 6.

The kit instructions list multiple cut-off definitions for the interpretation of generated Ct-values, of all control types and the unknown samples (Table 8). Cut-off settings are necessary to substract default background signals created by the delivered PCR chemistry and DNA extraction kit solutions. An automated analysis of the data is only possible using the real-time PCR cycler and software of the kit manufacturer, for all other real-time PCR cyclers the complex analysis is done manually.

Table 8: Interpretation of Results for Controls and Unknown Samples for the Alternative Solution

Sample Type	Channel	Ct-value definitions
NTC	FAM™	≥ 36
	VIC™	≥ 35.5
	Alexa Flour™ 647	no signal
	ABY ™	27.5 – 30.5
NEC	FAM™	≥ 33
	VIC™	≥ 34
	Alexa Flour™ 647	no signal
SNEC	FAM™	≥ 33
	VIC™	≥ 34
	Alexa Flour™ 647	no signal
PTC	FAM™	≤ 25
	VIC™	≤ 26
	Alexa Flour™ 647	≤ 25
PEC	FAM™	≤ 33
	VIC™	≤ 34
	Alexa Flour™ 647	no criteria listed
SPEC	FAM™	≤ 33
	VIC™	≤ 34
	Alexa Flour™ 647	no criteria listed
Unknown sample	FAM™	< 33 fungi present ≥ 33 no contamination
	VIC™	< 34 bacteria present ≥ 34 no contamination
	Alexa Flour™ 647	no criteria listed
All samples except NTC	ABY ™	Ct ≤ NTC ABY™ mean Ct + 2

Aim of This Study

Within this study the two PCR-based rapid sterility kits, Microsart® ATMP Sterile Release from Sartorius (SMB95-1007) and an alternative solution were compared. Microsart® ATMP Sterile Release is a fully validated solution. It includes all necessary PCR reagents and DNA extraction material with a respective protocol. In contrast, the alternative solution does only provide reagents for the PCR reactions but no validated DNA extraction. It must be combined with third party extraction kits (MolYsis™ Complete5 from Molzym or DNeasy PowerSoil extraction kit from QIAGEN) and validated from scratch.

Focus of the study is the PCR sensitivity and product convenience. PCR sensitivity was analyzed using *Bacillus subtilis* and *Candida albicans* genomic DNA dilution series (10,000, 1,000, 100, 10, 1 GC/µL) without prior DNA extraction. Detection sensitivity of the complete workflow including DNA extraction was assessed using *Bacillus subtilis* and *Candida albicans* EZ-CFU™ dilution series (100, 50, 25, 10 and 5 CFU/mL). User friendliness and convenience was evaluated during both experimental set-ups.

Materials and Methods

All protocols were performed according to the respective instructions for use provided by the manufacturers under sterile and DNA-free conditions. Separate laminar flow cabinets were used to perform DNA extraction and PCR set-up. The working area and equipment were rigorously cleaned with chlorine-based cleaning reagents. Protective clothing included lab coat, gloves, and arm sleeves. Positive Template Controls were added in a separate room to avoid any cross contamination of samples.

Sample Preparation

PCR sensitivity on DNA level was tested using quantified genomic DNA. As bacterial spike *Bacillus subtilis* and as fungal spike *Candida albicans* were chosen. Microsart® Calibration Reagents (Sartorius, SMB95-2044, SMB95-2023) of both species were diluted according to the manufacturers manual and tested in duplicates. The dilutions used for PCR were 10,000 GC/µL, 1,000 GC/µL, 100 GC/µL, 10 GC/µL and 1 GC/µL.

PCR sensitivity on CFU level was investigated by using *Bacillus subtilis* and *Candida albicans* EZ™-CFU material (0443C, 0486C; Microbiologics). Both species were rehydrated following the instructions for use. The CFU standards were diluted in DMEM (FG0415-500ML, Life Technologies limited) supplemented with 10% FBS (S0615, Merck Millipore) to 100, 50, 25, 10 and 5 CFU/mL. Calculated CFU counts were confirmed by plating on tryptic soy agar plates (11860842, Fisher Scientific).

DNA Extraction

Microsart® ATMP Sterile Release

The DNA extraction was performed following the product manual. Two NECs of 1 mL DMEM 10% FBS were prepared and processed together with 1 mL of previously prepared 100, 50, 25, 10 and 5 CFU/mL dilutions of *Bacillus subtilis* and *Candida albicans*. The DNA extraction included a centrifugation step to eliminate free microbial DNA, addition of the IC (that was rehydrated according to the instructions for use), a 10-minute lysis step combined with heating and a subsequent rehydration of the DNA pellet. The time needed to perform the extraction was 1h. Each sample was extracted in duplicate.

Alternative Solution - MolYsis™ Complete5

The DNA extraction was performed following the instructions for use provided with the MolYsis™ Complete5 kit (D-321-050, Molzym). As recommended in the alternative solution instructions for use a NEC, SNEC, PEC and SPEC were included during the extraction process. This preparation requires the dilution of the provided Discriminatory Positive Control and the correct pipetting of the extraction controls. SNEC and SPEC were prepared using DMEM 10% FBS as a sample matrix. For PEC and SPEC preparation the so-called Spike100 (see instructions for use) was chosen. MolYsis™ Complete5 is a DNA extraction kit designed for differential lysis. The combined instructions for use of MolYsis™ Complete5 kit and the alternative solution include a centrifugation step of 1 mL sample, a lysis step for potential host cells or cell background, a degradation of the released cell DNA followed by a lysis for microbial organisms. As soon as bacterial and fungal cells are disrupted the DNA was extracted using a column-based extraction. Used enzymes should be kept as cold as possible using freezing racks and immediate storage at -20 °C after usage. The time needed to perform the extraction was approx. 4h. All controls were extracted once, and all samples were extracted in duplicate.

PCR Set-Up

Microsart® ATMP Sterile Release

The PCR reagents (Master mix, i.e. Bacteria Mix and Fungi Mix, and Positive Control) were rehydrated following the instructions of the provided user manual (SMB95-1007, Sartorius). All necessary steps were performed at room temperature since the PCR is based on a Hot-start DNA-polymerase. To set up the PCR reaction, 0.2 mL PCR tubes (710970, Biozym) were filled with 15 µL Bacteria Mix or Fungi Mix. Enough tubes were prepared to measure every extracted sample (including NECs) in duplicate and additionally two NTCs and PCs were added to the PCR experiment. For NTC and PC reactions 1 µL IC was added to the Bacteria or Fungi Mix. 10 µL of the extracted samples were pipetted to the respective Master mixes. For NTCs the Suspension Buffer from the extraction process served as input and the rehydrated Positive Control was used for PC reactions. Subsequently, closed PCR tubes were vortexed and centrifuged briefly. Cycler settings and temperature profile were entered and used according to the manual.

Alternative Solution

Frozen qPCR Master mix and the Assay mix were thawed on ice and subsequently prepared on ice as described in the instructions for use. To set up the PCR reaction, 0.2 mL PCR tubes (710970, Biozym) were filled with 17 µL of the prepared Master mix. Enough tubes were prepared to measure every extracted sample in duplicate including NEC, SNEC, PEC and SPEC. 13 µL of each sample and control was added to the previously pipetted Master mix. The SDDB (dilution buffer) was used as a template for NTCs. PTC was generated by pipetting the so-called dilution D2 of the provided Discriminatory Positive Control into the Master mix. This dilution already was prepared following the instructions for use earlier during the extraction control preparation. All PCR tubes were vortexed and centrifuged briefly. Cycler settings and temperature profile were entered and applied according to the manual.

Results

PCR Sensitivity on DNA Level

To generate data on the PCR sensitivity, quantified genomic DNA of *B. subtilis* and *C. albicans* was measured according to the manual for both PCR solutions. The DNA dilution series was reliably detected for both species using the Microsart® ATMP Sterile Release chemistry (Table 9, Table 10).

A concentration-dependent change in the Ct-value was observed as expected. All control reactions (PCs, NTCs, ICs) fulfilled the acceptance criteria and showed results according to the expectations.

Table 9: Generated Ct Values and Interpretation of Results for Measured *B. subtilis* Genome Copies/µL (10,000, 1,000, 100, 10, 1 GC/µL) by Using Microsart® ATMP Sterile Release (FAM™-Channel) and Alternative Solution (VIC™-Channel)

		Microsart® ATMP Sterile Release				Alternative solution			
Sample	Expected result	Ct values			Number of replicates according to expected results	Ct values			Functional replicates according to expected results
NTC	Negative	ND	ND	ND	3/3	36.49	ND	37.09	3/3
PC/ PTC	Positive	28.80	26.54	/	2/2	24.44	24.44	/	2/2
B. subtilis 10,000 GC/µL	Positive	19.45	19.47	/	2/2	17.45	17.44	/	2/2
B. subtilis 1,000 GC/µL	Positive	22.07	22.09	/	2/2	21.08	21.07	/	2/2
B. subtilis 100 GC/µL	Positive	25.63	25.60	/	2/2	24.39	24.59	/	2/2
B. subtilis 10 GC/µL	Positive	28.99	29.03	/	2/2	27.92	27.67	/	2/2
B. subtilis 1 GC/µL	Positive	32.41	32.58	/	2/2	30.83	31.00	/	2/2

Note: Each sample was tested in duplicate. (ND = not detected).

Table 10: Generated Ct Values and Interpretation of Results for Measured *C. albicans* Genome Copies/µL (10,000, 1,000, 100, 10, 1 GC/µL) by Using Microsart® ATMP Sterile Release (FAM™-Channel) and Alternative Solution (FAM™-Channel)

		Microsart® ATMP Sterile Release				Alternative solution			
Sample	Expected result	Ct values			Number of replicates according to expected results	Ct values			Functional replicates according to expected results
NTC	Negative	ND	ND	ND	3/3	ND	ND	ND	4/4
P/ PTC	Positive	25.96	25.83	/	2/2	24.53	24.46	/	2/2
C. albicans 10,000 GC/µL	Positive	14.97	15.00	/	2/2	14.78	14.88	/	2/2
C. albicans 1,000 GC/µL	Positive	18.18	18.18	/	2/2	18.47	18.58	/	2/2
C. albicans 100 GC/µL	Positive	21.62	21.65	/	2/2	21.75	21.59	/	2/2
C. albicans 10 GC/µL	Positive	24.70	24.40	/	2/2	24.85	25.22	/	2/2
C. albicans 1 GC/µL	Positive	28.21	28.19	/	2/2	27.99	28.30	/	2/2

Note: Each sample was tested in duplicate. (ND = not detected)

Also the alternative solution detected the DNA dilution series of both species successfully (Table 9, Table 10). The assay showed expected Ct-value shifts due to the serial dilution of the genomic DNA standards.
The PTC revealed reliable results proving the assay functionality as well as the IC measured in the ABY™-Channel which fulfilled the acceptance criteria in all samples. The NTCs generated Ct values for two out of three replicates (36.49 and 37.09) for the bacterial target which are still considered as negative due to the kit specific Ct-value cut-offs shown in Table 8.

When focusing on the detection of bacterial targets, exemplified by *B. subtilis*, both kits detected down to 1 GC/µL successfully, but for all GC/µL concentrations the alternative solution delivered lower Ct values. Given that the NTCs also generated significant Ct-values, this is rather due to a higher initial bacterial DNA load in the PCR chemistry, than due to a higher PCR sensitivity.

PCR Sensitivity on CFU Level

Spikes of 100, 50, 25, 10, 5 CFU/mL *B. subtilis* or *C. albicans* diluted in DMEM supplemented with 10% FBS were extracted and subsequently measured with both Microsart® ATMP Sterile Release and the alternative solution PCR chemistry.

Candia albicans Detection

Processing the Microsart® ATMP Sterile Release workflow, including DNA extraction and PCR, all concentrations of *C. albicans* dilutions were detected reliably. The Ct-values shifted according to the dilution levels from ~ 28 for 100 CFU/mL to ~ 33 for 5 CFU/mL (Table 11). The controls met the acceptance criteria. A signal was detected in the FAM™ channel for the PC, while no signal was detected in this channel for the NTC, confirming the absence of contamination. The Internal Control behaved as expected, with a signal detected in the PC, NTC, NEC and in the sample reactions.

Table 11: Generated Ct Values (FAM™-Channel) and Interpretation of Results for Measured *C. albicans* CFUs/mL (100, 50, 25, 10, 5 CFU/mL) by Using Microsart® ATMP Sterile Release After Extracting the DNA With the Kit Included Extraction Material

Sample	Expected result	Ct value				Number of replicates according to expected results
		Extraction 1		Extraction 2		
C. albicans 100 CFU/mL	Positive	28.95	28.95	29.65	29.27	4/4
C. albicans 50 CFU/mL	Positive	28.84	29.69	29.47	29.17	4/4
C. albicans 25 CFU/mL	Positive	30.79	30.36	30.59	30.51	4/4
C. albicans 10 CFU/mL	Positive	32.06	32.07	31.82	31.59	4/4
C. albicans 5 CFU/mL	Positive	33.76	33.84	32.48	33.25	4/4
NEC	Negative	ND	ND	ND	ND	4/4
PCR controls (no extraction)						
NTC	Negative	ND	ND	ND	ND	4/4
PC	Positive	25.96	25.83	24.86	24.89	4/4

Note: Each sample was extracted in duplicate, and each extract was then PCR-tested in duplicate. (ND = not detcted)

The alternative solution successfully detected all concentrations of *C. albicans*, with Ct-values ranging from ~26 to ~30, which were within the cut-off specification (Ct < 33 indicates fungi present; Ct ≥ 33 indicates no fungal contamination). All NECs and three out of four SNECs had Ct-values (Table 12) that were considered negative based on the cut-off specifications (Table 8). NTCs showed no signal, confirming the cleanliness of the reagents. However, none of PECs or SPECs met the cut-off (Ct < 33), so these controls were classified as failed. Even after re-extraction, including spiking PEC and SPEC samples with Spike1,000, the Discriminatory Positive Control could not be detected via PCR for these samples. According to the alternative solution's manual, there was no inhibition, as the ABY™ signal difference was within acceptable limits (Ct > 2.0 compared to other samples). However, the manual provided no further guidance on resolving the failed PEC and SPEC detection. Lastly, the PTC functioned correctly, confirming the overall PCR functionality.

Bacillus subtilis Detection

When it comes to the sensitivity of *B. subtilis* using the Microsart® ATMP Sterile Release workflow 25 CFU/mL were still reliably detected for all four PCR replicates. For the 10 CFU/mL and 5 CFU/mL concentrations only one out of four replicates remained undetected (Table 13). Again, all NECs were clean as well as the NTCs. Expected amplification of the PC proved the assay functionality.

Regarding the sensitivity of *B. subtilis* using the alternative solution two out of four replicates failed the detection of a contamination of 50 CFU/mL. Referring to the alternative solutions' manual samples that are positive for contamination in more than one-third of the tested wells can be determined to be contaminated. For all lower concentrations no detection was possible (Table 14). Even though NECs, SPECs and NTCs were detected negative, and no bacterial contamination was detected after applying the cut-off specifications (Table 8), the PECs and SPECs again did not meet the required Ct value of ≤ 34 to be considered as positive. The PC Ct-values < 26 confirmed PCR functionality.

Summarized the PCR sensitivity on CFU level was equal for both kit solutions in case of *Candida albicans* detection but revealed the inability of the alternative solution to detect < 50 CFU/mL for *Bacillus subtilis*.

Table 12: Generated Ct Values (FAM™-Channel) and Interpretation of Results for Measured *C. albicans* CFUs/mL (100, 50, 25, 10, 5 CFU/mL) by Using Alternative Solution After Extracting the DNA With the Recommended Extraction Solution

Sample	Expected result	Ct value				Number of replicates according to expected results
		Extraction 1		Extraction 2		
C. albicans 100 CFU/mL	Positive	26.11	26.33	26.03	26.12	4/4
C. albicans 50 CFU/mL	Positive	27.16	27.16	26.72	26.93	4/4
C. albicans 25 CFU/mL	Positive	28.19	28.21	28.19	28.07	4/4
C. albicans 10 CFU/mL	Positive	29.32	29.24	28.33	28.37	4/4
C. albicans 5 CFU/mL	Positive	30.26	30.13	30.20	30.33	4/4
NEC	Negative	36.89	35.93	38.97	38.59	4/4
SNEC	Negative	36.05	38.95	38.27	ND	4/4
PEC (Spike 100)	Positive	ND	35.81	39.6	38.7	0/4
SPEC (Spike 100)	Positive	ND	36.98	ND	ND	0/4
PCR controls (no extraction)						
NTC	Negative	ND	ND	ND	ND	4/4
PTC	Positive	24.53	24.46	24.34	24.32	4/4

Note: Each sample was extracted in duplicate, and each extract was then PCR-tested in duplicate. Values marked in grey varied from expected results. (ND = not detected)

Table 13: Generated Ct Values (FAM™-Channel) and Interpretation of Results for Measured *B. subtilis* CFUs/mL (100, 50, 25, 10, 5 CFU/mL) by Using Microsart® ATMP Sterile Release After Extracting the DNA With the Kit Included Extraction Material

Sample	Expected result	Ct value				Number of replicates according to expected results
		Extraction 1		Extraction 2		
B. subtilis 100 CFU/mL	Positive	32.94	33.21	33.55	33.60	4/4
B. subtilis 50 CFU/mL	Positive	34.76	34.56	34.08	33.75	4/4
B. subtilis 25 CFU/mL	Positive	36.31	36.23	35.02	35.80	4/4
B. subtilis 10 CFU/mL	Positive	ND	37.01	36.83	36.01	3/4
B. subtilis 5 CFU/mL	Positive	37.85	39.54	ND	39.20	3/4
NEC	Negative	ND	ND	ND	ND	4/4
PCR controls (no extraction)						
NTC	Negative	ND	ND	ND	ND	4/4
PC	Positive	28.80	26.54	25.40	25.36	4/4

Note: Each sample was extracted in duplicate, and each extract was then PCR-tested in duplicate. Values marked in grey varied from expected results (ND = not detected)

Table 14: Generated Ct Values (VIC™-Channel) and Interpretation of Results for Measured *B. subtilis* CFUs/mL (100, 50, 25, 10, 5 CFU/mL) by Using Alternative Solution After Extracting the DNA With the Recommended Extraction Solution

Sample	Expected result	Ct value				Number of replicates according to expected results
		Extraction 1		Extraction 2		
B. subtilis 100 CFU/ mL	Positive	31.40	31.89	33.04	32.47	4/4
B. subtilis 50 CFU/ mL	Positive	34.71	35.54	32.01	32.15	2/4
B. subtilis 25 CFU/ mL	Positive	34.40	34.25	34.29	34.83	0/4
B. subtilis 10 CFU/ mL	Positive	36.77	35.66	34.55	35.72	0/4
B. subtilis 5 CFU/ mL	Positive	35.46	37.31	35.48	36.38	0/4
NEC	Negative	35.61	34.79	34.34	35.13	4/4
SNEC	Negative	36.64	35.69	37.81	37.15	4/4
PEC (Spike 100)	Positive	36.87	34.45	37.39	35.85	0/4
SPEC (Spike 100)	Positive	37.06	35.95	ND	37.47	0/4
PCR controls (no extraction)						
NTC	Negative	36.49	ND	37.09	ND.	4/4
PTC	Positive	24.44	24.44	24.08	24.01	4/4

Note: Each sample was extracted in duplicate, and each extract was then PCR-tested in duplicate. Values marked in grey varied from expected results. (ND = not detected)

Discussion

PCR Sensitivity on DNA Level

As shown in the results, both kits detected genomic DNA dilutions of *B. subtilis* and *C. albicans* down to 1 GC/µL (Tables 9 and 10). The Ct-values for fungal target detection demonstrated similar PCR sensitivity between the Microsart® ATMP Sterile Release kit and the alternative kit. However, the bacterial assay identified background bacterial DNA in the alternative solution reagents, potentially leading to false positives. In contrast, the Microsart® ATMP Sterile Release kit's DNA-free reagents avoided such background signals, ensuring no bacterial detection in NTCs. Thus, the lower Ct-values observed in the alternative bacterial assay stem from reagent background, not increased sensitivity. Overall, Microsart® ATMP Sterile Release provides more reliable PCR data by using ultra-clean, DNA-free reagents.

Sensitivity on CFU Level for *B. subtilis* and *C. albicans*

For *C. albicans* detection, both workflows demonstrated high sensitivity, reaching 5 CFU/mL (Tables 11 and 12). While the Microsart® ATMP Sterile Release workflow validated all controls as expected, the alternative workflow yielded negative PEC and SPEC results, with no guidance in the manual for interpretation. Though fungal spikes were detected in the PCR, these negative PEC and SPEC results suggest suboptimal assay functionality.

Sensitivity for *B. subtilis* varied significantly between the two rapid sterility testing kits. The Microsart® ATMP Sterile Release kit consistently detected 25 CFU/mL (Table 13), and even detected three out of four samples with 10 or 5 CFU/mL. By contrast, the alternative solution struggled with consistent detection of 50 CFU/mL (Table 14) and missed lower contamination levels altogether due to strict cut-off settings (Table 8). These cut-offs, intended to compensate reagent contamination, instead led to false negatives, ultimately demonstrating reduced sensitivity compared to Microsart® ATMP Sterile Release.

Convenience

All components in the Microsart® ATMP Sterile Release kit are room temperature stable or lyophilized, allowing for shipment without dry ice and immediate use after rehydration. In contrast, the alternative solution requires shipment on dry ice, thawing on ice before use, and handling on ice throughout the experiment, which necessitates laboratory ice availability and very precise timing for reagent thawing. In addition, there is a significantly greater effort involved in the safe delivery of the kit. Even though the possibility of performing the bacterial and fungal contamination test within one sample as offered by the alternative solution is advantageous, still, the higher multiplex level in combination with the cut-off criteria complicate the data analysis when no automated analysis tool is used.

Since MolYsis™ Complete5 is a kit designed for differential lysis, it includes much more steps than the minimalistic designed extraction protocol included in the Microsart® ATMP Sterile Release kit. This results in a total hands-on time of 5 – 6 h compared to 1.5 h when using Microsart® ATMP Sterile Release kit.

The much longer hands-on time and time-to-result for the competitor workflow is also driven by the preparation of various controls (NEC, SNEC, PEC, SPEC). To prepare these controls it is of great importance to combine the protocols of the alternative solution instruction for use and the recommended DNA extraction kit MolYsis™ Complete5. Both manuals must be understood separately and both complex protocols need to be combined by the operator, as no clear combined guidance is given. For example, it is necessary to prepare the Discriminatory Positive Control and all extraction controls according to the instruction for use prior to focusing on the MolYsis™ Complete5 kit. In contrast, when using the Microsart® ATMP Sterile Release kit all experimental steps are harmonized, and the operator only needs to follow one straight protocol.

Talking about controls and recommendations according to the manuals, the alternative solution foresees a higher number of reactions per tested sample than the Microsart® ATMP Sterile Release kit, leading to a higher workload and total PCR reactions needed in order to release one sample (Table 15).

Finally, one of the most important things in sterility testing is avoiding bacterial and fungal DNA contamination while performing the test leading to false-positive signals. This can only be achieved by using microbial DNA-free reagents, consumables and working in a suitable laboratory set-up. Within the Microsart® ATMP Sterile Release kit all working steps are reduced to an absolutely necessary minimum while the alternative solution's kit includes various dilution steps of the Positive Control, mixing of two components to create the final Master mix and an elongated lysis protocol which bears a high risk of contamination.

Table 15: Extraction Reactions and PCR Reactions Needed to Perform Microsart® ATMP Sterile Release Workflow and Alternative Solution Workflow for One Unknown Sample

	Microsart® ATMP Sterile Release workflow		Alternative solution Workflow	
	Extraction	PCR	Extraction	PCR
NTC	-	2	-	3
NEC	1	2	1	3
SNEC	-	-	1	3
PC | PTC	-	2	-	3
PEC	-	-	1	3
SPEC	-	-	1	3
Unknown Sample	2	4	3	9
Total	**3**	**10**	**7**	**27**

Conclusion

The Microsart® ATMP Sterile Release kit demonstrates superior reliability, sensitivity, and convenience over the alternative kit in detecting low levels of bacterial and fungal contamination. It is a fully validated, end-to-end solution that includes both DNA extraction and PCR detection components. In contrast, the alternative solution lacks a validated extraction protocol, requiring users to pair it with external kits, which introduces variability and potential for error.

With minimal handling requirements and a straightforward, single-protocol workflow, Microsart® ATMP Sterile Release significantly reduces contamination risks and workload, offering an efficient and user-friendly sterility testing solution. Its DNA-free reagents avoid false positives and provide consistent sensitivity without reagent background interference.

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