	Title	Revision n°	date	Document No
asean	**MICROBIAL LIMIT TEST FOR COSMETIC PRODUCTS**	0	2/12/2005	ACM THA 06

1 SCOPE AND FIELD OF APPLICATION

The method specifies the procedure for enumeration of aerobic bacteria, yeast, and mold present in cosmetic products.

2 PRINCIPLE

Method for enumeration of microorganisms from cosmetic products is direct colony counts. Products that are not soluble in water are initially treated to render them miscible before enumeration procedures are conducted. The possible inhibition of microbial growth by the sample shall be neutralized to allow the detection of viable microorganism. In all cases and whatever the methodology, the neutralization of the antimicrobial properties of the product shall be checked and validated. The scheme for these analyses is summarized in Fig 1.

3 MEDIA, REAGENTS, AND APPARATUS

3.1 Media and Reagents :

The following culture media and diluents are suitable for enumeration of aerobic mesophilic bacteria, yeast, and mold. Other culture media and diluents may be used if they have been demonstrated to be suitable for use.

3.1.1 Media for enumeration of bacteria and fungi

3.1.1.1 Malt extract agar (MEA) or other suitable media
3.1.1.2 Potato dextrose agar (PDA) or other suitable media
3.1.1.3 Modified letheen agar (MLA) or other suitable media
3.1.1.4 Sabouraud's dextrose broth or other suitable media

3.1.2 Other media and reagents

3.1.2.1 Aqueous solution of 70% ethanol and 1% HCl (v/v) or 4% iodine in 70% ethanol solution or 2% glutaraldehyde solution
3.1.2.2 Tween 80 (Polysorbate 80)
3.1.2.3 Ethanol, 95% (v/v)

3.2 Apparatus

3.2.1 Pipets, sterile, 1, 5, and 10 mL, graduated
3.2.2 Gauze pads, sterile, 4 x 4 inch
3.2.3 Gauze pads, sterile, 4 x 4 inch
3.2.4 Sterile instruments: forceps, scissors, scalpel and blades, spatulas, and microspatulas
3.2.5 Test tubes, screw-cap, 16 x 125, and 20 x 150 mm
3.2.6 Dilution bottles, screw-cap
3.2.7 Balance, sensitivity of 0.01 g
3.2.8 Petri dishes, sterile, plastic, 15 x 100 mm
3.2.9 Incubators, 30 ± 2°C and 35 ± 2°C
3.2.10 Laminar flow hood with HEPA filter (if available)

4 SAMPLE HANDLING

4.1 Analyze samples as soon as possible after receiving them. If necessary, store samples at room temperature. Do not incubate, refrigerate, or freeze sample before or after analysis.

4.2 Inspect samples carefully before opening and note any irregularities of sample container.

5 PROCEDURE

5.1 General Recommendation

5.1.1 Before opening and removing sample contents, disinfect surface of sample container with aqueous mixture of 70% ethanol (v/v) and 1% HCl (v/v). Use laminar flow hood if possible. Leave the cleaned surface to dry before opening.

5.1.2 Weigh 10 g or mL of sample from representative portion of contents for microbial analysis.

5.1.3 For products weighing less than 1 g or mL, analyze entire contents. If only one sample unit is available and multiple analyses are requested (i.e., microbial, toxicological, and chemical), take sub-sample for microbiological examination before those for other analyses. In this situation, amount of sub-sample used for microbial analysis will depend on other analyses to be performed. For example, if total sample content is 5 mL, use 1 or 2 mL portion for microbial analyses.

5.1.4 The amount of sample and diluent given here can be adjusted according to amount of sample available. If sample has many sub-samples, amount of test material can be increased and workload streamlined by compositing. Analysts should use their best judgment as to when and how much material to composite.

5.2 Sample preparation

5.2.1 **Liquids**. Decimally dilute 1 mL liquid directly into 9 mL modified letheen broth (MLB) in 20 x 150 mm screw-cap test tube for the 10^{-1} dilution.

5.2.2 **Solids and powders**. Aseptically remove and weigh 1 g sample into 20 x 150 mm screw-cap test tube containing 1 mL sterile Tween 80. Disperse product in Tween 80 with sterile spatula. Add 8 mL sterile MLB and mix thoroughly. This will be the 10^{-1} dilution.

5.2.3 **Waxy/Fatty products** (Lipstick). Aseptically remove and weigh 10 g sample into a sterile mortar containing 2 mL sterile mineral oil and 10 mL sterile Tween 20. Disperse with a sterile spatula to form a paste. Add 78 mL sterile MLB to make a 10^{-1} dilution.

5.2.4 **Cream and oil-based products**. Aseptically remove and weigh 1 g sample into 20 x 150 mm screw-cap tube containing 1 mL sterile Tween 80 plus five to seven 5-mm glass beads

(or ten to fifteen 3-mm glass beads). Mix total contents with Vortex mixer. Adjust total volume to 10 mL with sterile MLB (8 mL) for the 10^{-1} dilution.

5.2.5 **Aerosols of powders, soaps, liquids, and other materials.** Decontaminate nozzle of spray can as much as possible by swabbing with gauze pad moistened with 70% (v/v) aqueous ethanol. Expel some product to flush out nozzle; then spray appropriate amount into tared dilution bottle, e.g., 1 g of product into 9 mL sterile MLB. Thoroughly mix product and broth, and reweigh. This will be a 10^{-1} dilution if exactly 1 g of sample was obtained.

5.2.6 **Anhydrous materials**. Treat as in 5.2.2 or 5.2.4, as appropriate.

Note : For water immiscible samples : Transfer the samples to a suitable container containing a suitable quantity of solubilizing agent (e.g. Tween 80). Disperse the sample within the solubilizing agent and add appropriate volume of diluents or neutralizer.

5.3 Neutralization of the antimicrobial properties of the Cosmetic product

5.3.1 Any cosmetic products with anti-microbial properties (or preservatives) must be appropriately neutralized before conducting the test. Any residual preservative should be checked by re-challenging with appropriate microorganisms. Information relative to suitable neutralizers is given in Annex A.

5.3.2 Validation of the efficiency of neutralizer.

5.3.2.1 The two strains, representative of both Gram negative and Gram positive microorganism (e.g. *Staphylococcus aureus* ATCC6538, *Pseudomonas aeruginosa* ATCC 9027) are used to demonstrate the validity of the neutralizer.

5.3.2.2 For each strain, mix the neutralized sample with a dilution of microorganism ($1x10^8$ CFU/mL) and plate on a Petri dish. After incubation, check the nature of the colonies and compare the count with a control (without sample). The count should be less than 50% of the control.

5.4 Enumeration

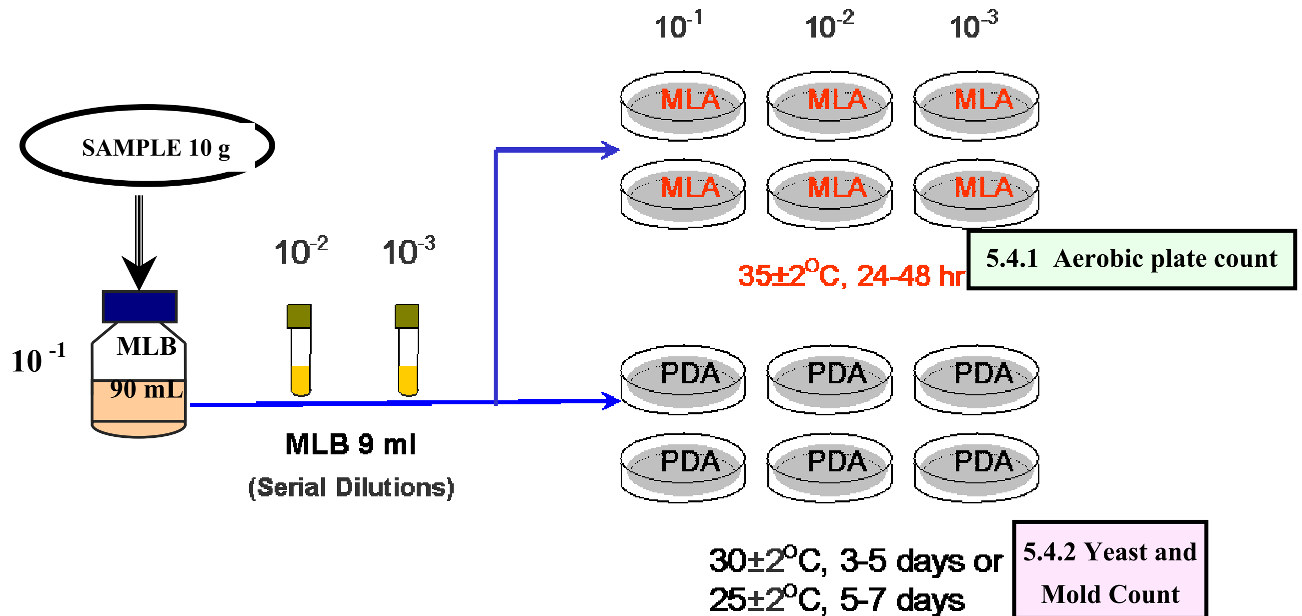

Fig.1 Enumeration diagram for cosmetic microbes. The diagram shows example of performing serial dilution up to 10^{-3} with duplicate plate counts for each dilution.

5.4.1 Aerobic plate count (APC).

5.4.1.1 Spread Plate Technique

5.4.1.1.1 Use spread plate technique to facilitate recognition of different colony types and, if necessary, for differential count. Prepare and label duplicate sets of petri dishes containing modified letheen agar (MLA) for samples at appropriate dilutions (10^{-1} to 10^{-6}). Add either 1 or 2 mL of the cosmetic preparation (refer 5.2 above) to 9 or 18 mL, respectively, of MLB, for 10^{-2} dilution.

5.4.1.1.2 Dilute samples decimally in MLB (NOTE: save dilutions for enrichment step) to obtain the appropriate dilution series (10^{-1} to 10^{-6}). Begin with 10^{-2} if all the 10^{-1} dilution is used up.

5.4.1.1.3 Thoroughly mix dilutions and pipet 0.1 mL of each dilution onto surface of solid media in prelabeled petri dishes.

5.4.1.1.4 Spread inoculum over entire surface with bent glass rod that was first sterilized by dipping in 95% ethanol and quickly flamed to remove the

ethanol. Use new spreader for each dilution (at low dilutions) because some product residue may carry over and adversely affect the flame-sterilization procedure. For effective inoculum absorption, be sure agar surface is dried (30 min at 35°C) when agar is freshly made.

5.4.1.1.5 Let medium absorb inoculum before inverting and incubating plates for 24-48 hours at 35 ± 2°C.

5.4.1.1.6 Count all colonies on plates containing 25-250 colonies, and record results per dilution counted. Calculate and report the result with reference to Annex B.

5.4.1.2 Pour Plate Technique

5.4.1.2.1 Use pour plate technique so that low level of microorganisms can be counted. Prepare and label duplicate sets of Petri dishes for samples with appropriate dilutions (10^{-1} to 10^{-5}).

5.4.1.2.2 Prepare serial dilution by transferring 1 mL of the first dilution into 9 mL suitable diluent. Mix the suspension using vortex mixer to ensure homogenous distribution.

5.4.1.2.3 Repeat the serial dilution process till the appropriate dilution is obtained.

5.4.1.2.4 Transfer 1 mL suspension from the serial dilution into the sterile petri dishes.

5.4.1.2.5 Add about 20 mL of melted MLA to the petri dishes. Cover the petri dish and carefully swirl the petri dish so that the inoculated sample mix well with the media.

5.4.1.2.6 When the agar is set, invert the petri dishes and incubate at 35 ± 2°C for 24-48 hours.

5.4.1.2.7 Count all colonies in plates containing 25-250 colonies, and record results per dilution counted. Calculate and report the result with reference to Annex B.

If no colonies are obtained on MLA, observe already prepared MLB dilutions while enriching them at 35 ± 2°C for 7 days. Examine enrichments daily for growth. After 7 days of incubation, or when growth is suspected, subculture all enrichments onto MLA plates. Incubate plates at 35 ± 2°C for 48 hours.

5.4.2 Fungi, yeast, and mold plate count.

5.4.2.1 Transfer 0.1 mL portions of dilution series above to appropriately labeled duplicate plates of either malt extract agar (MEA) or potato dextrose agar (PDA), both containing 40 ppm chlortetracycline.

5.4.2.2 Spread inoculum over surface of medium with sterile glass spreader rod. After inoculum is absorbed by medium, invert plates.

5.4.2.3 Incubate at 25 ± 2°C, and observe daily for 7 days.

5.4.2.4 Average the counts obtained on duplicate plates, multiply by 10 to allow for the volume plated (eg. 0.1 mL), multiply by the dilution factor, and report as yeast or mold count/g (mL) sample.

5.4.2.5 For fungal enrichments (optional), dilute prepared sample decimally in Sabouraud's dextrose broth and incubate as described above for MLB dilutions.

5.4.2.6 If growth occurs, streak on Sabouraud's dextrose agar, MEA, or PDA. The latter two agars should both contain 40 ppm chlortetracycline.

REMARKS

Cosmetic products are not expected to be aseptic. However, they must be completely free of high-virulence microbial pathogens, and the total number of aerobic microorganisms per gram must be low. The microorganisms which may be specified as pathogenic differ from country to country according to the national practices or regulations. Pathogens or opportunistic pathogens whose incidence would be of particular concern, especially in eye-area cosmetic products, include *S. aureus*, *Streptococcus pyogenes*, *P. aeruginosa* and other species, and *Klebsiella pneumoniae*. Some microbes normally regarded as nonpathogenic may be opportunistically pathogenic.

6 REFERENCES

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6.2 Hitchins, A.D., T.T. Tran, and J.E. McCarron. 2001. Chapter 23 Microbiological Methods for Cosmetics. *In*: U.S. FDA Bacteriological Analytical Manual Online.

6.3 Singer, S. 1987.The use of preservative neutralizers in diluents and plating media, Cosmetics and Toiletries, 102, 55.

6.4 Compendium of methods for the microbiological examination of foods.4th ed. 2001. pp. 56-57.

Harmonised method:

- **Issued by the microbial analysis group at the harmonization workshop in Kuala-Lumpur, on September 13th to 17th, 2004**
- **Approved by the harmonization workshop delegates workshop in Kuala-Lumpur, on September 13th to 17th, 2004,**
- **Modified after the Bangkok training, Nov 29th to Dec 3rd, 2004**
- **Modified (format only) in Brunei workshop Aug 30th to 31 st, 2005**
- **Modified and approved after the final review in Singapore, Nov 30th to Dec 2nd, 2005**

ANNEX A
(Informative)

Table A1. Neutralizers of antimicrobial activity of preservatives (ref. 5.3)

Preservative	Chemical compounds able to neutalize preservative's antimicrobial activity	Examples of suitable neutralizers
Phenolic compounds : Parabens, phenoxyethanol, phenylethanol, etc..Anilides	Lecithin, Polysorbate 80, Ethylene oxide condensate of fatty alcohol Non-ionic surfactants	Polysorbate 80, 30 g/l + lecithin, 3 g/l. Ethylene oxide condensate of fatty alcohol, 7 g/l + lecithin, 20 g/l + polysorbate 80, 4 g/l. D/E Neutralizing Broth
Quaternary ammonium compounds, Cationic surfactants	Lecithin, Saponine, Polysorbate 80, Sodium dodecyl sulphate, Ethylene oxide condensate of fatty alcohol	Polysorbate 80, 30 g/l + sodium dodecyl sulphate, 4 g/l + lecithin, 3 g/l. Polysorbate 80, 30 g/l + saponin, 30 g/l + lecithin, 3 g/l. D/E Neutralizing Broth
Aldehydes Formaldehyde-release agents	Glycine, Histidine	Lecithin, 3 g/l + polysorbate 80, 30 g/l + L-Histidine, 1 g/l. Polysorbate 80, 30 g/l + saponin, 30 g/l + L-Histidine, 1 g/l + L-cysteine, 1 g/l. D/E Neutralizing Broth
Oxidizing compounds	sodium thioglycollate	Sodium thiosulphate, 5 g/l.
Isothiazolinones, Imidazoles	Lecithin, Saponine, Amines, sulfates,mercaptans, Sodium Bisulfite, sodium thioglycollate	Polysorbate 80, 30 g/l + saponin, 30 g/l + lecithin, 3 g/l.
Biguanides	Lecithin, Saponine, Polysorbate 80,	Polysorbate 80, 30 g/l + saponin, 30 g/l + lecithin, 3 g/l.
Metallic salts (Cu, Zn, Hg) Organo-mercuric compounds	Sodium bisulphate, cysteine Sulfhydryl compounds, Thioglycollic acid	Sodium thioglycollate, 0.5 g/l or 5 g/l. L-cysteine, 0.8 g/l or 1.5 g/l. D/E Neutralizing Broth

ANNEX B

Guidelines for counting, calculating and reporting the amount of microorganisms (ref. 5.4)

To compute colony counts, multiply the total number of colonies (or the average number if replicate plates of the same dilution are used) per plate by the reciprocal of the dilution used. Record the dilution used and the number of colonies counted or estimated on each plate. To avoid giving false ideas of precision and accuracy when computing colony counts, record only the first two left-hand digits. Raise the second digit to the next highest number only when the third digit from the left is 5,6,7,8 or 9; use zeros for each successive digit to the right of the second digit (Table B1). Report counts (or estimates thereof) as CFU per g or mL, as applicable.

When counts on duplicate plates or consecutive dilutions are averaged, round off counts to two significant figures only at the time of conversion to the CFU per g (Table B1, Sample No. 1117).

The appropriate number of colonies to count on a plate is a function of colony size, plate size, and size of differential properties produced on the medium. Typically, 25 to 250 colonies per plate yield reliable results. Use this as a guide unless an alternate range is indicated for specific methods. The following guidelines or "rules" should be used for selecting plates and calculating the CFU per g or mL, as applicable:

1. *One plate with 25 to 250 colonies*. Select a plate with 25 to 250 colonies unless excluded by spreaders or lab accidents (see Item 5). Count all colonies, including those of pinpoint size and record the dilution used and the total number of colonies counted (Table B1, Sample Nos. 1001, 1004, 1011 and 1012)

2. *Duplicate plates.* Count plates with 25 to 250 colonies and average the counts to obtain the colony count (Table B1, Sample No. 1112). If only one plate of a duplicate pair yields 25 to 250 colonies, count both plates unless excluded by spreader and average the counts (Table B1, Sample Nos. 1113 and 1114). When counting duplicate plates from consecutive decimal dilutions, compute the count per g for each dilution and proceed as in Item 3 (Table B1, Sample Nos. 1111, 1115, 1116 and 1117)

3. *Consecutive dilutions with 25 to 250 colonies*. If plates from two consecutive decimal dilutions yield 25 to 250 colonies each, compute the count per g for each dilution and report the arithmetic average as the CFU per g, unless the higher compute count is more than twice the lower one. In that case, report the lower computed count as the CFU per g (Table B1, Sample Nos.1002, 1003, 1111, 1115, 1116 and 1117).

4. *No plate with 25 to 250 colonies*. If there is no plate with 25 to 250 colonies and one or more plates have more than 250 colonies, select plate(s) having nearest to 250 colonies and count as in Item g, for crowed plates. Report count as the estimated (est.) CFU per g (Table B1, Sample Nos. 1009 and 1118)

5. *All plates have fewer than 25 colonies.* If plates from all dilutions yield fewer than 25 colonies, record the actual number of colonies on the lowest dilution (unless excluded by spreaders) and report count as est.CFU per g (Table B1, Sample Nos. 1007 and 1119)

Table B1 Examples for computing colony count per gram or milliliter

Sample no.	Colonies counted Dilution 1:100	Colonies counted Dilution 1:1000	Count ratio[a]	Colony count[b] (CFU/g or mL)	Rule
Common application, one plate from each of two dilutions					
1001	234	23	—	23,000	1
1002	243	34	1.4	29,000	3
1003	140	32	2.3	14,000	3
1004	Spr[d]	31	—	31,000	1, 8
1005	0	0	—	<100 est.	6
1006	TNTC	7150	—	>5,600,000 est.	7
1007	18	2	—	1,800 est.	5
1008	Spr	Spr	—	Spr	8
1009	325	20	—	33,000 est.	4, 7
1010	27	215	—	LA	8
1011	305	42	—	42,000	1
1012	243	LA	—	24,000	1, 8
1013	TNTC	840	—	840,000 est.	7
Procedure where two plates per dilution are poured					
1111	228	28	1.2	25,000	2, 3
	240	26			
1112	175	16	—	19,000	2
	208	17			
1113	239	16	—	28,000	2
	328	19			
1114	275	24	—	30,000	2
	280	35			
1115	138	42	2.4	15,000	2, 3
	162	30			
1116	228	28	1.1	24,000	2, 3
	240	23			
1117	224	28	1.4	24,000	2, 3
	180	Spr			
1118	287	23	—	28,000 est.	4, 7
	263	19			
1119	18	2	—	1,700 est.	5
	16	0			
1120	0	0	—	<100 est.	6
	0	0			

[a] Count ratio is the ratio of the greater to the lesser plate count, as applied to plates from consecutive dilutions having between 25 to 250 colonies.

[b] All count should bemade in accordance with rules listed or given in the text.

[c] Underlined figures used to calculate count.

[d] spr = Spreader and adjoining area of repressed growth covering more than one-half of the plate.

[e] LA = Laboratory accident. .

6. *Plates with no colonies.* If plates from all dilutions have no colonies and inhibitory substances have not been detected, report the estimated count as less than (<) one times the corresponding lowest dilution (Table B1, Sample Nos. 1005 and 1120)

7. *Crowed plates (more than 250 colonies).* If the number of colonies per plate exceeds 250, count colonies in portion of the plate that are representative of colony distribution to estimate the aerobic colony count. If there are fewer than 10 colonies per cm^2 count the colonies in 12 cm^2, selecting six consecutive squares horizontal across the plate and six consecutive squares

8. *Spreaders.* There are three distinct types of spreaders. The first type is a chain of colonies, not too distinctly separated, that appears to be caused by disintegration of a bacteria clump when the inoculum is dispersed in or on the plating medium. If one or more chains appear to originate from separate sources, count each as one colony. Do not count each individual colony in such chain(s) as separate colonies.

The second type of spreading colony develops in a film of water between the agar and the plate. The third type forms in a film of water at the edge or over the surface of the agar. These two types develop mainly because of moisture accumulation at the point from which the spreader originates, and these spreaders may repress the growth of individual colonies. When dilution water is uniformly distributed throughout the medium, bacteria rarely develop into spreading colonies. Steps to eliminate spreaders of this type should be taken if 5% of a laboratory's plates have spreaders covering 25% of the plate.

If spreaders occur on the plate(s) selected, count colonies on representative portions thereof only when colonies are well distributed in spreader free areas and the area covered by spreader(s), including the total repressed growth area if any, does not exceed 50% of the plate area. Calculate the estimated count by multiplying the average count per cm^2 by the area of the plate. Where the repressed growth area alone exceeds 25% of the total area, report as "spreaders"(spr) or "laboratory accident" (LA) (Table B1, Sample Nos. 1008 and 1010)

Inhibitory substances in a sample may be responsible for the lack of colony formation. The analyst may suspect the presence of inhibitory substances in the sample under examination when plates show no growth or show proportionately less growth in lower dilutions. Such developments cannot, however, always be interpreted as evidence of inhibition, and unless inhibition is demonstrated, should be reported as LA.

ANNEX C

Media and Reagents, (ref 3.1)

Letheen Agar (Modified)

Letheen agar (Difco or BBL)	32	g
Trypticase peptone	5	g
Thiotone peptone	10	g
Yeast extract	2	g
NaCl	5	g
Sodium bisulfite	0.1	g
Agar	5	g
Distilled water	1	liter

Heat with agitation to dissolve agar. Autoclave 15 min at 121°C. Aseptically dispense 20 mL into 15 x 100 mm petri dishes. Final pH, 7.2 ± 0.2.

Letheen Broth (Modified)

Letheen broth	25.7	g
Trypticase peptone	5	g
Thiotone peptone	10	g
Yeast extract	2	g
Sodium bisulfite	0.1	g
Distilled water	1	liter

Dispense 90 mL into screw-cap bottle. Autoclave 15 min at 121°C. Final pH, 7.2 ± 0.2.

Malt Extract Agar (Cosmetics-General Microbiology)

Malt extract	30	g
Agar	20	g
Distilled water	1	liter

Boil to dissolve ingredients. Avoid overheating, which causes softening of agar and darkening of medium color. Autoclave 15 min at 121°C. Dispense 20-25 mL into sterile 15 x 100 mm petri dishes. Final pH, 5.5 ± 0.2.

For cosmetics use : Cool medium to 47-50°C after autoclaving. Dispense 4 mL stock filter-sterilized chlortetracycline HCl solution (1 g/100 mL) per liter of medium to yield final concentration of 40 ppm chlorotetracycline HCl. Mix thoroughly and dispense 20 mL portions into 15 x 100 mm petri dishes.

	Title	Revision n°	date	Document No
	MICROBIAL LIMIT TEST FOR COSMETIC PRODUCTS	0	2/12/2005	ACM THA 06

Potato Dextrose Agar

Potato infusion	200	g
Dextrose	20	g
Agar	20.0	g
Distilled water	1	liter

To prepare potato infusion, boil 200 g sliced, unpeeled potatoes in 1 liter distilled water for 30 min. Filter through cheesecloth, saving effluent, which is potato infusion (or use commercial dehydrated form). Mix in other ingredients and boil to dissolve. Autoclave 15 min at 121°C. Dispense 20-25 mL portions into sterile 15 x 100 mm petri dishes. Final pH, 5.6 ± 0.2.

Medium should not be re-melted more than once. Medium powder is available commercially but may require supplementing with extra agar to a final concentration of 20 g/liter. To BBL or Difco dehydrated medium, add 5 g of agar.

For cosmetics, cool medium to 47-50°C after autoclaving. Add 40 ppm (final concentration) chlortetracycline. Mix thoroughly and dispense 20 mL portions into 15 x 100 mm petri dishes. Dispense 4 mL of stock filter-sterilized chlortetracycline HCl (1 g/100 mL) per liter of medium.

Sabouraud's Dextrose Broth and Agar

Polypeptone or neopeptone	10	g
Dextrose	40	g
Distilled water	1	liter

Dissolve completely and dispense 40 mL portions into screw-cap bottles. Final pH, 5.8. Autoclave 15 min at 118-121°C. Do not exceed 121°C.

For Sabouraud's dextrose agar, prepare broth as above and add 15-20 g agar, depending on gel strength desired. Final pH, 5.6 ± 0.2. Dispense into tubes for slants and bottles or flasks for pouring plates. Autoclave 15 min at 118-121 °C.