



Standard Test Method for Evaluation of Antimicrobial Formulations by the Agar Patch Technique¹

This standard is issued under the fixed designation E1882; 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 test method determines the antibacterial activity and persistence of test formulations, as measured by the inhibition of a test organism on an agar surface exposed to test sites on human skin treated with the formulations.

1.2 A knowledge of microbiological techniques is required for these procedures.

1.3 It is the responsibility of the investigator to determine if Good Laboratory Practice (GLP) and Good Clinical Practice (GCP) are required and to adhere to these practices, as appropriate.

1.4 In this test method, SI units are used for all applications except linear measure. In that case, inches are used and SI units follow in parentheses.

1.5 *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.* Performance of this procedure requires the knowledge of regulations pertaining to the protection of human subjects (see 21 CFR, Ch. I, Parts 50 and 56).

2. Referenced Documents

2.1 Federal Standard ²

21 CFR, Ch. I, Parts 50 and 56 Protection of Human Subjects

3. Terminology

3.1 *active test formulation*—a substance containing active ingredient(s).

3.2 *active ingredient*—a substance added to a formulation specifically for the inhibition or inactivation of microorganisms.

3.3 *active plate*—inoculated agar plate that has been attached to a skin site treated with an active formulation.

3.4 *antibacterial activity*—killing of bacteria or suppression of their growth or reproduction.

3.5 *control formulation*—a formulation that does not contain an active ingredient.

3.6 *control plate*—inoculated agar plate that has been attached to an untreated skin site, or one treated with a control formulation.

3.7 *inhibition*—prevention of bacterial population growth, either through lethality or through prevention of bacterial reproduction.

3.8 *inoculum determination plate*—an inoculated plate that has not been exposed to any skin test site.

3.9 *persistence*—effectiveness of a test formulation in inhibiting bacteria, defined in terms of time elapsed between application of test formulation and application of test plates.

3.10 *resident microorganisms*—microorganisms that survive and multiply on the skin, forming a stable population.

3.11 *transient microorganisms*—microorganisms that contaminate the skin, but do not form a stable population.

3.12 *volar aspect of the forearms*—the surface of the forearm on the same side as the palm of the hand.

4. Summary of Test Method

4.1 This test method is conducted on subjects selected from a group of volunteers who have refrained from using topical antimicrobials for at least one week and have minimal hair on the test site. The test site should normally have a low number of resident microorganisms (approximately 10⁴ CFU/cm² or fewer) and be easily sampled.

4.2 The surfaces of agar contact plates are inoculated with the selected organism and placed in contact with skin sites that have been treated with active or control formulations, or left untreated. After contact with the treated skin sites, these plates are incubated and the colonies enumerated. Inhibitory activity of the active test formulation is measured by comparing

¹ This test method is under the jurisdiction of ASTM Committee E35 on Pesticides and Alternative Control Agents and is the direct responsibility of Subcommittee E35.15 on Antimicrobial Agents.

Current edition approved April 1, 2010. Published April 2010. Originally approved in 1997. Last previous edition approved in 2005 as E1882 – 05. DOI: 10.1520/E1882-10.

² Available from Superintendent of Documents, U.S. Government Printing Office, Washington, D.C. 20402.

differences in microbial colony counts between plates that were in contact with sites treated with an active formulation and plates that were in contact with untreated sites, or sites treated with a control formulation. Results are expressed as percent inhibition.³

5. Significance and Use

5.1 This procedure can be used to evaluate formulations containing ingredients intended to inhibit growth of bacteria on intact skin and measures the difference, post-product-exposure, between numbers of bacterial colonies on active test formulation plates and numbers on control plates, expressed as percent inhibition.

5.2 This procedure may also be used to test for persistence of activity, as a function of time elapsed between application of active test formulation and application of active test plates.

5.3 Because no procedure for neutralization of the antimicrobial action of active ingredients can be included in the test, the agar patch method is limited to the extent that results expressed as percent inhibition do not differentiate between bacteriostatic and bactericidal effects and, hence, must not be portrayed as “reductions.”

6. Apparatus

6.1 *Colony Counter*—Any of several types may be used. A magnifying device, such as a dissecting microscope, may be used for manual enumeration of colonies.

6.2 *Incubator*—Any incubator capable of maintaining a suitable temperature $\pm 2^{\circ}\text{C}$ may be used.

6.3 *Sterilizer*—Any steam sterilizer capable of producing the conditions of sterility.

6.4 *Timer (Stop Watch)*—One that can be read for hours, minutes, and seconds.

7. Reagents and Materials

7.1 *Bacteriological Pipettes*, 10.0 and 2.2 or 1.1 mL capacity.

NOTE 1—Presterilized/disposable bacteriological pipettes are available from most laboratory supply houses.

7.2 *Pipetter*, with disposable tips capable of delivering 10 μL .

7.3 *Plating Medium*, soybean-casein digest agar, or equivalent.⁴

7.4 *Dilution Fluid*, Butterfield’s phosphate buffer⁵, or equivalent.

7.5 *Isopropanol or Ethanol*, 60 to 75% (v/v)

7.6 *Sterile Disposable Culture Dishes*, 1.4×0.4 in. (35 by 10 mm) and 4.0×0.8 in. (100 by 20 mm).

7.7 *Sterile Test Tubes*.

7.8 *Surgical Adhesive Tape*, or equivalent.

³ Yackovich, F., Wagner, C. A., and Heinze, J. E., “Validity of the agar patch test with an antibacterial liquid soap and comparison with the finger imprint method,” *J. Soc. Cos. Chem.* Vol 40, 1989, pp. 263–271.

⁴ U.S. Pharmacopeia XXIV, NF 19. 2000. United States Pharmacopeial Convention Inc., Rockville, MD. Chapter 61, entitled “Microbial Limits Test.”

⁵ Horowitz, W. (Ed.), *Official Methods of Analysis of the AOAC*, 13th Ed. Sec. 46.013(m), p. 825. Assoc. of Official Analytical Chemists, Washington, D.C. 1980, 1018 pp.

7.9 *Disposable Examining Gloves*.

7.10 *Inoculating Loop or Glass Spreader*.

7.11 *Appropriate Bacterial Cultures*.

7.12 *Test Formulations*—Directions for application of active and control formulations should be followed, if available. If directions are not available, the directions provided in this test method may be applied.

8. Test and Control Skin Sites

8.1 The volar aspect of the forearm is commonly used as the location of the skin sites, but other areas such as the back or forehead may be used for test sites. Application of active test and control formulations (or no treatment) will be assigned by a predetermined randomization so that either forearm (or either side, right or left, of other anatomical areas) may receive active or control formulations (or none). (**Warning**—Application of agar patch plates and alcohol to the forehead risks contaminating the eyes, and extra precautions must be exercised.)

9. Subjects

9.1 *Number of Subjects*—The number of subjects used in the test depends on the statistical significance required for the expected results, the sampling variability encountered in the study, and the relative efficacy of the active formulation being evaluated.

9.1.1 Recruit a sufficient number of healthy adult subjects who have no clinical evidence of dermatoses, open wounds, or other skin disorders that may affect the integrity of the test.

9.2 Instruct the subjects to avoid contact with antimicrobials for at least the week prior to testing and, other than the active formulation, for the duration of the test. This restriction includes spray antiperspirants and deodorants, shampoos, lotions, dishwashing detergents, and soaps containing antimicrobial compounds, and materials such as acids, bases, and solvents. Bathing in biocide-treated pools, hot tubs, or spas should be avoided. Subjects may be provided with a kit of non-antimicrobial personal hygiene products for exclusive use during the test period and rubber gloves to be worn when contact with antimicrobial agents cannot be avoided.

10. Procedure

10.1 *Preparation of Agar Patch Contact Plates*:

10.1.1 Aseptically place three 35 by 10 mm Petri dishes with lids removed into a sterile 100 by 20 mm Petri dish.

NOTE 2—Three 35 by 10 mm plates are needed per active or control formulation per organism per subject. Three additional plates are needed per organism for an inoculum determination count.

10.1.2 Using a sterile pipette, aseptically dispense approximately 11.5 mL of soybean-casein digest agar (or other appropriate solid medium) into each of the small Petri dishes. The dishes are filled to form a convex meniscus elevated above the rim of the plate.

10.1.3 Allow the agar to solidify and the surface to dry before inoculation.

10.2 *Preparation of Test Organisms*:

10.2.1 The test organisms selected should be representative of the microbial flora of the skin or of transient microorganisms that may contaminate human skin under certain conditions. A

partial list of organisms that have been used or recommended for use in this test includes *Staphylococcus epidermidis* ATCC 14990, *S. epidermidis* ATCC 12228, *S. aureus* ATCC 6538, *Escherichia coli* ATCC 11229, and *Klebsiella pneumoniae* ATCC 10031. This list is not intended to be exhaustive. Other organisms can be used at the discretion of the investigator. The organisms used should be differentiable from the test subject's own normal resident microorganisms by using the inoculum determination plate as a resource for colony morphology or by using differential or selective media. However, if no difference between resident and test microorganisms can be detected (e.g., *S. epidermidis*), then it may be assumed that the colony counts on the control plates due to contamination with resident microorganisms are in relative proportion to those on the active plates, and effectively cancel out.

NOTE 3—A recent antibiotic sensitivity profile for each test organism used in testing is required for purposes of subject safety.

10.2.2 Sequentially transfer culture(s) twice (once every 18 to 24 h) into appropriate liquid growth media. The second transfer must be into a volume of medium sufficient to perform the test.

10.2.3 Alternatively, the second transfer may be onto an agar plate.

10.2.4 If preparing inoculum from an agar plate, suspend organisms in dilution fluid before use.

10.2.5 The final concentration of inoculum should be adjusted to $1.0\text{--}3.0 \times 10^8$ CFU/mL. Inoculum should be well mixed to break up clumps.

NOTE 4—If the inoculum is from a plate, additional care must be taken to mix the sample well.

10.2.6 Prepare ten-fold serial dilutions from the $1.0\text{--}3.0 \times 10^8$ CFU/mL suspension using 9 mL of dilution fluid to achieve a final inoculum of $1.0\text{--}3.0 \times 10^4$ CFU/mL.

10.2.7 Pipette 10 μ L of the final inoculum preparation onto the surface of each of the prepared agar plates.

10.2.8 Spread the drop evenly across the surface of the plate using a bent sterile glass spreader or other suitable device. The inoculum should be allowed to soak into the agar.

NOTE 5—Steps 10.2.7 and 10.2.8 should be performed no more than 30 min before applying plates to the test sites.

11. Decontamination of Test Sites

11.1 Prior to testing, apply ethanol or isopropanol (60 to 75 % [v/v]) to the test sites to reduce populations of resident or transient microorganisms.

11.2 Allow the alcohol to air-dry completely before proceeding with testing.

12. Application

12.1 Application sites will be located on the volar aspects of the forearms or on other suitable areas such as the forehead or back. Application will be assigned by a predetermined randomization such that the forearms (or either side, right or left, of other anatomical areas) of each subject are equally likely to be used for treatment with the active or control formulation (or left untreated).

12.2 Treatment will consist of one or more washes or applications with either active test formulation or control formulation.

NOTE 6—More than one treatment may be required for some test formulations to display antibacterial activity.

13. Application of Bar-Form Wash Products (to forearm sites, for example)

13.1 Place a clean disposal glove on the right hand. Briefly wet the volar aspect of the left forearm with warm ($38 \pm 2^\circ\text{C}$) tap water.

13.2 Wet the bar (active or control formulation) with tap water, using only the gloved hand.

13.3 Rub the bar on the left volar forearm for 15 ± 2 s. Place bar aside. Lather arm with gloved hand for 45 ± 5 s. If lather becomes too dry, a small amount of water may be added to maintain lather.

13.4 Rinse the arm under warm ($38 \pm 2^\circ\text{C}$) tap water for approximately 15 s to remove all lather and allow arm to air-dry.

13.5 If a control formulation is used, repeat procedure for right forearm using a new, clean disposable glove on the left hand to apply the formulation (active or control) not used in Section 13.2.

14. Application of Liquid or Gel Wash Products (to forearm sites, for example)

14.1 Place a clean disposable glove on the right hand. Briefly wet the volar aspect of the left forearm with warm ($38 \pm 2^\circ\text{C}$) tap water.

14.2 Deliver the amount of product specified by label instructions into the gloved hand. If instructions are not available, 2.0 mL is a commonly used amount.

14.3 Lather the volar aspect of the left forearm for 60 ± 5 s.

14.4 Rinse the arm under warm tap water ($38 \pm 2^\circ\text{C}$) for approximately 15 s to remove all lather and allow arm to air-dry.

14.5 If a control formulation is used, repeat procedure for right forearm using a new, clean disposable glove on the left hand to apply the formulation (active or control) not used in Section 14.2.

15. Application of Leave-On (No-Rinse) Products (to forearm sites, for example)

15.1 It is suggested that preliminary testing be performed to determine the amount of product (active or control formulation) that covers the test site adequately, without excess. That amount may then be expressed as volume (or weight) per cm^2 of test site surface area.

15.2 Place a clean disposable glove on the right hand.

NOTE 7—Because of higher rates of evaporation, an alcohol-based product usually can be applied in larger volume, without excess, to a skin site than can a non-alcohol-based product. Volumes of 0.5 mL for alcohol-based products and 50 μ L for non-alcohol-based products have been used successfully in testing.

15.3 Gently massage the product into the test site for 30 ± 5 s.

15.4 Allow site to dry for 60 ± 5 s, or until dry, as determined for the product.

15.5 If a control formulation is used, repeat procedure for right forearm using new clean disposable glove on the left hand to apply the formulation (active or control) not used in Section 15.1.

16. Attachment of Plates

16.1 Place three previously inoculated agar plates, without lids, agar-side against the skin of the volar aspect of each forearm (or other site) treated with active or control formulation.

16.2 Secure each plate to the site using surgical adhesive tape, or equivalent.

16.3 Allow the plates to remain in place for 30 ± 2 min. During this time, subjects are instructed to sit with their arms resting on a bench top or similar surface with the volar aspect of their forearms up (or for other test sites, in a manner such that the plates are not disturbed).

16.4 Plates should be removed carefully by trained personnel following the 30 min period. Place uncovered inoculated active or control plates inside a 100 by 20 mm Petri dish and cover for incubation.

16.5 The plates removed from the forearms (or other sites), along with three inoculated but unused plates/organism (inoculum determination plates), are incubated at appropriate growth temperature $\pm 2^\circ\text{C}$ for the shortest time necessary to allow growth of countable colonies, usually 24 to 48 h.

16.6 Immediately following the removal of the agar plates, wash the forearms (or other test sites) for 30 s with 70 % isopropanol or equivalent, air-dry, and then wash thoroughly with a known antimicrobial wash product to decontaminate the test site(s).

17. Data Collection

17.1 The colony-forming units (CFU) of each plate are counted, and the average number of CFU are determined for each site. These are the mean viable cell counts (VCC).

17.2 The three inoculum determination plates must each contain 100 to 300 CFU/plate for the test to be valid.

17.3 The percent inhibition, is calculated using mean viable cell counts (VCC), as follows:

$$\% \text{ inhibition} = \frac{(\text{Mean VCC of control plates} - \text{Mean VCC of test plates}) \times 100}{\text{Mean VCC of control plates}} \quad (1)$$

18. Interpretation of Results

To test for statistical significance, the mean number of colony-forming units (CFU) for individual test and control agar patch plates should be compared per subject using a paired *t* test with critical level set at $\alpha = 0.05$.

NOTE 8—The agar patch method, used largely as a screening test for antibacterial activity, cannot distinguish between bacteristatic and bactericidal effects.

19. Precision and Bias

A precision and bias statement cannot be made for this test method at this time.

20. Keywords

20.1 agar patch; antibacterial; antimicrobial; control formulation; resident microorganism; persistent activity; transient microorganism

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