

RiboNAT Rapid Sterility Test: Using rRNA-Based Nucleic Acid Test Methods for Results in as Little as Seven Hours

INTRODUCTION

Modern medicine is predicated on the safety provided by sterility assurance and aseptic practices. The United States Pharmacopeia (USP) defines these two important practices as followed:

Sterility: The complete absence of viable microorganisms or organisms that have the potential to reproduce.

Aseptic: The processes for handling sterilized materials in a controlled environment designed to maintain microbial contamination at levels known to present minimal risk.¹

These properties are central to the quality control regulations that guide the medical and pharmaceutical worlds today. Manufacturing contaminant-free products requires the practice of aseptic techniques, which includes using adequate facilities that are clear of any lingering particles that may affect product sterility, regular environmental monitoring, and bioburden testing of the raw materials being used. Once finished, the final product is still tested for sterility before being released for delivery.

TRADITIONAL STERILITY TESTING METHODS

The sterility test is a simple and accurate test that has remained consistent since being adopted by the British Pharmacopoeia in 1932. Examples of its requirements can be found in USP <71>, European Pharmacopoeia (Ph. Eur.) 2.6.1, and ICH Q4B Annex 8. Traditional sterility tests require an inoculation of the sample in a growth medium and an incubation period of 14 days. The medium is then visually inspected for growth, with a negative test indicating that the sample tested is free of viable microorganisms.

Although the sterility test is an excellent test to ensure the safety of a product, several considerations must be made to ensure the efficacy of the test. First, a negative test result of the sample does not confirm that the entire manufactured lot of products are sterile. USP <71> makes it clear that the test results

only apply to the sample that was tested. This limitation emphasizes the importance of maintaining aseptic practices during the entire manufacturing process. This leads to the second consideration, which is that sterility is never proven by a specific test, rather, it is assured by the entire contamination control techniques used during manufacturing. Not following aseptic practices can lead to false positives or other contaminations that could affect product safety. Lastly, the suitability of the test must be proven for each product tested. USP guidance makes it clear that the test results are not reliable unless samples spiked with known microorganisms have been detected by the test, confirming test efficacy.

CHALLENGES WITH TRADITIONAL STERILITY TESTING METHODS

With the correct contamination control strategies and test suitability considerations, sterility tests become a highly effective method for screening pharmaceutical injectables, medical devices, ophthalmic solutions, and other topical solutions needing sterility assurance. However, despite these strengths, traditional sterility tests have one major unmitigable limitation—the 14-day incubation period. For many products, this is not an issue as this hold time is miniscule compared to the shelf life of the product. But for products that require a short turnaround, there is a risk when they are administered before the final metric of sterility assurance has been determined.

The regulatory agencies refer to products that involve modifications of cells, proteins, and tissues that are then administered to the patient as Advanced Therapy Medicinal Products (ATMPs). The need

to produce a metric of sterility for ATMPs within only several hours rather than the 14-day period of other products has led the USP and Ph. Eur. to draft chapters that outline considerations for using Rapid Microbiological Methods (RMMs). These corresponding chapters are USP <1071> Rapid Microbial Tests and Ph. Eur. 5.1.6 Alternative Methods for Control, which include the following elements as needed for a rapid sterility test: produce results in less than 24 hours, the ability to detect both a low quantity and wide range of viable microorganisms, and to test low sample volumes while also testing multiple samples at once. These requirements are crucial to reduce administering at-risk products to patients and detect microorganisms in samples that would not be otherwise detected by traditional sterility tests, including antibiotic-containing samples and culture-negative infectious agents.

Incubation timelines for traditional versus rapid sterility test methods

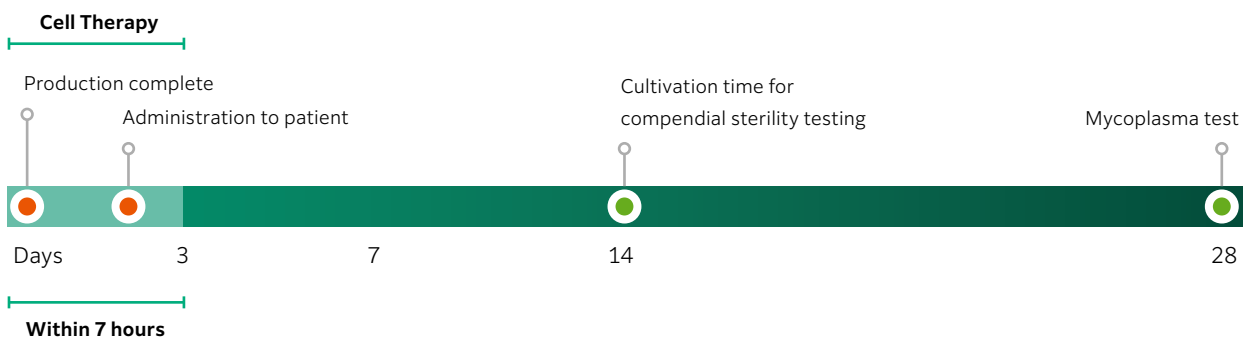


Figure 1. Traditional sterility tests generally require a 14-day incubation period. However, <USP>1071 Rapid Microbial Tests and Ph. Eur. 5.1.6 Alternative Methods for Control outline the requirements for the use of rapid sterility test methods that can deliver results within 7 hours, leading to reduced delivery of at-risk drugs and improving patient safety.

RAPID STERILITY METHODS

Growth-based rapid sterility tests

In response to the growing need of delivering drugs with a shorter shelf life to patients, several solutions have been presented as rapid microbiological methods for sterility testing. These solutions can be categorized as growth-based methods or non-growth-based methods. These solutions all report in Colony Forming Units (CFU), which correspond to the ability of the traditional sterility test to detect a single microorganism that propagates.

The USP has accepted two growth-based alternatives to the sterility test, including respiration-based techniques for detecting contamination and the other involving ATP detection.² The respiration-based test is similar to traditional sterility tests in that the sample is inoculated in growth media and then incubated. However, the microorganisms are not detected by just visible growth, but by the presence of carbon dioxide, essentially making it a test of all respiring, and therefore, viable microorganisms. This test method reports between 1–10 CFU/mL per sample and provides results of sterility assurance within 7 days.³

The second growth-based method involves the detection of ATP using a luminescence indicator and allowing sterility assurance within 5 days to a detection of 1–10 CFU/mL. Because ATP is a result of cellular respiration, this test provides a broad, accurate test of viable microorganisms.⁴

Although these methods provide an equivalent measure of sterility in less than half of the time of the traditional test, these methods still fall short of a 24 hour results that many RMMs require. Additionally, these tests are still susceptible to the potential interferences and contaminants that can appear in a growth sample.

Non-growth-based rapid sterility tests

Although the USP has not currently issued a compendial chapter on any non-growth methods for sterility assurance, USP <1071> and Ph. Eur. 5.1.6 do provide guidance on the adoption of several non-growth techniques for rapid sterility indication. One such finding is that solid phase cytometry is an extremely useful tool for visually scanning an entire sample for microorganisms. This method gives rapid results within a few hours, and provides results with a detection level of 1–10 CFU/mL—on par with the USP's recommended sensitivity for rapid methods. Although this method is simple, reliable, sensitive, and rapid, it performs best for product samples that are free of other cells in suspension. This means that for many of the cellular and gene therapies that make up the majority of ATMPs, this method can have difficulties.

Another method mentioned as a solution includes nucleic acid amplification tests (NAT) using PCR analysis. PCR technology amplifies target genes that are conserved among bacterial and fungal DNA, and these tests can use conventional qPCR analysis and provide a broad-coverage test within a few hours. However, these tests do have a few limitations. Since DNA is a stable molecule, these tests are susceptible to false positives caused by residual DNA. Secondly, these methods do not have the same sensitivity as the other RMMs with quantification limits typically in the range of 10–100 CFU/mL.

Many alternatives to the traditional sterility test exist. However, for an ATMP that: **(1)** contains a high concentration of cells, **(2)** needs results within 24 hours, **(3)** needs detection below 10 CFU/mL, and **(4)** potentially contains residual bacterial or fungal DNA, a perfect alternative method does not conventionally exist. With these limitations in mind, FUJIFILM Wako Pure Chemical Corporation has developed a test to meet these conditions.

RIBONAT RAPID STERILITY TEST

Methodology

The RiboNAT Rapid Sterility Test kit utilizes the NAT method in which ribosomal RNA (rRNA) is detected using RT-rt PCR. Rather than detecting genomic DNA (gDNA) like other DNA-based microbial methods, the detection of rRNA allows for a higher sensitivity and reduced false positives from residual DNA of dead microorganisms. Highly conserved ribosomal subunits are targeted for both bacteria and fungi, which provides specificity to prevent cross-reactions with human cells while also supporting a wide detection range of microorganisms. With this rapid microbial test method, sterility assurance for short shelf-life products can be confirmed prior to patient application to eliminate at-risk treatment.

Workflow

The RiboNAT Rapid Sterility Test kit consists of three distinct parts, including a pre-treatment step, an RNA isolation step, and a final measurement and detection step, allowing for the full assay flow to be completed within 7 hours. In the pre-treatment process, the sample is cultured both aerobically and anaerobically while inactivating free DNA and DNA from dead microorganisms. The second step involves a two-stage DNA degradation process with the use of DNase as well as magnetic beads for the extraction and purification of RNA from microorganisms. In the final detection step, the purified ribosomal RNA is detected using one-step RT-rt PCR. The reagents employed in the RiboNAT Rapid Sterility Test are divided into three individual kit components, RNA Isolation Kit 1, RNA Isolation Kit 2, and the Detection kit, to accommodate the distinguished stages of the assay.

Table 1. Comparison of Sterility Test Methods

Method	Time to Result	Culture	Sensitivity (CFU/mL)	Samples in Cellular Suspension	Dead Microorganisms
Compendial (cultural) method	14 days	14 days	1-3	●	Viable microorganisms only
Respiration	7 days	7 days	1-10	●	Viable microorganisms only
ATP detection	5 days	5 days	1-10	◆	Viable microorganisms only
Solid phase cytometry	3 hours	0 to a few days	1-10	◆	Viable microorganisms only
NAT	4 hours	0 to a few days	10-100	●	Both live and dead microorganisms
RiboNAT	7 hours	3 hours	<9	●	Reduce detection of dead microorganisms

● Method can be applicable for cellular samples ◆ Method is not suitable for cellular samples

Table 1. While other sterility tests utilizing the NAT method produce results faster than traditional methods, the RiboNAT Rapid Sterility Test maintains a higher test sensitivity and reduces the risk of false positives caused by dead microorganisms.

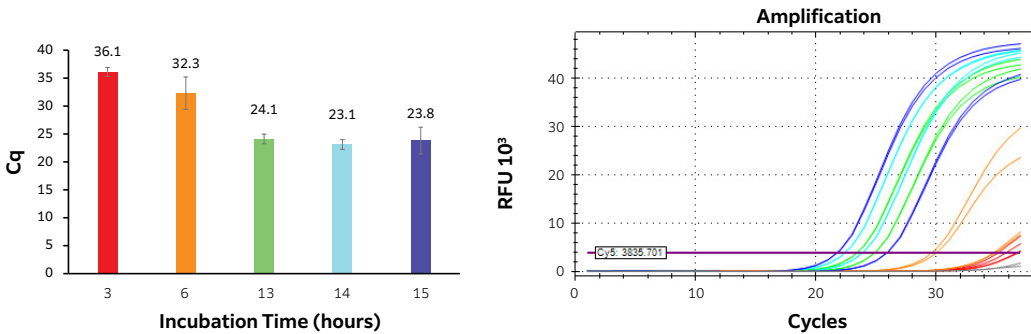
Effects of an increased incubation period on test sensitivity

Through the detection of rRNA rather than gDNA, the RiboNAT Rapid Sterility Test is able to provide a wider, more sensitive detection range of both anaerobic and aerobic bacteria as well as fungi in a single assay. Due to the larger relative quantity of RNA when compared to DNA, the RiboNAT Rapid Sterility Test has an enhanced detection sensitivity when compared to conventional DNA-based NAT methods, validated to as low as 9 CFU/mL.

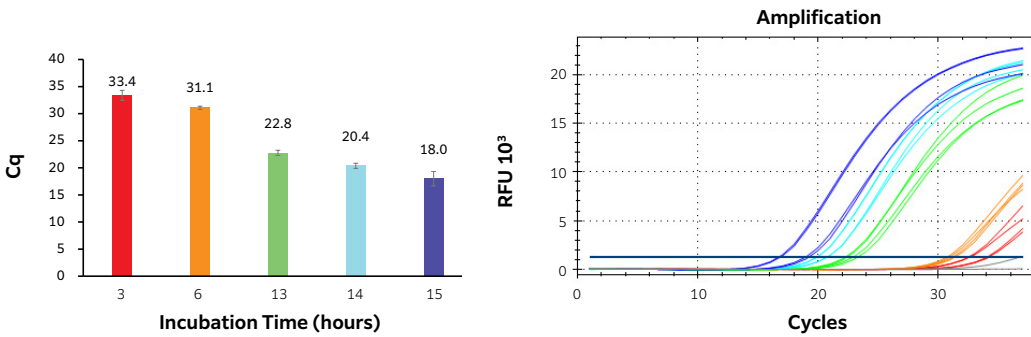
The standard protocol for RiboNAT Rapid Sterility Test involves incubating the samples for 3 hours, however, with an extended incubation period, sensitivity can be increased for certain samples. With a 14-hour incubation period, three microorganisms tested—*Aspergillus brasiliensis*, *Clostridium sporogenes*, and *Cutibacterium acnes*—were detectable at 2 CFU/mL.



Aspergillus brasiliensis



Clostridium sporogenes



Cutibacterium Acnes

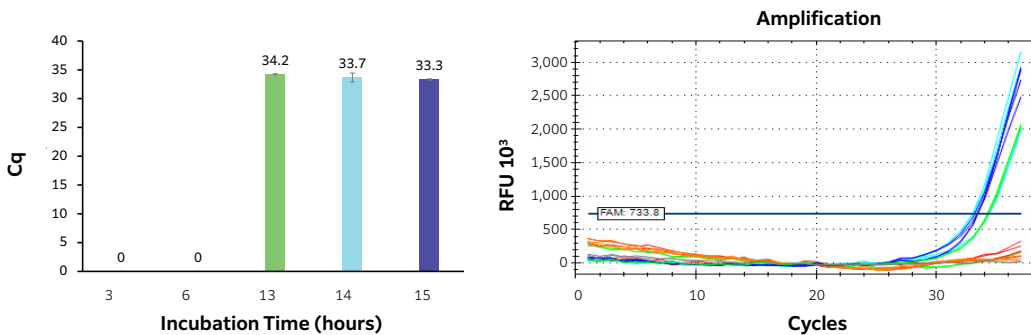


Figure 2. Microbial suspensions were prepared at 2 CFU/mL for *Aspergillus brasiliensis*, *Clostridium sporogenes*, and *Cutibacterium acnes* with incubation times ranging from 3 to 15 hours. After incubation, RNA was extracted using RiboNAT Rapid Sterility Test, and rRNA was detected according to the protocol. All three strains, including strictly anaerobic bacteria (*Clostridium sporogenes*) and slow-growth bacteria (*Cutibacterium acnes*), were successfully detected with Ct values below 35 after 14 hours or longer incubation.

Test sensitivity results following standard protocol

One study took the six microorganisms specified in the *Strains of the Test Microorganisms Suitable for Use in the Growth Promotion Test and the Method Suitability Test of USP Chapter <71>* and used them to validate the sensitivity of the RiboNAT Rapid Sterility Test assay. It then found rRNA from all six strains.

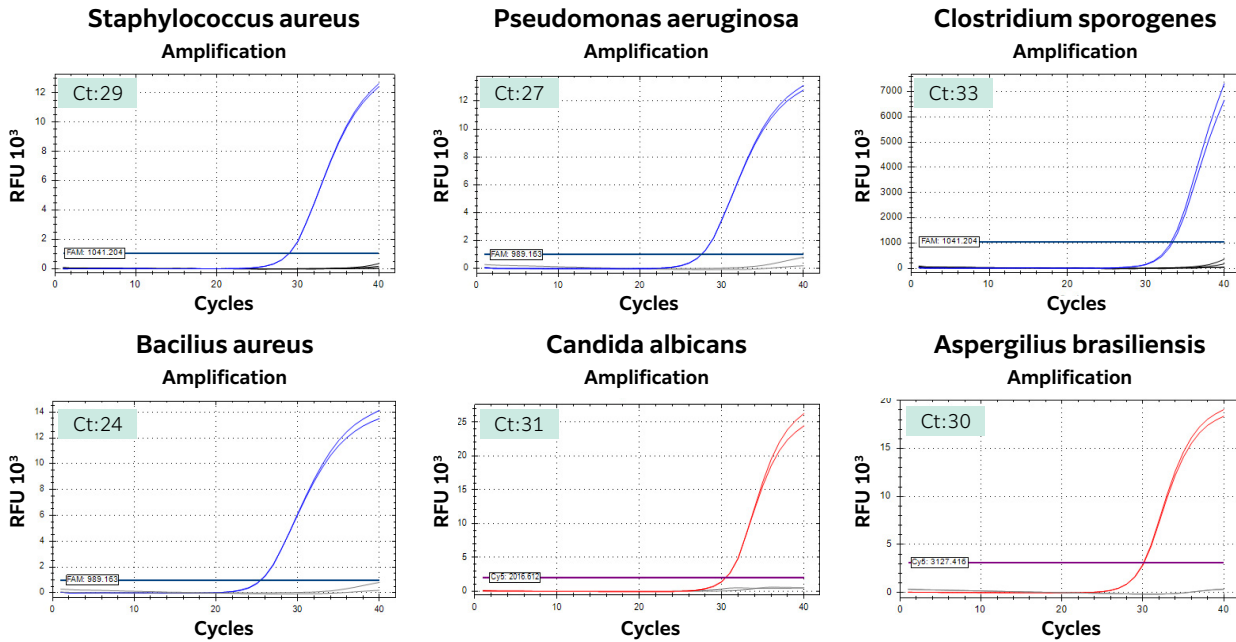


Figure 3. RNA was extracted from prepared microbial suspensions at 9 CFU/mL for the six microorganisms specified in USP <71> using the RiboNAT Rapid Sterility Test according to protocol. Subsequently, rRNA from all microorganisms was successfully detected with Ct values below 35.

Test Sensitivity Results in Cell Suspension Samples

In addition, the six strains were tested in HEK293, MSC, and T-cell suspension samples, which were again detected following the RiboNAT Rapid Sterility Test assay protocol. All six strains were detectable at 9 CFU/mL in these cell suspension sample preparations.

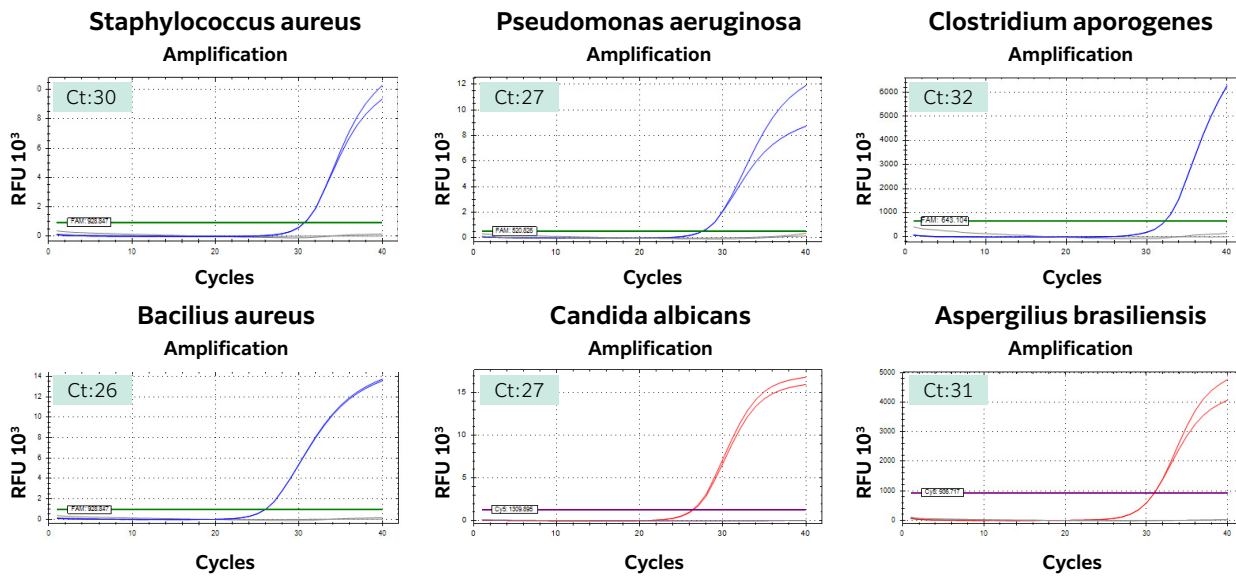


Figure 4. RNA was extracted from prepared microbial suspensions at 9 CFU/mL for the six microorganisms specified in the USP, each mixed with 0.25×10^6 cells/mL of HEK293, again using RiboNAT Rapid Sterility Test according to protocol. Subsequently, rRNA from all microorganisms was successfully detected with Ct values below 35.*

*All six microorganism strains were also detected in a mesenchymal stem cells suspension of 0.5×10^6 cells/mL, and a T-cell suspension of 1.0×10^6 cells/mL.

Effects of RNA-based detection methods on false positives

When using an RNA-based detection method, false positives were significantly minimized when compared to conventional DNA-based NAT methods for sterility assurance. RiboNAT Rapid Sterility Test not only includes a DNA inactivation step but also targets rRNA instead of gDNA to reduce false positives from dead microorganisms and residual DNA.

When sterile PBS was tested with both a commercial DNA extraction kit and the RiboNAT Rapid Sterility Test kit, it was found that amplification curves were observed in the DNA-based method, but no amplification was detected using RiboNAT Rapid Sterility Test.

Results of RiboNAT Rapid Sterility Test Versus Traditional DNA Detection Kit

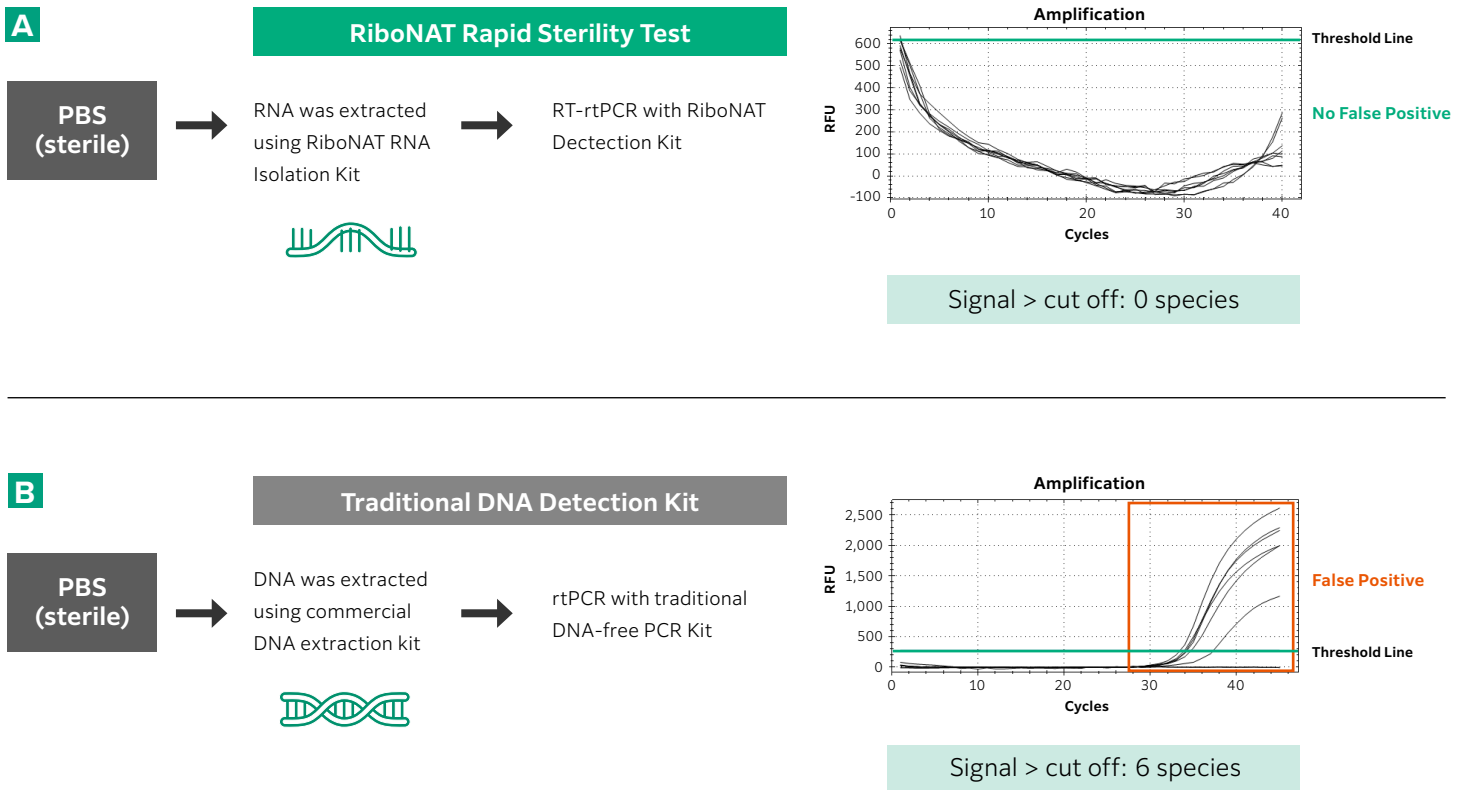


Figure 5. In the one set **(A)**, RNA was extracted using RiboNAT Rapid Sterility Test and subjected to reverse transcription real-time PCR. While six amplification curves were observed in the DNA-based method, no amplification was detected using RiboNAT Rapid Sterility Test. In another set **(B)**, nucleic acid testing (NAT) for bacteria was performed using commercially available sterile PBS as the test sample. DNA was extracted using a commercial kit followed by bacterial-targeted real-time PCR.

RiboNAT Rapid Sterility Test applications

Of the traditional growth-based sterility test methods included in the USP <71> compendial, the recommended 14-day incubation period is not suitable for products that have short shelf lives or are prepared for immediate use. These products, which include compounded sterile preparations (CSPs), nuclear medicine products, and ATMPs, can pose a risk to patient safety if administered before sterility testing is completed if not tested with a rapid microbial method for sterility assurance.⁵

Manufacturers of ATMPs, including cell and gene therapy products, as well as other immediate-use and short shelf-life products that are typically

administered at-risk can benefit from the accelerated sterility assurance timeline provided by the RiboNAT Rapid Sterility Test kit. With only a 3-hour incubation period required, the entire assay can be completed in one day, within 7 hours.

RiboNAT Rapid Sterility Test can also be beneficial for those products that experience difficulties with other DNA-based NAT methods. Additionally, products that have small batch volumes may find increased ease benefits with RiboNAT Rapid Sterility Test, as only 1 mL of product is required for each aerobic and anaerobic sample preparation. Additionally, multiple samples can be tested within one assay, allowing for increased throughput if necessary.

CONCLUSION

Compendial sterility testing requires a 14-day incubation period, which can be incompatible with products that have short shelf-lives or are manufactured for immediate use. For products that are not suitable for traditional growth-based sterility testing and are administered at-risk,

RiboNAT Rapid Sterility Test kit offers a rapid microbial method to assure product sterility before patient application. RiboNAT Rapid Sterility Test retains the benefits of NAT for sterility assurance while also minimizing the drawbacks associated with DNA-based methods, including false positives and decreased sensitivity.

References

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