

## (1035) BIOLOGICAL INDICATORS FOR STERILIZATION

A biological indicator is broadly defined as a characterized preparation of a specific microorganism that provides a defined and stable resistance to a specific sterilization process. Microorganisms widely recognized as suitable for biological indicators are spore-forming bacteria, because, with the exception of ionizing radiation processes, these microorganisms are significantly more resistant than normal microflora. A biological indicator can be used to assist in the performance qualification of the sterilization equipment and in the development and establishment of a validated sterilization process for a particular article. Biological indicators are used in processes that render a product sterile in its final package or container, as well as for the sterilization of equipment, materials, and packaging components used in aseptic processing. Biological indicators may also be used to monitor established sterilization cycles and in periodic revalidation of sterilization processes. Biological indicators may also be used to evaluate the capability of processes used to decontaminate isolators or aseptic clean-room environments.

The principles and requirements for these applications are described under *Sterilization and Sterility Assurance of Compendial Articles* (1211).

### TYPES OF BIOLOGICAL INDICATORS

There are at least three types of biological indicators. Each type of indicator incorporates a known species of a microorganism of known sterilization resistance to the sterilization mode. Some biological indicators may also contain two different species and concentrations of microorganisms.

One form of biological indicator includes spores that are added to a carrier (a disk or strip of filter paper, glass, plastic, or other materials) and packaged to maintain the integrity and viability of the inoculated carrier.

Carriers and primary packaging shall not contain any contamination (physical, chemical, or microbial) that would adversely affect the performance or the stability characteristics of the biological indicator. The carrier and primary packaging shall not be degraded by the specific sterilization process, which is used in a manner that will affect the performance of the biological indicator. The carrier should withstand transport in the primary and secondary packaging and handling at the point of use. The design of the carrier and primary packaging should minimize the loss of the original inoculum during transport, handling, and shelf life storage.

Another form of biological indicator is a spore suspension that is inoculated on or into representative units of the product to be sterilized. This represents an inoculated product; however, a simulated inoculated product may be used if it is not practical to inoculate the actual product. A simulated product is a preparation that differs in one or more ways from the actual product, but performs as the actual product using test conditions or during actual production sterilization processing. Spore suspensions with a known D value should be used to inoculate the actual or simulated product. If a simulated inoculated product is used, it must be demonstrated that it will not degrade the sterilization resistance of the bioindicator. The physical design of actual or simulated product can affect the resistance of spore suspensions that are inoculated on or into the products. In the case of liquid inoculated products, it is often advisable to determine both the D value and z value of the specific biological indicator microorganism in the specific liquid product. The population, D value, z value where applicable, and endpoint kill time of the inoculated actual or simulated product should be determined.

A third form of biological indicator is a self-contained indicator. A self-contained biological indicator is designed so that the primary package, intended for incubation following sterilization processing, contains the growth medium for recovery of the process-exposed microorganisms. This form of biological indicator together with the self-contained growth medium can be considered a system. In the case of self-contained biological indicators, the entire system provides resistance to the sterilization process.

If the biological indicator is a paper strip or disk in a self-contained package that includes an available culture medium, the package design should be readily penetrable by the sterilizing agent. To allow for the time lag that may occur while the sterilizing agent reaches the contained microorganisms in the system, the D value, process endpoint kill time, and the survival time should be characterized for the system and not solely for the paper strip in the self-contained unit. Following the sterilizing treatment, the spore strip or disk is immersed in the self-contained medium by manipulation, which allows contact with the culture medium.

Self-contained biological indicators may also consist of a spore suspension in its own medium, and they often also contain a dye, which indicates positive or negative growth following incubation. Resistance of the self-contained system is dependent upon penetration of the sterilant into the package. Penetration may be controlled by the manufacturer through varying designs and composition of the self-contained biological indicator package, ampul, or container. Self-contained ampul biological indicators may be incubated directly following exposure to the sterilization process. The entire system is then incubated under the specified conditions. Growth or no growth of the treated spores is determined visually (either by observing a specified color change of an indicator incorporated in the medium or by turbidity) or by microscopic examination of the inoculated medium.

The self-contained system resistance characteristics must also comply with the labeling of the self-contained system and the relevant biological indicator monograph. The self-contained biological indicator system should withstand transport in the secondary packaging and handling at the point of use without breakage. The design of the self-contained system should be such to minimize the loss of the original inoculum of microorganisms during transport and handling. During or after the sterilization process, the materials used in the self-contained system shall not retain or release any substance that can inhibit the growth of low numbers of surviving indicator microorganism under culture conditions. Adequate steps must be taken to demonstrate that the recovery medium has retained its growth support characteristics after exposure to the sterilization process.

### Preparation

All operations associated with the preparation of biological indicators are controlled by a documented quality system. Traceability is maintained for all materials and components incorporated in or coming into direct contact with the microorganism suspension, the inoculated carrier, or the biological indicator.

The preparation of stock spore suspensions of selected microorganisms used as biological indicators requires the development of appropriate procedures, including mass culturing, harvesting, purification, and maintenance of the spore suspensions. The stock suspension should contain predominantly dormant (nongerminating) spores that are held in a nonnutritive liquid.

The finished product (microbial suspension, inoculated carriers, or biological indicators) supplied by commercial manufacturers shall have no microorganisms, other than the test microorganism, present in sufficient numbers to adversely affect the product. The system to minimize the presence of microorganisms other than the biological indicator

microorganism in the product will be validated, monitored, and recorded.

Selection for Specific Sterilization Processes

The selection of a biological indicator requires a knowledge of the resistance of the biological indicator system to the specific sterilization process. It must be established that the biological indicator system provides a challenge to the sterilization process that exceeds the challenge of the natural microbial burden in or on the product.

The effective use of biological indicators for the cycle development, process, and product validation, and routine production monitoring of a sterilization process requires a thorough knowledge of the product being sterilized, along with its component parts (materials and packaging). Only the widely recognized biological indicators specified in the particular biological indicator monograph should be used in the development or validation of a sterilization process. This will ensure that the biological indicator selected provides a greater challenge to the sterilization process than the bioburden in or on the product. Some users may require biological indicators with characteristics that differ from those widely available commercially. In such cases, users may grow their own spore cultures for the express purpose of preparing in-house biological indicators for their specific use. In such a case, the user is well advised to use organisms already described in the scientific literature as indicator organisms, and the user must have the capability of determining D and z values for in-house biological indicators. When biological indicators are prepared in-house, users must confirm the population, purity, and shelf life of the biological indicator to ensure the validity of any test conducted using the in-house biological indicator. When a bioburden-based sterilization process design is used, data comparing the resistance of the biological indicator to that of bioburden are essential. Enumeration of the bioburden content of the articles being sterilized is also required. The process must result in a biologically verified lethality sufficient to achieve a probability of obtaining a nonsterile unit that is less than one in a million.

Alternatively, the overkill method may be used in the design of a sterilization process. In this case, specific assumptions are made regarding the resistance assumption used in establishing sterilization process lethality requirements. In general, all overkill processes are built upon the assumption that the bioburden is equal to one million organisms and that the organisms are highly resistant. Thus, to achieve the required probability of a nonsterile unit that is less than one in a million, a minimum 12 D process is required. A 12 D process is defined as a process that provides a lethality sufficient to result in a 12 log reduction, which is equivalent to 12 times a D value for organisms with sufficiently higher resistance than the mean resistance of bioburden. Because the bioburden is assumed to be one million, an overkill pro-

cess will result in a probability of nonsterility at much less than 10<sup>-6</sup> in actual practice. Overkill process design and evaluation may differ depending upon the sterilization process under test. The use of an overkill design and validation approach may minimize or obviate the need for bioburden enumeration and identification.

**Moist Heat**—For moist heat sterilization process, spores of suitable strains of *Bacillus stearothermophilus* are commercially available as biological indicators and frequently employed. Other heat-resistant spore-forming microorganisms such as *Clostridium sporogenes*, *Bacillus subtilis*, and *Bacillus coagulans* have also been used in the development and validation of moist heat sterilization processes.

**Dry Heat**—For dry heat sterilization, spores of *Bacillus subtilis* spp. are sometimes used to validate the process. During the validation of dry heat sterilization processes, endotoxin depyrogenation studies are frequently conducted in lieu of microbial inactivation studies during the establishment of sterilization cycles because the inactivation rate of endotoxin is slower than the inactivation rate of *Bacillus subtilis* spores. In practice the reduction of endotoxin titer by three or more logs will result in a process that also achieves a probability of nonsterility substantially lower than 10<sup>-6</sup>.

**Ionizing Radiation**—Spores of *Bacillus pumilus* have been used to monitor sterilization processes using ionizing radiation; however, this is a declining practice. Radiation dose-setting methods that do not use biological indicators have been widely used to establish radiation processes. Furthermore, certain bioburden microorganisms can exhibit greater resistance to radiation than *Bacillus pumilus*.

**Ethylene Oxide**—For ethylene oxide sterilization, spores of a subspecies of *Bacillus subtilis* (*Bacillus subtilis* var. *niger*) are commonly used. The same biological indicator systems are generally used when 100% ethylene oxide or different ethylene oxide and carrier gas systems are used as sterilants.

**Vapor-Phase Hydrogen Peroxide (VPHP)**—This process has been shown to be an effective surface sterilant or decontaminant. VPHP is capable of achieving sterilization (probability of nonsterility of less than one in a million) when process conditions so dictate and if the target of sterilization is suitably configured. However, VPHP is also commonly used as a surface decontaminating agent in the treatment of sterility testing, biological and chemical containment, manufacturing isolators, and clean rooms.

Surface decontamination is a process that is distinct from sterilization of product contact materials, container-closure systems, or product. It is a process designed to render an environment free of detectable or recoverable microorganisms. Biological indicators are widely used to verify the efficacy of the decontamination process. However, in the case of decontamination, a spore log reduction value of three to four is adequate because the goal is decontamination rather than sterilization.

Table 1. Typical Characteristics for Commercially Supplied Biological Indicator Systems

Sterilization Mode	Example of a Typical D value (minutes)	Range of D values for Selecting a Suitable Biological Indicator (minutes)	Limits for a Suitable Resistance (depending on the particular D value [minutes])	
			Survival Time	Kill Time
Dry heat <sup>a</sup>	1.9	Min. 1.0	Min. 4.0	10.0
160°		Max. 3.0	Max. 14.0	32.0
Ethylene oxide <sup>b</sup>				
600 mg per L	3.5	Min. 2.5	Min. 10.0	25.0
54°		Max. 5.8	Max. 27.0	68.0
60% relative humidity				
Moist heat <sup>c</sup>	1.9	Min. 1.5	Min. 4.5	13.5
121°		Max. 3.0	Max. 14.0	32.0

<sup>a</sup> For 1.0 × 10<sup>6</sup> to 5.0 × 10<sup>6</sup> spores per carrier.

<sup>b</sup> For 1.0 × 10<sup>6</sup> to 5.0 × 10<sup>7</sup> spores per carrier.

<sup>c</sup> For 1.0 × 10<sup>5</sup> to 5.0 × 10<sup>6</sup> spores per carrier.

*Bacillus stearothermophilus* is the most prevalently used biological indicator for validating VPHP. Other microorganisms that may be useful as biological indicators in VPHP processes are spores of *Bacillus subtilis* and *Clostridium sporogenes*. Other microorganisms may be considered if their performance responses to VPHP are similar to those of the microorganisms cited above.

These spores may be inoculated on the surface of various gas-impervious carrier systems having glass, metal, or plastic surfaces. Highly absorbent surfaces, such as fibrous substrates, or any other substrate that readily absorbs VPHP or moisture may adversely influence the VPHP concentration available for inactivation of inoculated microorganisms. Paper substrates are not used because VPHP will degrade cellulose-based materials.

For representative characteristics of commercially supplied biological indicators, see Table 1.

The biological indicator may also be individually packaged in a suitable primary overwrap package that does not adversely affect the performance of the indicator, and is penetrable by VPHP. Spunbound polyolefin materials have proven to be well suited as an overwrap of biological indicators intended for use in evaluation of VPHP processes. The overwrap material may facilitate laboratory handling of the biological indicators following exposure to VPHP. Also, the use of an overwrap material to package VPHP biological indicators must be carefully assessed to ensure that, following VPHP exposure, residual hydrogen peroxide is not retained by the packaging material, possibly inducing bacteriostasis during the recovery steps. Microbial D values will be influenced by the presence of a biological indicator overwrap material relative to the rate of inactivation and the potential presence of residual VPHP. In cases where biological indicators (inoculated carriers) are being used without the primary package, stringent adherence to aseptic techniques is required.

## PERFORMANCE EVALUATION

### Manufacturer's Responsibility

The initial responsibility for determining and providing to the users the performance characteristics of a biological indicator<sup>1</sup> lot resides with the manufacturer of biological indicators. The manufacturer should provide with each lot of biological indicators a certificate of analysis that attests to the validity of biological indicator performance claims cited on the biological indicator package label or in the package insert of the label package. The manufacturer should define the sterilization process that the biological indicator will be used to evaluate. The characterization of each type of biological indicator, which provides the basis for label claims, should be performed initially by the manufacturer of the biological indicator using specialized and standardized apparatus under precisely defined conditions.<sup>1</sup> The manufacturer should also provide information concerning the D value, the method by which the D value was determined, and microbial count and resistance stability of the biological indicator throughout the labeled shelf life of the indicator. Optimum storage conditions should be provided by the manufacturer, including temperature, relative humidity, and any other requirements for controlled storage. The data obtained from the various required performance assays should be cited in a package insert or on the label of the biological indicator package. The manufacturer should provide directions for use, including the medium and conditions to be used for the recovery of microorganisms after exposure to the sterilization process.

<sup>1</sup>See Apparatus under Biological Indicators—Resistance Performance Tests (55). These apparatuses have been designed to provide consistent physical conditions applicable to the characterization of biological indicators. The required performance characteristics are also indicated.

zation process. Disposal instructions should also be provided by the manufacturer of the biological indicator.

### User's Responsibility

**Commercial Product**—When biological indicators are purchased from a commercial source, their suitability for use in a specific sterilization process should be established through developmental sterilization studies unless existing data are available to support their use in the process. The user should establish in-house acceptance standards for biological indicator lots and consider rejection in the event the biological indicator lot does not meet the established in-house performance standards. A Certificate of Performance should be obtained for each lot of indicators, and the user should routinely perform audits of the manufacturer's facilities and procedures. If certificates are not obtained and audits have not been performed, or if the biological indicators are to be used outside of the manufacturer's label claims, verification and documentation of performance under conditions of use must exist.

Upon initial receipt of the biological indicator from a commercial supplier, the user should verify the purity and morphology of the purchased biological indicator microorganisms. Verification of at least the proper genus is desirable. Also, a microbial count to determine the mean count per biological indicator unit should be conducted. The manufacturer's comments relative to D value range, storage conditions, expiration dating, and stability of the biological indicator should be observed and noted. The user may consider conducting a D value assessment before acceptance of the lot. 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. Particularly important is the verification of the D value and count stability of the biological indicator system if long-term storage is employed.

In the event the spore crop is maintained for longer than 12 months under documented storage conditions, both spore count and resistance analysis must be conducted, unless performance of an original parent crop has been validated for a longer storage period. The result of spore count and resistance assays should be within the range of acceptability established during initial acceptance of the spore crop lot.

**Noncommercial Product**—A user of biological indicator systems may elect to propagate microorganisms for developing in-house biological indicators to develop or validate sterilization processes. In the event a user becomes a "manufacturer" of biological indicators, biological indicator performance requirements must be met. If the biological indicator system is used for the development of new sterilization processes or validation of existing processes, the same performance criteria described for commercial manufacturers of biological indicators must be followed.

### Spore Crop Preparation

Because most biological indicators use microbial spores, accurate records of spore crop identification must be maintained by commercial and noncommercial biological indicator manufacturers. These records should include records pertaining to the source of the initial culture, identification, traceability to the parent spore crop, subculture frequency, media used for sporulation, changes in media preparation, any observation of crop contamination, and pre- and post-heat shock data. Records of usage of the spore crop and resistance to sterilization (namely, D values and z values where applicable) should also be maintained.

## Instrumentation

The instrumentation used to evaluate the sterilization resistance of spore crops must be consistent with existing standards<sup>2</sup> related to the performance evaluation of biological indicator systems.

Equipment for the determination of D values of microorganisms exposed to VPHP should be able to closely control equipment operating parameters as described for other biological indicator systems under *Biological Indicators—Resistance Performance Tests* (55). Particularly important is the assurance of a consistently reproducible VPHP concentration, delivered within a finite time, and maintained within a specified concentration range or VPHP pressure range for a defined increment of time. Introduction of biological indicators into a stabilized concentration of VPHP conditions should be via a system that permits rapid entry and removal of the test units from the chamber. Also, the design of the test chamber should allow for the attainment of steady-state VPHP concentrations and pressure, or the use of a defined amount of cubic feet of free flowing VPHP at a standardized pressure and temperature. Currently, VPHP concentration measurement devices may not be widely used. Therefore, exposure conditions may need to be based on the maintenance of steady-state VPHP pressures or flow rates resulting from a known initial weight of hydrogen peroxide, admitted to the chamber in a defined unit of time. Using this information, together with the known fixed volume of the chamber environment, a calculation of the approximate VPHP concentration can be made. If conditions are maintained constant throughout each D value assessment run, comparisons of relative resistance among different biological indicator lots may be readily determined.

## USE FOR IN-PROCESS VALIDATION

Regardless of the mode of sterilization, the amount of the initial population of the microorganisms, its resistance to sterilization, and the site of inoculation on or in the product can all influence the rate of biological indicator inactivation.

During product microbial challenges, various areas of the product should be inoculated with biological indicators. If, for example, a container with a closure system is sterilized, both the product solution and the closure should be challenged to ensure that sterilization equivalent to a  $10^{-6}$  (one in a million probability of a nonsterile unit) sterilization assurance level (SAL) will be obtained in the solution as well as at the closure site.

One may need to determine through laboratory studies whether product components are more difficult to sterilize than, for example, a solution or drug within the product. Depending on the locations of the product components most difficult to sterilize, different process parameters may be involved in assuring microbial inactivation to an SAL of  $10^{-6}$ . The product performance qualification phase should identify the most important process parameters for inactivation of microorganisms at the sites most difficult to sterilize. Once these critical processing parameters are determined, during sterilization in-process validation of the product, they should be operated at conditions less than the conditions stated in the sterilization process specifications. Biological indicator survival is predicated upon both resistance and population. Therefore, a  $10^6$  biological indicator population is not always required to demonstrate a  $10^{-6}$  SAL. The appropriate use for biological indicators is to employ them to confirm that the developed process parameters result in the desired SAL. In moist heat sterilization, the biological indicator is used to establish that physically measured lethality can be verified biologically. Biological indicators with substantive D values and populations substantially less than  $10^6$  are adequate to validate many sterilization and decontamination

processes. It is important that the users be able to scientifically justify their selection of a biological indicator.

## 〈1041〉 BIOLOGICS

Products such as antitoxins, antivenins, blood, blood derivatives, immune serums, immunologic diagnostic aids, toxoids, vaccines, and related articles that are produced under license in accordance with the terms of the federal Public Health Service Act (58 Stat. 682) approved July 1, 1944, as amended, have long been known as "biologics." However, in Table III, Part F, of the Act, the term "biological products" is applied to the group of licensed products as a whole. For Pharmacopeial purposes, the term "biologics" refers to those products that must be licensed under the Act and comply with Food and Drug Regulations—Code of Federal Regulations, Title 21 Parts 600-680, pertaining to federal control of these products (other than certain diagnostic aids), as administered by the Center for Biologics Evaluation and Research or, in the case of the relevant diagnostic aids, by the Center for Devices and Radiological Health of the federal Food and Drug Administration.

Each lot of a licensed biologic is approved for distribution when it has been determined that the lot meets the specific control requirements for that product as set forth by the Office. Licensing includes approval of a specific series of production steps and in-process control tests as well as end-product specifications that must be met on a lot-by-lot basis. These can be altered only upon approval by the Center for Biologics Evaluation and Research and with the support of appropriate data demonstrating that the change will yield a final product having equal or superior safety, purity, potency, and efficacy. No lot of any licensed biological product is to be distributed by the manufacturer prior to the completion of the specified tests. Provisions generally applicable to biologic products include tests for potency, general safety, sterility, purity, water (residual moisture), pyrogens, identity, and constituent materials (Sections 610.10 to 610.15 and see *Safety Tests—Biologicals* under *Biological Reactivity Tests, In Vivo* (88), *Sterility Tests* (71), *Water Determination* (921), and *Pyrogen Test* (151), as well as *Bacterial Endotoxins Test* (85)). Constituent materials include ingredients, preservatives, diluents and adjuvants (which generally should meet compendial standards), extraneous protein in cell-culture produced vaccines (which, if other than serum-originating, is excluded) and antibiotics other than penicillin added to the production substrate of viral vaccines (for which compendial monographs on antibiotics and antibiotic substances are available). Additional specific safety tests are also required to be performed on live vaccines and certain other items. Where standard preparations are made available by the Center for Biologics Evaluation and Research (Section 610.20), such preparations are specified for comparison in potency or virulence testing. The U.S. Opacity Standard is used in estimating the bacterial concentration of certain bacterial vaccines and/or evaluating challenge cultures used in tests of them. (See also *Units of Potency* in the *General Notices*.)

The Pharmacopeial monographs conform to the Food and Drug Regulations in covering those aspects of identity, quality, purity, potency, and packaging and storage that are of particular interest to pharmacists and physicians responsible for the purchase, storage, and use of biologics. Revisions of the federal requirements affecting the USP monographs will be made the subjects of *USP Supplements* as promptly as practicable.

<sup>2</sup>BIER/Steam Vessels, American National Standards, ANSI/AAMI ST45 : 1992.