Sterility Testing Made Simple

Key Strategies for Ensuring Reliable and Compliant Sterility Testing in Pharmaceutical Labs

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Sterility Testing: Where Convenience Meets Compliance

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Experience effortless compendial sterility testing with the Sterisart® Universal | Gen 4 pump—engineered for precision and contamination-free sample transfer in any environment. Integrating state-of-the-art hardware with intuitive software, it offers compliant, paperless documentation and advanced connectivity for modern labs.
Enjoy a high-performance touchscreen, electronic documentation built for 21 CFR part 11, and enhanced cleanability, making Sterisart® pump and canisters the complete solution for all your sterility testing needs.

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Sterility Testing

A Best Practice Guide

Sterility Testing is designed to confirm that sterile products, such as sterile pharmaceuticals and medical devices, do not contain contaminating viable microorganisms. This essential test determines if a manufactured batch of a sterile product is suitable for release.

In this guide, we walk you through how best to perform your membrane filtration based sterility test

Preliminary Set-up

- omponents are suitably decontaminated. All elements of the Sterisart® universal pump are easily detachable for efficient sterilisation.
- Open the sterile packaging under sterile/aseptic conditions.
- *Note:* It is recommended that sterility testing be performed in advanced aseptic processing systems such as an isolator, ideally located in a controlled environment, or a grade A laminar airflow cabinet located within a grade B clean room.
- Acquire material data of the canisters and reagents used via the integrated barcode scanner
- Firmly seat the canisters in the drain tray/container making sure that they do not wobble
- The outlet plugs are located in an easily accessible pocket of the blister packet and the filter plugs are pre-tethered to the canister

Thread the tubing between the bow and rotor. Begin insertion at the tubing guide. Ensure that there is sufficient tubing on either side

Membrane Pre-Wetting

Briefly pre-wet the membrane (a couple of ml will suffice) with the rinsing fluid. The Sartochem® membrane has a wetting time of less than 1 second. A low pump speed is recommended for this step (speed setting 50).
The rinsing fluid can be filtered directly through the membrane (by closing the venting filter with the tethered plug) OR the canister filled with a small volume of rinsing fluid (by leaving the venting filter open) and subsequently filtered (by closing the venting filter with the tethered plug).

Procedure :

1. Set the pump speed to 50
2. Puncture the septum or surface of the upright rinsing fluid container. Piercing is eased by the puncture guard. Pinch-press or drive-down the needle as depicted.
3. Switch on the pump.
4. Invert the rinsing fluid container and place it in the bottle holder only after the pump is in operation for a couple of seconds. This facilitates equal distribution of the liquid through the Y-connector.

For non-dual-needle canister types (for e.g 16467--------GBD/ 16477--------GBD) ensure that the sterile venting needle is used to vent the rinsing fluid bottle.

Note: a residual volume of the rinsing fluid can also remain in the container to help resuspend the product. If this is required fill the canister with approx. 25 ml (half way mark to the first graduation) and then briefly filter a small volume of the rinsing fluid (by switching on the pump for 1-2 seconds). This step is not mandatory.

Product Filtration

Test the prescribed number of product containers prescribed by the pharmacopeia. Speed settings are dependent on the type of product being sampled:

Recommended speed setting	Product
> 50 (50-100)	Viscosity is similar to that of water
100	For substances with antibiotic/anti-microbial properties (to minimize contact time with the membrane)
< 70	For products with a tendency to foam
< 70	For viscous products or poorly filterable products (to avoid clogging and a build-up of excessive pressure)

1. The venting filter plugs should remain in the closed position. Products are directly filtered through the membrane (i.e no filling followed by filtration)
2. Puncture the septum or surface of the upright product container (in the case of a closed pierceable container). Make sure you have chosen the right type of sterility test canister for each application/ product container (refer to our Sterility Testing guide). Pinch-press or drive-down the needle as depicted.
3. Switch on the pump.
4. Invert the product container and place it in the bottle holder (or bag holder, in the case of collapsible bags) only after the pump is in operation for a couple of seconds.
5. Filter the volume prescribed by the pharmacopeia or your internal SOP. Please refer to the respective pharmacopeial recommendations on minimal volumes to be tested. Oftentimes, the entire volume of the product need not be filtered. The remaining volume in the product container will serves as a proxy for the approximate volume transferred.

Note: more than one sample can be pooled through one sterility testing unit provided that the product does not clog/block the membrane. For such difficult to filter products it is advisable to use one sterility testing unit per product container.

Ointments and emulsions can be diluted to 1 % in sterile isopropyl myristate by heating. Heating should not exceed 40°C. Only in exceptional circumstances, pending approval, can a product be heated to not more than 44°C. For liquids that are difficult to filter use Sterisart® Cellulose Acetate canisters (white base).

Membrane Rinsing

Rinsing fluid	Application
Fluid A (standard)	Compatible with most samples
Fluid D (standard)	For antibiotics and the rinsing of medical devices
Fluid K (occasional)	For oily solutions and products that are difficult to filter and dissolve

The membrane filter is typically rinsed not less than 3 times, with a volume of 50-100 ml

For products with known antimicrobial properties wash the membrane thoroughly. The maximum limit of each rinse cycle being 5 times 100 ml per filter/canister.

A low pump speed <70 is recommended to a) prevent potential foaming and b) to maximise contact of the rinsing fluid with the membrane

1. Uncap the venting filters (open position) .
2. Puncture the septum or surface of the upright rinsing fluid container. Piercing is eased by the puncture guard. Pinch-press or drive-down the needle as depicted.
3. Switch on the pump.
4. Invert the rinsing fluid container and place it in the bottle holder only after the pump is in operation for a couple of seconds. This facilitates equal distribution of the liquid through the Y-connector.
5. Fill the canisters with 50 -100 ml

   Note: do not exceed the 100 ml graduation mark in the canister. Overfilling the canister can result in clogging of the venting filter and this can compromise the sterility test by preventing the canister from venting

6. Cap the venting filter (closed position) and filter the rinsing fluid through the membrane.
7. Repeat step as required
8. At the ned of rinsing, uncap the venting filter (open position)

   Note: in select cases there can be a product interaction with the rinsing fluid, leading to the formation of a white precipitate. Such precipitates/ aggregates can clog the membrane. In such an event, try different rinsing fluids and ideally rinsing fluids sourced from different vendors. Differences do exist despite having the same composition.

Media Fill

Media sourced from some vendors can be darker than others. Ideally opt for a media where visual changes in the media (i.e. turbidity) are easily recognized.

1. Check that the venting filters are uncapped (open position)
2. Unseat the canisters and close the outlets with the outlet plugs. Close the outlet by pushing the plugs upwards with a half-turn anti-clockwise twist. Take care not to touch the inside of the filter plug or the outlet while executing this.
3. Firmly seat the canisters in the drain tray/container making sure that they do not wobble
4. Close both clamps on one tubing. The clamps on the same tubing are colour coded (i.e. close either the yellow clamps and leave the white clamps open, or vice versa)
5. Puncture the septum or surface of the upright growth media container (e.g. TSB). Pinch-press or drive-down the needle as depicted.
6. Switch on the pump.
7. Invert the growth media container and place it in the bottle holder only after the pump has run a couple of seconds. Growth media are typically supplied in 100 ml containers. Transfer the entire contents of the growth media bottle into one canister. If this is not the case, avoid filling the canister over the 100 ml graduation. Make sure to transfer the contents of the tubing into the container as well.
8. Release/open the closed clamps and close the open clamps on the second tubing.

9. Puncture the septum or surface of the upright growth media container (e.g. FTM). Pinch-press or drive-down the needle as depicted.
10. Switch on the pump.
11. Invert the growth media container and place it in the bottle holder only after the pump has run a couple of seconds. Growth media are typically supplied in

100 ml containers. Transfer the entire contents of the growth media bottle into one canister. If this is not the case, avoid filling the canister over the 100 ml graduation. Make sure to transfer the contents of the tubing into the container as well.

Note: use a pump speed setting < 50 for FTM to avoid aeration of the media. Excessive aeration during pumping may compromise the properties of the media required for anaerobic growth.

12. Release/open the closed clamps on the tubing.
13. Open the pump lever
14. Do not open the pump lever without releasing/ opening the closed clamps. Do not open the pump lever until completion of the test.
15. After freeing the tubing, close the clamps closest to the canister inlet.
16. Detach the venting filter plugs if required.
17. With a pair of scissors, cut the tubing 3-5 cm away from the clamp and loop the cut end back onto the luer-slip connector of the sterile venting filter.

Incubation, Inspection, and Readout

The sterility testing canisters are incubated at the temperatures prescribed by the Pharmacopeia for 14 days:

- TSB: 20-25° C
- FTM: 30-35° C

The canisters are periodically checked every 3-5 days.

If there is no visual change in the growth medium after 14 days of growth, the product is sterile and the sterile product is suitable for release.

If a visual change (e.g. turbidity) is observed, implying microbial growth, the test is positive and a detailed root cause analysis should be undertaken.

The test may be considered invalid and repeated only if it can be clearly demonstrated that the observed growth/contamination is unrelated to the product under examination. Typically, corrective action must be taken before the test can be repeated. A sterility test may be repeated only one time. If a contamination is detected in the repeat test the product is not in compliance and the entire batch should be rejected.

Note: in select cases there can be a product interaction with the growth media, leading to the formation of a precipitate. If the product renders the media turbid upon completion of the test, incubate the canisters for the 14 days, aseptically transfer a portion not less than 1 ml to fresh vessels of the same medium and incubate the original and transfer vessels for not less than 4 days. In this case we recommend that you use sterility testing canisters equipped with a sampling septum. The Sterisart® septum canisters facilitate aseptic sampling from a canister in a non-destructive manner

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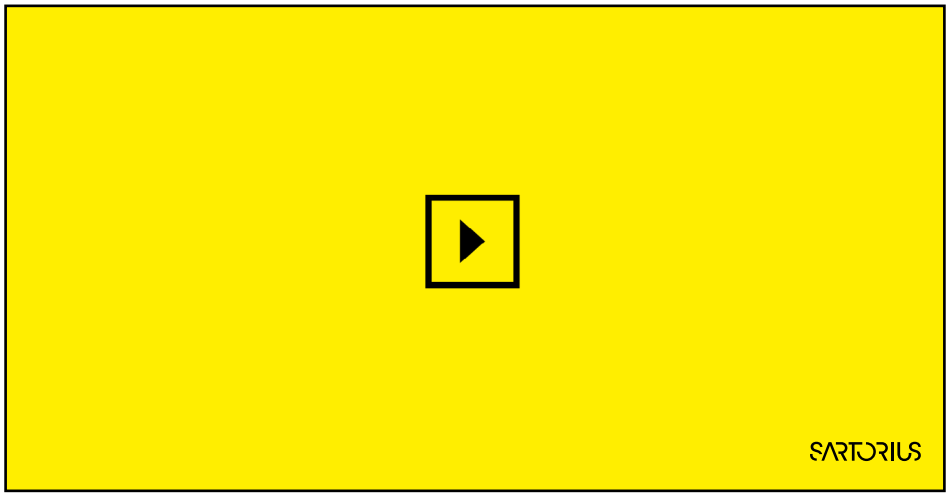
The Versatile Role of the Sterisart® Septum

Click to view application note.
Sterisart Septum: Sterility Testing

Method Validation/Revalidation

- Samples for sterility testing should be representative of the whole batch. For products that are aseptically filled samples should include containers filled at the beginning and at the end of the batch and after any significant interruption of work. For products that have been heat sterilized samples should be taken from the part of the load that is potentially the coolest.
- During method suitability testing, the growth media is spiked with an inoculum of viable microorganisms (not more than 100 CFU) and the containers are incubated for not more than 5 days. If clear visible growth is observed the product possesses no antimicrobial activity, the sterility test can be carried out with no further modification. If growth is not observed after 5 days, the rinsing step require further optimization or a neutralizing agent may have to be used in the test (via supplementation or using pre-supplemented media)
- Negative product controls are typically similar in type and packaging to the product under test. It is recommended that at least 10 negative product controls be tested to simulate manipulations.
- Although not a pharmacopeial requirement, it is good practice to revalidate test methodology every 12 months
- Although not mandatory a stasis test is recommended to be repeated every 12 months on product categories involving antibiotic substances. If conspicuous growth is not apparent within 5 days, the test is considered invalid.



Watch video

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Application Note

The Sterisart® System Simplifies the Sterility Testing of Therapeutics with Antimicrobial Properties

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Abstract

Membrane filtration-based sterility testing is particularly suited for products with microbial growth-inhibiting properties, such as antibiotics. These compounds need to be purged from the system to mitigate the incidence of false negatives. This is achieved by constructing the sterility testing canisters with materials that exhibit minimal to no non-specific binding. In this study, the recovery of microorganisms following the filtration of ciprofloxacin for intravenous administration and gentamicin for intramuscular injection using the Sterisart® closed system sterility testing device was evaluated. The results demonstrate that the Sterisart® canisters containing regenerated cellulose membranes are optimal for the sterility testing of antibiotics.

Introduction

Pharmaceutical products are routinely manufactured under strict GMP guidelines. Despite these strict codes, as a fail-safe prior to batch release, not all pharmaceutical products undergo stringent sterility testing to identify the potential presence of viable microorganisms. It is crucial that pathogenic microbes, such as bacteria and fungi, are detected in contaminated products before patients come in contact with. There have been rare instances where compromised drugs have been released to the market with devastating consequences for patients, and pharmaceutical companies.

Sterility tests are performed in accordance with the regulatory requirements defined by the International Pharmacopeia (USP <71>, Ph. Eur. 2.6.1., and JP 4.06) and harmonized in ICH Q4B Annex 8. According to these requirements, sterility testing can be performed either by direct inoculation, or by membrane filtration, which is the method of choice. Products are tested for sterility by direct inoculation only when the properties of the product do not permit membrane filtration. The membrane filtration approach typically relies on a closed filtration unit containing a membrane with a pore size not greater than 0.45 µm and that has reliably demonstrated the retention of microorganisms. Other components of the system include a suitable pressure supply (such as a peristaltic pump) that drives the sample across the membrane filter, an appropriate membrane rinsing solution, and growth media. This closed setup is conventionally cleanroom compliant to eliminate any contamination risks and consequent false positives.

Once sample filtration is complete, the closed system is incubated, typically for 14 days, and screened for turbidity as an indicator of microbial contamination. Sterisart® canisters are a closed system for sterility testing based on the membrane filtration method. This closed system excludes the need for physically manipulating membrane filters and thereby mitigates the risk of secondary contamination and false positives.

Being a method based on the evaluation of microbial growth, it is crucial to distinguish between true product sterility and a false negative. Different ingredients of a pharmaceutical formulation can possess innate bacteriostatic or fungistatic properties that can negatively influence the results of a sterility test. Antibiotics possess such growth-inhibiting antibacterial and antifungal properties. It is therefore recommended to use the membrane-filtration method for the sterility testing of antibiotics or use a suitable sterile inactivating agent (such as penicillinase or cephalosporinase) to supplement the growth media.

However, some antibiotics cannot be effectively neutralized. In such cases, non-specific adsorption of inhibitory compounds to the components of the sterility testing system is a major cause for concern. It is therefore critical that the physicochemical properties of the materials used in the construction of the sterility testing canisters must exhibit negligible non-specific adsorption and facilitate thorough rinsing to purge all traces of the antibiotic, and yet deliver on microbial retention.

In this report, a method suitability test was performed using the two antibiotics ciprofloxacin, for intravenous administration, and gentamicin, for intramuscular injection. Ciprofloxacin is a fluoroquinolone antibiotic effective against most Gram-negative bacteria such as *Pseudomonas aeruginosa*. Gentamicin is a type of aminoglycoside used in the treatment of infections mainly caused by Gram-negative bacteria as well as some Gram-positive bacteria such as *Staphylococcus aureus*. The susceptibility of *P. aeruginosa* and *S. aureus* to ciprofloxacin and gentamicin respectively was the main rationale for choosing these antibiotics.

Following dilution and membrane filtration of the antibiotics, the canisters were rinsed, inoculated with microorganisms listed in USP <71>, filled with growth medium and incubated at the prescribed temperature. Uninhibited microbial growth was observed in all the samples well before the prescribed 3-day maximum for bacteria and 5-day maximum for fungi recommended for growth promotion testing. Our results demonstrate that the Sterisart® canisters are optimal for the sterility testing of antibiotics and comply with all pharmacopeial requirements.


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Pump & Scan
Time
0:00
min:sec
Duration
off
Speed
100
FINISH
SCAN (0 / 7)
START
SARTORIUS
STERISART® UNIVERSAL

Materials and Methods

Product Name	Order No.
Sterisart® Universal Pump	16420
Ampoule breaker for Sterisart® Universal Pump	1ZW---0002
Sterisart® Transfer-Kit for liquids	16472--------GBD
Sterisart® system for liquids in open containers	16467--------GBD
Sterisart® system for closed large volume containers, with septum	16466--------GSD

Table 1 : Equipment and consumables.

Product Name	Order No.
Ciprofloxacin (200 mg / 100 mL)	/
Gentamicin (80 mg / 2 mL)	/
Tryptic Soy Broth (TSB) 100 mL	BD-257247
Fluid Thioglycollate Medium (FTM) 100 mL	BD-257246
Fluid A (peptone water) 300 mL	BD-254979
Tryptic Soy Agar (TSA)	BD-254086

Table 2 : Chemicals, media and rinsing fluids.

Test Strains
Pseudomonas aeruginosa ATCC® 9027™
Bacillus subtilis ATCC® 6633™
Staphylococcus aureus ATCC® 6538™
Clostridium sporogenes ATCC® 19404™
Aspergillus brasiliensis ATCC® 16404™
Candida albicans ATCC® 10231™

Table 3 : Certified microorganisms used in this study.

Sample Preparation

International pharmacopeias, including USP <71>, Ph. Eur. 2.6.1., and JP 4.06, recommend that for liquid antibiotics, a minimum sample volume of 1 mL from 20 distinct containers should be tested when the batch size exceeds 500 units. Accordingly, 20 mL of the respective antibiotic was tested per Sterisart® canister. Ampoules containing gentamicin were opened with the Sterisart® ampoule breaker. Using the Sterisart® Transfer-Kit-for liquids, 40 mL of either gentamicin or ciprofloxacin were aseptically pre-diluted in 200 mL of Fluid A.

Membrane Filtration

Step	Description	Pump Speed
1	Pre-wetting with 50 mL Fluid A per canister	50%
2	Filtration of pre-diluted antibiotics	90%
3	Rinse 4x with 100 mL Fluid A per canister	50%
4	Rinse 1x with 100 mL Fluid A spiked with test strain	50%
5	Add either 100 mL FTM or TSB per canister	50%
6	Incubate at 32.5 °C (±2.5 °C) for FTM and 22.5 °C (±2.5 °C) for TSB for 3 to 5 days	/

Table 4 : Schematic workflow for filtration of antibiotics with the Sterisart® Universal Pump.

Two Sterisart® canisters were positioned in the canister holder and the Sterisart® tubing system was threaded through the pump head. The sterile venting filters were left open, the needle was inserted into the septum of the container containing Fluid A and the pump was switched on (see Table 4). Once 50 mL of Fluid A were transferred into the canisters, the sterile venting filters were sealed using the tethered filter plugs. Pre-wetting the membrane with a rinsing fluid limits non-specific adsorption and is strongly recommended. Next, the needle was inserted into the container containing the pre-diluted antibiotics. To reduce the contact time of the antibiotic-containing solution with the membrane, the tethered filter plugs were left on the sterile venting filters. The pump was switched on and the entire content of the bottle was pumped in equal volumes between the two Sterisart® canisters. The pump was switched off and the sterile vent filters were uncapped. To eliminate potential droplets of antibiotics at the canister walls, the needle was inserted into the container with Fluid A and the two canisters were filled with a pre-defined volume of 100 mL, by switching the pump on. The sterile vent filters were capped, and the membrane was rinsed with the contents of the canister. As recommended by the international pharmacopeia, the membranes were washed not more than 5 times with 100 mL per canister and filter.

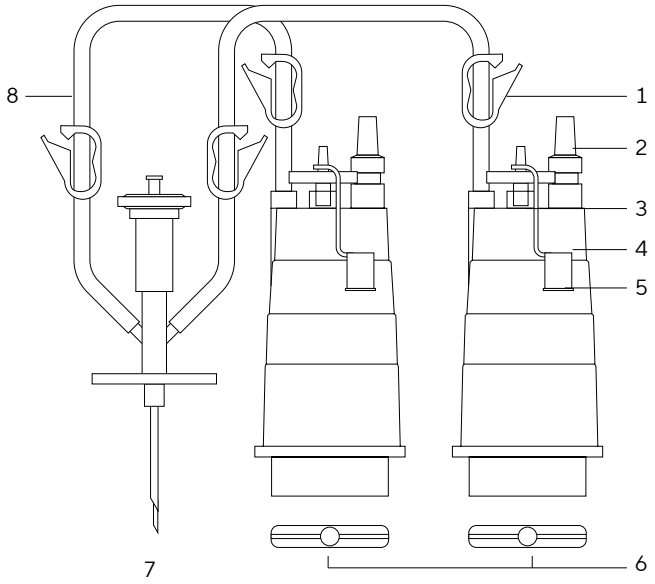
The fifth and final rinsing volume was spiked with <100 colony forming units (CFU) of certified test microorganisms and filtered through the Sterisart® canisters. For cell number quantification, the same amount of the spiked test microorganisms was transferred onto Tryptic Soy agar plates (TSA) using spread plate method and incubated under the same conditions as the respective spiked Sterisart® canisters.

After the last rinsing step, the outlet of each Sterisart®

canister was sealed using the enclosed wing nut plugs. The two sterile vent filters were uncapped. The yellow tube clamp at the outlet of the Y-distributor was opened and the adjacent white tube clamp closed. The needle was inserted into a bottle containing either 100 mL Fluid Thioglycollate Medium (FTM) or Tryptic Soy Broth (TSB) and the Sterisart® Universal pump was switched on. The Sterisart® canisters were filled according to Table 5. For negative controls, 50 mL of fluid A was filtered through Sterisart® canisters and then filled with either 100 mL FTM or TSB. For the positive controls, 50 mL of fluid A were first filtered, then 100 mL of fluid A was filtered with spiked test strain and filled with either TSB or FTM (see Table 5). The tubing was sealed using the two clamps above the inlets of the canisters. The tubing was cut at an appropriate distance away from the clamp and slid onto the slip-connector of the sterile venting filters. Canisters containing FTM were incubated at 32.5 °C ± 2.5 °C while canisters containing TSB were incubated at 22.5 °C ± 2.5 °C. The canisters were visually inspected daily.

Growth promotion tests were performed in compliance with USP <71>, Ph. Eur. 2.6.1. and JP4.06. The experiments were carried out in triplicate with three different lots of Sterisart® canisters.

Please refer to our Sterisart® user manual for a pictorial depiction of the described workflow.



No.	Component
1.	Pre-installed tube clamp
2.	Connector with septum for sterile sampling
3.	Vent filter
4.	Sterisart® container
5.	Tethered filter cap
6.	Wing nut plug
7.	Dual-needle metal spike for closed containers (16466)
8.	Tubing

System	Canister	Test Strain	Medium	Filtered Sample
1	A	/	TSB	/
	B		FTM	
2	A	*C. albicans*	TSB	Gentamicin
	B	*P. aeruginosa*	FTM	
3	A	*C. albicans*	TSB	Ciprofloxacin
	B	*P. aeruginosa*	FTM	
4	A	*C. albicans*	TSB	/
	B	*P. aeruginosa*	FTM	
5	A	*B. subtilis*	TSB	Gentamicin
	B	*S. aureus*	FTM	
6	A	*B. subtilis*	TSB	Ciprofloxacin
	B	*S. aureus*	FTM	
7	A	*B. subtilis*	TSB	/
	B	*S. aureus*	FTM	
8	A	*A. brasiliensis*	TSB	Gentamicin
	B	*C. sporogenes*	FTM	
9	A	*A. brasiliensis*	TSB	Ciprofloxacin
	B	*C. sporogenes*	FTM	
10	A	*A. brasiliensis*	TSB	/
	B	*C. sporogenes*	FTM	

Table 5: Experimental overview of the growth promotion tests (n=3).

Results

Although both gentamicin and ciprofloxacin are highly potent against Gram-negative microorganisms, the canisters containing FTM spiked with *P. aeruginosa* ATCC® 9027™ exhibited clearly visible growth that was no different from the positive control (see Figure 1). Canisters containing FTM medium spiked with *C. sporogenes* ATCC® 19404™ turned fully turbid after 3 days of growth. FTM medium containing *S. aureus* ATCC® 6538™ exhibited clearly visible growth throughout the culture media column within the canister, with colonies growing directly on the membrane.

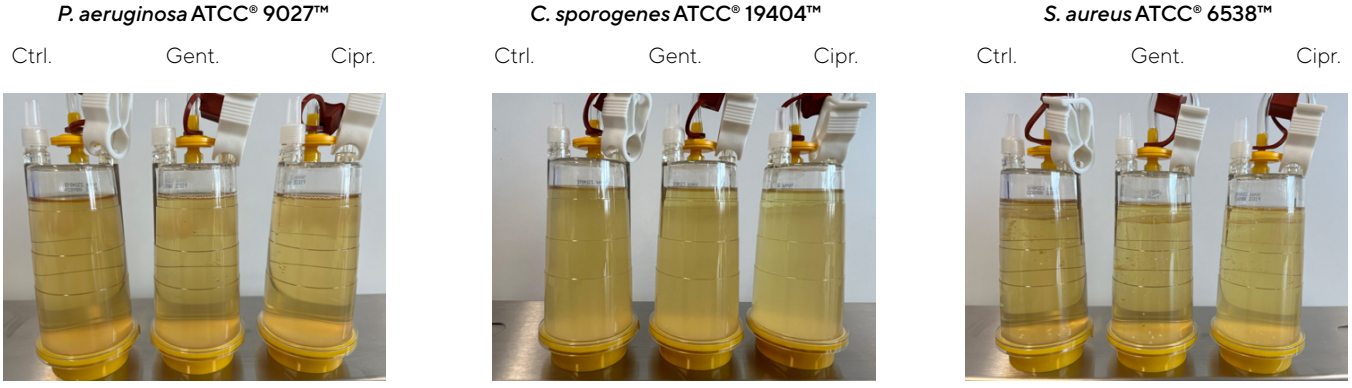


Figure 1 : Exemplary results of test strains grown in FTM after filtration of gentamicin (Gent.) and ciprofloxacin (Cipr.). Growth was always compared to positive control (Ctrl.) where no antibiotic was filtered.

Without prior agitation, all canisters filled with TSB showed clearly visible growth throughout the culture media within the canister or directly above the membranes for *A. brasiliensis* ATCC® 16404™, *C. albicans* ATCC® 10231™, and *B. subtilis* ATCC® 6633™ (see Figure 2).

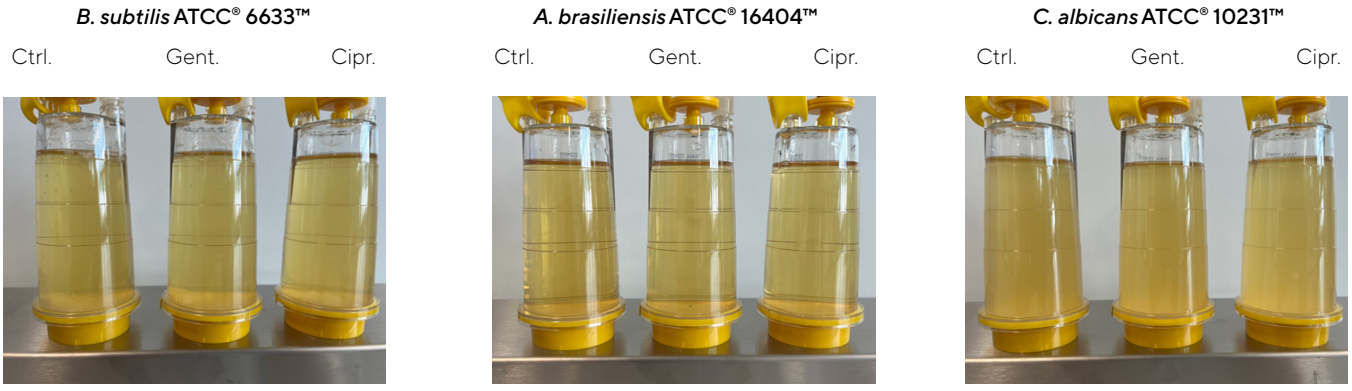


Figure 2: Exemplary results of test strains grown in TSB after filtration of gentamicin (Gent.) and ciprofloxacin (Cipr.). Growth was always compared to positive control (Ctrl.) where no antibiotic was filtered. Samples were agitated for better visibility.

In summary, no growth inhibition was observed in any of the canisters used for filtration of either gentamicin or ciprofloxacin, compared to the positive controls, where no antibiotics were filtered. Growth was not observed in any of the negative controls (see Figure 3).

Figure 3: Exemplary results of negative canisters filled with FTM (left) or TSB (right).

Conclusion

This study demonstrates that Sterisart® canisters are optimal for testing sterile pharmaceuticals with antimicrobial properties, including those products that cannot be effectively neutralized. Rinsing each membrane with 5x100 mL of Fluid A solution guarantees adequate removal of gentamicin and ciprofloxacin. The Sterisart® Sartochem® Regenerated Cellulose Membrane stands as an universal membrane, characterized by minimal to no non-specific binding properties. As a result, there is no imperative need for segregation into products with or without antibiotic properties before conducting sterility tests.

In addition to the tests performed in this study, we have conducted detailed adsorption and desorption tests of compounds with antimicrobial properties using Reverse Phase HPLC. Please see sections 5.1-5.2 of our validation guide for further details.

Our extensive Sterisart® sterility testing portfolio has been designed for simplicity and is fully compliant with every pharmacopeial need. The unique Sterisart® septum port eliminates the risk of false positives and eases aseptic supplementation for antibiotic inactivation or aseptic sampling for identification, sub-culturing or rapid microbial release. **Scan the QR code below** to learn more on a study demonstrating that the integrity of the sterility testing canisters is maintained even after more than 100 repeated septum sampling events via the Sterisart® septum port.

References:

1. US Pharmacopoeia (USP) <71> - Sterility Tests
2. European Pharmacopoeia (Ph. Eur.) 2.6.1. - Sterility
3. Japanese Pharmacopoeia (JP) 4.06 – Sterility Test

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Application Note

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The Sterisart® Septum Enables Reliable Sampling from a Closed System Sterility Testing Unit

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Abstract

In this study, we evaluated the Sterisart® closed system sterility testing device, with a septum, for the recurrent sterile extraction of samples. The results demonstrate that even after more than 100 repeated septum sampling events, which far exceeds any foreseeable sampling requirements, the septum remains intact and the growth media contained in these canisters remains sterile.

The Sterisart® septum allows easy inoculation and sampling, and enables the coupling of the conventional closed system sterility testing with rapid detection methods.

Find out more: sartorius.com

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Introduction

Pharmaceutical products are routinely manufactured under strict GMP guidelines. Despite these strict codes, as a fail-safe prior to batch release, all pharmaceutical products undergo stringent sterility testing to identify the potential presence of viable microorganisms. It is crucial that pathogenic microbes, such as bacteria, viruses and fungi, are detected in contaminated products before patients come in contact with them. There have been rare instances where compromised drugs have been released to the market with devastating consequences, for the patients and also the pharmaceutical companies.

Sterility tests are performed in accordance with the regulatory requirements defined by the International Pharmacopeia (USP <71>, EP 2.6.1, JP 4:06). Sterility testing can be performed either by direct inoculation | transfer, or membrane filtration, which is the method of choice. Products are tested for sterility by direct inoculation only when the properties of the product do not permit membrane filtration. The membrane filtration approach typically relies on a closed filtration unit containing a membrane with a pore size not greater than 0.45 µm and that has reliably demonstrated the retention of microorganisms. Other components of the system include a suitable pressure supply (such as a peristaltic pump) that drives the sample across the membrane filter, an appropriate membrane rinsing solution, and growth | culture media. This closed setup is conventionally cleanroom compliant to eliminate any contamination risks and consequent false positives. Once sample filtration is complete, the closed system is incubated, typically for 14 days, and screened for turbidity as an indicator of microbial contamination.

Sterisart® canisters are a closed system for sterility testing based on the membrane filtration method. This closed system excludes the need for physically manipulating membrane filters and thereby mitigates the risk of secondary contamination and false positives. However, sample extraction is a prerequisite, when the growth media is rendered turbid by microbial growth, following the prescribed 14 days of incubation. If microbial growth is detected, the identity of the microorganism and the source of the contamination is determined, and the sterility test is declared invalid and then repeated. Aseptic sample withdrawal or aseptic enzyme supplementation, for instance to deactivate antibiotics that might result in false negatives, may also be required after filtration or during incubation.

Precipitation of the filtered test sample, or an adverse color change due to the inherent properties of the compound, can also render the growth media turbid, even prior to incubation at the prescribed temperatures. This convolutes the interpretation of the sterility test and the certification of the batch for release; the batch may require additional testing by sample extraction from the canister and subsequent sub-culturing.

Sample extraction in conventional sterility test systems involves puncturing or cutting the tubing leading to the inlet of the canister and then attempting to carefully extract a sample, without compromising the integrity of the canister or its contents. Sample extraction by cutting the tubing precludes repeated sampling. Multiple sampling using other approaches can increase the risk of contamination by compromising the closed system.

The Sterisart® septum was designed to facilitate repeated sampling during incubation of the growth promotion test. In this report, we show that multiple sampling performed through the Sterisart® canister septum – over 100 times – exceeding any conceivable requirement for aseptic sampling, does not lead to the contamination of the system.

Reasons for septum usage:

a)	The growth media is rendered turbid by microbial growth, following incubation, and necessitates the identification of the micro-organism as part of a root cause analysis.
b)	The product renders the growth medium turbid, prior to incubation, and requires sub-culturing	dilution.
c)	Samples are drawn to test for microbial contamination by rapid detection methods.
d)	Samples are supplemented with agents to counteract anti-microbial components of the tested product.

Materials and Methods

Consumables
Tryptic soy broth (TSB) (Gila/BD), Fluid thioglycollate medium (FTM) (Gila/BD), TSB (Merck), FTM (Merck), Tryptic soy agar (TSA) (Merck), Glass reaction tubes, 30 ml (Borosil), Needle – 0.90 × 70 mm, 20G × 2 ¾ (Sterican – B. Braun), Syringe – F Luer (Omnifix – B. Braun).

Equipment
Sterisart® universal pump, Incubator (Sartorius Stedim Biotech GmbH), Combisart® 3-branch filtration manifold (Sartorius Stedim Biotech GmbH), e.jet Pump (Sartorius Stedim Biotech GmbH).

Sterisart® NF sterility testing system
16467--------GSD, 16475--------GSD, 16466--------GSD

Also available: versions pre-qualified for optimal use with Celsis® rapid microbial detection instruments:
- 16466CR-GSD, Celsis® Qualified Sterisart® NF Septum with 4 cm dual-needle
- 166467CR-GSD, Celsis® Qualified Sterisart® NF Septum with 5.2 cm needle

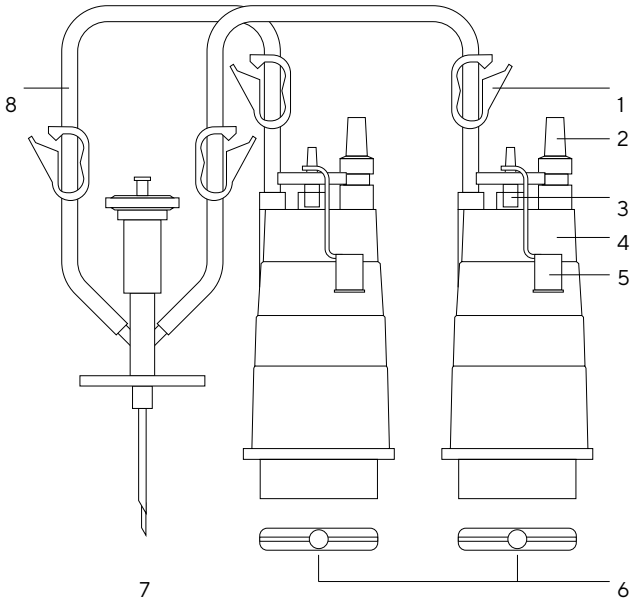
Membrane Filtration:
Ten individual Sterisart® canisters from the three types of septum variants (30 in total) were analyzed in the septum sampling tests. One of the ten canisters (from each Sterisart® canister type) served as a negative control (i.e. samples were not extracted from this canister until day 24 of the test).

The Sterisart® canisters were filled with growth media under aseptic sterile conditions in a biosafety cabinet. The two Sterisart® canisters were positioned in the pump holder and the Sterisart® tubing system was thread through the pump head. The outlet of each Sterisart® canister was sealed using the enclosed wing nut plugs. The two sterile vent filters were left uncapped. The yellow tube clamp at the outlet of the Y-distributor was opened and the adjacent white tube clamp closed. The dual-needle metal spike was inserted into a bottle containing FTM and the Sterisart® Universal pump was switched on. The pump was switched off once a predefined volume (75 ml) of medium was transferred into the first canister. The white tube clamp at the outlet of Y-distributor was opened and the adjacent yellow clamp closed. The dual-needle metal spike was inserted into a bottle containing TSB and the Sterisart® Universal pump was switched on. A similar volume (75 ml) of medium was transferred into the second canister. The tubing was sealed off using the two clamps above the inlets of the canisters and the tubing was cut off. Please refer to our Sterisart® NF gamma user manual for a pictorial depiction of the described process.

Sterisart® canisters containing TSB, the recommended growth media used in the detection of low incidence fungi and aerobic bacteria, were incubated at 22.5° C for 24 days. Sterisart® canisters containing FTM, the recommended growth media for cultivating aerobic, microaerophilic, and anaerobic microorganisms were incubated at 32.5° C for 24 days.

Septum Sampling:
Samples were extracted from the Sterisart® canisters under sterile conditions in a biosafety cabinet. Three samples of 100 µl each were extracted twice a day from the top, middle, and bottom of the Sterisart® canister, over a period of 17 days (3 × 2 × 17 = 102 samples). The extracted samples were transferred into the glass reaction tubes containing the sterile liquid media, FTM and TSB. The vials containing TSB were incubated at 22.5° C for 14 days, and the vials containing FTM were incubated at 32.5° C for 14 days. The results were recorded by photographing each Sterisart® unit and the corresponding extracted sample.

Microbial Enumeration:
A final inspection was performed using a black gridded membrane filter placed in a sterile Sartorius Combisart® filtration unit and connected to an e.jet pump. 60 – 70 ml of TSB (following the 24 day incubation period of the Sterisart® canisters) was filled into the funnel and filtered through the black membrane filter. The filter was transferred using sterile forceps onto a TSA plate, and the plate was incubated at 36° C for 3 – 5 days. These plates were then inspected for microbial contamination.



No.	Component
1.	Pre-installed tube clamp
2.	Connector with septum for sterile sampling
3.	Vent filter
4.	Sterisart® container
5.	Tethered filter cap
6.	Wing nut plug
7.	Dual-needle metal spike for closed containers (16466)
8.	Tubing

Results and Discussion

After 102 septum piercings and repeated sample withdrawals, it was established that all Sterisart® canisters (3 × 9 containing FTM, and 3 × 9 containing TSB; the 10th canister containing FTM and TSB serving as their respective controls) were sterile and showed no detectable microbial contamination after 24 days. (Figure 1)

Similarly, the extracted samples were likewise sterile and free of microbial growth demonstrating that the Sterisart® septum promotes efficient and highly reliable aseptic sampling. (Figure 2)

Figure 1: No microbial contamination after repeated sample extraction. Representative images of the Sterisart® 16466 GSD version filled with TSB (A and B) FTM (C and D) incubated for 24 days at 22.5° C and 32.5° C respectively. Negative controls are shown in A and C.
Canisters in B and D were pierced 102 times for sample extraction.

Figure 2: No microbial contamination in samples extracted from Sterisart® 16466 GSD after incubation in glass vials. Samples were inoculated into glass vials containing TSB (upper panel) and FTM (lower panel) were grown at 22.5° C and 32.5° C, respectively, for 14 days.

Coring can occur when a septum has been punctured multiple times or if an inappropriate needle type is used. Only after 36 piercings were small particles observed in some Sterisart® canisters. These particles were collected after 24 days on a black membrane filter and monitored for their ability to form colonies on TSA plates. These particles did not demonstrate any growth even after an incubation period of five days, suggesting that these particles are not biological in nature. Based on their morphology, we conclude that these inert particles are fragments of rubber that are sheared off the septum during repeated piercing with syringe needles. These fragments do not influence the efficacy of the sterility test and are barely visible in the growth medium.

We recommend that septum sampling be performed only after unplugging the sterile vent (i.e. uncapped) in a controlled environment.

Our results demonstrate that Sterisart® canisters remain a closed and sterile unit, even after successive sampling for a tested total of 102 extractions.

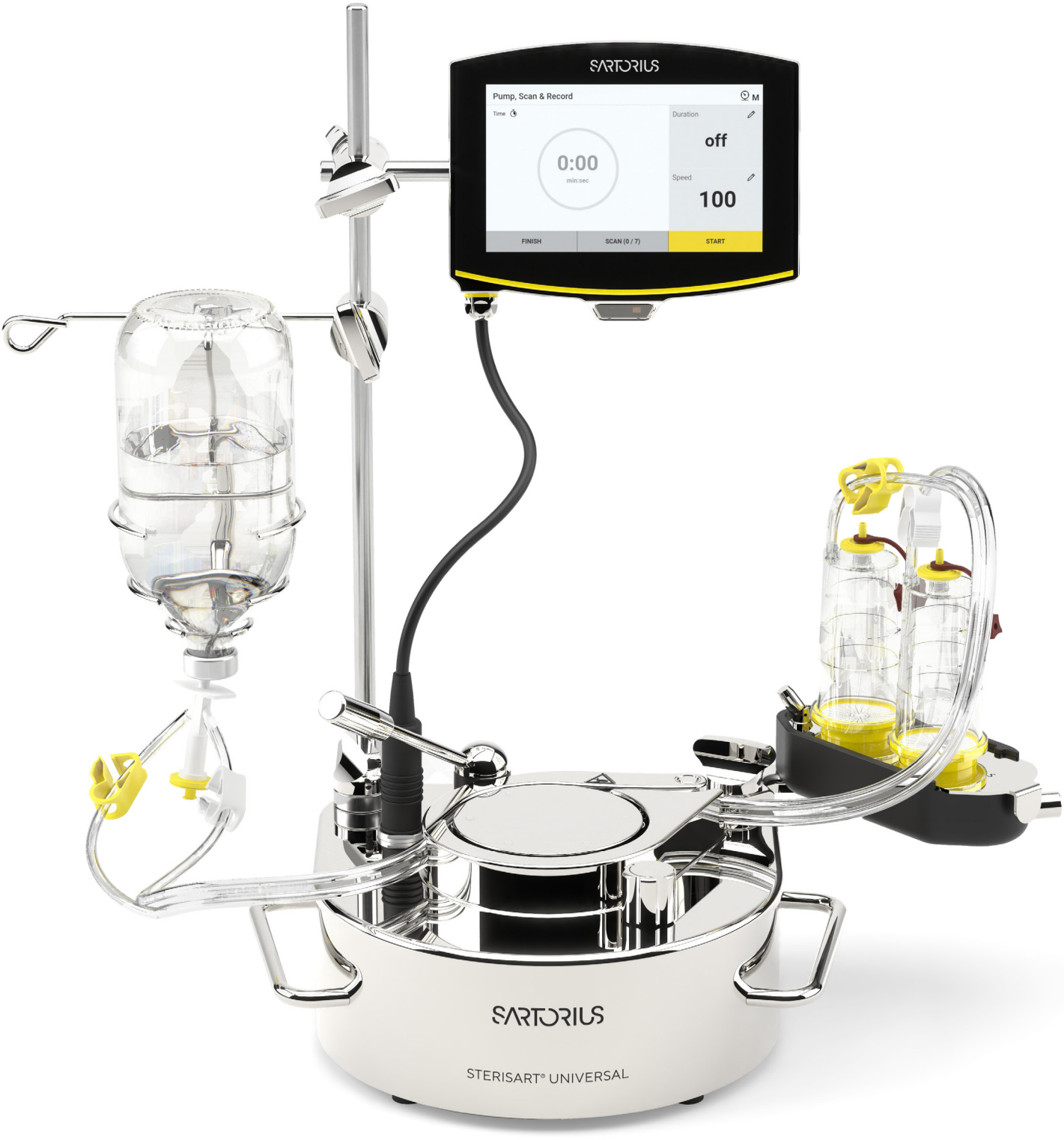
Figure 3: Representative image of the Sterisart® septum after 102 sample extractions.

Conclusion

In summary, we demonstrate that the Sterisart® septum is exceedingly robust and maintains an intact sterile environment even after more than a hundred sample extractions.

The presence of preservatives and anti-microbial agents, in products tested for sterility, have to a great extent impeded the adoption of rapid detection methods that rely on direct inoculation. Membrane filtration, and subsequent membrane rinsing, of such products curtails the risk of false negatives during sterility testing. By also facilitating the analysis of large volumes through membrane filtration and by enabling the extraction of samples, we afford our users the ability to integrate closed system sterility testing with rapid sterility testing methods.

However, some slow growing anaerobes can be difficult to detect using some rapid sterility methods. Given that septum sampling does not compromise the integrity of the closed system sterility test, we provide our customers with the potential to sample for rapid sterility testing, yet re-incubate the sterility tests for the stipulated 14-day period of incubation.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Points to Consider When Validating Your Sterility Testing Canisters

Sterility testing is an integral part of all pharmaceutical microbiology laboratories and is designed to detect the presence of viable microbial contaminants in sterile pharmaceuticals. Being a method based on the evaluation of microbial growth, it is crucial to distinguish between true product sterility and a false negative (Aseptic Guideline 2004). Certain ingredients used in the formulation of drugs can possess innate anti-microbial properties and prevent a sterility test from reliably reporting on the presence of viable microorganisms..

Validation is therefore performed for all new product formulations, whenever there is any change made in product formulation or if there are changes in experimental conditions. This includes selecting or making a change between a primary | secondary supplier of your sterility testing equipment. Even in the absence of a change, it is recommended to routinely revalidate all processes on a regular basis.

This involves the continual collection, evaluation and documentation of data. As a general rule, it is advisable to seek guidance and feedback from local or international regulatory bodies or advisors on the proposed methodology early in the process and prior to undertaking a validation exercise to ensure these will comply with their requirements.

There are several detailed guidelines for sterility testing, besides the pharmacopeial chapters. We have compiled the following points to be considered during the validation | revalidation of your sterility testing canisters.

Validation of Sterisart® Sterility Testing Canisters

Sterility testing canisters must be compliant with the pharmacopoeia guidelines used in the facility, and a manufacturer's validation guide should be available. Ultimately, methods validation studies should demonstrate that the method does not provide an opportunity for false negatives (Aseptic Guideline 2004). The following points should be considered when selecting or making a change to suppliers of sterility testing canisters or any other critical component
of manufacture or testing of a product.

1. Approval
This Sterility Testing Canister Validation Protocol should be reviewed by the head of Microbiology or an authorised QC microbiologist and approved by the head of Quality Assurance or their designated authority.

2. Objective
The object of this protocol is to validate Sartorius Sterisart® Canisters as approved canisters for use during quality control and lot release testing in this facility.

3. Scope
This protocol is relevant for the sterility testing team in the microbiology laboratory of the quality assurance department of the facility.

4. Reason for Validation
All critical materials and assays used in the manufacture and release of parenteral products must be validated for suitability. Validation of Sartorius Sterisart® canisters should be performed as indicated by the appropriate pharmacopoeia, regulatory guidelines, or both.

Manufacturers may want to consider validating materials and consumables from alternate suppliers for mission critical testing consumables to provide protection against process interruptions due to supply outages. These outages could be due to logistics issues, where the consumable | test item is not available locally, or if there is a failure in the manufacturing process. Examples of causes for these outages include fire, earthquake, or other natural or man-made disaster at the factory manufacturing the test consumable, lack of raw material(s) required to manufacture the test consumable, logistics issues caused by a break-down in the logistics chain due to strike action, a pandemic, or a similar interruption to freight.

5. Revalidation Criteria
Test methods should be revalidated if there is a change in manufacturing procedures, testing procedures or any of the test items, including the consumables or growth media used. The PIC/S 11.6.2.4 and the TGA guidelines on sterility testing (407) also recommends revalidating test methods every 12 months, although this is not a pharmacopoeial requirement. Revalidation may be required if a manufacturer of a consumable or media changes the construction, materials used, functionality of the consumables, or the formulation or ingredients in a media type.

6. Responsibilities
The head of Quality Assurance is responsible for approving the validation protocol, and for accepting changes to the testing procedure once equivalence of the second supply has been demonstrated. The head of the Microbiology Laboratory is responsible for overseeing the implementation of this validation protocol, and the sterility test team is responsible for performing the associated tasks. Sartorius will provide its in-house validation document. This document demonstrates that the Sterisart® canisters meet or exceed the requirements for use in a compendial sterility test in terms of the materials and methods used in their manufacture, assembly, packaging, and sterilization.

7. Reference Documents
In-house standard operating procedures that comply with relevant pharmacopoeia, cGMP and PIC/S guidelines, and any other appropriate guidelines or regulations should be referenced to perform this validation protocol. Validation experiments will be based on the current, validated work methods and SOPs in the first instance, and varied as required to achieve product validation. These internal reference documents may need updating to include any

necessary variations from the current methodology required to successfully utilize the Sterisart® canisters. Established change-management procedures for updating documents should be followed if changes are required.

8. Procedure

The following is a general overview of the procedure involved in the process of validating a sterility test, and is by no means exhaustive. Please refer to the appropriate pharmacopoeia document or local authority guidelines for a more complete description.

For new products, validation of the inactivation of product ingredients having anti-microbial activity, or rinsing them from the membrane will be required. For most re-validations of existing assays due to any of the changes listed above, existing inactivation, filtration, and rinse parameters should be suitable, but must be re-validated using bacteriostasis and fungistasis tests.

This should be performed generally by spiking the final rinse solution with <100 CFU of test organisms. In the case of products having no demonstrable anti-microbial activity, and that require no rinsing of the membrane (for example isotonic saline solutions), the product itself may need to be spiked. Growth promotion tests (positive controls) should be performed alongside sterility testing to validate the growth of test species in this assay. Bacteria should grow within 3 days and fungi within 5 days for valid results, and there should be little visible difference in microbial growth between the positive controls and the test canisters. Occasionally, there are regional differences in recommendations and practices. For instance, the TGA guideline specifies that growth promotion tests should be performed after 14 days on un-spiked sterility test samples to show the media are still capable of supporting growth. Bacteria should grow within 3 days and fungi within 5 days for valid results.

Growth promotion tests (positive controls) will require three canister sets for confirming the growth rates and patterns of the six standard species in the assay being validated, and bacteriostasis and fungistasis tests will require three canister sets per batch of product tested. Negative controls (one canister set) and negative product controls (one canister set per product) should also be conducted. While a definitive number of batches of product are no longer specified for assay validation, regulators expect manufacturers to use a science-based approach to determine how many batches will be used during validation or revalidation, and to have sound rationale for this decision. Consequently, a typical validation experiment will consist of eight or more canister sets as defined in Table 1.

Negative product controls should have all filtration and rinse steps performed apart from the actual product filtration, which is substituted by product or simulated product of known or undoubted sterility. Negative product controls should be exposed to a terminal sterilisation process, such as exposure to steam sterilisation, gamma-irradiation etc., and be packaged in a similar manner to the test sample. Alternatively, distilled water in the same or similar container could be used. Growth results of all canisters should be recorded as pass | fail. Photographic records are desirable. Where necessary, inactivation and rinse steps may need to be re-optimised to achieve the desired growth results in the bacteriostasis | fungistasis tests. These should be recorded as deviations from the existing test method.
In urgent situations, concurrent validation of alternative

Canister set	Contents	TSB inoculum	FTM inoculum
1	Growth promotion assay (positive control)	*Bacillus subtilis*	*Clostridium sporogenes*
2	Growth promotion assay (positive control)	*Candida albicans*	*Staphylococcus aureus*
3	Growth promotion assay (positive control)	*Aspergillus niger*	*Pseudomonas aeruginosa*
4	Bacteriostasis	fungistasis test (per batch)	*Aspergillus niger*	*Clostridium sporogenes*

Table 1. Species inoculation scheme for sterility canister testing

sterility test canisters can be performed as part of routine testing. Preferably, this should run alongside existing sterility tests, using consumables from the current supplier(s) to demonstrate equivalence to existing methods and

9. Deviations

Any and all deviations from the written procedure occurring during the validation activity should be recorded.
Any deviation occurring in inactivation or rinse steps due to changes required to achieve acceptable growth rates in the bacteriostasis | fungistasis tests in a revalidation should be recorded.

10. Conclusions

The conclusion should include the overall results of the validation process indicating if validation passes or fails for each product and should note any changes required to the test method for the tests to successfully pass the assessment criteria.

11. Report

A report including the raw data for each product evaluated should be attached as annexure. Data should be compiled by the operator performing the validation and should be checked by the head of Microbiology or their designated authority.

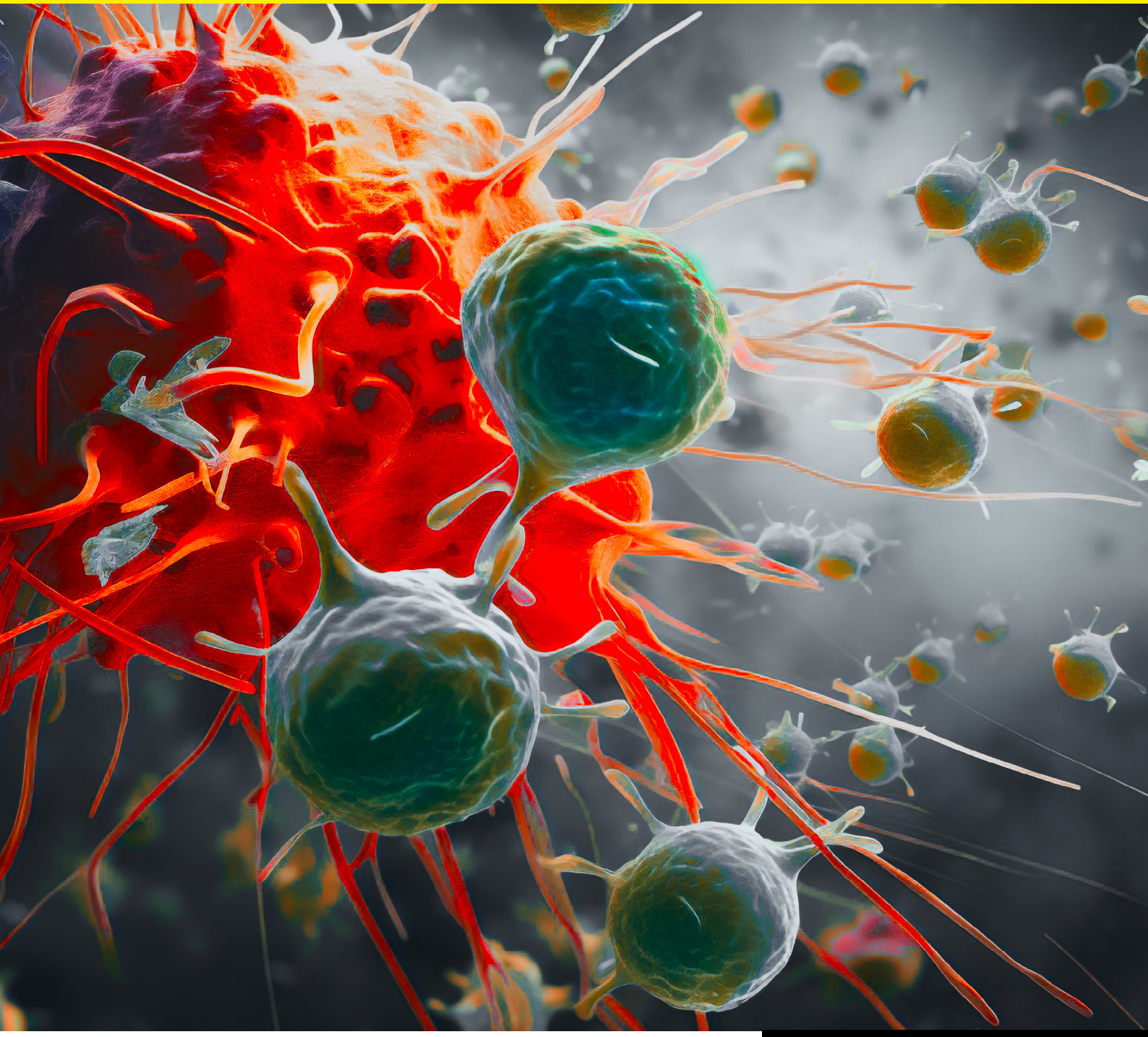
12. Report Approval

Reports should be reviewed by concerned departments and approved by the head of Quality Assurance or their designated authority. Report approval shows that the validation was completed successfully and according to the validation protocol.

References

U.S. Pharmacopeia. USP <71> Sterility Test
European Pharmacopoeia. Ph. Eur. 2.6.1 Sterility
Japanese Pharmacopoeia. JP 4.06 Sterility test
World Health Organization (WHO); 3.2 Test for sterility

TGA guidelines for sterility testing of therapeutic goods, 2006
21 CFR 610.12 – General Provisions
PIC/S PI 012-2 Recommendation on Sterility Testing
FDA Aseptic Guideline (Sterile Products Produced by Aseptic Processing, 2004)



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Application Note

Rapid, Real-Time PCR-Based Detection of Microbial Contaminations in High Cell Density Jurkat-, HPBMC- and CHO- Cultures using Microsart® ATMP Kits

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Abstract

In this study, we used Sartorius Real-time PCR kits for the rapid detection of bacteria, fungi, and mycoplasma to establish that these assays can detect microbial contaminants, even in the presence of high cell backgrounds, ranging from 10 to 40 million cells per milliliter (cells/mL). We observed that the cell density limit and assay robustness depend on cell types and media compositions. The detection limits for bacteria, fungi, and mycoplasma in Jurkat cells, HPBMC and CHO cells varies between 10 and 25 x 10^6 cells/mL.

This study clearly demonstrates that Sartorius Microsart® ATMP kits tolerate higher cell density, still reaching the required sensitivity criteria (≤ 99 CFU, or colony forming units, for bacteria and fungi and ≤ 10 CFU for mycoplasma). The study also demonstrates the capability of Microsart® ATMP kits to sensitively detect microorganisms, even in the presence of high-density cell cultures, and thus contributes to risk-reduction and patient safety of cell therapy products.

Find out more: sartorius.com/en/applications/quality-control-testing/microbiological-quality-control/rapid-testing

Introduction

Sterility testing is a critical component of the release testing for any cell therapy product since microbial contamination of cell therapy products can potentially kill recipients. The current compendial sterility test for most bacteria takes 14 days and 28 days for mycoplasma testing before contamination can be ruled out with certainty[1,2,3]. However, time-to result is an important attribute of testing or short shelf-life cellular therapeutics, especially for autologous cell therapies intended for terminally ill patients.

As a result, growth-independent rapid assays are in increasing demand. Therefore, to fulfill this demand, we developed and comprehensively validated a highly sensitive and broad range microbial detection system, consisting of an efficient Microsart® ATMP Extraction DNA isolation protocol, and followed by a real-time PCR assay using the Microsart® ATMP Bacteria/Fungi/Mycoplasma kit. In-silico sequence alignment analysis demonstrated that the Microsart® ATMP Bacteria/Fungi/Mycoplasma assays detect a broad range of microorganisms, including species difficult to detect by classical culture methods, as well as dormant contaminants. In addition, the typically very high cell densities of cellular therapeutics can pose challenges to the sample preparation used in rapid, nucleotide amplification (NAT)-based assays.

In this study, we assessed the detection capability of Microsart® ATMP Extraction, combined with Microsart® ATMP Bacteria/Fungi/Mycoplasma assays, in high-density cell cultures. We used Human Peripheral Blood Mononuclear Cells (HPBMC), Jurkat cells, and Chinese Hamster Ovary (CHO) cells as representative background matrices. We chose HPBMC and Jurkat cells lines because the product was developed primarily for QC testing of ATMPs, and therefore, the Real-time PCR detection kits mainly target applications with human cell lines. We chose CHO cells because they can be cultivated in high densities, making them valuable for tests with very high cell densities[4].

Methods

Preparation of eukaryotic cell samples
Jurkat cells were grown in T-flasks in RPMI medium (RPMI 1640, Thermo Fisher/Gibco), supplemented with 2 mM GlutaMAX (Thermo Fisher/Gibco), 10 % fetal calf serum (Thermo Fisher/Gibco), 1 mg/mL Penicillin, and 104 U Streptomycin (Thermo Fisher/Gibco). Media was exchanged every three to four days. Jurkat cell density was counted with a Cedex HiRes analyzer (Roche).

CHO cells were diluted with Dulbecco's modified Eagle's medium (DMEM) to required concentrations. HPBMC and Jurkat cells were further concentrated by centrifugation for 10 min at 200 x g or 300 x g, and supernatant discarded until the required concentration was reached. Cell pellet was resolved gently.

To prepare cell culture samples with defined microbial spikes, Microsart® Validation Standards, which contain a specific number of CFU of inactivated bacteria or mycoplasma cells, were used according to the instructions[5,6]. Validation standards for Bacillus subtilis, Pseudomonas aeruginosa, Kocuria rhizophila, Candida albicans, Mycoplasma arginini, Mycoplasma orale and Mycoplasma s ynoviae were used in this study. These standards contain 99 CFU of lyophilized, inactivated bacterial particles or 10 CFU lyophilized, inactivated mycoplasma particles. For fungal contaminations, the EZ-CFU™ standards from Microbiologics® with a spike level of 50 CFU were used. The working suspension was prepared according to the EZ-CFU™ protocol (one CFU pellet, 1:20 diluted)[7].

DNA extraction
DNA was extracted with the Sartorius Microsart® ATMP Extraction kit, suitable for DNA isolation of gram-positive and gram-negative bacteria, fungi, and mycoplasma prior to PCR testing[8]. According to the protocol, 1 mL sample material was added to a DNA free 1.5 mL tube. The tube was centrifuged for 15 min at 16,200 g. Supernatant was discarded, then 500 µL Lysis Buffer added to the pellet. To enable better lysis, the pellet was dissolved with a filter tip or by vortexing. After vigorously vortexing for 30 s, the sample was incubated for 10 min at 80° C (combined with shaking at 1,500 rpm) and centrifuged for 10 min at 16,200 g. Supernatant was discarded, 100 µL Suspension Buffer added, and the tube vortexed vigorously for 30 s. DNA was used for PCR directly.

Quantitative real time polymerase chain reaction (qPCR)
For PCR testing, the Sartorius Microsart® ATMP Bacteria, Microsart® ATMP Fungi, or Microsart® ATMP Mycoplasma kits were used[9,10,11]. These PCR kits use FAM™ and ROX™ fluorescence dyes for real-time detection. The dyes are fused to ssDNA probes, which are complementary to the amplified target DNA (FAM™) or the Internal Inhibition Control (ROX™). The use of these TaqMan® probes enhances the specificity of the assay.

The lyophilized Bacteria/Fungi/Mycoplasma Mix, the Internal Control, and Positive Control were centrifuged briefly to collect the lyophilized material on the bottom of each tube. Reagents were rehydrated by adding 390 µL Rehydration Buffer to the Mastermix (MM), 800 µL PCR grade water to the Internal Control, and 300 µL to the Positive Control. The reagents were incubated for 5 min and briefly vortexed. 26 µL Internal Control was added to the 390 µL MM. Afterwards, the PCR reaction mix was prepared by adding 10 µL sample DNA extract, PCR grade water (no template control), or Positive Control to 15 µL MM in a PCR reaction tube. A total reaction volume of 25 µL per sample was amplified according to the following thermal protocol:

1. 95° C for 3 min
2. 95° C for 30 s
3. 55° C for 30 s
4. 60° C for 45 s

Steps 2 to 4 were repeated 39 times.

Fluorescence of ROX™ and FAM™ was measured during the elongation in step 4.

For most experiments, all pipetting steps were conducted inside a closed glovebox with airlock to avoid contaminations. The BIO-RAD PCR cycler CFX96 Deep Well cycler instrument was used, data were analyzed with the Bio-Rad CFX Manager 3.1 software. Experiments that were conducted without the glovebox, were performed at a sterile bench and with a Stratagene Mx3005p PCR cycler. Data were analyzed with the MxPro software. Ct threshold and baseline were manually adjusted for ROX™ and FAM™ in both software systems. The baseline start was set where fluorescence signal levelled off at a constant level. The baseline end was set before the fluorescence signal of the positive control increased. The threshold was set to a tenth of the mean maximum fluorescence of the no template controls (for ROX™) or the Positive Control (for FAM™) for the bacteria and fungi PCR. Resulting Ct values < 40 were considered positive, CT values > 40 were considered negative. Automatic software settings (baseline and threshold) were used for Mycoplasma detection using the Stratagene Mx3005p PCR cycler.

Results

As summarized in Table 1, we detected 10 CFU M. arginini or M. orale spikes or 99 CFU of B. subtilis in a background of 15 to 19 million CHO cells per ml, using Microsart® Validation Standards. In the presence of 25 million Jurkat cells per ml, we detected 99 CFU of K. rhizophila and in presence of up to 20 million Jurkat cells per ml, we detected 50 CFU of C. albicans. We did not detect 10 CFU of M. orale or M. synoviae in the presence of Jurkat cells, due to PCR inhibition.

In up to 10 million HPBM cells per ml, we detected 99 CFU of K. rhizophila. In a background of 15 and 19 million cells per ml, we detected 10 CFU of *M. orale* and *M. arginini*. In presence of 20 and 25 million HPBM cells per ml, we detected 99 CFU of *P. aeruginosa*.

Detection limits in different cell types

Cell Type Background	Microorganism Spike	Background Cells /mL (In 10^6)	Detection
CHO	99 CFU *B. subtilis*	19.0	Successful
CHO	10 CFU *M. arginini*	15.0 and 15.6 (two individual experiments)	Successful
CHO	10 CFU *M. orale*	15.5 and 16.3 (two individual experiments)	Successful
Jurkat	99 CFU *K. rhizophila*	10 to 40	Successful up to 25×10^6 c/mL
Jurkat	50 CFU *C. albicans*	10 to 40	Successful up to 20×10^6 c/mL
Jurkat	10 CFU *M. orale*	10 to 40	Not successful: PCR inhibition > 15×10^6 c/mL No detection of Mycoplasma spike
Jurkat	10 CFU *M. synoviae*	10 to 35	Not successful: Partial PCR inhibition No detection of Mycoplasma spike
HPBMC	99 CFU *K. rhizophila*	10 to 40	Successful only up to 10×10^6 c/mL
HPBMC	10 CFU *M. arginini*	15.0	Successful
HPBMC	10 CFU *M. orale*	19.1	Successful
HPBMC	99 CFU *P. aeruginosa*	20 and 25	Successful

Table 1: Results of Real-Time PCR detection of respective microbial spikes in varying cell types with respective cell densities.

Conclusion

Short shelf-life therapeutical products require rapid, alternative methods for sterility testing to rule out contamination before administration. Rapid detection methods, such as Real-time PCR-based approaches, contribute to patient safety; however, the typically very high densities of cellular therapeutics pose challenges to sample preparation protocols. Here, we tested the tolerance of Microsart® ATMP kits to different cell densities and cell types.

We found that the cell density limits and assay robustness varied, depending on cell type and media compositions. While the detection limit in Jurkat cells seems to be around 20 to 25 x 10^6 cells/mL for fungi and bacteria, the PCR reaction itself was inhibited at lower cell densities for the mycoplasma PCR. The limit for HPBMC varies between 10 and 25 x 10^6 cells/mL for bacteria, fungi, and mycoplasma detection. We successfully detected mycoplasma and bacteria in the presence of about 15 to 19 million CHO cells per ml.

The performance of these kits was validated using a maximum cell concentration of 10^6 cells/ml; however, our study demonstrates that even higher cell numbers can be processed without losing sensitivity. The maximum cell number for testing can vary according to specific characteristics of the sample (e.g. medium, cell type) and may require adaptation of the procedure to the specific matrices.

Rapid, sensitive, and robust detection of bacteria, fungi, and mycoplasma in the presence of a dense cell background requires an efficient lysis step, followed by a suitable DNA isolation and a robust PCR assay as offered by Microsart® ATMP kits.

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Simplifying Progress

At the Borderline of Sensitivity and Noise

Detection of Nucleic Acid Traces – A PCR Kit Manufacturer Perspective

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Abstract

The bio-pharmaceutical world has moved drastically towards short-lived personalized cell and gene therapy products in recent years. With the new quality control requirements, several new rapid microbial detection methods have been developed. USP <1071> states, "The ability to detect contamination, in real-time, prior to the administration of the shortlife product may be considered more important than detection of a single colony-forming unit (CFU) in the product."

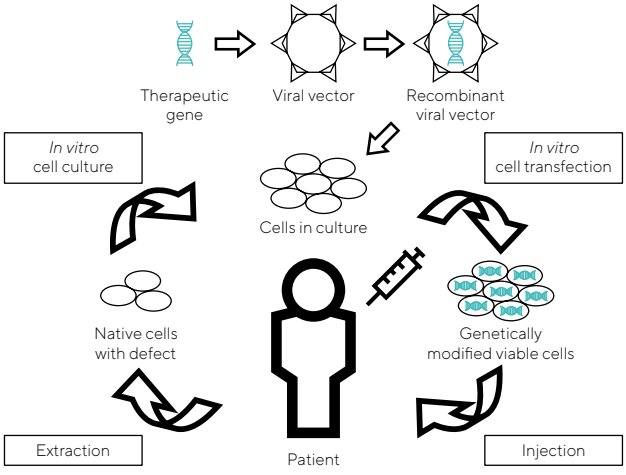
However, the new methods strive to detect the holy grail of 1 CFU. Will this ever be possible, or is it even necessary?

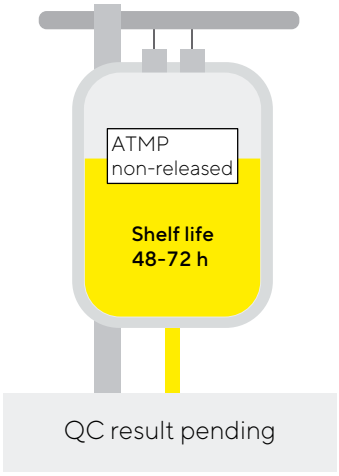
In our presentation, we will walk you through state-of-the-art nucleic acid detection, elucidate the critical steps and highlight the many benefits of this approach. In doing so, we will address key requirements, such as the limit of detection and how to deal with "false" positives. As groundbreaking technology digital PCR (dPCR) allows a new level of precise quantification.

We quantified the positive control (PC) of our Microsart® ATMP Sterile Release Kit that allows us now to shed light on the borderline between sensitivity and noise. We propose an open discussion on this advanced method and the importance of understanding standards in QC testing and release.

1. Advanced Therapy Medicinal Products (ATMPs)

ATMPs[1] are a new class of complex medicinal products associated with viable cells and tissue. An example of a cell-based medicinal product is an "Ex Vivo Autologous Gene Therapy", where cells of the patient are genetically modified *ex vivo* and introduced back to the patient to fight the illness (Cell and Gene Therapy = CGT).



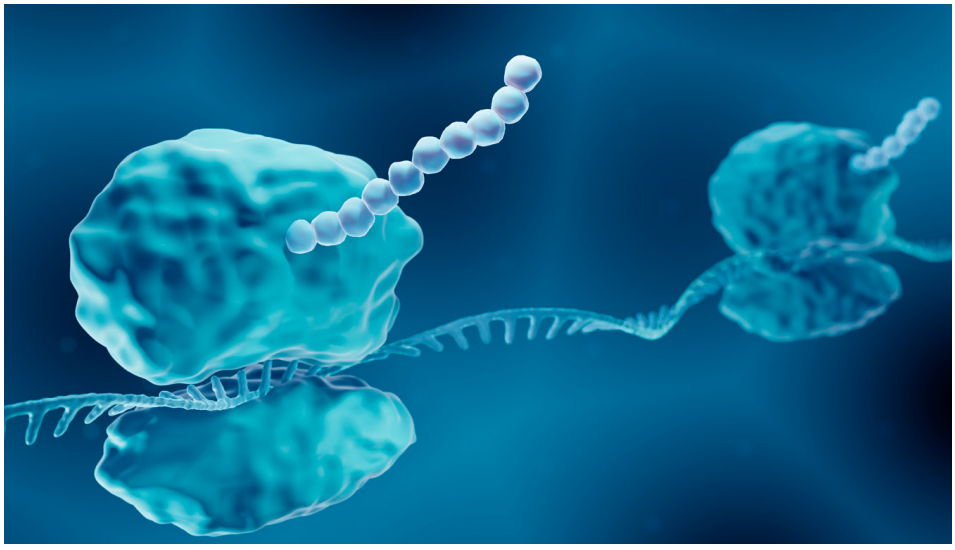


2. Sartorius' Real-Time PCR-Based Sterile Testing Solutions

Microsart® ATMP Sterile Release kit allows bacteria and fungal presence/absence tests within one working day. The included Microsart® ATMP Extraction kit enables the processing of two 1 mL samples and one negative extraction control (NEC). The extracted DNA can be further analyzed with the included two real-time PCR kits, Microsart® ATMP Bacteria and Microsart® ATMP Fungi, enabling the fast and precise detection of bacterial and fungal contaminants by targeting ubiquities ribosomal genes.

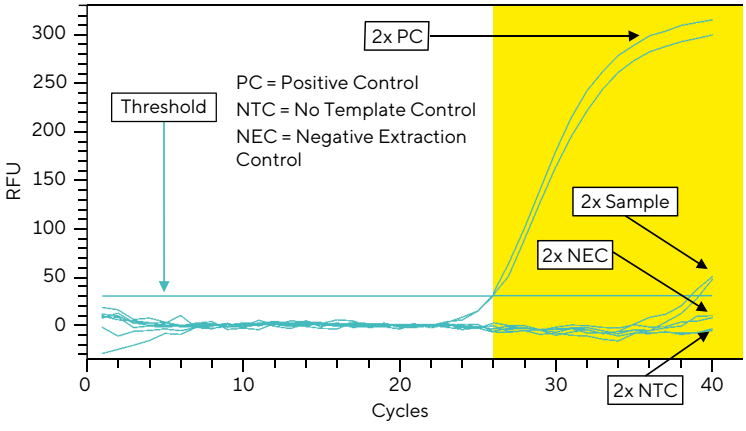
3. Ribosomal Genes as Real-Time PCR Target

Ribosomes translate genomic information into live maintaining proteins. Thus, all living entities, including bacteria and fungi, have ribosomal genes, which can be targeted by real-time PCR. Biological contamination can be excluded by experimentally verifying the absence of the bacterial 16S and fungal 18S rRNA gene. However, the ubiquity of ribosomal genes is also a challenge. Omnipresent bacteria lead to a high basic load of bacterial DNA, which must be removed at great expense and, in some cases, can lead to false-positive results in PCR-based tests.



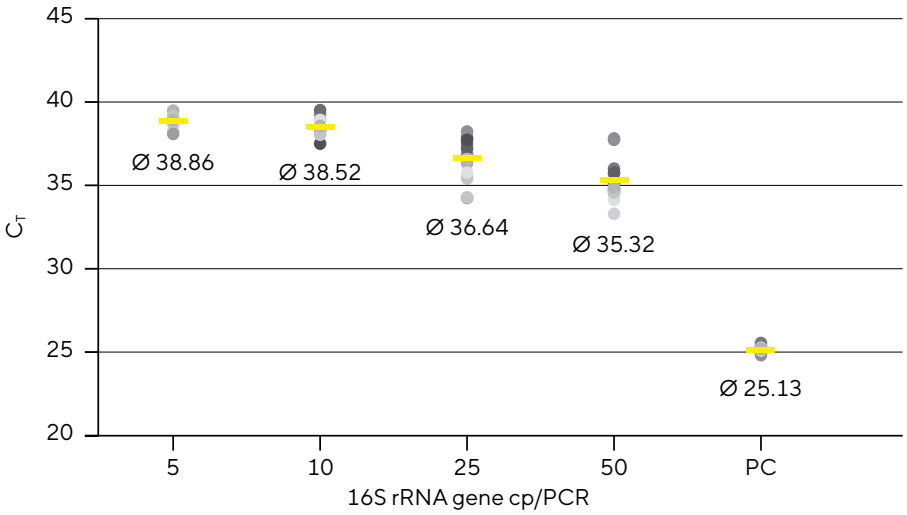
4. Challenges of a Real-Time PCR Test

The Microsart ® ATMP Bacteria real-time PCR assay addresses the universal bacterial 16S rRNA gene and detects the presence of almost any bacterial DNA. However, in rare cases, the signal crosses the threshold and product sterility can no longer be guaranteed. The arising question is how to interpret the observation and what consequences it has for the Advanced Therapy Medicinal Product (ATMP) and ultimately for the patient. [2,3]



5. A Quantified DNA Standard

The dPCR quantified positive control (PC) can be used as a DNA standard to set a desired concentration to estimate the 16S rRNA gene copy number behind a Ct value (Cycle threshold value where the signal crosses the threshold) or can be used further as a positive control (PC). The Ct value in the shown example (Section 4) is about 38. With the help of the dPCR quantified PC, we now can estimate about 5-10 copies of 16S rRNA gene to be responsible for it. Assessing the potential contamination load becomes possible now.



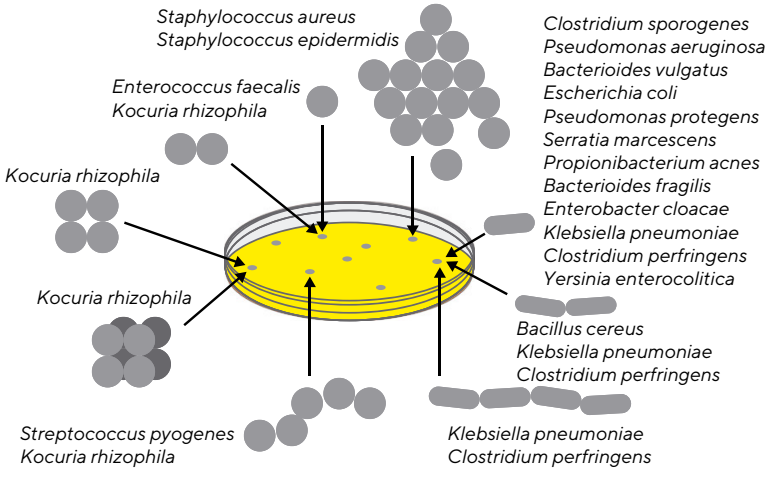
6. 16S rRNA Gene Copies ≠ Genome Copies ≠ CFU

Bacterial genomes usually contain several 16S rRNA copies. A metabolically active bacterium usually holds several genome copies per cell, enabling fast cell division. Daughter cells often stick to each other and can form a conglomerate of several cells. Thus, already one CFU can hold a two-to-three-digit number of 16s rRNA gene copies.[6] The following Table shows the average number of 16S rRNA gene copies of QC-relevant bacterial strains (USP<71>) with the corresponding genome equivalent when 5, 10 or 50 16S rRNA gene copies are detected.

		Genome equivalents considering the detected 16S rRNA gene copies		
P <71> Compendial bacteria species	**16S cp/genome**	**5 cp**	**10 cp**	**50 cp**
illus subtilis	10	0.5	1	5
stridium sporogenes	9	0.6	1.1	5.6
udomonas aeruginosa	4	1.3	2.5	12.5
phylococcus aureus	7	0.7	1.4	7.1

7. Microbial Colony Forming Units – CFU

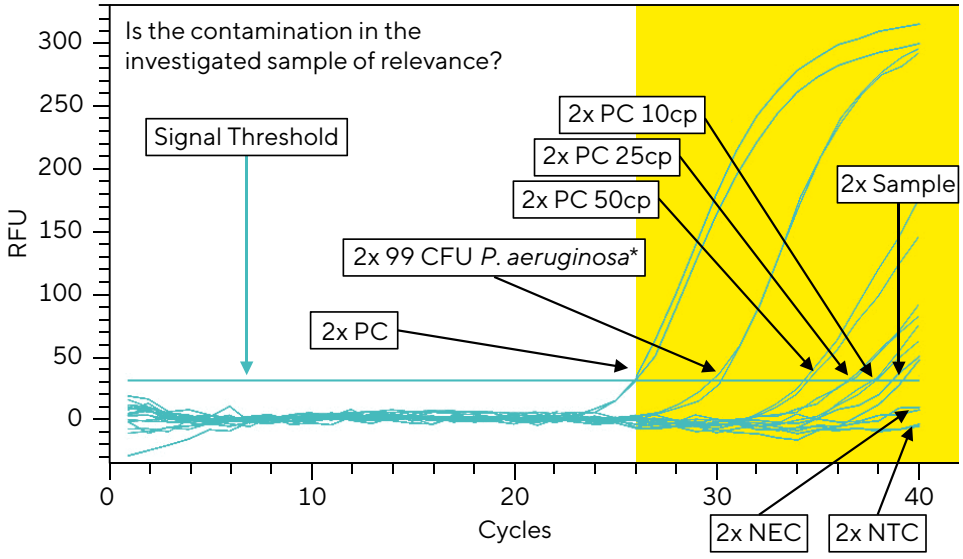
The smallest unit leading to a bacterial colony is called the "colony forming unit or CFU". In an ideal case, one bacterial cell is sufficient to form a CFU. However, in real life, bacterial cells often stick together in several units or even form a biofilm with a consortium of bacteria. Consequently, a CFU consists of several cells, with very species-specific numbers. [4,5]



8. At the Borderline of Sensitivity and Noise

Microbial Colony-Forming Units (CFUs) display diverse characteristics influenced by growth conditions and division status. This inherent genomic diversity poses challenges for precise detection. Moreover, a single genome can have differing numbers of 16S rRNA gene copies, further complicating detection efforts.

Regulatory guidelines, such as the United States Pharmacopeia (USP<1071>), suggest a detection limit of at least 100 CFUs for risk-based release assessments. But what does this mean for the experiment?



Using our Microsart® Validation Standard 99 CFU *P. aeruginosa* to spike a sample, we detect it with a Ct value of 30. If *P. aeruginosa* was the only possible contaminant to expect, one might risk stating the observed contamination is uncritical (Sections 2 and 8).

However, rarely is the contaminant known, and its exclusivity guaranteed. Thus, the question arises of how many 16S rRNA gene copies are the basis of an observed PCR signal and what is the worst-case scenario for a potential contaminant. In the most challenging scenario, an organism may possess one genome copy with only one 16S rRNA gene copy. Consequently, detecting 10 16S rRNA gene copies in a real-time PCR would correspond to 10 genome copies (GC) per PCR and 100 GC per 1 mL investigated sample when using our Microsart® ATMP Sterile Release kit.

We addressed these questions using our new quantified PC to replicate the worst-case scenario. By knowing the 16S rRNA gene copies underlying specific Ct values (Section 5), we could estimate that our sample was contaminated with about 10 copies of a 16S rRNA gene (Sections 4 and 8). With such information, one can now assess the GC of relevant contaminants (Section 6) and potential CFUs (Section 7).

In light of these findings, critical questions arise:

Should we employ worst-case scenarios to define the boundary between sensitivity and noise in microbial detection?

How relevant is such a cut-off in a clinical context?

How would you address this borderline?

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