	Title	Revision No	date	Document No
	EVALUATION OF THE ANTIMICROBIAL PROTECTION OF A COSMETIC PRODUCT	1	28/11/13	ACM 008

1. SCOPE AND FIELD OF APPLICATION
   To evaluate the overall antimicrobial protection of a cosmetic product.

2. PRINCIPLE
   The test involves, for each test microorganism, placing the formulation in contact with a calibrated inoculum, and then measuring the changes in the microorganism count at set time intervals for a set period and a set temperature.

3. APPARATUS
   - 3.1 Biohazard cabinet
   - 3.2 Autoclave
   - 3.3 Hot Air Oven
   - 3.4 Incubator: 32.5 ± 2.5°C and 22.5 ± 2.5°C
   - 3.5 Vortex mixer
   - 3.6 Glass beads
   - 3.7 Pipettes
   - 3.8 Petri dishes
   - 3.9 Haemocytometer & Phase-contrast Microscope (if available)
   - 3.10 Spectrophotometer / Colorimeter (if available)
   - 3.11 Colony counter
   - 3.12 Media bottles
   - 3.13 Sterile universal bottles / Test tubes
   - 3.14 Inoculating loops / spreader
   - 3.15 Bunsen burner (or Bacticinerator)
   - 3.16 Waterbath
   - 3.17 Top pan balance
   - 3.18 pH meter

4. MEDIA AND REAGENTS
   For convenience, dehydrated media of any brand equivalent in function may be used.
   Media should be tested for sterility and growth promotion using suitable organisms.
   - 4.1 Nutrient Agar (or other suitable equivalent media)
   - 4.2 Letheen Agar (or other suitable equivalent media e.g. TSA with 1% Tween 80, “TSA t”)
   - 4.3 Mycophil Agar, pH 4.7 (or other suitable equivalent media e.g. “PDA a” / “SDA a”, SDA with 1% Tween 80 “SDA t”)
   - 4.4 Letheen Broth (or peptone saline with 1% Tween 80)
   - 4.5 Chloride buffer (or similar buffer)
   - 4.6 Diluent 1 – Sterile solution containing 0.9% sodium chloride and 0.1% peptone
   - 4.7 Diluent 2 – Sterile solution containing 0.9% sodium chloride and 0.05% Tween 80

   Note: "a" means with antibiotic

5. TEST ORGANISMS
   - 5.1 *Pseudomonas aeruginosa* ATCC 9027, CIP 82.118, or equivalent
   - 5.2 *Staphylococcus aureus* ATCC 6538 (NCIMB 9518, CIP 4.83, NCTC 10788)
   - 5.3 *Candida albicans* ATCC 10231 (NCPF 3179, IP 48.72)
   - 5.4 *Enterobacter aerogenes* ATCC 13048
   - 5.5 *Aspergillus brasiliensis* (previously *A. niger*) ATCC 16404, IP 1431, IMI 149007 or equivalent

6. PREPARATION AND ENUMERATION OF INOCULA

6.1 Bacteria and yeast

6.1.1 Prepare a subculture from the stock culture by streaking slant tubes or plates (TSA for bacteria, SDA for *Candida albicans*) in order to obtain a confluent culture. Incubate at (32.5 ± 2.5) °C for 18 – 24 hours.

6.1.2 Wash each slant of bacteria and yeast culture with 10 mL of diluent 1, loosening the culture from the agar surface with the help of sterile glass beads. Mix by using mechanical mixer to disperse evenly and transfer the suspension into sterile universal bottle.

6.1.3 Adjust the number of cells in the suspension to 1 X 10^7 cfu/mL to 1 X 10^8 cfu/mL (bacteria) or 1 X 10^6 cfu/mL to 1 X 10^7 cfu/mL (yeast) using diluent 1 either by using McFarland $BaSO_4$ standard No.2, direct microscopic count, turbidimetry, absorbance or other method correlated to an aerobic plate count.

6.1.4 At the time of the test, check the initial capacity of the suspension, N. Make successive tenfold dilutions of the calibrated suspension in the diluent 1. Perform the enumeration by duplicating 1 mL of the suitable dilutions into TSA for bacteria and into SDA for *Candida albicans*. Incubate the dishes at (32.5 ± 2.5) °C for 24-48 hours.

6.2 *Aspergillus brasiliensis* spore suspension

6.2.1 Streak *Aspergillus brasiliensis* culture onto SDAa slant and incubate at (22.5 ± 2.5) °C for 7 – 11 days or until full sporulation is achieved.

6.2.2 Wash each slant of fungi culture with 10 ml of diluent 2, loosening the spores from the culture surface with the help of sterile glass beads. mix by using mechanical mixer to disperse evenly and transfer the suspension into sterile universal bottle.

6.2.3 Adjust the number of spores in the suspension to a value about 1 X 10^6 spores/mL to 1 X 10^7 spores/mL using diluent 2 and any appropriate means.

6.2.4 At the time of the test, check the initial capacity of the suspension, N. Make successive tenfold dilutions of the calibrated suspension in the diluent 2. Perform the enumeration by duplicating 1 mL of the suitable dilutions into SDA plates. Incubate the dishes at (22.5 ± 2.5) °C for 3-5 days.

7. DEMONSTRATION OF THE NEUTRALIZER EFFICACY

7.1 In the test, the neutralization of the possible antimicrobial activity of the tested sample shall be checked and demonstrated.

7.2 The suitability and effectiveness of the neutralizing agent with respect to the test strains used and to the tested formulation shall be demonstrated.

7.3 A calibrated suspension of microorganisms (about 10^3 cfu/mL) is inoculated in the neutralizer in the presence (test) and in the absence (control) of the formulation.

7.4 The neutralizer efficacy is demonstrated if the counts performed on the inoculum, N_v, and on the control, N_{vn} (mixture of the neutralizer and diluent) are equivalent and if the count in the test, N_{vf} (mixture of the neutralizer and the formulation) is at least 50% of N_{vn}.

7.5 If the results do not comply with the requirements, it is necessary to either modify the neutralizer **or** make a further dilution of the sample **o**r carry out a membrane filtration, if possible**.**

7.6 If the results still do not comply with the requirements, it is unlikely that the formulation can be contaminated by the strain concerned.

8. DETERMINATION OF THE PRESERVATION EFFICACY OF THE FORMULATION

8.1 Procedure

8.1.1 Run the test separately for each strain.

8.1.2 Aliquoting of Test Product

8.1.3 For each strain, dispense 20 gram or 20 mL of the test formulation into a sterile container.

8.2 Inoculation of the test microorganisms

8.2.1 Add to each container 0.2 mL of calibrated inoculum to obtain 1 X 10^5 cfu/mL and 1 X 10^6 cfu/mL or gram for bacteria, and between 1 X 10^4 cfu/mL and 1 X 10^5 cfu/mL or gram for *Candida albicans* and *Aspergillus brasiliensis* in the formulation (final concentration).

8.2.2 Mix thoroughly to ensure a homogeneous distribution of the inoculum.

8.2.3 The initial concentration of microorganisms present in the inoculated product, N0, is calculated using the results of the enumeration of the calibrated inoculum, N.

8.3 Incubation of the Inoculated Formulation

8.3.1 Store the containers holding the inoculated formulation at (22.5 ± 2.5) °C.

8.4 Sampling and Enumeration

8.4.1 Remove 1 gram or 1 mL of sample at each specified sampling interval, 7 days (T7), 14 days (T14) and 28 days (T28) according to the test strain into peptone saline containing 1% Tween 80. Mix until homogeneous.

8.4.2 Leave in contact for 15-45 minutes at room temperature. Proceed with 10-fold serial dilutions using peptone saline containing 1% Tween 80.

8.4.3 Determine the number of viable microorganisms in duplicate by surface spread technique on TSA for bacteria, and pour plate technique using SDA for yeast and fungi.

8.4.4 Incubate at (32.5 ± 2.5) °C for 48-72 hours for the bacteria and *C. albicans* and at (22.5± 2.5) °C for 3-5 days for *A. brasiliensis*.

8.4.5 Count the number of surviving microorganisms per gram or ml of product.

9. CALCULATIONS

9.1 Determination of the initial numbers of microorganisms, N and N_0

9.1.1 Calculate N, the number of microorganisms present in the calibrated suspensions in colony-forming units per millilitre, using Equation:

$$N = Ć / (V \times d)$$

Where,

Ć is the mean number of colonies counted in duplicate over the plates.
V is the volume of inoculum applied to each dish, in millilitres.
d is the dilution factor of the counted dilution.

N shall be between 1 X 10^7cfu/mL and 1 X 10^8 cfu/mL for bacteria, and between 1 X 10^6cfu/mL and 1 X 10^7 cfu/mL for *C.albicans* and *A.brasiliensis.*

9.1.2 Determine N_0, the number of microorganisms inoculated in the formulation at time t_0 using Equation:

$$N_0 = N/100$$

N_0 shall be between 1 X 10^5cfu/mL and 1 X 10^6 cfu/mL or gram for the bacteria, and between 1 X 10^4cfu/mL and 1 X 10^5 cfu/mL or gram for *C.albicans* and *A.brasiliensis.*

9.2 Enumeration of the microorganisms at each sampling time, N_x

9.2.1 Calculate N_x the number of surviving microorganisms in the contaminated formulation, in colony-forming units per millilitre or grams, at each sampling time, t_x, (T7, T14 or T28) using Equation:

$$N_x = C / (V \times d)$$

Where

C is the mean number of colonies counted in duplicate over the plates.
V is the volume of inoculum applied to each dish, in millilitres.
d is the dilution factor corresponding to the retained and counted dilution.

9.3 Reduction in Microbial Counts

9.3.1 Calculate the reduction values, R_x, expressed in log units, obtained at each sampling time using Equation:

$$R_x = \lg N_0 - \lg N_x$$

Where

N_0 is the number of microorganisms inoculated at time t_0
N_x is the number of surviving microorganisms at each sampling time, t_x

There may be no reduction and there may be an increase in the microorganism count.

10. INTERPRETATION OF TEST RESULTS

10.2 Criteria

10.2.1 The obtained log reduction values, R_x are compared to the minimum values required for evaluation criterion A or B (Table 1).

10.2.2 The criterion representing the protection capacities are:
Criterion A, whereby the formulation is protected against microbial proliferation that may present a potential risk for the user and no additional factors are considered.
Criterion B, whereby the level of protection is acceptable if the risk analysis demonstrates the existence of control factors not related to the formulation indicating that the microbiological risk is tolerable for the cosmetic product.

10.2.3 The criteria are expressed either by a minimum log reduction value or by “NI” when the requirement is that there be no increase in the microbial population.

Table 1

Log Reduction Values ($R_x = lgN_0 - lgN_x$) required[a]								
Microorganisms	Bacteria			*C.albicans*			*A.brasiliensis*	
Sampling Time	T7	T14	T28	T7	T14	T28	T14	T28
Criteria A	≥3	≥3 and NI[b]	≥3 and NI	≥1	≥1 and NI	≥1 and NI	≥0[c]	≥1
Criteria B	Not performed	≥3	≥3 and NI	Not performed	≥1	≥1 and NI	≥0	≥0and NI
[a] In this test, an acceptable range of deviation of 0.5 log is accepted								
[b] NI: no increase in the count from the previous contact time								
[c] $R_x = 0$ when $lgN_0 - lgN_x$(no increase from the initial count)								

11. REFERENCES

11.1 The American Society for Testing and Materials, Designation: E 640-78 (Reapproved 1998), Standard Test Method for Preservatives in Water-Containing Cosmetics, pp.141-142.

11.2 AOAC Official Methods of Analysis (2000), Chapter 15, Efficacy of Preservation of Non-Eye Area Water Miscible Cosmetic and Toiletry Formulations, pp 3-5

11.3 British Pharmacopoeia 2012, Volume 4, Appendix XVI C, Efficacy of Antimicrobial Preservation

11.4 ISO 11930, Cosmetics-Microbiology-Evaluation of the antimicrobial protection of a cosmetic product, First Edition 2012-04-01

Harmonised method:

- *Issued by the microbiological analysis group at the harmonization workshop in Kuala-Lumpur, September 13th to 17th 2004*
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- *Modified and approved after the final review in Singapore, Nov 30th to Dec 2nd, 2005*
- *Modified and approved after the Regional Cosmetic Workshop in Malaysia, July 10th to 12th 2006*
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