Objectives
After completion of this self-study activity, the learner will be able to:
1. Identify the performance characteristics of a biological indicator.
2. Explain how Rapid Readout biological indicators (biological indicators with enzyme-based early-readout capability) detect sterilization process failures.
3. Discuss what a marginal sterilization process is.
4. Develop policy and procedures for the appropriate use of biological indicators.

Test Questions
True or False:
1. A positive biological indicator (BI) result should be investigated to determine the cause.
2. D-values, survival and kill times, and population are considered performance characteristics of BIs.
3. A marginal sterilization cycle is one that fails to kill all organisms and can yield both positive or negative BI results.
4. Sterilization processes are designed to destroy spores within the first half of the exposure cycle.
5. A Class 5 integrating indicator can replace the use of a BI.
6. Recalls only need to extend back to the last acceptable integrator result and do not need to be based on the last negative BI result.
7. Rapid Readout BIs contain a fluorescent indicator dye that is both faster and more sensitive than visual pH indicator dyes at detecting sterilization process failures.
8. BI testing does not need to be done in both gravity-displacement and dynamic-air-removal cycles if they are done in the same sterilizer.
9. Loads containing implantable devices should be monitored with a BI in an appropriate test pack or process challenge device (PCD), and quarantined whenever possible until the BI results are available.
10. Whenever a BI delivers a positive result it should be considered a false positive and disregarded.
Introduction

Positive biological indicators set in motion the recall of all medical devices processed since the last negative biological indicator (BI) (no matter what the results of other monitoring tools such as Class 5 integrating indicators), an analysis of what caused the failure, correction of those causes, and retesting of the sterilizer before it is put back into routine use. Unfortunately, sometimes the first question asked is, what is wrong with the BI?

The purpose of the BI is to identify when microorganisms are not killed which is a sterilization process failure. So when you get a positive BI the more appropriate question to ask is what changed or was different about this sterilization process that the microorganisms were not killed?

In order to appreciate the value of the information provided by the BI, let’s review the definition and performance characteristics of BIs, take a walk through the history of BI development, discuss when a BI is doing its job, and review the recommended practices for using biological indicators. Before beginning, there is one important word of advice: A BI is the best friend you have for detecting sterilization process failures—so don’t shoot the messenger.

Definition and Performance Characteristics of Biological Indicators

The Association for the Advancement of Medical Instrumentation (AAMI) defines a biological indicator as a “sterilization process monitoring device consisting of a standardized, viable population of microorganisms (usually bacterial spores) known to be resistant to the mode of sterilization being monitored. Biological indicators are intended to demonstrate whether the conditions were adequate to achieve sterilization.”

A BI consists of a calibrated population of bacterial spores of a high resistance to the mode of sterilization being monitored. For example, Geobacillus stearothermophilus is the most resistant spore for steam sterilization, hydrogen peroxide gas plasma and ozone sterilization. Bacillus atrophaeus is the most resistant spore for ethylene oxide (EO) sterilization. In healthcare settings, spores are coated on a paper strip, which is enclosed in a plastic vial containing a crushable glass media ampoule and cap that allows the sterilant to penetrate into the plastic vial, killing the spores and demonstrating whether sterilization conditions were met. This is called a self-contained biological indicator; Figure 1 shows its components.

The performance characteristics of a BI are defined in the Association for the Advancement of Medical Instrumentation (AAMI) standards. BI performance is based on spore population, D-value and Survival/Kill values. See Figure 2 on page 94 for an example of biological indicator performance data for steam sterilization and Figure 3 on page 95 for biological indicator performance data for ethylene oxide (EO) sterilization. This data is included in a Quality Assurance Certification that is found in each box of product.

Figure 1 Components of a Self-Contained Rapid Readout Biological Indicator
The labeling of a BI will state which sterilization cycle the BI can be used for, which spore is contained on the strip and what the population of the spores are. The population is expressed as the mean number of spores per strip and the term colony forming unit (C.F.U) is used. If the population is listed as $3.7 \times 10^6$ C.F.U., there are 3,700,000 spores on the strip. In order to meet the AAMI standards and be an appropriate challenge for the sterilization process, the population of spores must not be less than $1 \times 10^6$ C.F.U. for ethylene oxide sterilization processes and $1 \times 10^5$ C.F.U. for steam sterilization processes. A BI with a spore count less than these would not be considered an appropriate challenge.

The D-value is defined as the decimal reduction value. This value indicates the resistance of the BI. D-value testing is determined in a BIER vessel using a gravity cycle. D-values are determined by a fraction negative procedure after graded exposures to sterilization conditions. D-value is reproducible only under the exact conditions under which it is determined. User would not necessarily obtain the same results and would need to determine the biological indicators suitability for their particular use.

**Figure 2: Biological Indicator Performance Data for Steam Sterilization Processes**

For use in monitoring the 250°F (121°C), gravity and 270°F (132°C) vacuum assisted steam sterilization process.
Organism: *Geobacillus stearothermophilus* ATCC 7953
*Population (mean/strip): 3.7 $\times 10^6$ C.F.U.

**Resistance Testing Data:**
**Test D-Value (121°C):** 1.6 minutes  
**Survival time (121°C):** 7.3 minutes  
**Kill time (121°C):** 16.9 minutes  
* Determined at time of manufacture. Population is reproducible only under the exact conditions under which it was determined.  
** Survival/kill is verified and D-value is determined in a BIER vessel using a gravity cycle. D-values are determined by a fraction negative procedure after graded exposures to sterilization conditions. D-value is reproducible only under the exact conditions under which it is determined. User would not necessarily obtain the same results and would need to determine the biological indicators suitability for their particular use.
test organisms under stated conditions. For example, if a BI used for steam sterilization states that the D-Value (121°C) is 1.6 minutes, it means 90 percent of the spore population is killed in the first 1.6 minutes of a 121°C steam sterilization cycle. During the next 1.6 minutes, 90 percent of the remaining spore population is killed. By this data you can tell that all spores do not die at the same time. There is a transition period between all spores surviving and all spores being killed. During this transition period, when some negative and some positive BIs are obtained, the cycle is described as consisting of marginal sterilization conditions.

Biological indicator performance is also defined by survival/kill values. This also relates to the resistance of the biological indicator. The survival time is the time at which all spores in the BI will still be alive. The kill time is the time at which all spores in the BI will be killed. The survival and kill value can be determined by testing in a BIER test vessel or can be calculated based on the spore count and the D-value.

### Figure 3: Biological Indicator Performance Data for EO Sterilization Processes

For use in monitoring the ethylene oxide sterilization process.
Organism: *Bacillus atrophaeus* ATCC 9372
*Population (mean/strip): 3.9 X10^6 C.F.U.*

**Resistance Testing Data:**
- **Test D-Value (54°C):** 3.4 minutes
- **Survival time (54°C):** 15.99 minutes
- **Kill time (54°C):** 36.99 minutes

* Determined at time of manufacture. Population is reproducible only under the exact conditions under which it was determined.
** Survival/kill is verified and D-value is determined in a BIER vessel at 54°C, 60 percent RH, 600mg EO/liter. D-values are determined by a fraction negative procedure after graded exposures to sterilization conditions. D-value is reproducible only under the exact conditions under which it is determined. User would not necessarily obtain the same results and would need to determine the biological indicators suitability for their particular use.
BI performance data must be included in each package of product, usually in a Certificate of Analysis. The information should also include a statement about which ANSI/AAMI standards this product meets.

A Walk through History

From Spore Strips to Self-Contained Biological Indicators to Rapid Readout Biological Indicators.

In 1950, healthcare facilities started using BIs consisting of a spore strip contained in a glassine envelope. The test BI strip in an envelope, along with a positive control BI strip in a separate envelope, was sent to the microbiological laboratory to be transferred to a test tube containing media and incubated. Contamination during the transfer of spore strips was a common occurrence. Media that became cloudy, indicating growth of spores, required subsequent gram staining and subculture for further identification before a result could be provided. Negative results were not available for a week or more. Frequent contamination and long incubation times were the major disadvantages of spore strips.

In the late-1960s the concept of self-contained BIs was conceived and became the BI of choice in the 1970s when 3M developed and introduced them into the marketplace. Self-contained BIs have three major advantages. First, they eliminated the need to aseptically transfer the spore strip to a liquid growth media by combining the spore strip and a crushable glass ampoule in the same container. This addressed the common contamination problem of spore strips. Second, the addition of a pH dye, which turned yellow when microbial growth produced acidic by-products, was used to detect positives in place of observing for cloudy media indicating microbial growth. This greatly simplified interpretation of the results and put BI testing in the hands of the sterilization departments rather than the microbiology laboratory. The third advantage is faster read-out times. As refinements in recovery media were developed they resulted in shorter required incubation times. These advantages have resulted in the elimination of spore strips that require aseptic transfer to media and incubation wherever possible. These advantages, plus labor and time savings have resulted in the widespread use of self-contained BIs.
The need to verify the efficacy of the sterilization process in a shorter time period was becoming more important because of the turnover demands on the sterilization department, the complexity of medical devices being introduced and the need to save time and control costs. These needs drove the development of Rapid Readout Biological Indicators by 3M.

Rapid Readout BIs (biological indicators with enzyme-based early-readout capability) are identical to the original self-contained BIs with one major exception: The glucose in the media has been removed and replaced by a similar glucoside with a fluorescent indicator dye attached. Spores that have not been destroyed by a sterilization process and are biologically active will be demonstrated in a much shorter period of time because, as soon as the glucoside is broken down, the fluorescent dye will become detectable in trace amounts. Spores do not need to multiply to release the dye from the glucose substrate. A proper sterilization process will sufficiently destroy cellular components so that microbes are no longer able to grow. Following a proper sterilization process, neither detectable enzymatic activity is present nor is the cell able to grow or multiply. The auto-reader detects the presence of the natural occurring enzyme, which is an intrinsic component of the spore, by reading a fluorescent product that is produced when this enzyme converts the non-fluorescent substrate in the media vial. The fluorescence indicates the presence of an active enzyme and a sterilization process failure. Non-fluorescence indicates inactivation of the enzyme and an effective sterilization process.

Figure 4


250°F Superheated steam using a prevacuum cycle with an 8 minute exposure in a BIER Vessel (% positive for biological indicators and % reject for chemical indicators.)

A sterilization process failure can be detected in as little as a few minutes rather than days. It still takes one to three hours to obtain a final negative result but this is much better than one to seven days. Obtaining results within a minimal incubation time allows sterilization process failures to be identified much sooner, instruments to be turned around faster, costs associated with inventory and recall to be reduced and improved patient outcomes.

Spore strips, self-contained and Rapid Readout BIs all contain spores that directly measure the combined lethality of an infinite variety of processing conditions.

According to Dr. Irvin Plug, “We use spores as BIs because they can integrate the sterilization effect of a lethal agent, whether it be wet heat, dry heat, a chemical such as EO or ionizing radiation.” This is why BIs (not chemical indicators, such as Class 5 integrating indicators) are the only acceptable monitor for determining the effectiveness of industrial steam and EO processes.

“The only currently accepted system capable of integrating all physical parameters responsible for lethality is a BI,” states the PDA Journal of Pharmaceutical Science and Technology, 2004:10 “The BI is the only tool that will accurately integrate the combined lethal parameters within the load… More weight must be given to biological results because all critical parameters cannot be measured by physical means…”

This is the advantage BIs have over chemical indicators that only measure a specific set of artificially designed conditions. Recent scientific studies have demonstrated that failures, due to marginal cycle conditions created by either inadequate air removal or superheated steam conditions, were not detected equally by chemical and biological indicators. Under these common failure conditions all BIs tested, which included spore strips,
self-contained BIs and Rapid Readout BIs, demonstrated failures. Integrating indicators failed to detect these same failure conditions in side-by-side testing. The conclusions reached showed only biological indicators consistently detected all of the sterilization process failure conditions evaluated and that both the fluorescent readout and visual readings detected these failure conditions.

Data from this study showing a cycle with an eight-minute exposure at 250°F using superheated steam is presented in Figure 4 on page 98. As superheated steam is not as effective as saturated steam for achieving sterilization, the expectation for a sterilization indicator would be to identify this cycle as failed and alert the user to the need for sterilizer maintenance. Note, however, that none of the three chemical integrators detected this condition at an acceptable frequency, while the BI responses indicated that there was a problem with this cycle.

The goal of sterilization is to kill microorganisms and the destruction of spores provides direct evidence of this. BIs, regardless of type, remain the most critical tool for sterilization monitoring.

**When a Biological Indicator Is Doing its Job**

BIs detect conditions that are not able to kill the spores. Since spores are more resistant than other microbes, they provide a safety margin. If the spores have been killed then, by inference, the other microbes on medical devices should have also been killed. Sterilization cycles are designed to kill spores within the first half of the exposure cycle. In a normally functioning cycle the spores should easily be destroyed.

See Figure 5 for a graphic representation of spore kill in a typical sterilization process.

At the beginning of the process all spores are expected to be alive or survive. By the middle of the process all spores should be killed. At some point, between seeing all spores survive and all spores being killed, marginal cycle conditions exist. A marginal cycle is one that fails to completely kill all spores. In a sterilization process failure (i.e., sterilizer not functioning, inadequate steam quality and quantity, or human errors due to incorrect packaging, loading or choosing the incorrect cycle for the load) the marginal part of the process may come at the end of the cycle. It is toward the end of these marginal cycle conditions where a more sensitive indicator, such as

![Figure 5: Spore Kill in A Sterilization Process](image-url)
a fluorescent dye, may detect a few more positives than a less-sensitive indicator, such as a pH dye. Detection of biologically active proteins, such as the intrinsic enzyme in the spore that breaks down the glucoside substrate containing the fluorescent dye, demonstrates a sterilization process failure. Whether the spore is able to multiply or not, the detection of biologically active proteins demonstrates a sterilization failure.

Rapid Readout BIs can detect marginal cycle conditions that other spore strips and self-contained BIs do not. Dr. Donald Vesley concluded in his evaluation of BIs that the Rapid Readout BI technology was a more sensitive indicator of marginal sterilization cycles than other self-contained BIs without any indication of false positive results. Dr. William Rutalla concluded in his study that the Rapid Readout BIs were an excellent monitor that ensures sterilization without inappropriately indicating failure. The better a product is at detecting failures, the more value it provides.

**Recommended Practices for Using Biological Indicators**

All the recommended practices require the use of biological indicators to monitor the effectiveness of a sterilization process.

AAMI and the Association for the periOperative Registered Nurses (AORN) recommend that a BI in a test pack (process challenge device or PCD) be run weekly—preferably

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**Answer Key**

every day—that the steam sterilizer is used.1,14,16,19 This testing should be done in each type of cycle (gravity-displacement, dynamic air-removal [prevacuum or steam-flush-pressure-pulse], flash) used.1,16 In addition when using the flash sterilization process, each type of tray configuration (e.g., open surgical tray, single-wrapped surgical tray, protective organizing case, rigid sterilization container) in routine use should be tested separately.1,16

Each load containing implantable medical devices should be monitored with a BI test pack/PCD, and the implantable device quarantined, whenever possible until the results of the BI testing are available.1,14,15,16,19 The AORN standard also states:14

“*If an implantable medical device is flash sterilized, the device must be used immediately after a negative biological readout. If not used, the device must be reprocessed before future use.*”

The American Society for Healthcare Central Service Professionals of the American Hospital Association (ASHCSP) has the most stringent recommended practices for routine BI testing:15

“*Biological monitoring should be used to challenge the performance of the sterilizer. They should be used:*”

- Routinely in steam sterilization loads, daily, preferable in each load that contains critical items, e.g., instrument sets, individual surgical instruments, or any item that comes into contact with sterile tissue.

  **Rationale:** Critical Medical Devices pose the greatest risk to the patient if the devices are not sterile. Monitoring each load containing critical medical devices allows for quicker detection of failures."

In addition to routine testing of steam sterilizers, a BI test pack/PCD should be used for qualification testing by the health care facility whenever a sterilizer is installed, relocated, after a sterilizer malfunction, after sterilization process failures, and after any major repairs.1,14,15,16,19 For qualification testing, each cycle type used (gravity-displacement, dynamic air-removal [prevacuum or steam-flush-pressure-pulse], flash), should be tested. BIs should also be used for product testing.1,16

For routine testing of low-temperature sterilization processes, a BI test pack/PCD should be used in each load and implantable medical devices quarantined, whenever possible until the results of the BI testing are available.9,14,17,18 BIs are used for qualification testing by the sterilizer manufacturer at time of installation, and by the healthcare facility for periodic quality assurance testing (quarterly), after major redesign, relocation, corrective maintenance or a sterilization process failure.17,18 BIs are also used for product testing.17

For more details on the usage of BIs and other monitoring tools, CEU accredited inservices are available from *Managing Infection Control*.20-23

**Summary**

Biological indicators (BIs) provide direct evidence that the sterilization process conditions are able to kill spores. BIs have evolved over the past 50 years. Results that once took seven days or more now are obtained in one or three hours. Cumbersome subculturing and long incubation times have been replaced by self-contained biological indicators with rapid readout techniques.
Sterilization assurance levels, used to establish sterilization parameters, dictate that there be a very low probability—one-in-one million or a SAL of $10^{-6}$—of any surviving spores at the end of the full cycle time. The ability to obtain results sooner has allowed faster turnaround times on medical devices and earlier detection of sterilization process failures. When a positive biological indicator occurs, don’t shoot the messenger.

Instead ask the question, what changed or was different about this sterilization process that the microorganisms were not killed?

**References**

Indicators and Five Chemical Indicators., Vol. 17, No. 7, July:1996.


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