As described in ISO 14161:2009, “Biological indicators are utilized to test the effectiveness of a given sterilization process and the equipment used, by assessing microbial lethality according to the concept of sterility assurance level.” Although the role of biological indicators (BIs) in sterility assurance is widely recognized, BIs are subject to a number of factors that may affect their performance and influence the outcome of testing. These factors include the handling, transport, and storage of the BI prior to use, the manner in which it is placed within the sterilization load, and the growth medium and incubation conditions used to culture the test organism following sterilization processing. Previous studies of the resistance performance of BIs have noted inconsistent results and poor reproducibility among testing laboratories.

Two important considerations with regard to verification of the resistance performance of a BI are as follows: 1) If the user chooses to empirically determine the D-value of a BI, the resistance performance testing should be performed under the same conditions used by the BI manufacturer. 2) The stringent measurement and control requirements of the resistometer in which the resistance performance characteristics of the BIs are determined preclude its validation using procedures similar to those used to validate conventional moist heat sterilizers. The first consideration requires that the conditions under which the label claims of the BI were established be clearly defined. The second consideration is challenging to address, as variability in resistometer design and performance makes it difficult to exactly reproduce the conditions used by the BI manufacturer to establish the resistance performance of the BI.

**Materials and Methods**

**BIs**
The BIs tested included self-contained BIs, with the test organisms inoculated onto paper strip carriers or suspended in growth medium within the BI ampule, and paper strip BIs, for which growth medium is provided separately. BIs were obtained from 3M, Steris, SGM Biotech (now Mesa Laboratories), Raven Labs (now Mesa Laboratories), SPS Medical (now Crosstex/SPS Medical), and Merck. Upon receipt, the BIs were stored under controlled conditions in accordance with the BI manufacturer’s recommendations and protected from excess temperature and humidity.

**Incubators**
BIs were processed and incubated in accordance with the BI manufacturer’s recommendations. Incubators were calibrated every six months and temperature mapped annually. Autoreaders used for the determination of the fluorescent response of 3M Attest 1292 rapid readout BIs were returned to 3M for temperature and fluorescence calibration on an annual basis.

**Growth media**
All growth media used with paper strip BIs was sterilized according to the media manufacturer’s recommendations and tested for sterility and growth promotion as per USP <71>. Unless otherwise specified by the BI manufacturer, paper strip BIs were tested in Soybean-Casein Digest Medium sourced from approved vendors.

**Resistometer**
Resistance performance testing was carried out in a Steris (Joslyn) Steam BIER (BI evaluator resistometer) vessel with a 5-inch width × 10-inch length circular chamber with approximately 3.2 L of usable chamber volume, an internal steam generator, and a
486 computer processor–based control system. The resistometer is maintained under service contract with the manufacturer, with calibration and preventive maintenance performed every six months. All testing was performed at a temperature setpoint of 121°C. Before initiating testing, two five-minute exposure cycles were processed with an empty chamber to preheat the unit. Cycles were processed sequentially with no greater than 15 minutes allowed to elapse between cycles without preheating the unit.

**Test Procedures**

Testing was performed by trained personnel according to the procedures outlined in ISO 11138-1 and ISO 11138-3 and as specified by the BI manufacturer. D-values were calculated with the Stumbo-Murphy-Cochran and Spearman-Karber methods, basing acceptance on the most conservative estimate. Statistical analysis was performed using Minitab version 17.3.1 (Minitab, State College, PA). The nonparametric Empirical Percentile Process Capability macro was downloaded from the Minitab website.

**Results and Discussion**

**BI Population and Resistance**

Tables 1–3 in the data supplement (available online at http://aami-bit.org) present the outcome of population and resistance performance testing conducted during a 10-year period of 92 lots of moist heat BIs. Of the 92 lots, 32 were self-contained BIs with the test organisms inoculated onto paper carriers (Table 1 in data supplement), 35 were self-contained BIs with the test organisms suspended within the growth medium (Table 2 in data supplement), and 25 were paper strip BIs with the test organisms inoculated onto paper carriers and packaged in glassine envelopes (Table 3 in data supplement). With the exception of one paper strip BI lot (Table 3 in data supplement), all self-contained BI lots complied with their labeled resistance performance claims.

The outcome of resistance performance testing found that with the exception of one lot, all self-contained BI lots complied with their labeled resistance performance claims. Specifically, the results showed $D_{121°C}$ values of 1.5 minutes or more within ±20% of the labeled $D_{121°C}$ values, complete outgrowth at the labeled survival times, and complete inactivation at the labeled kill times. This corresponds to a verification success rate of 100% for self-contained BIs with the test organisms inoculated onto paper carriers (Table 1 in data supplement) and 97.1% for self-contained BIs with the test organisms suspended in growth medium (Table 2 in data supplement). Six paper strip BI lots were rejected, four of which were sourced from the same manufacturer (Table 3 in data supplement). Five of the paper strip BI lots tested failed to comply with a minimum $D_{121°C}$ value requirement of 1.5 minutes, as well as failed to exhibit complete recovery at their labeled survival parameters. Further, one lot exhibited a confirmed $D_{121°C}$ value of greater than 1.5 minutes but outside ±20% of its labeled value, for a verification success rate of 76% for paper strip BIs. The $D_{121°C}$ values of lots that failed to exhibit complete recovery at their labeled survival times ranged from 0.7 to 1.4 minutes.

Factors to consider regarding the difference in outcome of the resistance performance testing of self-contained and paper strip BIs include the following. 1)
differences between the performance of the resistometer used by the BI manufacturer and the resistometer in a given laboratory should be considered. 2) Self-contained BIs are packaged with the same growth medium used by the manufacturer to qualify the lot, which diminishes the impact of the quality of the growth medium on the recovery of the test organism. 3) Paper strip BIs have less mass than self-contained BIs and generally respond more rapidly to changes in heating conditions. The latter factor may compound the differences in heating characteristics among resistometers when paper strip BIs are evaluated.

**Rapid Readout BIs**

In conjunction with the determination of the viable outgrowth of the test organism, the fluorescent (enzyme) response of 20 lots of 3M Attest 1292 Rapid Readout self-contained BIs also was evaluated after three hours of incubation at 60 ± 2°C in either a 3M model 290 or 390 Autoreader. For all lots tested, the three-hour fluorescent response was found to be conservative or equivalent (lots 15 and 19) to that of the viable outgrowth response (Figure 1). The readout reliability testing (as per manufacturer’s instructions on 100 samples each) of lots 15 and 19 returned values of 100% for each lot, which complies with the manufacturer’s claim of a readout reliability of 97% or greater.

**Process Capability Analysis**

Although the potency of a BI may decline following initial qualification, another possibility is that the resistometer used to verify the resistance performance of the BI may exhibit performance that is not consistent with the resistometer used by the BI manufacturer. As noted, the stringent measurement and control requirements of resistometers preclude their validation using procedures similar to those for conventional moist heat sterilizers, such as heat distribution and heat penetration studies. Therefore, process capability analysis was evaluated as a means to characterize the consistency of the performance of the resistometer used to qualify BIs in our laboratory.

Process capability analysis is a widely used tool for evaluating the performance of a process against a predetermined set of criteria. Essentially, process capability is a measure of the statistical variation of a process from its pre-established specifications. In this case, we sought to evaluate the capability of the resistometer to consistently reproduce the desired exposure conditions based on the temperature and pressure measurements recorded during the exposure phase of the cycle.

The equipment manual provided with the resistometer stated that the process of charging the chamber with steam can be

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**Figure 1.** Comparison of the fluorescent (enzyme) response and viable outgrowth of biological indicators processed at 121°C. Data are presented as the number of minutes exposure required to yield no recovery of enzyme fluorescence or viable spore outgrowth from 10 samples tested per time point.
controlled by one of three methods; however, it is always based on pressure determined either directly from the measurement of pressure within the chamber via a pressure transducer or indirectly from temperature measured by either a resistance temperature detector (RTD) placed in the jacket of the vessel or a load probe thermocouple placed within the chamber. Control of the resistometer based on temperature measurements taken from either the jacket RTD or load probe thermocouple requires that the measured temperature be converted to pressure by means of a preprogrammed algorithm. Each cycle printout provides a record of the chamber pressure, boiler temperature, control temperature (jacket RTD), and load probe temperature measured at predetermined intervals.

Use of the load probe temperature for determining the process capability of the resistometer was considered and rejected in view of the stated accuracy of the load probe (±1.0°C), as compared with the process control requirement (±0.5°C) for moist heat resistometers. In our application, control of the resistometer was based on the jacket RTD (stated accuracy of ±0.5°C). However, use of the jacket RTD to assess the consistency of performance of the resistometer may be misleading, as the measurement is taken outside of the chamber where the BIs are placed.

As noted by Mosley and Gillis, temperature measurements taken from within the jacket of the resistometer may act as a signal-averaging system and bias the data. Therefore, the use of chamber pressure as a means to assess the process capability of the resistometer was used. This presents a number of advantages, including 1) the fact that the relationship between the temperature and pressure of saturated steam is well documented, 2) the measurement is taken from within the chamber where the BIs are placed, 3) pressure is uniform throughout the chamber of the vessel, and 4) the resistometer is equipped with a pressure transducer, with a stated accuracy of ±0.5 psia compliant with the ISO 18472 process control specification of 3.5 kPa at 121°C. Disadvantages include the potential for residual air within the chamber or noncondensible gasses within the steam being admitted to the chamber, thereby introducing error into the pressure measurements.

Methods for process capability analysis generally fall into one of two categories: 1) parametric methods (used with data that fit a normal distribution) or 2) nonparametric methods (used with data that do not fit a normal distribution). Initial analysis of the resistometer data indicated that they were not normally distributed and could not be transformed to a normal distribution using any of the more common statistical transformations.

Although valid reasons may account for the data not being normally distributed, the lack of normality does not necessarily affect the outcome or reliability of the process. Therefore, to assess the consistency of the performance of the resistometer, nonparametric process capability analysis was used. This method requires a minimum of 100 data points to provide an accurate analysis. With chamber pressure readings taken every five seconds and the first 10 seconds of the exposure phase excluded from the analysis to account for the stabilization period (per ISO 18472), only cycles of 8.5 minutes duration or greater could be evaluated using this approach.

As the resistometer is regulated based on pressure, the upper specification limit (USL) and lower specification limit (LSL) were based on a ±3.5 kPa tolerance (corresponding to a tolerance of ±0.5°C) during the steady-state period (per ISO 18472) and a saturated steam pressure of 205.039 kPa (29.73 psia) at 121°C, yielding saturated steam values of 29.23 psia (LSL) and 30.25 psia (USL), respectively. Based on a yield of 99.73% or greater (3 sigma) for a two-sided process, the minimum acceptable results would correspond to a capability index (Cnpk) value of 1.0.

To gain an understanding of the potential application of process capability analysis for the evaluation of resistometer performance, 30 cycles of 12-minute exposures were processed at a temperature setpoint of 121°C during a five day period, processing six consecutive cycles daily. Testing was performed with a load of 10 paper strip BIs placed within the chamber of the resistome-
ter. All testing was performed by the same operator. The results (Table 1 and Figures 2–4) suggested that the resistometer was capable of complying with a minimum Cnpk value of 1.0 with an average Cnpk value of 1.655863 over 30 cycles. However, for one cycle (cycle 5521; Table 1 and Figure 4), the Cnpk value (0.74510) was less than the minimum acceptance criteria of 1.0.

These results demonstrate that although the resistometer is capable of performing within the established limits of 29.23 and 30.25 psia with a reasonable degree of consistency, occasional minor excursions in pressure above or below the desired limits may occur, affecting the resulting Cnpk values adversely.

It can be shown by calculation that these minor variations in pressure, and thus temperature, have little impact on overall cycle lethality due to their short duration and limited magnitude. A 10-second excursion to 30.38 psia (121.68°C) contributes approximately 0.008 F0 minutes (1.1695 min– 1.1220

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Table 1. Empirical process capability analysis: moist heat resistometer. All cycles reflect 12 minutes of exposure at 121°C. Data in italics indicate failure to meet specification. Abbreviations used: Cnp, empirical process capability analysis for two-sided specification limit (lower specification limit [LSL] and upper specification limit [USL]) without regard for process centering; Cnpl, empirical process capability relative to the LSL; Cnpu, empirical process capability relative to the USL; Cnpk, empirical process capability relative to the LSL and USL, accounting for process centering.
min) × 0.166) of additional lethality to the cycle with respect to the USL of 30.25 psia (121.5°C), assuming a z-value of 10°C. This is unlikely to affect the resulting $D_{121C}$ value of the BI. Nevertheless, these excursions are sufficient to lower the resulting Cnpk value below the minimum acceptable value of 1.0. As these pressure excursions above the USL of 30.25 psia were most frequently noted within the first 60 seconds of the exposure phase, they may reflect either the purging of small amounts of residual air from the chamber or a slight leakage of steam from the chamber solenoid valve.

For each of the rejected BI lots, retrospective process capability analysis was undertaken for each cycle within the range of exposure times, where fraction negative (partial growth) data would be anticipated based on the labeling of the BI. The results of this analysis are presented in Table 4 in the data supplement. The highest and lowest Cnpk values noted were 1.54 and 0.69, respectively. For those cycles with Cnpk values less than 1.0, only a single pressure excursion (of five seconds duration) above the desired USL of 30.25 psia (maximum excursion of 30.47 psia) was noted within the first 60 seconds of the exposure phase; all other pressure readings during the remainder of the cycle remained within the desired limits.

Despite the low Cnpk values encountered with some cycles, we believe that the five paper strip BI lots for which the verified $D_{121C}$ values were less than 1.5 minutes represent a true loss of potency of the BIs compared with the labeled values, as they also failed to exhibit complete growth at the labeled survival parameters. Thus, of 92 total BI lots tested, five paper strip BI lots and one self-contained BI lot failed to comply with a minimum $D_{121C}$ value of 1.5 minutes, resulting in an overall verification success rate of 93.5% for all moist heat BI lots.

Conclusion
Determining the resistance performance of BIs requires the use of sophisticated test equipment and is beyond the capability of many users. Users, therefore, must rely on the information provided in the labeling of the BI to determine whether the resistance performance characteristics of BIs are suitable for given applications. The data reported here suggest that although this practice is generally acceptable, there may be a greater risk of paper strip BIs failing to comply with their labeled resistance perfor-
mance characteristics compared with self-contained BIs.

We believe that for most moist heat sterilization applications with an “overkill nature,” this risk is acceptable. Based on their confirmed population and estimated $D_{121\text{C}}$ values (i.e., >0.7 min; Table 3 in data supplement), even the moist heat BI lots that failed to comply with a minimum $D_{121\text{C}}$ value of 1.5 minutes when tested in our laboratory would still present a considerably greater challenge to moist heat sterilization than most common product bioburden. This is important, as many complex medical devices processed in healthcare may exhibit substantial levels of bioburden pre- and postcleaning.

For industrial BI users, verification of the labeled BI population in conjunction with a review of the labeled resistance performance claims, as indicated in USP <1229.5>, should be adequate to establish the suitability of the BI for use with most moist heat sterilization applications. The industrial BI user may wish to consider verification of the resistance performance of the BI when reduced sterilization processing conditions are used or when qualifying a new BI supplier. Conducting routine audits of the BI manufacturer, as described in ISO 14161, is strongly recommended. These audits should include a review of the BI manufacturer’s sampling plan.

For healthcare applications, the user must rely almost exclusively on the labeling of the BI to determine its suitability for use. In this situation, the risk of loss of population or potency of the BI is mitigated to some extent by the classification of BIs as medical devices under 21CFR880.2800, when used by a healthcare provider. Due to their classification as Class II medical devices, BIs are subject to recall should they fail to meet their performance claims or other critical quality attributes.

When distributed for use in healthcare applications, each manufacturer must ensure the stability and potency of its BI throughout the expiration dating period. Thus, in healthcare applications, the burden of compliance of the BI to the labeled population and resistance performance characteristics falls on the BI manufacturer, not the user.

In summary, we believe our data support the practice of determining the BI population—coupled with a review of the labeled resistance performance—as a means of establishing the suitability of a particular BI for use in industrial moist heat sterilization applications. In addition, process capability analysis may prove to be a useful means for evaluating the consistency of resistometer performance, though further evaluation over a range of different resistometers and resistometer designs would be necessary to establish the validity of this approach.

References


