Designation: E 1882 – ~~97~~00

Standard Test Method for Evaluation of ~~Antibacterial Washes~~ Antimicrobial Formulations by the Agar Patch Technique[1]

This standard is issued under the fixed designation E 1882; 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 ~~covers determining~~ determines the ~~residual~~ antibacterial activity ~~(substantivity)~~ and persistence of ~~an antibacterial wash formulation~~ test formulations, as measured by the ~~reduction~~ inhibition of a test organism on an agar surface ~~following use.~~ 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 ~~g~~ Good ~~l~~ Laboratory ~~p~~ Practice (GLP) and ~~g~~ Good ~~c~~ Clinical ~~p~~ Practice ~~is~~ (GCP) are required and ~~follow it~~ to adhere to these practices, as appropriate.

1.4 In this test method, metric units are used for all applications except ~~distance.~~ linear measure. In that case, inches are used and metric 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, Part ~~2~~s 50 and 56).

2. Referenced Documents

2.1 *Federal Standard:*

~~Title 21, Code~~ Protection of ~~Federal Regulations (CFR), Food~~ Human Subjects, 21 CFR Ch. I, Parts 50 and ~~Drug Administration, Part 250, Protection~~ 56[2].

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[1] This test method is under the jurisdiction of ASTM Committee E-35 on Pesticides and is the direct responsibility of Subcommittee E35.15 on Antimicrobial Agents . Current edition approved May 10, ~~1997.~~ 2000. Published ~~February 1998.~~ August 2000. Originally published as E 1882 – 97. Last previous edition E 1882 – 97.

2.2 Yackovich, F., C.A. Wagner, and J.E. Heinze. 1989. Validity of ~~Human Subjects~~ [2] the agar patch test with an antibacterial liquid soap and comparison with the finger imprint method. *J. Soc. Cos. Chem.* 40:263-271.

2.3 *U.S. Pharmacopeia* XXIV, NF 19. 2000. United States Pharmacopeial Convention Inc., Rockville, MD. Chapter 61, entitled "Microbial Limits Test."

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

3. Terminology

3.1 *active formulation~~Definitions:~~*

~~3.1.1~~ —a formulation containing active ingredient(s).

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

~~3.1.2~~

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

~~3.1.3~~ formulation.

3.4 *antibacterial activity*—bacteriostatic and/or bacteriocidal activity.

3.5 *control formulation*—a formulation ~~which~~ that does not ~~exhibit antimicrobial activity in the agar patch test.~~ contain an active ingredient.[3]

~~3.1.4~~

3.6 *control plate*—inoculated 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 attached to any skin test site.

~~3.1.5 *placebo plate*~~

3.9 *persistence*~~—inoculated plate that has been attached to~~—effectiveness of a test ~~site washed with a control~~ formulation in inhibiting bacteria, defined in terms of time elapsed between application of test formulation and application of test plates.

3.1.~~6~~0 *resident microorganisms*—microorganisms that normally live and multiply on skin, forming a permanent population.

~~3.1.7 *test plate*—inoculated plate that has been attached to a test site washed with an active test formulation.~~

~~3.1.8~~

3.11 *transient microorganisms*—organisms ~~from the environment~~ that contaminate, but do not normally ~~permanently~~ colonize skin permanently.

3.1.~~9~~2 *volar aspect of the forearms*—the inside of the forearm~~. T~~ 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 ~~(including spray on antiperspirants)~~ 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 10^4 CFU/cm^2 ~~or less) number of resident microorganisms~~ or fewer) and be easily sampled.

4.2 The surfaces of agar contact plates are inoculated with the selected organisms and placed in contact with skin sites ~~which~~ that have been treated with active or control ~~test~~ formulations, or left untreated. After contact with the treated skin sites, these plates are incubated and the colonies enumerated. ~~Residual~~ Inhibitory activity of the ~~antibacterial wash~~ active formulation is measured by comparing differences in microbial colony counts between plates that were in contact with sites treated with an active formulations and plates that were in contact with ~~sites treated with control formulations~~ untreated sites, or ~~by comparing microbial counts between plates that were in contact with the panelist(s) test~~ sites treated with ~~test formulations and plates that were not in contact with~~ a ~~panelist (inoculum determination plates (see 3.1.4)).~~ control formulation. Results are expressed as percent inhibition.

5. Significance and Use

5.1 The~~is~~ procedure can be used to ~~test~~ evaluate formulations containing ~~antibacterial~~ ingredients ~~that are~~ intended to ~~reduce the number~~ inhibit growth of ~~organisms~~ bacteria on intact skin and ~~to~~ measures the ~~antibacterial activity~~ difference, post-product-exposure, between numbers of bacterial colonies on active 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 formulation and application of 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

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

[3] Yackovich, F., ~~et al, "Validation of the Agar Patch Test With an Antibacterial Liquid Soap and Comparison With the Finger Imprint Test," *J. Soc. Cos. Chem.*, 40, 1989, pp. 265-271.~~ Wagner C.A., Heinze J.E. 1989.

bacteriocidal 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 ~~also~~ be used ~~if desired.~~ for manual enumeration of colonies.

6.2 *Incubator*—Any incubator capable of maintaining a suitable temperature ± 2°C may be used.

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

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

7. Reagents and Materials

7.1 *Bacteriological Pipettes*, 10.0 and 2.2 or 1.1 mL capacity.[4]

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

7.3 *Plating Medium*, soybean-casein digest agar, or equivalent.[5]

7.4 *Dilution Fluid*, Butterfield's phosphate buffer[6], or equivalent.

7.5 ~~*Sterile Disposable Culture Dishes*, 35 by 10 mm and 100 by 20 mm.~~ Isopropanol or Ethanol, 60 to 75% (v/v)

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

7.7 *Sterile Test Tubes*.

~~7.7 *Surgical Adhesive Tape*.~~

7.8 *Surgical Adhesive Tape*, or equivalent.

7.9 *Disposable Examining Gloves*.

~~7.9 *Inoculating Loop* or glass spreader.~~

7.10 ~~*Appropriate Bacterial Cultures*~~ *Inoculating Loop or Glass Spreader*.

7.11 *Appropriate Bacterial Cultures*.

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

8. Test and Control Skin Sites

~~8.1 The~~

The volar aspect of the forearm is ~~utilized,~~ commonly used as the location of the skin sites, but ~~additional sites~~ other areas such as the back or forehead may be used for test sites.[7] Application ~~of test materials~~ of test and control formulations (or no treatment) will be assigned by a predetermined randomization ~~such~~ so that either ~~the left or right~~ forearm ~~will receive~~ (or either side, right or left, of other anatomical areas) may receive active or control formulations (or none).

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 ~~antibacterial agent~~ active formulation being evaluated.

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

9.2 Instruct the ~~volunteers~~ subjects to avoid contact with antimicrobials ~~(other than the test formulation)~~ for ~~the duration of the test and for~~ at least the week prior to testing and, other than the active formulation, for the duration of the test. This restriction includes ~~antimicrobial containing antiperspirants,~~ spray antiperspirants and deodorants, shampoos, lotions, dishwashing detergents, and soaps containing antimicrobial compounds, and materials such ~~material~~ as acids, bases, and solvents. Bathing in biocide-treated pools, hot tubs, ~~spas, etc.~~ or spas should be avoided. ~~Volunteers~~ Subjects may be provided with a kit of non-antimicrobial personal ~~care~~ 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 mm by 10 mm ~~petri dishes, whose~~ Petri dishes with lids ~~have been~~ removed into a sterile 100 mm by 20 mm ~~p~~ Petri dish.

NOTE 1—Three 35 mm by 10 mm plates are needed per actives or control formulation~~/~~ per organism~~/~~ per subject. ~~An~~ Three additional ~~three~~ plates are needed ~~for each~~ per organism for an inoculum determination count.

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[4] Presterilized/disposable bacteriological pipettes are available from most laboratory supply houses.

[5] "Microbial Limits Test," *U.S. ~~Pharmacopeia, XXIII:~~ Pharmacopeia.* ~~United States Pharmacopeial Convention, Inc. Rockville, MD, 1995.~~ 2000.

[6] ~~Butterfield's Phosphate Buffer, *Journal of the Association of Official Analytical Chemists*, Vol. 22, No. 635, 1939.~~

[6] Horowtiz, W. (Ed.). 1980.

[7] Application of agar patch plates and alcohol to the forehead risks contaminating the eyes, and extra precautions must be exercised.

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

10.1.3 Allow the agar to ~~solidify. The agar should be allowed~~ solidify and the surface to dry before ~~use.~~ inoculation.

10.2 *Preparation of Test Organisms*:

10.2.1 The test organisms selected should be representative of the ~~bacterial~~ microbial flora of the skin or of transient ~~organisms typically found on~~ 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 155, *~~S.~~ 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 ~~found on human skin~~ can be used at the discretion of the ~~individual performing the test.~~ investigator. The organisms used ~~must~~ should be ~~able to be differentiated~~ 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 2—A recent antibiotic sensitivity profile for each test organism used in testing is required for purposes of subject safety.

10.2.2 ~~T~~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 or slant.~~ plate.

10.2.4 If preparing inoculum from an agar ~~plate or slant,~~ plate, suspend organisms in ~~Butterfield's phosphate buffer.~~

~~10.2.5 Final~~ dilution fluid before use.

10.2.5 The final concentration of inoculum should be adjusted to 1.0–3.0 × 10^8 ~~cfu/mL.~~ CFU/mL. Inoculum should be well mixed to break up clumps.

NOTE ~~2~~3—If the inoculum is from a ~~plate or slant,~~ plate, additional care must be taken to ~~adequately~~ mix the sample well.

10.2.6 Prepare ten-fold serial dilutions from ~~this~~ the 1.0– 3.0 × 10^8 CFU/mL suspension using 9 mL of ~~Butterfield's buffer or equivalent~~ dilution fluid to achieve a final inoculum of 1.0 – 3.0 × 10^4 ~~cfu/mL.~~ CFU/mL.

10.2.7 Pipette 10 µL 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 ~~rod~~ spreader or other suitable device. The inoculum should be allowed to soak into the agar.

NOTE ~~3~~4—Steps 10.2.7 and 10.2.8 should be ~~done~~ 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

1~~1~~2.1 Application sites will ~~consist of~~ 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 ~~contralateral~~ 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 ~~subjects will receive~~ active or control ~~app~~ formul~~i~~cations (or left untreated).

1~~1~~2.2 Treatment will consist of one or more washes or applications with either ~~test~~ active or ~~placebo product.~~ control formulation.

NOTE ~~4~~5—More than one treatment ~~will~~ may be required for some ~~active~~ test formulations to display ~~residual~~ antibacterial activity.

~~12. Application of Bar-Form Wash Products~~

~~12.1 Place a clean disposable glove on the right hand. Briefly wet the volar aspect of the left forearm with warm (38 ± 2°C) tap water.~~

~~12.2 Wet the bar with tap water, using only the gloved hand.~~

~~12.3 Rub the bar on the left volar forearm for 15 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.~~

~~12.4 Rinse the arm under warm (38 ± 2°C) tap water for approximately 15 s to remove all lather and allow arm to air dry.~~

~~12.5 Repeat procedure for right arm using new clean disposable glove on the left hand.~~

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

13.1 Place a clean disposa~~b~~le glove on the right hand. Briefly wet the volar aspect of the left forearm with warm (38 ± 2°C) ~~running~~ tap water.

13.2 Deliver 2.0 mL of

13.2 Wet the product bar (active or amount specified by label instructions into control formulation) with tap water, using only the gloved hand.

13.3 Lather

13.3 Rub the bar on the left volar forearm for 60 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 ± 2°C) tap water for approximately 15 s to remove all lather and allow arm to air-dry.

13.5 RIf 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. AttachmentApplication of P Liquid or Gel Wash Products (to forearm sites, for example)

14.1 Place three previously inoculated agar plates, without lids, agar side against a clean disposable glove on the skin, onto right hand. Briefly wet the volar aspect of each washed forearm.

14.2 Secure each plate to the arm using surgical adhesive tape.

14.3 Allow the plates to remain in place for 30 left forearm with warm (38 ± 2 min. During this time subjects 2°C) tap water.

14.2 Deliver the amount of product specified by label instructions into the gloved hand. If instructions are instructed to sit with their arms resting on not available, 2.0 mL is a bench top or similar surface with commonly used amount.

14.3 Lather the left volar aspect of their forearms up.

14.4 Plates should be carefully removed by trained personnel following the 30 min period. The uncovered 35 by 10 mm petri dishes will be placed inside forearm for 60 ± 5 s.

14.4 Rinse the arm under warm tap water (38 ± 2°C) for approximately 15 s to remove all lather and allow arm to air-dry.

14.5 If a covered 100 by 20 mm petri dish.

14.5 Immediately following removal of control formulation is used, repeat procedure for right forearm using a new, clean disposable glove on the agar plates, left hand to apply the forearm will be gently rubbed with 70 % isopropanol formulation (active or equivalent. control) not used in Section 14.2.

15. Data Collection

15.1 Plates removed from the forearms, along with three inoculated but unused plates/organism (inoculum determination plates), are incubated at appropriate growth temperature ± 2°C for the shortest time necessaryApplication to allow control samples to grow countable colonies.

15.2 The three inoculum determination plates must contain between 100 to 300 cfu/plate Leave-On (No-Rinse) Products (to forearm sites, for the test to be valid.

15.3 Using example)

15.1 Place a colony counter, count the surviving bacteria clean disposable glove on test plates. A magnifying device may the right hand.

15.2 It is suggested that preliminary testing be needed performed to help differentiate contaminating normal flora from determine the test organisms seeded onto the agar surface.

15.4 The cfu amount of each plate will be counted and product (active or control formulation) that covers the average cfu of each test site (subject's forearm) will adequately, without excess. That amount may then be determined.

15.5 Colony forming unit (cfu) counts expressed as volume (or weight) per cm^2 of test and placebo plates will site surface area.

NOTE 6—Because of higher rates of evaporation, an alcohol-based product usually can be compared using applied in larger volume, without excess, to a paired T-test. The percent reduction, if needed, will be calculated skin site than can a non-alcohol-based product. Volumes of 0.5 mL for alcohol-based products and 50 µL 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 geometric mean (antilog of left hand to apply the mean log cfu/plate). P-values ≤ 0.05 will be considered statistically significant:

$$\text{\% reduction} = \frac{\textit{VCC of placebo plate or average of plates} - \textit{VCC of test plate}(s) \times 100}{\textit{VCC of placebo plate}(s)}$$

where:

VCC = viable cell count.

formulation (active or control) not used in Section 15.2.

16. Precision and Bias

16.1 A precision and bias statement cannot be madeAttachment 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 mm 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 ± 2°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, the forearms (or other test sites) are washed for 30 s with 70 % isopropanol or equivalent, air-dried, and then washed 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:

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

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 7—The agar patch method, used largely as a screening test for antibacterial activity, cannot distinguish between bacteriostatic and bacteriocidal effects. For determination of bacteriocidal effectiveness of a product, immediate or persistent, it is recommended that another method — e.g., ASTM Standard Methods E 1174 or E 1874 — be used.

19. Precision and Bias

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

20. Keywords

~~17~~20.1 agar patch; antibacterial; antimicrobial; control formulation; resident microorganism; ~~residual~~ persistent activity; ~~substantivity;~~ transient microorganism