

## INTRODUCTION

Biological indicators (BI) are routinely used by medical device manufacturers and healthcare providers to monitor the efficacy of different sterilization processes (e.g., dry heat, ethylene oxide, hydrogen peroxide, moist heat, peracetic acid). Users of BIs are dependent upon manufacturers performance certification (label claims) to provide enough details to ensure they choose the correct indicator for the given application to be monitored. Per the United States Pharmacopeia (USP 26, 2003), <1035>, a Certificate of Performance should be obtained for each lot of biological indicators. Because of the critical nature associated with the use of BIs and a desire to follow the USP guidelines some users choose to have the BI manufacturers label claims verified by third party, independent testing laboratories. For several reasons, replicating results can be a challenge. Sufficient detail regarding BI characterization should be provided by the BI manufacturer allowing for label claims such as population and resistance to be duplicated.

Per the ANSI/AAMI/ISO 11138 Parts 1-5 (2006) BI standards, population should be replicated within 50% to 300% of manufacturers label claims and resistance (e.g., D-value) should be within  $\pm 20\%$  of manufacturers label claims.

There are several potential sources of variability that may adversely affect population and resistance values when comparing third party, independent testing laboratory results to BI manufacturers certification values. These sources of variability fall into three primary categories: 1) equipment, 2) materials, and 3) methods. We will now investigate these different sources of variability and attempt to provide insights as to why these errors may be occurring and what can be done to help minimize and/or eliminate them.

## EQUIPMENT Resistometers

Resistometers are specialized test vessels that are used to rapidly produce and precisely control critical variables associated with a given sterilization process. An ANSI/AAMI/ISO 18472 (2006) compliant resistometer is required to characterize the performance of BIs. The resistometer is also an important tool allowing for the accurate comparison of performance between products. There are some specific steps that must be followed to ensure consistency when characterizing the performance of BIs. Those developing standards on resistometers and BIs realize this fact and during the standards development process are defining the important steps necessary to achieve consistent results. Issues such as pre-vacuum depth, length of time permitted during pre-vacuum step (too long can lead to product desiccation), come up time, come down time and quality of steam are all important considerations which need to be consistent run after run in order to properly compare results.

In the past, resistometers used for characterizing BI performance were referred to as a Biological Indicator Evaluator Resistometers, or BIER vessels. But because the same piece of equipment can be used for a variety of applications (BI characterization, chemical indicator characterization, medical device exposures, reusable studies, etc.), the standards development organizations such as Association for the Advancement of Medical Instrumentation (AAMI) and International Standards Organization (ISO) now simply refer to this equipment as resistometers. Different resistometers are used for different sterilization processes.

Resistometer performance has been identified as a significant source for inconsistency within and between laboratories. A 1990 publication of an AAMI round-robin study authored by Oxborrow et al. summarizes the characterization of BIs exposed to both to ethylene oxide and steam, by Mosley, Mosley and Gillis, respectively. There was observed to be a greater than 20% variation in results within a lab and between labs which can be anticipated when using resistometers. The standards development organizations (AAMI & ISO) recognize resistometers as a potential source of variability and are thus in process of revising BI & CI standards to provide greater detail in test methods using resistometers in an attempt to help eliminate sources of variability caused by resistometers. Revised test methods should help allow for resistometers to provide more consistent results.

One identifiable source of variability is the depth of vacuum that a resistometer should pull before charging the chamber with sterilizing agent. In the most recent ANSI/AAMI/ISO BI standards (11138 Parts 1-5) the depth of vacuum for EO and steam cycles is identified as 10 kPa and 4.5 kPa, respectively. Unless such depths of vacuum are pulled, air and other non-condensable gasses remain in the resistometer chamber, the presence of which can create a micro-environment whereby the sterilizing agent is not optimally in contact with the BI, thus a potential source in variability leading to different responses each time a test is run.

The ranges and tolerances that the resistometer must meet for the critical variables associated with each different sterilization process are also getting better definition by the standards development organizations. This, too, shall help ensure better uniformity in the operation of resistometers.

The take home point is that domestic and international BI working groups responsible for standards development realize such issues exist with resistometers and are active in taking steps to help eliminate potential sources of variability when using such test equipment.

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# BIOLOGICAL INDICATOR CHARACTERIZATION (con't...)

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## Spore Strip Maceration Process

There are three primary methods for physically macerating a spore strip prior to performing population verification:

1) stomacher lab blender, 2) blender cups, and 3) glass beads and/or vortex. While each method has certain pros and cons, the ultimate purpose is to adequately macerate the carrier such that a fine pulp consisting of single-spores is obtained so that subsequent plating and enumeration is performed on individual spores.

Inadequate maceration of an inoculated carrier can leave "chunks" of paper onto which a large number of spores could adhere, such that when plated, appear as a single colony. If inadequate maceration occurs, this could lead to a lower plate count.

Clumping of spores leads to a lower number of colonies counted. Clumping of spores does not occur during the maceration process. Clumping generally occurs during the propagation or inoculation process due to inadequate stirring, use of ethyl alcohol, or inadequate spreading of spores over the length of an inoculated carrier.

Let's move on to investigate the pros and cons associated with each of the different maceration processes.

## Stomacher Lab Blender (Tekmar Company, Cincinnati, OH)

Maceration via this process is recommended for "standard" size BI strips with the approximate dimensions of 1 3/8" x 1/4". The length of time to obtain a fine pulp varies with carrier type (paper thickness and density). The maceration process should be continued until only loose, single fibers are visible. Overprocessing resulting in spore death is typically not a concern when using this method.

## Blender Cups (Eberbach)

Validation of the length of processing time to achieve complete maceration is recommended, as the time required will vary based on age and manufacturer of blender (e.g., amount of shear being delivered) and the type of inoculated carrier. Spore recovery can be impacted by incomplete maceration of the carrier and by over processing as spores can adhere to foam in cup if processed too long. Blender cups are not recommended.

## Glass Beads

This method of maceration can be used if a stomacher lab blender is not available. This is the recommended method for maceration of 2 mm x 10 mm inoculated carriers and paper discs of 1/4" or 1/8" diameters.

## MATERIALS

### Agar/Media

The importance of the type of agar and/or media used to culture spores should not be underestimated. Studies in our and other

laboratories have demonstrated that different sources of agar and media can dramatically affect results, even by as much as more than one order of magnitude! This seems to be applicable more to *Geobacillus stearothermophilus* than it is to *Bacillus atrophaeus*.

If you are purchasing media (prepared or dehydrated), be sure to test your organism(s) in the different medias available and determine which one works optimally for you.

## METHODS

### Heat Shocking

Following maceration of the inoculated carrier, it is necessary to heat shock the organisms to stimulate their growth. There are a couple of fine points that should be observed to ensure consistency and efficacy of the heat shock procedure: 1) be sure the heat shock period is conducted for the correct length of time, and 2) perform heat shock at the appropriate temperature for the duration of the heat shock period (measuring temperature in a blank tube filled with water as a surrogate).

Perform the heat shock procedure using a water bath by placing a thermometer in a test tube (of the same size as that containing an aliquot of the macerated inoculated carrier) containing blank diluent. Place this tube into a hot water bath. Start timing when the thermometer reaches 80°C for heat shocking at 80 to 85°C, or start timing when the thermometer reaches 95°C for heat shocking at 95 to 100°C. Do not to overheat. Heat shock as follows for each organism:

*Geobacillus stearothermophilus*  
95 to 100°C, 15 minutes

*Bacillus atrophaeus*  
80 to 85°C, 10 minutes

*Bacillus pumilus*  
80 to 85°C, 10 minutes

After heat shocking, rapidly cool tubes in an ice bath (0 to 4°C). Plate within 20 minutes after heat shocking using molten soybean-casein digest agar.

### Agar Temperature

When creating a pour plate using hot agar, be sure to temper the agar to 45°C prior to pouring the plate. Agar that is too hot will damage and/or kill the spores thus reducing the recovery. Agar that is too cool will clump and not distribute evenly resulting in clumping and again, reduce recovery.

For additional information:

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## Growth Promotion Testing

When using media (whether purchasing prepared media or making your own using dehydrated media) test for growth promotion capabilities of that media using less than 100 colony forming units of each of the organisms of interest as the inoculum. Using media that can consistently support growth is one key to obtaining consistent results. It is also important not to over sterilize your media after it is made as this can reduce the nutrient value.

## Incubation Temperatures

Be sure to follow manufacturers recommendations for the incubation temperature for the specific organism being tested. If the manufacturers instructions say to incubate at 30 - 35°C, then don't incubate at 37°C with a tolerance of  $\pm 2^\circ\text{C}$  and assume that you'll get similar results.

## Vials of Spore Suspensions and Spore Ampules

Vials of Spore Suspensions and Spore Ampules are notorious for spores settling over time. Be sure to shake well before opening. Do not vortex suspensions as this type of agitation can cause the spore coat to become sticky and cause the spores to adhere to the container wall. Be especially cautious about spores settling in the apex of a spore ampule. Also, due to the taper of the spore ampule, be sure sufficient liquid gets into the upper half of the spore ampule to ensure any spores present are dislodged and washed down to the lower half of the ampule.

## Aseptic Technique

Use sterile materials and aseptic technique throughout conduct of the various processes. The introduction of unwanted microorganisms can adversely affect results.

## SUMMARY

Either alone, additively, or synergistically, the equipment, materials and methods selected to replicate population and resistance claims can affect the outcome of your studies and thus influence your ability to replicate BI manufacturer label claims. Every good BI manufacturer should provide the user of their products with technical bulletins or the like, that provide sufficient detail allowing any third-party testing facility to satisfactorily replicate population and resistance claims.

## REFERENCES

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