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Standard Test Method for Microbial Ingress Testing on Single-Use Systems[1]

This standard is issued under the fixed designation E3251; the number immediately following the designation indicates the year of original adoption or, in the case of revision, the year of last revision. A number in parentheses indicates the year of last reapproval. A superscript epsilon (ε) indicates an editorial change since the last revision or reapproval.

1. Scope

1.1 The microbial test method outlined in this document applies to microbial ingress risk assessment of a single-use system (SUS) or its individual components that require integrity testing either by the assembly supplier or the end user of the assembly based on a potential risk of a breach to the product or manufacturing process.

1.2 The aim of microbial ingress testing of sterile SUSs used in biopharmaceutical manufacturing is two-fold:

1.2.1 Firstly, it is used to evaluate the ability of a SUS fluid path to remain sterile after a SUS has been challenged by microbial exposure. Microbial exposure is achieved either by directly placing a SUS into a container of microbial challenge solution, or by delivering an aerosolized microbial challenge onto a SUS that is placed inside a test chamber designed to generate and deliver the aerosol. The choice of the test challenge organism should be justified based on a risk assessment of the SUS and conditions of use.

1.2.2 Additionally, microbial ingress testing can be used to determine the maximum allowable leakage limit (MALL) that does not allow microbial ingress under specific test conditions. The defect size that can be detected by specific physical integrity testing methods can be correlated to this MALL in order to claim microbial integrity. Test articles bearing calibrated defects over a range of dimensions, including up to a defect size expected to consistently allow microbial ingress as a positive control (defect-based positive control), may be tested to determine the MALL.

1.3 Both purposes for microbial ingress testing as described in 1.2.1 and 1.2.2 can either be conducted by liquid immersion or aerosol exposure. For the purpose described in 1.2.2, the type of exposure should be determined according to the SUS's use-case conditions and a risk assessment.

1.4 The method used to create a breach, hole or defect in single-use film or in a SUS test article, as well as the analytical method used to physically characterize the defect size is outside of the scope of this document. The sampling plan for a given test article should be justified with the rationale of sampling size to obtain a statistically meaningful effect (Practice E3244). Determining the appropriate number of SUS test articles will depend on a risk assessment of the SUS and the conditions of its use and is also outside of this document's scope.

1.5 The values stated in SI units are to be regarded as standard. No other units of measurement are included in this standard.

1.6 *This standard does not purport to address all of the safety concerns, if any, associated with its use. It is the responsibility of the user of this standard to establish appropriate safety, health, and environmental practices and determine the applicability of regulatory limitations prior to use.*

1.7 *This international standard was developed in accordance with internationally recognized principles on standardization established in the Decision on Principles for the Development of International Standards, Guides and Recommendations issued by the World Trade Organization Technical Barriers to Trade (TBT) Committee.*

2. Referenced Documents

2.1 *ASTM Standards:*[2]

E3244 Practice for Integrity Assurance and Testing of Single-Use Systems

2.2 *Other Documents:*

USP <1207> Sterile Product Packaging — Integrity Evaluation, 2016[3]

ISO 15747 Plastic Containers for Intravenous Injections[4]

3. Terminology

3.1 *Definitions:*

3.1.1 *(calibrated) artificial defect, n*—an artificial breach or defect (that is, laser-drilled hole, capillary) representing typical failure modes, intentionally introduced into a test article.

[1] This test method is under the jurisdiction of ASTM Committee E55 on Manufacture of Pharmaceutical and Biopharmaceutical Products and is the direct responsibility of Subcommittee E55.04 on General Biopharmaceutical Standards.

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[2] For referenced ASTM standards, visit the ASTM website, www.astm.org, or contact ASTM Customer Service at service@astm.org. For *Annual Book of ASTM Standards* volume information, refer to the standard's Document Summary page on the ASTM website.

[3] Available from U.S. Pharmacopeial Convention (USP), 12601 Twinbrook Pkwy., Rockville, MD 20852-1790, http://www.usp.org.

[4] Available from International Organization for Standardization (ISO), ISO Central Secretariat, BIBC II, Chemin de Blandonnet 8, CP 401, 1214 Vernier, Geneva, Switzerland, http://www.iso.org.

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3.1.2 *challenge solution, n*—a liquid suspension containing a selected microorganism used to generate an aerosol or used for liquid immersion.

3.1.3 *defect-based positive control, n*—a test article exposed to a challenge solution with a calibrated breach or defect. The size of the breach or defect will depend on a previous determination of the defect size that can be consistently detected under given conditions. This positive control is used as a control to ensure that the microorganism can pass through a defect and can be detected by the test method.

3.1.4 *exposed negative control, n*—a test article without defects exposed to a challenge solution. The purpose of the exposed negative control is to confirm the correct preparation and assembly of the test article.

3.1.5 *growth promotion test, n*—a test, using a negative control after the complete incubation time, by inoculating ≤100 CFU of the microbial challenge organism and incubating at the appropriate temperature until either visible growth is seen, or a maximum of 7 days is reached. The purpose of the growth promotion test is to demonstrate that the selected solution can support microbial growth.

3.1.6 *non-exposed negative control, n*—a test article that is not exposed to a challenge solution. The purpose of the non-exposed negative control is to validate the test system's sterility. This could be accomplished by filling a SUS control with growth medium and incubating for several days to ensure that the SUS test article was not contaminated upon filling.

3.1.7 *viability-based positive control, n*—a test article directly inoculated with a test organism. The purpose of this positive control is to validate the viability of the test organism under the test conditions, throughout the test.

3.2 *Acronyms:*

3.2.1 *CFU*—colony forming unit.

3.2.2 *IQ*—installation qualification.

3.2.3 *IT*—integrity test.

3.2.4 *OQ*—operational qualification.

3.2.5 *PQ*—performance qualification.

3.2.6 *SUS*—single-use system.

3.2.7 *TSA*—tryptic soy agar.

3.2.8 *TSB*—tryptic soy broth.

4. Significance and Use

4.1 Single-use systems (SUSs) used for biopharmaceutical manufacturing must maintain sterility and product quality of the fluid inside. Such articles or systems should therefore be validated as providing an effective barrier against microbial ingress. The microbial barrier properties of a SUS may be demonstrated using deterministic physical tests that have been correlated to microbial integrity. Two test methods (aerosol exposure and immersion exposure) are described that can be used to demonstrate microbial integrity of a SUS or determine the MALL, the maximum defect size that does not allow microbial ingress, into a SUS.

4.2 It is important to note that the results of microbial ingress tests are heavily dependent on the conditions under which the test is performed and are not suitable for routine checking of a SUS due to the test's destructive nature.

4.2.1 Any size defect may be forced to fail under sufficiently aggressive conditions (including a large enough sample size, high differential pressure, or high hydrostatic pressure, for example) that would not ordinarily reflect normal use conditions. Thus, it is necessary to clearly define the relevant conditions for a test through a risk assessment of both the actual SUS claims and its final use (Practice E3244). Once that is established, the size of defect that can be detected under those conditions can be determined, if required, using defined defects.

4.2.2 "Relevant conditions" refers to worse-case actual use conditions but does not mean that a SUS must be tested under theoretically absolute (extreme) "worst-case" conditions.

4.2.3 Testing may be performed on individual components or entire systems. Considerations for defining "relevant conditions" and testing design should be based on a risk assessment for the SUS intended use and should include:

4.2.3.1 A channel created by a defect or breach through the film thickness or through a seam or connection which must be filled with liquid to allow microbial passage.[5, 6]

4.2.3.2 Factors that could influence liquid filling of a channel, including a liquid's viscosity, defect size and type, plastic materials and pressure applied inside the SUS.

4.2.3.3 Rationale for selecting a defect type should be based on the probable type of defect(s) that could occur during the SUS life cycle

4.2.3.4 Rationale for selection of defect sizes should be based on a deterministic physical testing method (detection limit)

4.2.3.5 Consideration of pressure(s) differential applied during testing to simulate conditions that a SUS may be subjected to during actual use conditions (Practice E3244).

4.3 The selection of challenge microorganism and minimum target challenge concentration should be based on a risk assessment, justified, and validated, as necessary, for the limit of detection. A minimum of 10^6 CFU/cm^2 surface area (aerosol) or 10^6 CFU/mL (liquid immersion) is typically used (ISO 15747 and Aliaskarisohi[7]).

4.4 SUS test articles bearing calibrated defects may be produced and tested to allow either the determination of the minimum defect size that can be detected by a microbial test method under given conditions (for example, microbial ingress) or to determine the MALL of SUSs under use-case conditions (for example, aerosol test).

4.4.1 If the test objective is to determine the MALL and demonstrate correlation between physical integrity test sensitivity and microbial ingress, selection of the artificial defect

[5] Keller, S., "Determination of the Leak Size Critical to Package Sterility Maintenance," in PhD dissertation, Virginia Polytechnic Institute State University, VA, 1998.

[6] Gibney, M., "Predicting Package Defects: Quantification of Critical Leak Size," MS thesis, Faculty of Virginia Polytechnic Institute and State University, 2000.

[7] Aliaskarisohi, S., Hogreve, M., Langlois, C., Cutting, J., Barbaroux, M., Cappia J.-M., and Menier, M.-C., "Single-Use System Integrity I: Using a Microbial Ingress Test Method to Determine the Maximum Allowable Leakage Limit (MALL)," *PDA Journal of Pharmaceutical Science and Technology*, April 2019.

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(laser-drilled hole, capillary, copper wire) should be based on the most probable type of defect that could occur during the SUS's life cycle.

4.4.2 The selection of defect sizes should be based on the expected transition from ingress to no ingress under the SUS's intended use-case conditions, alternatively, worst-case conditions can be selected. As described in the Practice E3244, a typical range is from 1 µm to 100 µm. The defect sizes should be calibrated by a defined method.

4.4.3 One approach for determining the MALL of a SUS film material is to test single-use film coupons with calibrated defects, in holders. This enables higher throughput testing; however, using coupons as test articles may not represent a scale-down model of an entire SUS.

4.4.4 Another approach is to validate the test method on alternative container-like vials. The principle remains the same. The alternative container must be able to hold the minimum size defect.

4.5 These procedures should be conducted in a microbiological laboratory by trained personnel. It is assumed that basic microbiological equipment and supplies for conducting routine microbiological manipulations (for example, standard plate counts, autoclave sterilization, etc.) are available.

MICROBIAL INGRESS TEST METHOD BY AEROSOL EXPOSURE

5. Summary of Test Method

5.1 Pre-treat SUS test articles or SUS internal fluid path with methods consistent to those used to sterilize the SUS according to process requirements (for example, sanitize, sterilize or receive pre-sterilized).

5.2 Fill the SUS test articles with sterile culture media, (appropriate to the test organism), sufficiently to wet all surfaces, and place filled test articles into the aerosol exposure chamber. The internal surface of all SUS test articles must be maintained wet with media during the whole exposure. Air inside the SUS must be removed to permit wetting of the entire SUS test article interior. All external surfaces should be exposed to the aerosol.

5.3 Prepare the challenge solution at the required microbial concentration to deliver the minimum target challenge.

5.4 Subject test articles to the aerosolized microorganism challenge solution within an exposure chamber, under system parameters (flow rate, exposure time) designed to deliver the minimum target challenge.

5.5 Remove the SUS from the aerosol chamber and incubate at appropriate conditions for the test microorganism. Visually examine the test articles for the presence or absence of growth.

6. Apparatus

6.1 Aerosol exposure equipment (an example of which is illustrated in Fig. 1) comprises an aerosol chamber, in which test articles are placed on a carrier plate and the challenge microorganism is aerosolized. HEPA filters are attached to the top of the chamber to maintain an atmospheric pressure. The bottom, underneath the aerosol chamber, contains equipment required for aerosolization and aerosol evacuation. The air

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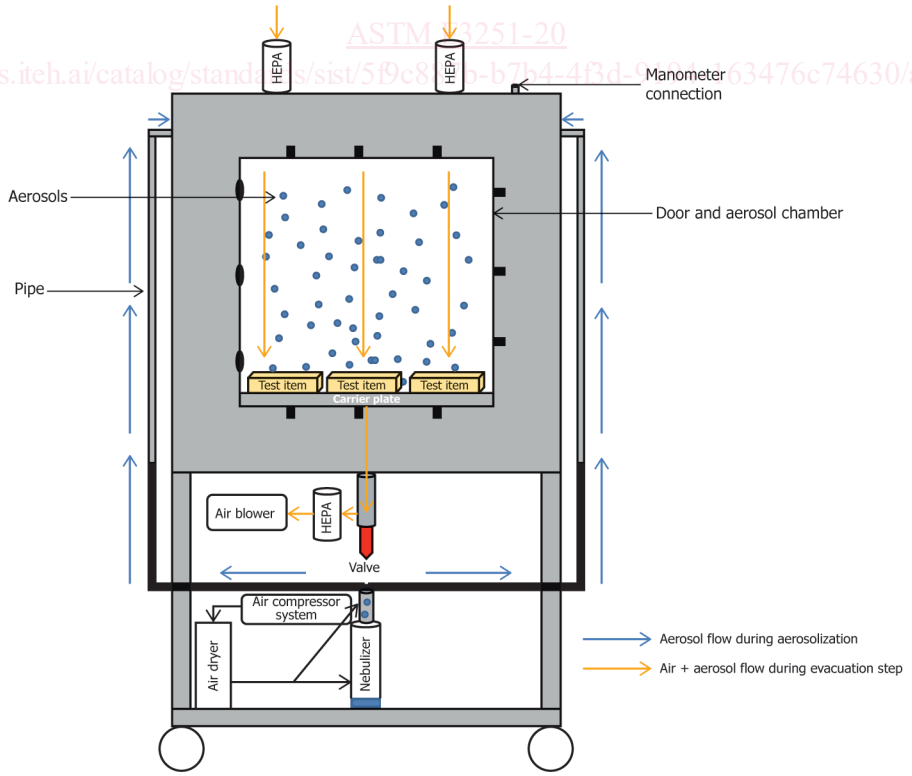

FIG. 1 Example of an Aerosol Exposure Chamber

compressor system, air dryer, and liquid nebulizer delivers aerosol formation and diffusion within the chamber. The air blower ensures evacuation of any remaining aerosol still in suspension at the end of the settling time.

6.2 To test film coupons bearing artificial calibrated defects, a single-use film coupon holder (an example of which is shown in Fig. 2) can be used. This comprises a holder that secures the film coupon and allows film coupon exposure to the aerosol challenge.

7. Materials

7.1 Example challenge microorganism: *Bacillus atrophaeus* (ATCC 9372), spore suspension. Alternative challenge microorganisms can be used with justification for their selection.

7.2 Laminar flow cabinet for aseptic filling of test articles. Incubator(s) large enough to contain SUS test articles, regulated at 30–35°C, or as appropriate to the chosen challenge microorganism.

7.3 Vessel to contain SUS during incubation.

7.4 Holder system for film coupons (if used).

7.5 Sterile TSB, or culture medium appropriate for culture of the chosen challenge microorganism, to fill SUS test and control articles.

7.6 Agar plates appropriate for culture of chosen challenge microorganism.

7.7 Pumps, fittings, hoses as needed to aseptically fill SUS test and control articles.

7.8 Dilution tubes for titration of culture suspensions and challenge solution.

7.9 Sterile pipettes.

7.10 Calibrated timer.

7.11 Device to apply pressure inside the test articles, if appropriate.

7.12 Calibrated flow meter.

7.13 Sterile forceps.

7.14 Sterile gloves.

7.15 Sterile water or suitable diluent for preparation of challenge solution.

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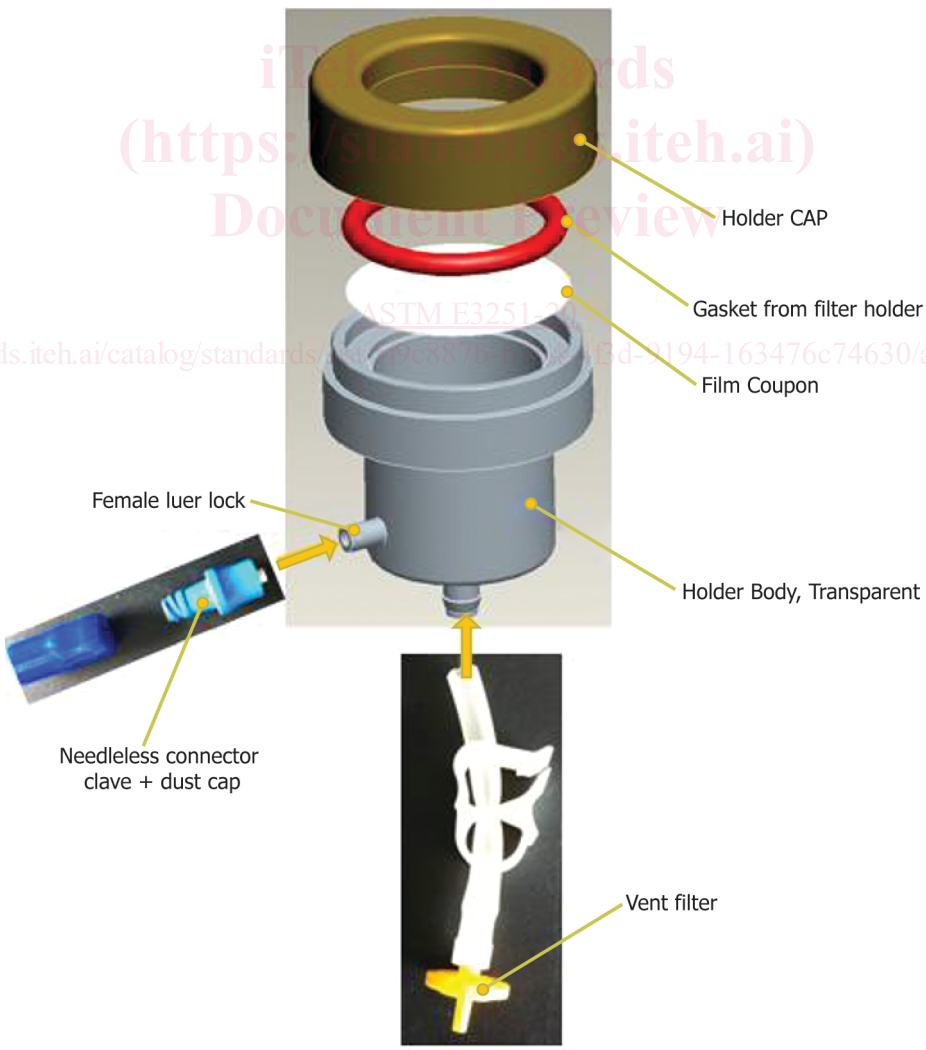

FIG. 2 Example of a Single-Use Film Coupon Holder

7.16 Sterile petri plates.

7.17 Pipettors (100 µL and 1000 µL) and sterile tips.

7.18 70 % alcohol.

7.19 Sterile three-lead transfer sets.

7.20 Manometer.

7.21 Glass beads (5 mm diameter), only for alternative recovery method.

7.22 Sterile glass containers and adapted caps, only for alternative recovery method.

7.23 Vortex mixer.

7.24 Rotary shaker.

8. SUS Test Articles

8.1 For test article description, refer to 4.4.

9. SUS Test Article Preparation

9.1 Sterilize the SUS test and control articles (if required) as in accordance with the manufacturer's recommendation. Alternatively, sterilize the SUS using conditions that reflect all the intended sterilization conditions to be employed for process use.

9.2 Include at least two SUSs for controls. As a minimum, perform at least one set of controls (one negative and one viability-based positive control) for each day of testing.

9.2.1 Negative controls as defined in 3.1.4 and 3.1.6.

9.2.2 Positive controls as defined in 3.1.3 and 3.1.7.

9.3 Perform physical integrity/leak testing on test articles (or controls) before filling with media to provide test sensitivity. The objective is to demonstrate that if there is microbial ingress, it is caused only by the artificial defect and not the test article itself. Suitable methods are described in Practice E3244. To reduce the risk of compromising the sterility of the test article, the integrity/leak test should be performed after assembly, but before sterilization.

9.4 Using aseptic technique, fill the SUS test articles with a sufficient amount of sterile culture medium to wet all interior surfaces.

9.5 If the objective of the test is to assess SUS integrity (not to define the MALL), the exposed negative control and defect-based positive controls are not necessary.

10. Apparatus and Method Validation

10.1 The test apparatus and method should be validated using approved procedures including IQ, OQ, and PQ protocols.

10.2 *Installation Qualification (IQ):*

10.2.1 To verify that all functional parts of the equipment are present, properly installed and work according to manufacturer's specifications.

10.2.2 A water aerosolization test should be performed to verify that the nebulizer is able to aerosolize liquid.

10.3 *Operational Qualification (OQ):*

10.3.1 To verify that the equipment is able to aerosolize in a reproducible and homogeneous manner $\geq 10^6$ CFU/cm^2. After initial experiments to determine the required challenge solution concentration and nebulizer parameters, a minimum of three qualification runs should be conducted. One approach to determine the challenge organism delivery is to use stainless steel coupons with a defined diameter to minimize bias: rigidity provides easy handling, and the smooth surface and weak electrostatic charge provides optimal organism recovery. Select representative locations within the chamber. An alternative method is to use flat collection vessels with a defined area (for example petri plates), filled with a known volume of collection fluid, to measure the number of organisms delivered per unit area.

10.3.2 At the end of each run, recover each stainless-steel coupon with sterile forceps and carry out the following protocol to determine the final challenge level (CFU/cm^2). The protocol described here for recovery from the stainless-steel coupon can be adapted. Validation of the microorganism recovery method is recommended to obtain a minimum ratio of 50 % recovery. The ratio obtained using this qualified recovery method should be applied to calculate the number of microorganisms during PQ.

10.3.3 A maximum of 1 log difference from target delivery of 10^6 CFU/cm^2 between selected locations is acceptable.

10.3.4 Suspend microorganisms by immersing the coupon in a sterile glass container containing sterile buffer (100 mL) and 15 glass beads (5 mm diameter). Orient the stainless-steel coupon challenge-side up to optimize organism recovery (Fig. 3).

10.3.5 Shake the container and its contents at 220 rpm for 30 min. Then perform serial dilutions in a sterile buffer and spread appropriate dilution(s) (100 µL) onto three agar plates for a standard plate count.

10.3.6 Incubate plates as appropriate for single colony enumeration.

10.3.7 After incubation, count and record the number of colonies and calculate the challenge concentration using the equation:

$$C = \frac{N \times F}{S} \quad (1)$$

where:

F = dilution factor,
S = size of the sample in cm^2,
N = average colony count, and
C = challenge in CFU/cm^2.

10.4 *Performance Qualification (PQ):*

10.4.1 To verify that the equipment is able, using OQ parameters, to aerosolize $\geq 10^6$ CFU/cm^2 at each run for each location within the chamber in a reproducible and homogeneous manner. A minimum of three runs should be conducted. Place a minimum of six coupons within the chamber per qualification run. This qualification should be conducted on the target material (or material category) as electrostatic forces and surface roughness can influence the ability of microorganisms to adhere to plastic during the aerosolization process. The tests described in 10.3 should be used.

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