Biological Indicators/Steam D-Value Verification

Requirement? Need? 'Problems That Lay Ahead'

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For many years now some of the end users of Biological Indicators have been routinely sending out samples of BI's (Biological Indicators) to Third Party Laboratories for both D-Value and population verification. On an initial validation or on an annual revalidation of Sterilization cycles, the cycles are challenged with Biological Indicators to demonstrate actual microorganism lethality produced during the sterilization a cycle. In order to present a cycle challenge, these 'resistant microorganisms' must be tough enough to meet specific standard requirements as set out by AAMI, ISO or USP. For example, with Steam Sterilization at 121C, AAMI, ISO and USP all state that if a BI is being used for a Validation, the minimum acceptable D-Value or Resistance for that BI is 1.5 minutes.[1] Thus the possible need for resistance verification prior to BI use.

This verification is often being done as part of a facility's 'acceptance criteria' for a new Lot of BI's coming into the facility and prior to acceptance and use of the Lot for Validation work or Routine Monitoring of the Sterilization Cycles used at that facility. *USP 28, 'User's Responsibility'* states that "*The user may consider conducting a D-Value assessment before acceptance of the lot." (of BI's).*[2] The wording '*may consider'* is important to note. This is not mandatory. But it may be mandatory according to the individual users or Pharma Company's procedure or protocol for 'BI Acceptance Criteria'. Under the area of '*User's Responsibility',* it does not mention as to 'testing result acceptance criteria'. What criteria do I use to accept a Lot of BI's if one did perform a 'D-Value Assessment?' What variation allowance is acceptable?

As part of verification, the Lot of BI's being tested are expected to meet specific requirements as set out in ISO or USP as to the accuracy of the 'label claimed' Resistance or D-Value and Population of the BI's. *USP, User's Responsibility* states that *'Laboratories that have the capability of performing D-Value assays could conduct a D-Value determination using one of the three methods cited in the general test chapter Biological Indicators-Resistance Performance Tests <55> and in the appropriate USP monographs for specific biological indicators'.*[2] This statement of 'methods allowed to be used' refers one back to the manufacturers section in <55> for the Test Method. If one accepts and performs the Test Method to be USP compliant, then like it or not, I would assert that the '*USP acceptance criteria'* goes with the method as per USP. The same would be true for *BI population verification.* If performing a population verification, per USP <55> *Total viable spore count*[10]*,* one also accepts the acceptance criteria of results not less than 50% or more than 300% of the labeled certified population.

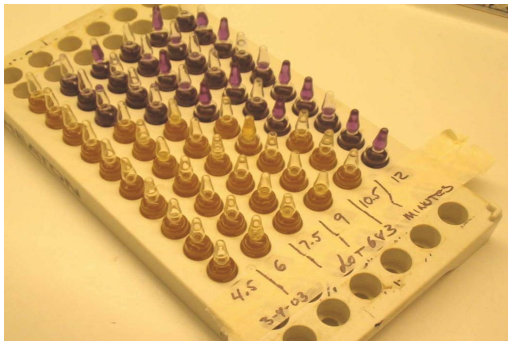
For example, if one were expecting to comply with USP 28 Official Monograph/ Biological Indicator, *Resistance Performance Tests*- "*The requirements of the test are met if the determined D value is within 20% of the labeled D value for the selected sterilizing temperature and if the confidence limits of the estimate are within 10% of the determined D value."*[3] Once the resistance or D-Value has been verified and is within acceptable limits of the Label Claim and it meets or exceeds minimum acceptance criteria for BI resistance as indicated in ISO or USP, that particular Lot of BI's can now be used for Validation work. This would be considered an acceptable biological challenge to the sterilization process.

To obtain a label claim D-Value, ISO *11138-1*[4] allows for the use of two of three methods. One may use the **Most Probable Number** method by direct enumeration, a **Fraction Negative** method (such as Spearman/Karber) or assess the D-Value accuracy by using the USP **Survive/Kill** calculated cycles. Regardless of which of the three Methods is used, one piece of equipment that will be needed is a **Resistometer.** A Resistometer, also known as a BIER Vessel (Biological Indicator-Evaluator Resistometer), is a piece of test equipment that can very quickly and accurately deliver and control very precise sterilization process parameters that are critical to the process. Various Standards developed by ANSI/AAMI, ISO and USP have very tight equipment or BIER Vessel operational capabilities that must be met. As an example, ANSI/AAMI ST44:2002[5] states that with a Steam BIER Vessel the equipment must be capable of hitting the target temperature set point within 10 seconds or less from the time 'steam charge' occurs, must maintain that set temperature to within + or – 0.5C and then at cycle end, the postvacuum time to reach atmospheric pressure must be within 10 seconds or less. ST44:2002 further states that the Steam Resistometer be capable of measuring such conditions as Time (resolution of 00:00:01 and accuracy within + or – 00:00:02), Temperature (resolution of 0.1C and accuracy of + or – 0.5C) and Pressure (range of 0 to 60psia, resolution of 0.1 and accuracy of + or – 0.5psia). The duration time of exposure at a given temperature is thus controlled as exact as possible for both time and temperature.

One can note that on the pictured Steam BIER Vessel shown at left, manufactured by the Steris Corporation, the Vessel Chamber is fairly small as compared to an autoclave chamber. The small chamber is part of the vessel design that allows for an extremely fast steam charge and rapid increase in chamber temperature. Rather than the normal autoclave 'come-up' time needed to reach set temperature, a Steam BIER Vessel should be very capable of hitting set point for temperature in less than 10 seconds and in many

cases closer to 6 seconds. This allows for the very exact 'exposure time' needed to determine BI resistance characteristics.

If one were performing a Fraction/Negative method of verifying BI resistance in such a BIER Vessel, one would expose multiple groups of BI's to varying cycle exposure times. For example, if one were attempting to verify the resistance of a particular BI in a Steam Vessel at 121C using the Limited Spearman-Karber Fraction Negative Method, one could expose 20 BI's per group to various exposure times at 121C.[6] The photo example shows exposure results for cycle exposures of 4.5 minutes, 6 minutes, 7.5 minutes, 9 minutes, 10.5 minutes and 12 minutes. After exposures, each group of BI's would be aseptically transferred to growth medium as with Spore Strips and incubated at the appropriate temperature.



In this shown example, self-contained ampoules were used. The ampoules are purple in color to start with. Bacterial Spores are suspended in the purple TSB. The color is from an added pH indicator. After exposure and incubation, if the spores in the ampoule survived the exposure and grow, the ampoule will turn a yellow color as the pH drops as an indication of growth. In the photo, one can see that all the ampoules exposed at a time exposure of 4.5 minutes survived and that at the exposure time of 12 minutes, all the BI's were killed and no signs of growth occurred and the ampoules remain a purple color. This gives us 4 exposures where a fraction of the ampoules were killed at each exposure, *a fraction were negative*. As the exposure time increased from the initial 4.5 min exposure, more and more BI's were killed. With fractional information such as this, one can use the Limited Spearman-Karber method to determine just how tough the BI's are or what their resistance is to this particular cycle.

Getting the above method accomplished in a BIER Vessel by a 3rd Party Laboratory for Resistance Verification may not look to be very difficult. Several laboratories offer this service. The price may range from $1,500.00 to $3,400.00 to do a full Fraction Negative D-Value assessment. The main area of concern is 'How competent is the Laboratory you have chosen to perform the D-Value Assessment?' A proper D-Value Assessment is not as straight forward as one may think. Getting a + or – 20% allowance variation may be extremely difficult. There are numerous critical components involved in performing such a test. The following will look at some areas of concern.

Equipment

Does the facility that you have contracted to do your D-Value Assessment have an ANSI/AAMI, ISO compliant BIER Vessel? This may seem like a mute point, but you would be surprised at the facilities that contract to do D-

Value Assessments and do not actually have compliant Vessels. Many are far from what you would consider a BIER Vessel. Some are no more than *a well maintained pressure vessel* that does not have the capability to measure and record temperature, pressure or even time to the accurateness required. This is an area that one should look into prior to contracting with a 3rd Party Lab for work to be done. Is the equipment to be used ISO, ANSI/AAMI compliant? Control capabilities and Specifications one should look at for a Steam Resistometer are (ANSI/AAMI ST44:2002)[5]:

- Capable of 'Time Resolution' of 00:00.01 and accuracy of + or – 00:00:02
- Temperature resolution of 0.1C and accuracy of 0.5C
- Pressure accuracy of 0.5 psia
- Temperature control of + or – 0.5C during exposure
- Vacuum level capable of 0.65 psia
- Steam Charge time from 100C to set temperature within 10 seconds or less

Along with an instrument being capable of meeting the above abbreviated list of equipment specifications, is the instrument capable of accurately documenting that the conditions, phases, time and temperatures that occurred during the cycle actually occurred and were within specs? I have seen 'Final D-Value Verification' reports where Time, Temperature and Pressure were 'hand-written' notations on a sheet of paper and not actual Resistometer Data printouts. Critical factors such as pre-vac, come-up time, actual temp and pressure during exposure and pos-vac were not even part of the report data. The performance accuracy of the unit is critical. The allowances seem very tight but widening tolerances will only make verification more difficult. A + or – 0.5C temperature variation actually allows for a difference of 1.0C if one BIER unit at the BI manufacturer was operating on the high side (121.6C) and the verification lab BIER unit was operating at the low end (120.6C). This variation alone could account for at least half of our 'D value variation allowance of + or – 20%'.

The final result report from your contract testing laboratory should include documentation to provide evidence that all critical cycle parameters were met.

Test Method Used

ISO 11138-1:1994(E) allows for the use of a Survivor Curve Method or use of Fraction Negative Methods to initially determine a D-Value or BI Resistance. To do a D-Value 'Assessment' or'verification', there are a number of Test Methods that can be used. One could perform one of several Fraction Negative methods, Direct Enumeration or Survive/Kill. In an effort to reduce variables and allow for the closest duplication of the BI Manufacturers procedure for D-Value certification, it is important that the same Test Method be used for D-Value Verification. USP 28 states- "*Indicate in the labeling that the stated D*

value is reproducible only under the exact conditions under which it was determined, that the user would not necessarily obtain the same result…."[3] If the BI Manufacturers certification states that Direct Enumeration Method was used to establish that BI's resistance, then the method that should be used for Verification would be Direct Enumeration (Survivor Curve). Using different methods for D-Value Certification and for Assessment only allows for the introduction of variables that could affect D-Value reproducibility. This strongly applies to situations where one is trying to reproduce the D-Value for verification.

If only *verificati*on is intended, *USP* allows for the use of the Survive/Kill Method[7]. This is pretty straight forward and will only involve two cycles being run. One would expose a minimum of 20 replicate samples for USP procedure or 50 samples for ISO procedure[8] to each of the USP calculated Survive/Kill time cycles. All samples exposed to the Survive time must survive the exposure and all exposed to the Kill time must not show growth. Using this method where only two cycles are involved may be much quicker and less expensive than running a full D-Value assessment. The Survive/Kill method calculation is a bit padded but it still can provide points to check for survivors and lethality. It provides a reference point for resistance. To assist in checking the consistent performance of all units within a particular Lot of BI's, Survival/Kill results will provide that additional information.

Recovery Media

Different Brands and different Lots of both TSB and TSA may not have the same degree of 'ability to promote growth of injured spores'. Brands 'X', 'Y' and 'Z' of tryptic soy agar may all perform in a very similar manner for a majority of typical laboratory tasks in obtaining growth for culture streaks, slants, etc. with most common laboratory microorganisms. Quantifying the presence of remaining CFU's from a BI of *Geobacillus stearothermophilus* 'injured spores' is a different matter. Not all Brands or Lots of recovery media have equal ability to accurately promote the growth of such injured spores. Several articles have been published that demonstrate as much as a 'full Log difference' in the recoverability of injured spores when comparing one Brand of TSA to another Brand.

Table 1: The Comparitive CFU Results

Lot # of BIs Tested	Average CFUs (of triplicate plates) Recovered with TSA BRAND A	Average CFUs (of triplicate plates) Recovered with TSA BRAND B
1A	0.3×10^6	1.4×10^6
1B	0.1×10^6	1.0×10^6
2A	0.1×10^6	0.6×10^6
2B	0.1×10^6	0.7×10^6
3A	0.3×10^6	0.9×10^6
3B	0.7×10^6	1.4×10^6

The table information shown at the left (*Infection Control Today, January 2000)*[9] demonstrates the variance that is possible in CFU recovery between two different Brands of TSA used in population assays of several different Lots of BI Spore Strips. *USP Total Viable Spore Count* procedure[10] was used to run population assay's

on 6 different Lots of Spore Strips containing *Geobacillus stearothermophilus* spores. From the last two dilution tubes in the dilution series while performing the assay, 1ml aliquots were added to 6 separate Petri dishes. To three of the plates TSA Brand A was added and to the remaining three plates, TSA Brand B was added. The only variable in this exercise was the Brand of TSA used for the pour plates. One can easily see the extreme difference in the 'spore recovery ability' between the two Brands used. A D-Value result based upon Direct Enumeration Method using Brand A media for several fractional cycles would provide a much different result than one produced using Brand B media. Using Brand A media would give one the impression of a faster Log Reduction in CFU population and thus calculate a 'lower D-Value' than if one used Brand B media.

Technique and Lab Utensils/Personnel

Under this heading one can add a score of additional variables that would make D-Value assessment success even more difficult. Besides recent BIER Calibration, what about utensils such as lab pipettes or repeaters? Are these as well as incubators in good calibration or within acceptable specs? As most would readily recognize, there can be a wide variability in accuracy between technicians doing serial dilutions or plating techniques. Are all BIER Cycles reviewed for compliance to specs prior to acceptance? Concerning BI placement into and removal from the BIER Vessel chamber; is placement consistent from one run to another, are the BI's removed immediately upon cycle completion and upon cycle initiation, are the BI's quickly inserted with little warm-up occurring. Does the BI holding rack offer little to no protection to the BI's being tested as compared to the holding rack used by the Manufacturer? All these factors can add up to a confirmation of a test result well outside the accepted + or – 20% variation needed.

All the above being considered, a D-Value Verification can be very difficult to accomplish and be within acceptable variation. Much discussion has occurred that involved the widening of this + or – 20% to an even wider margin due to the lack of success in hitting the current range. Skill and Technique variability from one Laboratory to another is definitely a contributing factor to the problem with obtaining a D-Value Verification that is within allowable limits. This is also compounded by equipment or BIER Vessel function differences, calibration and equipment maintenance as a contributing factor.

All in all, if one is contracting for a D-Value Verification, some problems may lay ahead. Even with all the possibilities mentioned, some Verifications are successful and well within the + or – 20% allowance. When this occurs and can be repeated with additional Lots of BI's, one can only assume that more than luck is taking place. Both the BI Manufacturer and the Testing Laboratory are running the resistance testing in a very similar manner and both are paying excellent attention to equipment function, methodology, calibration/maintenance and have at some point communicated with each other to duplicate as much as possible the

initial testing including all factors involved. Even if the same Lab were to do back-to-back D-Value Testing on the same Lot of BI's with the same equipment, result differences are still going to occur. Small variations we can live with, the larger differences we must work together to avoid.

With this in mind, when trying to verify a D-Value and problems occur, arrange so that the Verifying Lab and the BI Manufacturer start communication to help resolve the problem. BI manufacturers want the verification to go well and the Verification Lab want to have a successful testing experience. They should work together to do all that is possible to help find what aspect of the testing is causing the difficulty. It is possible to get verification within the 20% area but many times some exposures may need to be run again after calibration and equipment is checked. Aspects such as differences in recovery media used, verification method used, equipment calibration, etc. are all likely contributing to a verification problem. This should not be an 'I'm right and you are wrong' issue. Communication and a willingness to look at test equipment and procedures used between those directly involved can usually solve the differences for inter-lab variation on verification issues.

[1]*USP 28, Pg. 2538, Chapter 1035, Typical Characteristics for Commonly Supplied Biological Indicator Systems.*

[2]*USP 28, Pg. 2539, Chapter 1035, General Information, User's Responsibility*

[3]*USP 28, Pg. 259, Biological Indicator for Steam Sterilization, Paper carrier, Resistance performance tests*

[4]*ISO 11138-1:1994(E), Pg. 6, 5.1.2- Resistance testing requirements*

[5]*ANSI/AAMI ST44:2002, Pg. 3, Performance requirements for resistometers.*

[6]*ISO 11138-1:1994 Annex C, Fraction negative analysis/MPN method for subsequent determination of D value by Limited Spearman-Karber Method.*

[7]*USP 28, <55>, Biological Indicators- Resistance Performance Tests, Survival Time and Kill Time.*

[8]*ISO11138-1, Pg. 13, Annex E, Survival-kill response characteristics*

[9]*Infection Control Today, January 2000, Biological Indicators and Population Verification.*

[10]*USP 28, Pg. 244, <55> Biological Indicators- Resistance Performance Tests, Total Viable Spore Count.*