Designation: ~~E3031 – 15~~ E3031 – 20

Standard ~~Test Method~~ Practice for Determination of Antibacterial Activity on Ceramic Surfaces[1]

This standard is issued under the fixed designation E3031; 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 method is designed to quantitatively evaluate the antibacterial activity of glazed ceramic surfaces that have been specifically designed to contain an antibacterial treatment as part of the glaze. This test method is meant to compare the efficacy of one ceramic surface to another ceramic surface using the stated conditions and is not meant to be extrapolated to other conditions.~~

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

~~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 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 limitations prior to use.*~~

~~2. Referenced Documents~~

~~2.1 *ASTM Standards:*[2]~~

~~E177 Practice for Use of the Terms Precision and Bias in ASTM Test Methods~~
~~E691 Practice for Conducting an Interlaboratory Study to Determine the Precision of a Test Method~~
~~E1054 Test Methods for Evaluation of Inactivators of Antimicrobial Agents~~
~~E2180 Test Method for Determining the Activity of Incorporated Antimicrobial Agent(s) In Polymeric or Hydrophobic Materials~~
~~E2756 Terminology Relating to Antimicrobial and Antiviral Agents~~
~~2.2 *ISO Standard:*[3]~~
~~ISO 22196 Measurement of Antibacterial Activity on Plastics and Other Non-porous Surfaces~~

~~3. Terminology~~

~~3.1 For definitions of terms used in this test method refer to Terminology E2756.~~

~~4. Summary of Test Method~~

~~4.1 This test method is used for evaluating the antibacterial effect of antimicrobials incorporated into a ceramic glaze. This~~

[1] This ~~test method~~ practice 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.

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

standard does not seek to imitate all possible real world scenarios but to provide a standardized method to compare multiple antimicrobial technologies that can be incorporated or coated on a ceramic surface. The inherent nature of the ceramic tile allows for desiccation, therefore each ceramic specimen is equilibrated to the testing environment for 18- 24 h. Once the tiles are equilibrated, bacteria are inoculated onto the surface followed by a 24-h exposure time. Bacteria are recovered in a neutralizer broth and enumerated according to a validated method. Log reductions are calculated for a treated versus an untreated sample.

5. Significance and Use

5.1 Current solid surface test methodologies, such as the Test Method E2180 and ISO 22196, do not take into account the complexities associated with a ceramic surface. This includes, but is not limited to, differing chemistries incorporated into the glaze and desiccation due to water absorption through the bisque body. Each point will be elaborated below:

5.1.1 The glaze composition of ceramic tiles can vary between manufacturers, lots, and product lines. Some glaze chemistries such as tin, silver and copper can negatively impact the testing conditions. Therefore, an untreated tile from the same lot is not always suitable for comparison. The control tile proposed herein is capable of supporting growth over the indicated time frame and nutrient level (see Section 9).

5.1.2 Desiccation is a common problem when testing tile surfaces. This can be overcome by pre-hydrating the tile by placing the specimen on a moistened wipe and allowing incubation for 18 to 24 h before beginning the test. This reduces the number of false positive results and more accurately measures the ability of the antimicrobial to inhibit growth.

5.2 This test method utilizes a low inoculum load and requires growth on the control substrate to demonstrate a valid testing environment. In addition, while some antimicrobials demonstrate activity against static cultures, others require growth of the bacteria to maintain activity. A low inoculum level will allow for both types of antimicrobials to be examined with the same testing conditions.

6. Apparatus

6.1 *Incubator*—capable of maintaining a temperature of 35 ± 2ºC and >75% RH.

6.2 *Pipetter*—continuously adjustable between 100 µL and 1000 µL.

6.3 *Sterilizer*—any suitable steam sterilizer with conditions that produce sterility of samples.

6.4 *Petri dish*—sterile 150 mm by 15 mm for holding the samples

6.5 *Culture tubes and closures*—any with a volume capacity of 10 mL and a minimum diameter of 16 mm. Recommended size is 16 mm by 125 mm borosilicate glass with a threaded opening.

6.6 *Cover film*—25 mm by 25 mm sterile polyethylene or other suitable material that does not impact bacterial growth.

6.7 *Large Water Absorbent Laboratory wipe*—to facilitate pre-hydration of samples similar to a Kimwipes Kimtech[4] delicate task wiper 30 cm by 30 cm.

6.8 *Vortex mixer*—to provide a homogenous bacterial suspension prior to inoculation of samples and prior to the enumeration technique that will be used.

6.9 *Plastic screw top jar*—150 ml capacity that has an opening large enough to insert the sample as a vessel for recovery.

6.10 *Wrist action shaker*—to recover bacteria from samples.

6.11 *Petri dish*—100 mm by 15 mm for enumeration.

[4] Kimwipe is a registered trademark of Kimberly-Clark Dallas TX, USA

6.12 *Shaking incubator*—capable of maintaining 35 ± 2ºC.

7. Reagents and Materials[5]

7.1 *Dilution fluid or diluent*—sterile Butterfield's buffered phosphate.

7.2 *Growth medium.*

7.2.1 *Overnight culture*—brain heart infusion broth prepared according to the manufacturer's instruction.

7.2.1.1 Alternative media may be used for overnight culture of the organism, such as tryptic soy broth, but details shall be included in the final report.

7.2.2 *Inoculation broth*—1:500 dilution of nutrient broth as defined below:

7.2.2.1 Prepare nutrient broth by dissolving 3.0 g of meat (beef) extract, 10.0 g peptone, and 5.0 g of sodium chloride in 1000 mL of distilled or deionized water.

7.2.2.2 Dilute the nutrient broth with distilled or deionized water to a 500-fold volume and adjust the pH to a value between 6.8 and 7.2 with sodium hydroxide or hydrochloric acid.

7.2.2.3 Sterilize by autoclaving at 120°C for 30 min.

7.3 *Solid growth media*—tryptic soy agar plates.

7.4 *Sterile deionized water*—or equivalent.

7.5 *Neutralizer*—A neutralizer should be selected that has been shown to effectively neutralize the active according to Test Methods E1054.

8. Culture Preparation

8.1 *Escherichia coli* American Type Culture Collection, ATCC No. 8739 is the organism to be utilized for this test. Grow a fresh 18 ± 1 h culture in sterile brain heart infusion broth at 35 ± 2ºC and shaking at 110 r/min prior to beginning the test. Dilute this suspension appropriately in the inoculation broth described in 7.2.2 to obtain 1-5 × 10^4 CFU/mL. This will be the working bacterial stock solution.

9. Untreated Control Specimen

9.1 Control tiles suitable for testing purposes may be prepared from glaze ingredients that are free of elements that contribute to antimicrobial activity. One example of a product that meets this criterion is F-524.[6] However, glazed tiles are generally acceptable as controls if they can be shown to meet the following criterion:

9.1.1 Can support > 1.5 log growth under the test conditions given herein as calculated in 12.4.

9.1.1.1 If a control tile, as described above, is not available then the use of borosilicate glass squares, cut to the same dimensions as described in 10.1, can be substituted as control specimens. Glass squares shall meet performance specifications indicated in 9.1.1.

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[5] *ACS Reagent Chemicals, Specifications and Procedures for Reagents and Standard-Grade Reference Materials*, American Chemical Society, Washington, DC. For suggestions on the testing of reagents not listed by the American Chemical Society, see *Analar Standards for Laboratory Chemicals*, BDH Ltd., Poole, Dorset, U.K., and the *United States Pharmacopeia and National Formulary*, U.S. Pharmacopeial Convention, Inc. (USPC), Rockville, MD.

[6] The sole source of supply of the of the control tiles (F-524) suitable for testing purposes prepared from glaze ingredients that are free of elements that contribute to antimicrobial activity and known to the committee at this time is Fusion Ceramics, Inc. (Carrollton, Ohio USA). If you are aware of alternative suppliers, please provide this information to ASTM International Headquarters. Your comments will receive careful consideration at a meeting of the responsible technical committee,[1] which you may attend.

10. Sample Preparation

10.1 Prepare five (5) replicates of each specimen, measuring 50 mm by 50 mm ± 1 mm^2 (see Section 9). Wipe test specimens to remove any debris from processing, place in a sterilization pouch/container and autoclave for at 120°C for 1 h.

NOTE 1—If the active ingredient is affected by autoclaving, then other types of sterilization can be used.

10.2 While test specimens are being sterilized, fold and place two large 1-ply laboratory wipes (30 cm by 30 cm) into a 150 by 15 mm sterile petri dish. Fold in such a way to get 18 layers in a 10 cm by 10 cm square. Moisten with sterile deionized water until the wipe is saturated.

10.3 Remove a sterile test specimen (10.1) from sterilization pouch aseptically, place onto the wipe in the petri dish (10.2). Visually monitor the dish during preparation to prevent excess water from accumulating in the dish.

10.4 Incubate the dish containing the sample at 35 ± 2ºC with >75% RH for 18 to 24 h.

11. Procedure

11.1 *Inoculation and Incubation:*

11.1.1 Remove test specimens from the incubator and proceed to 11.1.2

11.1.2 Pipette 100 µL of the prepared bacterial stock solution (8.1) onto each pre-hydrated test specimen (see Section 10). The test specimen will remain on the moistened wipe for the duration of the test. Addition of water may be necessary if the saturated wipe has become dry.

11.1.3 Enumerate the inoculum by spread or pour plate.

11.1.4 Place a 25 mm by 25 mm polyethylene film on top of the inoculum to ensure even contact with the surface. Make sure that no inoculum leaves the surface of the ceramic tile.

11.1.4.1 In accordance with 6.6, the cover film to be utilized should not affect bacterial growth or absorb water. If the test specimen size is increased, the volume of inoculum and cover film shall be increased proportional to the surface area of the sample (a ratio of 1µL:6.25 mm^2).

11.1.5 Place each petri dish containing inoculated samples in the incubator at 35 ± 2ºC with >75% RH for 24 ± 1 h.

11.2 *Recovery:*

11.2.1 After the specified incubation time, remove the test specimen or control from the petri dish and loosen the cover film. Note any desiccation that is observed for each sample.

11.2.2 Place the film and ceramic test specimen into a sterile 150-mL plastic screw top jar containing 100 mL of neutralizer. Shake for 1 min on a wrist-action shaker set to the maximum speed.

NOTE 2—Alternative vessels and volumes may be utilized but their description will be included in the report. In addition, alternative recovery techniques, such as vortex and sonication, may also be utilized. Use of other recovery methods should be noted in the test report.

11.2.3 Recover culturable organisms from appropriate dilutions by use of spread- or pour plate, spiral plate, or by other valid microbial enumeration methods.

11.2.4 Incubate plates at 35 ± 2°C for 24 h.

11.2.5 Count and record colony numbers for each dilution.

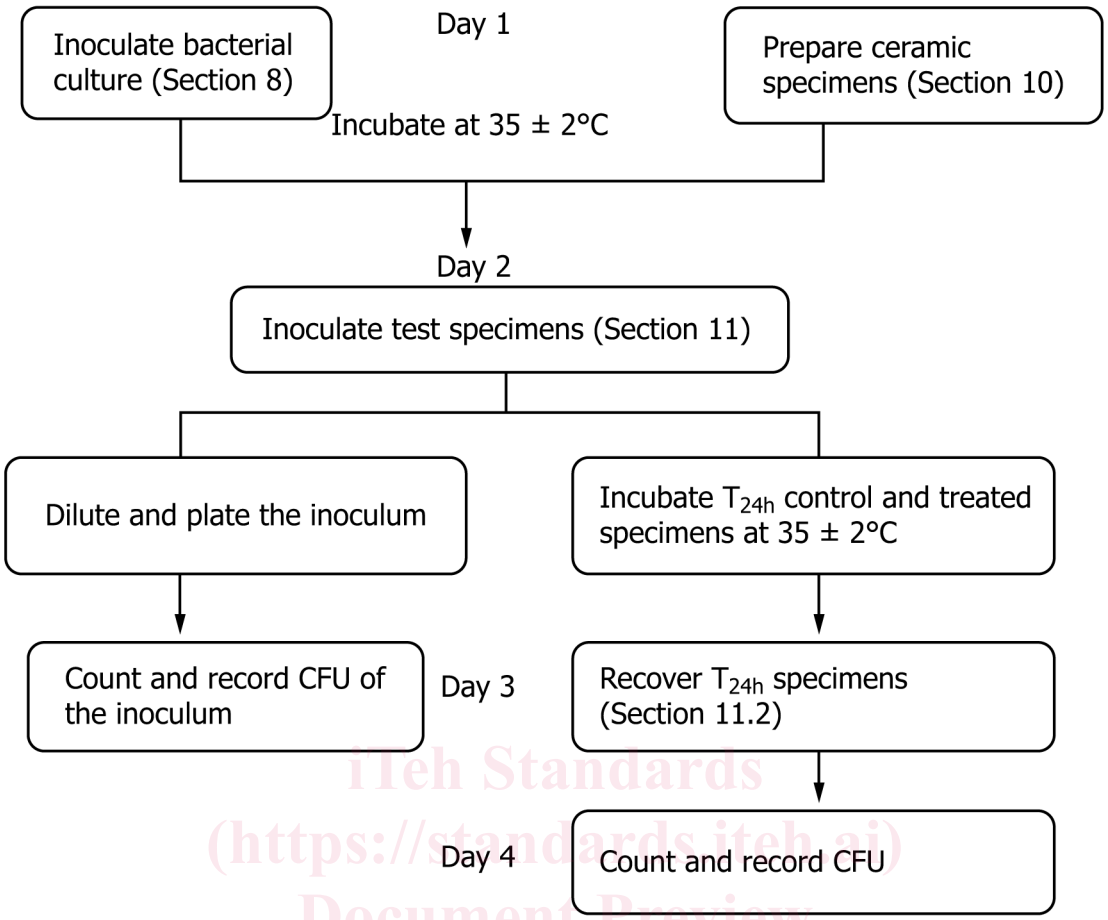

FIG. 1 Flowchart of Testing Operation

~~**12. Calculation or Interpretation of Results**~~

~~12.1 For each test specimen, determine the number of viable bacteria per specimen:~~

$$N = (C \times D \times V) \quad (1)$$

~~where:~~

~~N = is the number of viable bacteria recovered from test specimen;~~
~~C = is the average plate count;~~
~~D = is the dilution factor for the plates counted;~~
~~V = is the volume, in ml, of neutralizer added to the specimen;~~

~~If no colonies were recovered in any of the agar plates for a dilution series, then record the number of colonies counted as "< V" (where V is the volume, in ml, of neutralizer added to the specimen). For calculating the average when there are no viable bacteria recovered in a dilution series, consider the number of viable bacteria to be "V".~~
~~EXAMPLE In the case of V = 100 ml, the number used for calculating the average will be 100.~~

~~12.2 Convert CFU to Log_{10} CFU for each specimen.~~

~~12.3 Calculate the geometric mean of the log value of the replicate samples.~~

~~12.4 Calculate the log difference between the Inoculum and T_{24h} control specimens~~

$$\Delta Log_{10}\ Control = A - B \quad (2)$$

where:

A = geometric mean of the Log_{10} T_{24h} control specimens
B = geometric mean of the Log_{10} Inoculum,

12.4.1 If ΔLog_{10} Control <1.5, the test is invalid and must be repeated.

12.4.2 If ΔLog_{10} Control ≥ 1.5, continue to 12.5.

12.5 Calculate the log reduction for all samples if the conditions for a valid test are met.

$$\text{LogReduction} = C - T \quad (3)$$

where:

C = geometric mean of the Log_{10} T_{24h} control samples
T = geometric mean of the Log_{10} T_{24h} treated samples

13. Interpretion

13.1 If Log reduction = ΔLog_{10} Control the treatment is bacteriostatic

13.2 If Log reduction > ΔLog_{10} Control the treatment is considered bactericidal

13.3 If Log reduction < ΔLog_{10} Control but >0.5 the sample is considered to display partial inhibition.

14. Precision and Bias

14.1 The precision of this test method is based on an intra-laboratory study of E3031 Test Method for Determination of Antibacterial Activity on Ceramic Surface, conducted in 2014. A single laboratory participated in this study, testing the Log_{10} reduction of *Escherichia coli* on ceramics treated with two different inhibitors. Every "test result" represents the average of five determinations. The laboratory reported ten replicate test results for each ceramic material, as well as two growth control samples. Except for the use of only one laboratory, Practice E691 was followed for the design and analysis of the data; the details are given in ASTM Research Report No. RR:E35-1010.[7]

14.1.1 *Repeatability (r)*—The difference between repetitive results obtained by the same operator in a given laboratory applying the same test method with the same apparatus under constant operating conditions on identical test material within short intervals of time would in the long run, in the normal and correct operation of the test method, exceed the following values only in one case in 20.

14.1.1.1 Repeatability can be interpreted as maximum difference between two results, obtained under repeatability conditions that is accepted as plausible due to random causes under normal and correct operation of the test method.

14.1.1.2 Repeatability limits are listed in Table 1 and Table 2 below.

TABLE 1 Repeatability Limits for Testing the Log_{10} Reduction of *Escherichia coli* on Ceramics Treated with Two Different Inhibitors

Treated Ceramics	Average[A]	Repeatability Standard Deviation	Repeatability Limit
	$\bar{x}$	s_r	r
R2BZ treated ceramic piece	3.519	0.757	2.119
A2B2Z2 treated ceramic piece	4.620	0.429	1.200

[A]The average of the laboratories' calculated averages.

[7] Supporting data have been filed at ASTM International Headquarters and may be obtained by requesting Research Report RR:E35-1010. Contact ASTM Customer Service at service@astm.org.

TABLE 2 Repeatability Limits for Testing the Log10 (CFU) Growth of *Escherichia coli* on Control Pieces

Control	Average[A]	Repeatability Standard Deviation	Repeatability Limit
	$\bar{x}$	s_r	r
Control untreated ceramic piece	1.624	0.964	2.700
Glass control piece	3.432	0.301	0.842

[A]The average of the laboratories' calculated averages.

14.1.2 *Reproducibility (R)*—The difference between two single and independent results obtained by different operators applying the same test method in different laboratories using different apparatus on identical test material would, in the long run, in the normal and correct operation of the test method, exceed the following values only in one case in 20.

14.1.2.1 Reproducibility can be interpreted as maximum difference between two results, obtained under reproducibility conditions that is accepted as plausible due to random causes under normal and correct operation of the test method.

14.1.2.2 Reproducibility limits cannot be calculated from a single laboratory's results.

14.1.3 The above terms (repeatability limit and reproducibility limit) are used as specified in Practice E177.

14.1.4 Any judgment in accordance with statement 14.1.1 would normally have an approximate 95% probability of being correct, however the precision statistics obtained in this ILS must not be treated as exact mathematical quantities which are applicable to all circumstances and uses. The limited number of laboratories reporting replicate results essentially guarantees that there will be times when differences greater than predicted by the ILS results will arise, sometimes with considerably greater or smaller frequency than the 95% probability limit would imply. Consider the repeatability limit as a general guide, and the associated probability of 95% as only a rough indicator of what can be expected.

14.2 *Bias*—at the time of the study, there was no accepted reference material suitable for determining the bias for this test method, therefore no statement on bias is being made.

14.3 The precision statement was determined through statistical examination of 40 results, from a single laboratory on two (2) treated ceramic materials and two (2) controls.

15. Keywords

15.1 antimicrobial; antibacterial; antibacterial glaze; ceramic; quantitative antibacterial assay; tile