Designation: ~~E2315 – 03 (Reapproved 2008)~~ E2315 – 16

Standard Guide for Assessment of Antimicrobial Activity Using a Time-Kill Procedure[1]

This standard is issued under the fixed designation E2315; 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 This guide covers ~~examples~~ an example of a ~~basic~~ method ~~to measure~~that measures the changes ~~of~~in a population of aerobic microorganisms within a specified sampling time when ~~tested against~~ antimicrobial test materials ~~in vitro.~~are present. Several options for organism selection and growth, inoculum preparation, sampling times and temperatures are provided. When the ~~basic~~ technique is performed as a specific test method, it is critical ~~when evaluating the results to ensure that such~~ that the above mentioned variables have been standardized. Antimicrobial activity of specific materials, as measured by this technique, may vary significantly depending on variables selected. It is important to understand the limitations of *in vitro* tests, especially comparisons of results from tests performed ~~under~~with different ~~circumstances.~~parameters. As an example, test results of microorganisms requiring growth ~~supplements,~~supplements or special incubation ~~conditions,~~conditions may not be directly comparable to ~~more robust organisms under the conditions of a single procedure.~~organisms evaluated without those stated conditions.

1.2 Knowledge of microbiological techniques is required for this ~~test.~~procedure.

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

1.4 *This standard ~~may involve hazardous materials, operations and equipment. 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 and health practices and determine the applicability of regulatory requirements prior to use.*

2. Referenced Documents

2.1 *ASTM Standards:*[2]

D1193 Specification for Reagent Water

E1054 Test Methods for Evaluation of Inactivators of Antimicrobial Agents

E2783 Test Method for Assessment of Antimicrobial Activity for Water Miscible Compounds Using a Time-Kill Procedure

3. Terminology

3.1 *Definitions:*

3.1.1 *inoculum suspension, n*—the initial suspension of test organism used to inoculate the test ~~material~~material. This may also be known as the organism inoculum (see ~~8.2~~8.3).

3.1.2 *microbial population, n*—the microbial count (cfu/mL) in the final volume of test material (see 9.4). This may also be known as the ~~"initial population" or "numbers control."~~"numbers control." The measurement may be taken at time zero which may be termed "Initial Population." Alternatively, the measurement may be taken at each exposure time or the longest exposure time used during testing to simulate the test procedure which may be termed "Final Population."

3.1.3 *neutralization, n*—~~a process which results in the inactivation or quenching of the antimicrobial~~ the process for inactivating or quenching the activity of a test material. This may be achieved through ~~dilution of the test material(s) or with the use~~physical means (for example, filtration, dilution) and/or the addition of chemical agents, called ~~neutralizers, to reduce or quench the antimicrobial activity.~~neutralizers.

---

[1] This guide is under the jurisdiction of ASTM Committee E35 on Pesticides, Antimicrobials, and Alternative Control Agents and is the direct responsibility of Subcommittee E35.15 on Antimicrobial Agents.

Current edition approved ~~April 1, 2008~~Jan. 15, 2016. Published ~~May 2008~~March 2016. Originally approved in 2003. Last previous edition approved in ~~2003~~2008 as ~~E2315 – 03.~~E2315 – 03(2008). DOI: ~~10.1520/E2315-03R08.~~10.1520/E2315-16.

[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.

3.1.4 *neutralizer, n*—a ~~procedure or~~ chemical agent used to inactivate, neutralize, or quench the ~~microbiocidal~~microbicidal properties of an antimicrobial agent.

3.1.5 *total test volume, n*—the volume of test material plus the volume of inoculum suspension.

4. Summary of a Basic Test Method

4.1 The test material or a dilution of the test material is brought into contact with a known population of microorganisms for a specified period of time at a specified temperature. ~~The~~ An appropriate and specified neutralization technique is applied to quench the antimicrobial activity of the test material ~~is quenched~~ at specified sampling intervals (for example, 30 s, 60 s, or any range covering several minutes or ~~hours) with an appropriate neutralization technique. The test material is neutralized at the sampling time~~ hours), and the surviving microorganisms are enumerated. The percent ~~or~~and/or $\log_{10}$ ~~reduction, or both, from either an initial microbial population, or test blank is calculated.~~ reduction is calculated by comparison with the microbial population.

5. Significance and Use

5.1 This procedure may be used to assess the *in vitro* reduction of a microbial population of test organisms after exposure to a test material.

6. Apparatus

6.1 *Sterile Vials or Test Tubes,* or equivalent.

6.2 *Timer ~~(Stop-clock),~~(Stop-clock)* ~~one~~ that displays minutes and seconds.

6.3 ~~*Shaking*~~ *Water ~~Bath or~~ Bath, Controlled Temperature Chamber,* or equivalent capable of maintaining test system at the specified exposure temperature ~~± 2°C.~~±2°C.

6.4 *Colony Counter,* any of several manual or automated types may be used.

6.5 *Incubator,* any ~~incubator~~ capable of maintaining a specified temperature ±2°C may be used.

6.6 *Sterilizer,* any ~~suitable~~ steam sterilizer capable of producing the conditions of sterilization.

6.7 *Vortex Mixer, Magnetic Stirrer,* or equivalent.

6.8 *Spiral Plating System,* (optional).

6.9 *Sterile Bacteriological Pipettes,* for viscous test materials, positive displacement pipettes or syringes may be necessary.

6.10 *Water Dilution Bottles,* any sterilizable container having appropriate capacity and tight closures may be used.

6.11 *Sterile Cotton Applicator Swabs.*

7. Reagents and Materials

7.1 *Dilution Fluid or ~~Diluent,~~ Diluent*—sterile water, ~~0.65 %~~ 0.9 % (w/v) saline, sterile Butterfield's buffered phosphate diluent,[3] or equivalent.

7.2 *Broth Growth ~~Medium,~~ Medium*—soybean-casein digest ~~broth,~~broth or equivalent ~~and other~~ liquid media appropriate to ~~support~~supporting growth of the test organism(s), with appropriate neutralizers, if required (see 3.1).

7.3 *Solid Growth and Plating ~~Medium,~~ Medium*—soybean-casein digest ~~agar,~~agar[4] or ~~equivalent, and other~~ equivalent solid media appropriate to support growth of the test organism(s), with appropriate neutralizers, if required (see 3.1.3 and 3.1.4).

7.4 *Sterile Deionized Water,* or equivalent (Specification D1193, Type III).

8. Test ~~Methods~~ Organism Preparation

8.1 The test organism selected may be representative of the microbial flora encountered under the conditions of use of a test material or may be standardized strains.

8.2 ~~*Test Organisms:*~~ *Organism Preparation—*

~~8.1.1 The test organisms selected may be representative of the microbial flora encountered under the conditions of use, or may represent standardized strains. The organism should be capable of providing reproducible results under specific test conditions.~~Transfer culture(s) from stock twice (once every 18 to 24 h or as appropriate for the test organism) into appropriate growth medium to maintain the organism in growth phase. The second transfer may be made into a volume of growth medium that will provide a microbial suspension sufficient to conduct testing and controls. Consider that additional volume may be needed to permit testing of multiple samples or time points.

---

[3] Horowitz, W., Ed., *Official Methods of Analysis of the AOAC, ~~17th Ed.~~18th Ed.,* ~~ch. 17, p. 4, sec. 17.2.01, A(m),~~ Association of Official Analytical Chemists, Washington, DC, 2000; ~~(As cited in, Butterfield's Phosphate Buffer,~~ Journal of the Association of Official Analytical Chemists. Vol 22, No. 635, ~~1939.)~~1939.

[4] *U.S. ~~Pharmacopoeia, 24th Revision,~~Pharmacopeia, 38–NF33,* The United States ~~Pharmacopoeia~~Pharmacopeial Convention, Inc. Rockville, MD, 2000.

8.2.1 ~~*Organism Preparation*—Transfer culture(s) from stock twice (once every 18 to 24 h or as appropriate for the test organism) into appropriate growth media. The second transfer~~ Alternatively, the transfers may be made ~~into a volume of growth medium to produce sufficient microbial suspension to inoculate. Volumes used should permit testing of multiple samples or time points.~~onto agar plates or slants, and the inoculum suspension prepared by washing the organism from the slant with an appropriate broth or diluent.

~~8.1.2.1 Alternatively, the transfers may be made onto agar plates or slants and the inoculum suspension may be prepared by washing the organism from the slant with an appropriate broth or diluent.~~

NOTE 1—Reports in the published literature have noted differences in microbial kill or ~~antimicrobial resistance~~ susceptibility as a result of ~~cell protection in broth or as a result of washing cells.~~ different propagation methods. It is recommended that tests be conducted ~~with either all cells prepared in broth dilutions or with all cells prepared by washing.~~using a consistent procedure for organism propagation.

8.3 *Inoculum Suspension Preparation and Determination of the Microbial ~~Population or Numbers Control:~~Population:*[5]

8.3.1 To prepare inoculum suspension directly from broth growth medium, a dilution in sterile broth (diluent is same as that used for broth growth medium) may be performed to achieve the desired concentration.

8.3.2 To prepare inoculum suspension ~~directly from broth, a dilution in sterile broth (the same as that used for growth medium)~~ in dilute broth, an up to 1:10 dilution of the suspension into Butterfield's buffered phosphate diluent or equivalent may be performed to reduce the concentration of the ~~microorganisms to the appropriate level.~~growth medium.

~~8.2.1.1 To prepare inoculum suspension in dilute broth, a 1:10 dilution of the suspension into Butterfield's buffered phosphate diluent or equivalent may be performed to reduce the concentration of the growth medium.~~

~~8.2.1.2 Inoculum suspensions grown from broth may be diluted to appropriate concentration or they may be centrifuged and reconstituted in Butterfield's buffered phosphate diluent, broth, saline, or equivalent, to the appropriate concentration.~~

8.3.3 Inoculum suspensions in broth may be diluted to achieve the desired concentration or they may be centrifuged and reconstituted in Butterfield's buffered phosphate diluent, broth, saline, or equivalent, to achieve the desired concentration.

8.3.4 To prepare the inoculum suspension from an agar plate or slant, wash microbial growth or transfer the growth aseptically using a sterile swab from the agar surface with Butterfield's buffered phosphate diluent, saline, or equivalent.

NOTE 2—Because certain antimicrobials (for example, alcohol and iodine) are sensitive to organic material and may have activity reduced by even the slightest organic load, washed inoculum suspensions, whether established initially in broth or from solid media, may be used.

~~NOTE 2—Antimicrobials sensitive to organic material (for example, alcohol and iodine) may have reduced activity by even the slightest organic load and therefore thoroughly washed inoculum suspensions only, whether grown initially in broth or from solid media, should be used.~~

8.3.5 The inoculum suspension should be prepared to achieve a minimum population concentration of 10^6 cfu/mL ~~microbial population~~ (see 9.4). ~~Results of tests where the initial microbial populations differ from the test population by greater than $2\log_{10}$ should be interpreted with care because of the exponential nature of the populations.~~ The final inoculum suspension should be ~~well mixed~~ well-mixed prior to ~~addition~~transfer to test ~~materials~~material (see 9.5).

8.3.6 The inoculum suspension should be ~~enumerated~~plated in duplicate by standard microbiological procedures at the initiation and completion of testing. Appropriate dilutions ~~are~~ should be prepared and enumerated by standard microbiological procedures ~~(Spread~~(spread- or ~~pour plating,~~ pour-plating, microbial filtration, or ~~spiral plating).~~ spiral-plating). The initial and final ~~count~~titer of the inoculum should be within ± 0.5 $\log_{10}$ for a valid test. This step may be omitted where Microbial Population enumeration is conducted.

~~8.2.4.1 To perform the population quantitation of the control blank, a volume of inoculum suspension equivalent to that inoculated into the test material is added to a dilution blank containing the same volume as used for the test material. The initial and final count of the population in the blank must be within ± 0.5 $\log_{10}$ for a valid test.~~

~~8.2.5 Incubate plates at the specified temperature ±2°C for 24 to 48 h or as appropriate for the test organism(s).~~

~~8.2.6 Count colonies and record raw data as cfu/plate to determine surviving organisms. Average duplicate plates (2 plates from each replicate dilution) and multiply by the dilution factor to calculate (cfu/mL) microbial population of both the control blank and test system.~~

8.4 To perform the Microbial Population (3.1.2) quantitation, a volume of inoculum suspension equivalent to that inoculated into the test material is added to a dilution blank containing the same volume as used for the test material. The Initial Population and Final Population counts must be within ± 0.5 $\log_{10}$ for a valid test.

8.4.1 Incubate plates at the specified temperature ±2°C for 24 to 48 h or as appropriate for a test organism.

8.4.2 Count colonies and record raw data as cfu/plate to determine the number of surviving organisms. Average duplicate plate counts (2 plates from each dilution) and multiply by the dilution factor to calculate cfu/mL of inoculum.

9. Basic Procedure

9.1 Select the ~~test~~ concentrations of the test ~~material. The concentrations selected may reflect the anticipated concentration of the test material during use.~~ material to be tested. Each concentration is tested ~~at least~~ in duplicate. Each recovery sample is plated in duplicate. See Fig. 1.

[5] Brown, M. R. W., Gilbert P., *Microbiological Quality Assurance: A Guide Towards Relevance and Reproducibility of Inocula*, CRC Press, New York, NY, 1995.