SARTORIUS

Application Note

November 2025

Keywords or phrases:
Sterility Testing, Bacteria, Fungi, ATMP, CGT, NAT, digital PCR, droplet digital PCR, Cyclus® dPCR Tool Box Bacteria Fungi, Rapid QC Release, Rapid Sterility Testing

Cyclus® dPCR Tool Box Bacteria Fungi

Cutting-Edge Digital PCR Technology Facilitating Rapid Sterility Testing in Cell and Gene Therapies and Short Shelf-Life Applications

Lisa Hollstein[1], Michel Gerke[1], Dr. Alexandra Müller-Scholz[1], Dr. Robert Hertel[1], Dr. Diana Patzelt[2]

[1] Sartorius, Goettingen, Germany

[2] Sartorius, Aubagne, France

Correspondence

*Email: PCR@sartorius.com

Abstract

Rapid sterility testing is essential for the release of products with short shelf lives, as microbial contaminations can be detrimental to recipients. The current compendial sterility test requires 14 days to rule out contaminations with certainty. However, for applications with limited shelf life, such as cell based therapeutics and other critical medicinal products, the time to result is a significant factor in ensuring timely delivery. Therefore, reliable and accurate rapid sterility testing methods are necessary to meet these demands efficiently. Cyclus® dPCR Tool Box Bacteria Fungi was designed to provide rapid QC results in short shelf life applications such as rapid sterility testing in cell and gene therapy products. It leverages advanced digital PCR technology to enhance the sensitivity and robustness of rapid testing, enabling the detection of as few as 10 CFU of a contaminant in one milliliter of product.

For further information, visit sartorius.com

Introduction

With Cyclus® dPCR Tool Box Bacteria Fungi, Sartorius brings the Microsart® ATMP Sterile Release TaqMan™ assay - validated according to EP 5.1.6.[1], EP 2.6.27[2]. and USP <1223>[3] - to the next level to facilitate digital PCR based rapid sterility testing. Cyclus® dPCR Tool Box Bacteria Fungi is optimized for use with digital PCR workflows, such as the QIAGEN QIAcuity and Bio-Rad QX systems™. It consists of a Bacteria Primer- & Probes Mix for the broad-range detection of bacterial contaminants, a Fungi Primer- and Probes Mix for the detection of clinical relevant fungal contaminants, an Internal Control DNA suitable for DNA extraction monitoring and serving as an amplification control to monitor PCR Inhibition, a Positive Control for conforming functionality of the bacteria and fungi assays, and PCR grade Water for the No Template Control (NTC) and for volume replenishment. One kit provides reagents for the testing of up to 10 samples.

Cyclus® dPCR Tool Box Bacteria Fungi is optimized for the detection of genomic bacterial and fungal DNA in cell culture samples. Bacteria are detected by amplifying a highly conserved area of the 16S rRNA coding region in the bacterial genome while fungal detection is realized by amplifying a fragment of the conserved 18S rRNA coding region, respectively.

The FAM™ labelled TaqMan™ probes allow the specific detection of ≥94% bacterial and USP/EP- and clinically relevant fungal species. False negative results due to PCR inhibitors or improper DNA extraction are detected by the internal amplification control in the HEX™ channel.

Cyclus® dPCR Tool Box Bacteria Fungi is designed to be used in digital PCR workflows (Figure 1) and has been successfully tested with the nanoplate-based systems from QIAGEN (QIAcuity) and Roche (Digital LightCycler®) and the droplet based digital PCR system from Bio-Rad, QX200™. Thus, compatibility with other dPCR systems, such as the QuantStudio Absolute Q™ from Thermo Fisher Scientific or the Nio®+ from Stilla (now QX700 E from Bio-Rad) is expected. The data presented in this Application Note were generated using the QIAGEN QIAcuity system.

The aim of this Application Note was to experimentally assess the minimum detectable microbial count considered significant under EP 2.6.27.

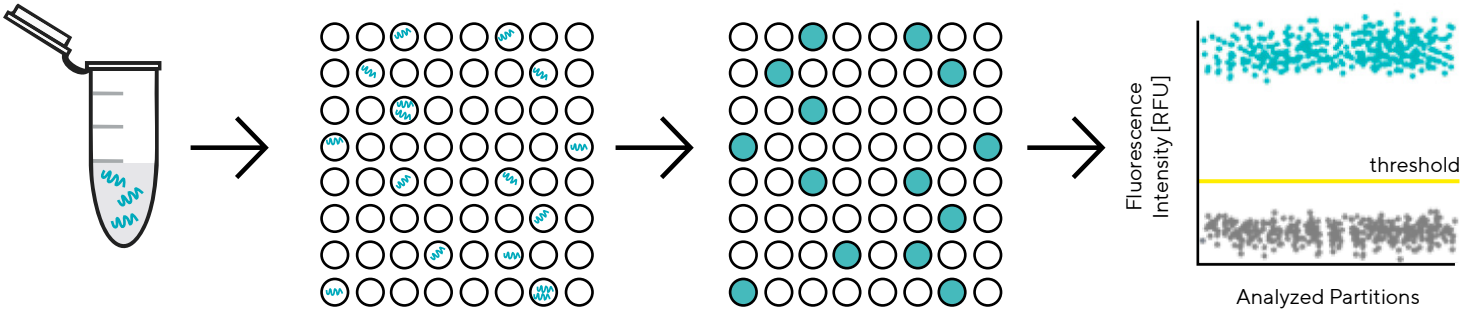

Figure 1: In digital PCR, a TaqMan™ assay is split into thousands of individual reaction chambers by means of droplet formation or separation into nano wells. The target molecules are thereby separated into individual reaction units, where they lead to a positive fluorescence signal after PCR. The positive and negative cavities are counted and plotted, allowing direct quantification of the target molecule.

Materials and Methods

DNA Extraction
- Microsart® ATMP Extraction (SMB95-2001)

Microbes and Media
The following microbes were purchased from Microbiologics®

Microbe	Kit	Reference
Pseudomonas aeruginosa	EZ-Accu Shot™	REF 0484A
Staphylococcus aureus	EZ-Accu Shot™	REF 0485A
Clostridium sporogenes	EZ-Accu Shot™	REF 0317A
Cutibacterium acnes	EZ-Accu Shot™	REF 0419A
Bacillus spizizenii	EZ-Accu Shot™	REF 0486A
Kocuria rhizophila	EZ-Accu Shot™	REF 0688A
Streptococcus pyogenes	EZ-CFU™	REF 0385C
Candida albicans	EZ-Accu Shot™	REF 0443A
Aspergillus brasiensis	EZ-Accu Shot™	REF 0392A

Media	Company	Reference
Trypticase™ Soy Agar (TSA)	BD	REF 254086
Dulbecco's Modified Eagle's Medium (DMEM)	Life Technologies limited	REF FG0415-500ML
Fetal Bovine Serum (FBS)	Merck Millipore	REF S0615

Consumables
- Laboratory gloves
- Cleaning Spray (SMB95-2001/SMB95-2003), Cleaning Wipes (SMB95-5003/SMB95-5004)
- Pipette filter tips free from bacterial and fungal DNA (Recommendation: Sarstedt Biosphere® filter tips: 20 µL, Prod. No. 70.3020.255; 100 µL, Prod. No. 70.3030.255; 300 µL, Prod. No. 70.3040.255; 1,000 µL, Prod. No. 70.3050.255)
- DNA-free 1.5 mL reaction tubes (Recommendation: Sarstedt Biosphere® plus Safe Seal reaction tubes 1.5 mL. PP, Order number: 72.706.200)

Equipment
- Laminar flow cabinet
- Heat block with optional shaking function
- Vortex
- Minicentrifuge for 1.5 mL reaction tubes
- Mechanical pipettes
  - 0.5-10 µL Sartorius Prod. No. LH-729020
  - 10-100 µL Sartorius Prod. No. LH-72905O
  - 100-1,000 µL Sartorius Prod No. LH-729070
- Pipette tips
  - Sarstedt Biosphere® Filter tip 20 µL REF 70.3020.255
  - Sarstedt Biosphere® Filter tip 100 µL REF 70.3030.255
  - Sarstedt Biosphere® Filter tip 1,000 µL REF 70.3050.255
- Rack for 1.5 mL reaction tubes
- QIAcuity One from QIAGEN (Cat. No. / ID: 911001 or 911021)

Digital PCR Specific Components
Reaction Chemistry
- Cyclus® dPCR Tool Box Bacteria Fungi (SMB95-6001)
- 4x QIAcuity UCP Probe PCR Kit (Cat. No. / ID: 250121)

Reaction Setup
- QIAcuity Nanoplate 26k 24-Well (Cat. No. / ID: 250001)
- Nanoplate Tray (supplied with the QIAcuity instrument - Cat. No. / ID: 250098)
- Plate roller (supplied with the QIAcuity instrument Cat. No. / ID: 911105)

Method
To test the performance of Cyclus® dPCR Tool Box Bacteria Fungi in the QIAcuity workflow, aliquots of 1 million HEK cells in DMEM with 5% FBS were spiked with approximately 10 CFU and 20 CFU of EP 2.6.27 relevant microbes to generate representative samples. As microbial spikes, certified viable cell materials from Microbiologics® were used in the product format EZ Accu Shot™ [4] or EZ-CFU™ [5]. The total nucleic acid of a spiked sample was extracted using Microsart® ATMP Extraction (SMB95-2001)[6]. It must be noted that the use of Microsart® ATMP Extraction in combination with Cyclus® dPCR Tool Box Bacteria Fungi (SMB95-6001) requires protocol modifications (20 minutes heat incubation, 20 minutes centrifugation, adding 200 µL Suspension buffer) as described in the Cyclus® dPCR Tool Box Bacteria Fungi user manual[7]. After DNA extraction the samples were processed and analyzed according to the instructions for use of Cyclus® dPCR Tool Box Bacteria Fungi. In parallel, the cell counts of the spiked samples were verified by spread plate method. Each microbial strain was plated on TSA agar from BD in triplicates and cultured at optimal strain specific conditions.

The results of the experiments were validated by the performance of the controls. Bacterial and fungal assays were considered functional when positive signal amplification was observed in the positive control. The purity of the used components was confirmed when there was almost no amplification in the NTC. The extraction was considered successful when amplification of the internal control was detected. All controls together confirmed the validity of the assay based on the performance described above. The negative extraction controls (NECs) indicated the background DNA load of the entire process and were used to calculate the Limit of Blank (LoB). The LoB was set in accordance with the recommendations of the guideline CLSI EP17-A2 ($LoB = mean_{NEC} + 1.645*(SD_{NEC})$)[8].

The calculation of the LoB for bacterial detection is based on 56 measurements and 12 replicates to determine the LoB for fungal detection. Signals higher than the determined LoB concentrations can be considered as positive samples.

The concentrations determined by the dPCR reaction were used directly for evaluation and were not converted back to the concentration of the template in the extract.

Results

In all experiments, the positive control (PC) and Negative Template Control (NTC) performed as expected, showed positive and negative results respectively and hence confirmed the functionality of the assay and the purity of the used components. Almost all samples revealed positive reaction cavities and therefore resulted in measurable concentrations.

Particularly the bacterial assay was highly affected even when analyzing the Negative Extraction Control (NEC). Thus, no direct presence / absence assessment of microbial DNA is possible. However, with digital PCR the creation of a LoB is possible and appropriate. Considering the NEC data and applying the recommendations of the CLSI EP17-A2 guideline, a LoB of 0.64 copies per microliter (cp/µL) for the bacterial assay and a LoB of 0.14 cp/µL for the fungal assay was calculated. Signals below the LoB can be considered as noise. Signals above the LoB can be considered as real signals.

The average value of the NECs of each test was below the LoB and was therefore considered negative. Almost all spiked samples yield values above the bacterial and fungal LoBs and are therefore considered positive. Only *Streptococcus pyogenes* yielded a value below the LoB in one sample and a value above the LoB in the other sample, indicating the limits were close.

This experiment clearly demonstrates the potential of Cyclus® dPCR Tool Box Bacteria Fungi to detect relevant bacterial and fungal EP 2.6.27 organisms at concentrations down to 10 CFU per mL.

			1. Dilution ~10 CFU			2. Dilution ~20 CFU		
		NEC	Detection Concentration (cp/µL)		Conformation CFU spike	Detection Concentration (cp/µL)		Conformation CFU spike
		Average (cp/µL)	1. Extract	2. Extract		1. Extract	2. Extract	
Bacteria	*Pseudomonas aeruginosa*	0.31	1.82	3.40	7	4.13	1.83	13
	Staphylococcus aureus	0.32	9.75	6.65	12	14.55	16.90	24
	Clostridium sporogenes	0.26	0.98	3.00	12	2.50	2.80	24
	Cutibacterium acnes	0.40	6.70	4.00	10	17.40	11.10	19
	Bacillus subtilis	0.14	1.50	1.50	9	2.40	1.75	18
	Kocuria rhizophila	0.16	0.99	2.15	9	1.30	2.45	17
	Streptococcus pyogenes	0.27	0.50	1.00	11	0.86	1.30	21
Fungi	*Candida albicans*	0.03	0.19	0.21	8	1.19	1.70	17
	Aspergillus brasiliensis	0.06	0.39	0.47	8	0.81	0.50	15

Table 1: Detecting 10 and 20 CFU of the EP 2.6.27 microbes with Cyclus® dPCR Tool Box Bacteria Fungi. Measurements were performed using QIAcuity® dPCR. DMEM + 5% FBS and 1 Mio. HEK cells were used as matrix. The viable cell count was determined on agar plates under culture conditions optimal for the respective organism. The determined colony forming units (CFUs) are presented as average value of three plates. The applied LoB for the signal interpretation of negative (highlighted in yellow) and positive (highlighted in grey) measurements is based on the LoB of 0.64 cp/µL for bacteria and 0.14 cp/µL for fungi.

Discussion

The NECs of the fungal assay frequently showed almost no signals, whereas the bacterial assay always revealed some background noise. This is due to the ubiquitous presence of bacterial DNA in the environment which is not the case for the fungal genomic DNA. This circumstance is a challenge for any NAT-based sterility test. Even if an assay itself is free of bacterial DNA, the lab environment, working materials and operators are part of the equation. If one template copy of bacterial DNA enters a real-time PCR reaction, it sooner or later will amplify and turn the reaction positive (Figure 2 A). This is why most commercially available real-time PCR assays use Ct cut-off values and do not run until enzyme collapse. Having a Ct cut off limits the detection of difficult targets which cannot compete with a common contaminant and are therefore detected only at high CFU levels.

By introducing the DNA extract into a digital PCR reaction with 20 to 26 K cavities, possible contaminants are isolated in individual nano wells (Figure 2 B). Those contaminants will lead to positive signals but remain isolated even with increased cycle numbers. The overall experiment remains valid, taking the positive signals of the background contamination as a LoB.

In the small reaction volume, the resulting signal stays concentrated and is not diluted by the large volume of a real-time PCR reaction (Figure 2 C). Thus, one template in a digital cavity reaches the signal intensity of a 25 µL real-time reaction already after about 25 cycles. Consequently, the additional 15 cycles serve to amplify difficult targets thereby increasing sensitivity.

With all the advantages of digital PCR technology Cyclus® dPCR Tool Box Bacteria Fungi enables detecting of almost all compendial microbes down to a concentration of about 10 CFU per mL. The inconsistent detection of *Streptococcus pyogenes* at such low concentrations may be due to several factors. Colony forming units are species-specific in their template counts. The fluctuating number of templates can be further impacted by the species-specific extraction efficiency. Extraction itself is also highly dependent on the processed matrix. All mentioned factors point to further potential which can be leveraged by optimizing extraction. In any case, it is essential to validate the process in all its facets at each specific customer site to determine the best possible performance under the given circumstances.

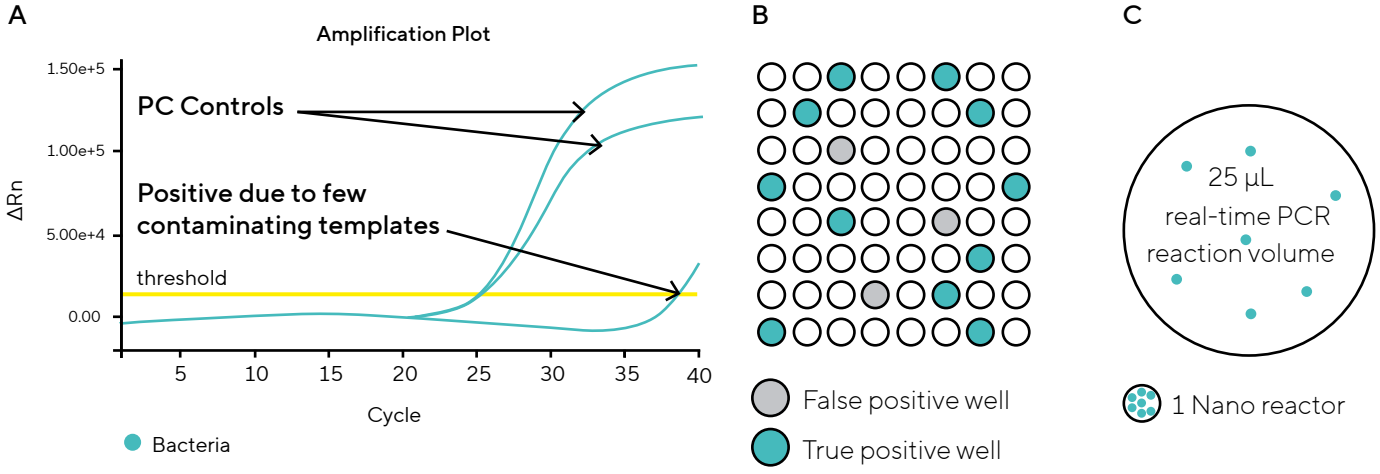

Figure 2: Principles of qPCR/dPCR based test.

A: A real-time amplification plot of two positive control reactions and a contaminated sample.

B: Schematic overview of contaminant distribution in a dPCR.

C: Signal accumulation with respect to the reaction volume.

Conclusion

With Cyclus® dPCR Tool Box Bacteria Fungi, Sartorius presents the first dPCR based product for the detection of relevant bacterial and fungal EP 2.6.27 organisms. At optimal conditions it can reach a sensitivity down to 10 CFUs previously unseen with NAT-based assays. Its power lies in handling low-level contamination in individual isolated reaction cavities. It requires a LoB value which reflects the lab specific DNA background contamination, while maintaining the validity of the experiment and making sterile testing feasible even outside clean room environments.

References

1. *European Pharmacopoeia (Ph. Eur.) 11th Edition, Section 5.1.6: Alternative methods for control of microbiological quality*, European Directorate for the Quality of Medicines & HealthCare (EDQM), Strasbourg, 2023

2. *European Pharmacopoeia (Ph. Eur.) 11th Edition, Section 2.6.27: Microbial examination of non-sterile products: Microbial enumeration tests*. European Directorate for the Quality of Medicines & HealthCare (EDQM), Strasbourg, 2023

3. *United States Pharmacopeia (USP) <1223>: Validation of Alternative Microbial Methods*. In. *United States Pharmacopeia and National Formulary (USP-NF)*, U.S. Pharmacopeial Convention, Rockville, MD, 2023

4. Instructions for use: EZ-Accu Shot™ Microorganisms, Microbiologics, PI.2383.ENG Rev B, 2022.MAR.23

5. Instructions for use: EZ-CFU™ Microorganisms, Microbiologics, IFU-01922-ENG.D, 2023.11.20

6. Instructions for use: Microsart® ATMP Extraction, Sartorius, Publication No.: SM-2001-e240901, Ver. 09 | 2024

7. Instructions for use: Cyclus® dPCR Tool Box Bacteria Fungi, Sartorius, Publication No.: SM-6001-e250902, Ver. 09 | 2025

8. 2008 David A Armbruster, Terry Pry, Limit of Blank, Limit of Detection and Limit of Quantitation, Clin Biochem Rev Vol 29

Germany
Sartorius Lab Instruments GmbH & Co. KG
Otto-Brenner-Straße 20
37079 Göttingen
Phone +49 551 308 0

USA
Sartorius Corporation
3874 Research Park Drive
Ann Arbor, MI 48108
Phone +1 734 769 1600

For further information, visit
www.sartorius.com/en/products/microbiological-testing/rapid-sterility-testing