THE SCANRDI® STERILITY TEST PROTOCOL

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The ScanRDI® – an Effective and Reliable Test for Sterile Compounded Preparations

SUMMARY:

This white paper demonstrates that the Eagle ScanRDI Sterility Test Protocol is an effective and reliable test for the detection of microorganisms in sterile compounded preparations. In addition, the procedure uses the same sampling protocols as the standard test method, has been shown to detect all of the standard USP test organisms, is more sensitive than the reference method, and when coupled with a standard control for each test run, it has been shown to provide consistent and reliable results. Therefore, the test method meets the requirements of USP <797> for the testing of sterile compounded preparations.

The testing of compounded sterile preparations has received renewed scrutiny in the past several months in light of the increased awareness of the need to improve quality control in the compounding of sterile preparations. This white paper will discuss the ScanRDI Sterility Test Protocol, explain how it is performed and show how it can be used to comply with USP <797> Pharmaceutical Compounding – Sterile Preparations for the sterility testing requirements outlined therein.

USP <797> allows an alternate sterility test to be used in lieu of the 14 to 18 day USP <71> Sterility Test procedure. The language of USP <797> states that a method not contained in the USP may be used if it can be shown that the alternative is at least as effective and reliable as the USP test procedure. To demonstrate that the ScanRDI is as effective and reliable, we have chosen a multi-faceted approach based on USP <1223> Validation of Alternative Microbiological Methods.

a. Explain the ScanRDI protocol with particular reference to the sampling procedures utilized as compared to the USP <71> procedure.
b. Explain how the ScanRDI meets the specificity requirements for sterility testing.
c. Demonstrate the superior limit of detection of the method.
d. Show how Eagle Analytical Services’ (Eagle) protocol accounts for day-to-day differences in any performance issues, which are usually referred to as Ruggedness.

Overall Description of the ScanRDI System

The ScanRDI system consists of a scanning laser cytometer that rapidly detects viable microbial cells down to one microorganism without the need for an extended incubation period. The system includes an analytical scan module, laser source, computer, microscope/camera with a motorized stage, and associated equipment.

The sample is aseptically filtered through a membrane filter with a nominal rating of 0.40 microns, which is then washed with Fluid D (peptone water with polysor-
bate 80). A counter stain solution is applied to reduce background fluorescence of particulates and the membrane surface. The membrane is then placed on a pad saturated with a nutrient-fortified pre-labeling buffer to promote the recovery of stressed cells and induce spore germination then is incubated for three hours. Following pre-labeling, the membrane is transferred to a second pad saturated with the fluorescent labeling solution and is incubated for an additional 45 minutes. The labeling solution diffuses passively across the cell membrane, and in viable cells, it is cleaved by ubiquitous esterase enzymes present in the microorganism and accumulates within the cell as fluorescein.

The membrane is then placed in the scan module and the number of cells is counted by exciting the fluorescein within the cells with a laser beam. The total surface area of the membrane is scanned by a laser spot six microns in diameter. The individual scan lines are 2.2 microns apart to ensure that each section of the membrane is illuminated twice by overlapping scan lines. Software in the instrument excludes any signal that is not seen by two consecutive scans in the same position on the membrane. Two photomultiplier tubes detect the emitted fluorescence as the laser scans the surface of the membrane. The signals produced by the photomultipliers are processed using a series of software discriminants that enable the instrument to differentiate between valid signals (labeled microorganisms) and background noise (electrical, optical, or auto-fluorescent particles). The results of the scan are displayed as fluorescent "Events."

The data also is displayed in the form of a membrane scan map, identifying the position of each labeled event on the membrane surface. Because the process does not destroy the cells, the operator can then visually confirm the presence of the labeled cells and their morphology by transferring the filter to an epifluorescent microscope linked to the cytometer, and, by manipulating the cytometer, return to the X Y coordinates of the fluorescent event on the membrane. The results are presented as the number of fluorescent Events, which is the total number of identified possible microorganisms on the membrane, and as "Verified Microorganisms," which are the number of Events that have been confirmed to be microorganisms by visual observation via the microscope.

Each data set includes an instrument calibration using fluorescent styrene beads and a positive and negative control microorganism sample. The complete data set, with quality control information and a statistical trend analysis, is then stored in a database on the Eagle server.

The basic technologies used in the ScanRDI are well documented. The viability substrate technique has been used in many guises and applications in microbiology, so it is also well documented. The fluorescein
esters employed with the ScanRDI technology have been used for many years and have well-documented applications for detecting a wide range of viable microorganisms. The novel component of the ScanRDI is the data-handling software that enables it to maximize the benefits of the viability label and the cytometer. The particular technological aspects of the ScanRDI have been accepted by the U.S. FDA as a Type V Drug master File (#14621) under the title, “Description of the data to support use of solid phase cytometer (Chem ScanRDI) for the measurement of viable micro-organisms in pharmaceutical water systems.”

Eagle’s standard operating procedure (SOP) for the ScanRDI Sterility Test requires that we report both the total fluorescent events that the instrument records and the number of microorganisms verified by visual observation through the microscope. For a sample to pass the test, we must observe each and every event and verify that it is not a microorganism. If we find one microorganism, or cannot visually confirm an event, the sample fails the test. In addition, if the events are greater than 100, we inspect the filter under the microscope to verify that the large number of events is not gross contamination as evidenced by numerous microorganisms. Given that the events are not microorganisms, we then report that the test is “Incompatible,” meaning it neither passes nor fails the test but that we cannot determine the sterility of that specific sample using the ScanRDI test protocol. In effect, we are actually performing a suitability test on each and every sample as it is tested using the ScanRDI protocol. In this case, since we cannot use the ScanRDI to ascertain the sterility of the sample, we may recommend that our client test the sample using the USP <71> Sterility Test procedure. We believe that this conservative approach is both necessary and prudent to assure the highest level of quality assurance for our clients.

**Preparation Sampling**

When considering microbiological sterility testing, one must take into account that the sample is actually quite nonhomogeneous. For example, if you were to prepare a beaker with 200 mL of sample, and it contained five microorganisms, the preparation would be considered non-sterile. Now, if you then distribute the 200 mL into 20 vials containing 10 mL each, you may have any random distribution of the five organisms from all in one vial to one each in five vials. The process of filling the vials included a filtering step that should have removed the organisms so that all the vials would be sterile, but how do we make sure that this has actually happened? Since the sterility test is a destructive test, we cannot test every vial, as we would have none to dispense after the test.

The acceptable answer to this question is verifying that our sterile process is working properly, thus assuring that the vials are sterile, but that is another discussion. What we want to determine here is how many of the vials need to be examined to give us an acceptable probability of detecting a possible organism in the vials that were just made. While there are a host of different sampling plans available – each with its own statistical assumptions and probabilities – the USP has chosen to provide us with a guideline in the testing procedure outlined in USP <71> Sterility Test. Not only do they specify how many units from a batch of a preparation to test, but they also specify how much of each vial must be sampled.

As the number of vials and amount of each vial can result in a considerable volume, the USP test recommends that the Membrane Filtration method of sampling be employed. This entails filtering the sample through a nominal 0.4 µm filter where microorganisms are trapped for further analysis. Another advantage of the filtering procedure is that the filter is subsequently washed with a rinsing fluid (Fluid D), which removes materials that may interfere with the subsequent analysis of the microorganisms. (For example, if your preparation includes an antimicrobial agent, by rinsing the filter we remove that substance from the presence of any microbes, thus removing its potential interference with the subsequent detection process.)

The importance of this discussion is that the Eagle ScanRDI Protocol, as it incorporates a sample filtering procedure, allows us to follow exactly the recommendations of USP <71> with regard to the number of vials and the sample volume to be tested in the sterility test.
Specificity Considerations
The Specificity of a qualitative microbiological method is its ability to detect a range of microorganisms that may be present in the test article. This has usually been shown by growth promotion testing, but for sterility testing technologies that do not require growth of the microorganism as an indicator of microbial presence, this test is superfluous. Rather, it is more useful to determine if the technology can detect the microorganism and if and when extraneous matter in the test material may interfere with the detection of the microorganisms.

Let us first consider the ability of the ScanRDI to detect a range of microorganisms. The range of microbes that may contaminate a sterile preparation could number in the thousands. Fortunately, the USP test procedures do not require us to determine if each and every one of these can be detected by a sterility testing protocol. The USP has specified six microorganisms that are representative of a wide range of contaminants that may be present in sterility testing, and requires that a test protocol detect the presence of these microbes. The test organisms are:

- **Staphylococcus aureus** (Aerobic bacteria)
- **Bacillus subtilis** (Aerobic bacteria)
- **Pseudomonas aeruginosa** (Aerobic bacteria)
- **Clostridium sporogenes** (Anaerobic bacterium)
- **Candida albicans** (Fungi)
- **Aspergillus brasiliensis** (Fungi).

At Eagle we have shown that we can detect each of these organisms at a very low concentration in a test solution. We can purchase certified samples of these organisms in a known concentration and use them in the test protocol with excellent results. In fact, one of these organisms is used as a “Control Test” for each batch of tests performed on the ScanRDI. This is done not only to confirm the operation of the basic ScanRDI instrumentation, but also to check the chemistry labeling solutions for each and every run. Not only have we demonstrated that the ScanRDI can detect these organisms, but a host of literature references\(^1\),\(^2\),\(^3\) have confirmed that the system is capable of detecting a wide range of microorganisms. In fact, there are known instances where the ScanRDI procedure detected organisms that were not detected by the reference USP <71> Sterility Test. These non-culturable but viable (NCBV) microbes are proof that the ScanRDI is actually superior to the USP <71> test in this respect.

The significance of this discussion is that the ScanRDI technology, both at Eagle Analytical and at numerous other sites, has been shown to detect a wide range of microorganisms, including those specified by the USP testing procedures.

The second question with regard to the detection of microorganisms has to do with the limit of detection. In other words, what is the minimum number of CFUs that can be detected in a sample? Due to the discrete nature of microbes, this is not an easy thing to measure. However, in an attempt to show that the claimed number of organisms that the ScanRDI protocol can measure is “one,” Eagle Analytical performed the following “successive dilution experiment.”

A sample of *Candida albicans* was prepared and measured to have 350 CFU present in a 100 mL sample. Samples of 10 mL, 5 mL, 2.5 mL and 1.25 mL of this mother solution were diluted to 10 mL and measured on the ScanRDI. The results of this test are shown in the graph below:

![Graph showing Candida Albicans dilution experiment](https://example.com/graph.png)

While the numbers do not fall exactly on the predicted line, for a microbiological test, the agreement is quite acceptable and elucidates the linearity and exceptional detection limits of the ScanRDI protocol.
While this simple test does not conclusively prove that the minimum detection limit for the technology is one microorganism, others have taken a more rigorous approach and have validated this claim.

One final comment about selectivity and the limit of detection of the ScanRDI in comparison to the USP Sterility Test is worth noting. A recent article in a peer reviewed journal stated:

“Not only was the ScanRDI better able to reveal the presence of bacteria when they were very rare, analysis of the data indicate that the limit of detection of the ScanRDI is about an order of magnitude lower than that of the reference sterility test method.”

The significance of the above discussion is the demonstration that the ScanRDI is not only equivalent to the USP Sterility Test with respect to limit of detection, but is superior to the reference standard.

Procedure Interferences
Understanding when and where a test procedure is not applicable is a key component of any microbiological test. More specifically, determining if the test is compromised by the material being tested is often a very difficult task. Fortunately, the ScanRDI protocol, because it ultimately is a visually confirmed test, makes this determination relatively straightforward. However, let us consider the following cases where the ScanRDI is incompatible and cannot be used:

a. **Preparations that are suspensions.** When filtered, the suspended chemical covers the filter, potentially hiding any organism.

b. **Preparations that are not filterable.** Again, if we cannot get a sample to pass through the filter, we cannot capture the microorganisms for labeling.

c. **Preparations that have a high particulate count.** Preparations that have a high number of sub-visible particulates can result in a high number of “Events” measured on the ScanRDI. The Eagle protocol specifies that if the number of events is greater than 100 – assuming that we’ve inspect the filter microscopically to ascertain that the high number of events are not microorganisms caused by gross contamination – we will classify the sample as incompatible and suggest that the preparation be tested using another technique. Our concern – since we must look at each and every event to classify it as a potential microorganism – is that if the number of events is more than 100, there’s the possibility of an event covering a microorganism on the filter, leading to a false negative in our test.

d. **Preparations that react with the labeling chemicals used in the test.** Even though we wash the filter with a rinsing fluid after the preparation has been filtered, there are cases where the preparation may still be present on the filter and subsequently, reacts with the labeling chemicals. Some phosphorus containing preparations and (in some cases) protein APIs with very high molecular weights are some of these chemicals. When this happens, the visual observation of microorganisms is impossible since the filter literally lights up with fluorescence. Since visual observation and confirmation of a microorganism is the final verification step in our analysis, this becomes impossible and the test for that preparation is noted as Incompatible.

Since the final verification step in the determination of microbial contamination is a visual confirmation of the microbe, potential interfering substances or materials are readily determined and result in an incompatible test report.

Protocol Ruggedness
In any test procedure, no matter how well the SOPs are written and followed, there are differences in the performance of the procedure. These day-to-day changes are often the result of small differences in technique of the microbiologist performing the analysis, or even in the normal precision differences in the measuring apparatus used in the protocol. A protocol is deemed to have Ruggedness if the results that it produces are insensitive to these small changes.
To minimize the effect of these the day-to-day ruggedness issues inherent in the ScanRDI protocol, Eagle has chosen to demonstrate the performance of the ScanRDI measurement and detection system on each and every measurement run. This is accomplished using a three-step procedure:

1. The basic instrumentation performance and calibration is performed using fluorescent styrene beads before any samples are processed through the system. The styrene beads are dispersed on a system filter and then laser scanned and counted. They are then visually verified at several locations on the filter using the microscope. This procedure checks the calibration and performance of the instrumental system (laser, scanner, microscope stage, microscope and software).

2. A zero control is performed. This checks not only the instrumentation, but the chemical incubating and labeling solutions to make sure that they are not adding extraneous substances to the filter that would potentially interfere with the analysis procedure.

3. A Positive control is performed. A sample of one of the USP standard microorganisms is tested. This again checks the instrumentation but also confirms that the chemicals being used in this specific run are capable of properly incubating and labeling microorganisms in the test samples.

If any of the above tests fail, the run is aborted and the failure is investigated to determine where the error occurred. No compounded sterile preparations can be reported until a successful three-point test (described above) can be successfully passed.

This test procedure – performed on each and every ScanRDI run – ensures that small changes in the day-to-day performance of the protocol do not materially affect the results of the test.

References


• Dedicated to the unique needs of compounding
• Microbial Detection using the bioMerieux ScanRDI* Protocol
• Potency to USP protocols for accurate compositional analysis performed using HPLC systems

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