Designation: E640 – 06 (Reapproved 2012)

Standard Test Method for Preservatives in Water-Containing Cosmetics[1]

This standard is issued under the fixed designation E640; 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 the determination of the suitability of preservatives for use in cosmetic formulations. It sets minimal requirements for preservative performance in model formulations.

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

1.3 *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]

E1054 Test Methods for Evaluation of Inactivators of Antimicrobial Agents

3. Summary of Method

3.1 This test method involves a microbiological challenge test of preservatives incorporated into model formulations at recommended efficacy levels. Routine microbiological procedures are used to determine the antimicrobial activity of preservatives in formulations. This method requires the knowledge of standard microbiological techniques.

4. Significance

4.1 This test method should be used to determine if a preservative or preservative system has application for the preservation of water-miscible cosmetic products.

5. Materials

5.1 *Test Formulations*—Formulations that the submitter feels are appropriate for demonstration of preservative activity shall be included in the test. Non-preserved (control) samples of these formulas shall also be included. Incompatibility of the preservative(s) with any of the formulations or formulation components shall be noted.

5.2 *Test Microorganisms (Suggested Panel):*

5.2.1 Other test microorganisms or equivalent species may be included as appropriate and if standardized cultures from cosmetic isolates become available. The primary function of these cultures is to provide a common basis for comparison of different preservatives.

5.2.1.1 *Pseudomonas aeruginosa* ATCC 9027.
5.2.1.2 *Burkolderia cepacia* ATCC 25416.
5.2.1.3 *Escherichia coli* ATTC 8739.
5.2.1.4 *Staphylococcus aureus* ATCC 6538.
5.2.1.5 *Candida albicans* ATCC 10231.
5.2.1.6 *Enterobacter gergoviae* ATCC 33028.
5.2.1.7 *Aspergillus niger* ATCC 16404.
5.2.1.8 *Eupenicillium levitum* ATCC 10464.

5.2.2 If available, cosmetic spoilage microorganisms and/or microorganisms obtained from the cosmetic manufacturing environment may be used in addition to those microorganisms suggested in 5.2.

5.3 *Culture Maintenance*—The microorganisms listed in 5.2.1 shall be maintained as specified by ATCC.

5.3.1 *Plating Diluents*—Plating diluents are used to disperse the test sample in preparation for plating and, if necessary, aid in neutralizing the preservative present to permit the optimum recovery of surviving microorganisms. The choice of diluents is dependent of the diluents ability to meet the neutralization requirements specified in 5.3.3. The following suggested diluents have been found to be suitable for this purpose:

5.3.1.1 Buffered 1 % Peptone in physiological saline (0.85 % NaCl).
5.3.1.2 Dey/Engley (D-E) neutralizing broth.
5.3.1.3 Eugon Broth.
5.3.1.4 Letheen Broth.
5.3.1.5 Modified Letheen Broth.
5.3.1.6 Nutrient Broth.
5.3.1.7 Phosphate Buffer (pH 7.0).
5.3.1.8 TAT Broth.
5.3.1.9 Trypticase Soy Broth.

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

Current edition approved April 1, 2012. Published June 2012. Originally approved in 1978. Last previous edition approved in 2006 as E640 – 06. DOI: 10.1520/E0640-06R12.

[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.

5.3.2 *Recovery Media*—A recovery medium should provide adequate nutritional support for the growth of the selected test microorganisms. The following suggested agar recovery media have been found to be suitable for this purpose:

5.3.2.1 *For Bacteria:*

Eugon Agar
Letheen Agar
Microbial Content Agar
Modified Latheen Agar
Plate Count Agar
Trypticase Soy Agar

5.3.2.2 *For Fungi:*

Malt Agar
Malt Agar Extract
Mycophil Agar
Potato Dextrose Agar

5.3.3 *Preservative Neutralization*—Neutralizing agents are incorporated into the plating diluent or the recovery medium, or both, in order to inactivate the preservatives and permit a more accurate enumeration of the microbial content. Where neutralizers are not available or are ineffective, physical dilution or membrane filtration may be necessary. (See Test Methods E1054.)

6. Procedures

6.1 *Preparation of Challenge Inocula*—Grow bacterial cultures at 35 ± 2°C for 24 to 28 h on slants of the appropriate solid media. Grow yeast cultures on the appropriate media at 25 ± 2°C for 48 to 72 h. Grow mold cultures on the appropriate media at 25 ± 2°C for 5 to 7 days or until full sporulation is achieved.

6.1.1 *Harvesting Bacterial Cultures*—Using a sterile inoculating loop, transfer the growth from each culture into tubes of sterile saline. Alternatively, wash culture from slant using sterile saline and transfer to a sterile tube. Adjust to yield a suspension of approximately 1 × 108 cfu/mL using a McFarland Barium Sulfate Standard #2, turbidimetry, optical density, or other technique that correlates to an aerobic plate count. Confirm culture standardization using a verified aerobic bacterial plate count.

6.1.2 *Harvesting Yeast Cultures*—Harvest yeast cultures as described in 6.1.1, however, adjust suspension to approximately 1×10^7 cfu/mL. Confirm culture standardization using a verified aerobic fungal plate count technique.

6.1.3 *Harvesting Mold*—Harvest mold spores by adding sterile saline containing 0.05 % Polysorbate 80 to the culture and rubbing the growth gently with a sterile inoculating loop or other appropriate sterile implement. Filter through sterile gauze, sterile glass wool, or sterile nonabsorbent cotton. Adjust the mold spore suspension to approximately 1×10^7 cfu/mL using a hemocytometer or other reproducible direct microscopic counting technique. Confirm culture standardization using a verified aerobic fungal plate count. Harvested mold spore cultures may be used immediately or stored at 2 to 5°C for up to four weeks.

6.1.4 *Preparation of Inocula:*

6.1.4.1 *Mixed Culture Method:*

(1) Mixed Bacteria—Mix equal portions of selected bacteria and label "Bacteria 1."

(2) Optional Mixed Bacteria Pools—Mix separate bacteria pools for gram-positive bacteria, gram-negative fermenter bacteria, and gram-negative non-fermenter bacteria. Pooled cultures may be used immediately or stored at 2 to 5°C for up to 72 hours.

(3) Fungi (Mold and Yeast)—Mix equal parts of fungal suspensions thoroughly and label "Fungi #2."

6.1.4.2 *Pure Culture Method*—Optionally, challenges may also be performed using single (pure) cultures. If this method is chosen, prepare the cultures used for challenges as described in 6.1 and use directly as described in 6.3. Label sample jars appropriately according to the test microorganisms used to challenge that sample.

6.1.5 *Determination of Challenge Inocula Levels*—Pre pare serial dilutions of the challenge inocula (6.1.4.1). Plate out in duplicate using Letheen agar. Incubate bacteria and yeast at 32°C for 24 h and fungi at 25°C for 72 h. Determine the number of colony-forming units (cfu) in each inoculum.

6.2 *Sample Preparation*—Weigh out 20 g aliquots of the test material into suitable glass containers and label appropriately. Cap and store at ambient temperature (20 to 25°C).

6.3 *Challenge of Test Formulation*—Inoculate each 20 g aliquot of the test material by adding 0.2 mL of the appropriate inoculum. Mix thoroughly by shaking, stirring, vortexing, or using a any other suitable mechanical mixing device. Store inoculated samples at ambient temperature (20 to 25°C).

6.4 *Microbiological Testing:*

6.4.1 Mix inoculated sample thoroughly. Prepare a 1:10 dilution (1 part test material plus 9 parts neutralizing diluent) and mix thoroughly. Additional serial 10-fold dilutions may be prepared as required. Plate diluted test samples in duplicate on the appropriate selected recovery agars for bacteria and fungi.

6.4.1.1 Invert bacterial plates and incubate at 35 ± 2°C for 48 to 72 hours. Count colonies on plates. Bacterial counts in the range of 25 to 250 cfu are considered acceptable. Verify the identity of the microorganisms by gram staining where appropriate.

6.4.1.2 Invert fungal plates and incubate at 25 ± 2°C for 3 to 5 days. Count colonies on plates. Fungal counts in the range of 8 to 80 cfu are considered acceptable.

6.4.1.3 Where no plates fall into the acceptable countable ranges, count the colonies on plate(s) nearest that range. Average the duplicate plate counts and record as cfu/g of test material.

6.4.2 *Test Time Intervals*—Test inoculated samples at the minimal suggested test intervals of 0, 7, 14, 21 (optional), and 28 days (additional test intervals may be selected as desired).

6.5 *Rechallenge*—If the preservative is intended for cosmetics that are subject to repeated insult by the consumer, the use of a rechallenge procedure may be considered. Rechallenge the test aliquots at 21 or 28 days and continue the test another 28 days, thereby repeating all procedures described for the initial challenge.

7. Interpretation of Data

7.1 The following may be used as the criteria for demonstrating the effectiveness of a preservative incorporated into the model cosmetic systems:

7.1.1 Bacteria and yeast should show at least a 99.9 % (3 log) reduction within seven days following each challenge and no increase thereafter for the remainder of the test within normal variation of the data.

7.1.2 Fungi should show at least a 90 % (1 log) reduction within seven days following each challenge and no increase thereafter for the remainder of the test within normal variation of the data.

7.2 Calculate the percent (%) reduction as follows:

$$\% \text{ Reduction} = \frac{\text{Inoculum Count} - \text{Sample Count}}{\text{Inoculum Count}} \times 100$$

7.3 The uninoculated test material must contain less than 100 cfu/g to proceed with the challenge test.

7.4 Inoculum counts must be in the prescribed ranges or the test is considered to be invalid and must be repeated.

7.5 If preservative neutralization is not demonstrated, the test is invalid and cannot be repeated until a suitable neutralizing agent or procedure is developed.

8. Precision and Bias

8.1 The precision and bias of this method have not been determined. Replicate samples are recommended.

9. Keywords

9.1 bacterial; cosmetics; microbial challenge; preservative performance criteria; preservatives