STANDARD OPERATING PROCEDURE	
Department: Microbiology	**SOP No.:**
Title: Procedure for Antimicrobial Preservative Effectiveness Testing	**Effective Date:**
Supersedes: Nil	**Review Date:**
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1.0 OBJECTIVE:

To lay down a procedure for Antimicrobial Preservative Effectiveness Test.

2.0 SCOPE:

This SOP is applicable for Antimicrobial Preservative Effectiveness Test in Microbiology Section of Quality Control Laboratory.

3.0 RESPONSIBILITY:

Officer / Executive – Microbiology

4.0 ACCOUNTABILITY:

Head – QC

5.0 ABBREVIATIONS:

µl	Micro liter
ml	Milliliter
No.	Number
QA	Quality Assurance
QC	Quality Control
SOP	Standard Operating Procedure

6.0 PROCEDURE:

6.1 ANTIMICROBIAL PRESERVATIVES:

6.1.1 Antimicrobial preservatives are substances added to non-sterile dosage forms to protect them from microbiological growth or from microorganisms that are introduced inadvertently during or subsequent to the manufacturing process.

6.1.2 In the case of sterile products packaged in multiple-dose containers, antimicrobial preservatives are added to inhibit the growth of microorganisms that may be introduced from repeatedly withdrawing individual doses. One or more antimicrobial preservatives area expected in all multidose units.

6.1.3 All useful antimicrobial agents are toxic substances. For maximum protection of patients, the concentration of the preservative shown to be effective in the final packaged product should be below a level that may be toxic to human beings based on the recommended dosage of the medicinal product.

6.2 PREPARATION OF TEST STRAINS:

6.2.1 Subculture the following microorganisms from working culture to the given medium and Incubate at Temperature as given in below table:

S.No.	Organism	Type	Medium	Incubation Temperature and Duration
1.	*Candida albicans*	ATCC10231	Sabouraud Dextrose Agar/Sabouraud Chloramphenicol Agar	20-25^{0}C for 3-5 Days
2.	*Aspergillus brasiliensis*	ATCC16404	Sabouraud Dextrose Agar/ Sabouraud Chloramphenicol Agar	20-25^{0}C for 3-7 Days
3.	*E. coli*	ATCC 8739	Soyabean Casein Digest Agar	30-35^{0}C for 3-5 Days
4.	*P. aeruginosa*	ATCC 9027	Soyabean Casein Digest Agar	30-35^{0}C for 3-5 Days
5.	*S. aureus*	ATCC 6538	Soyabean Casein Digest Agar	30-35^{0}C for 3-5 Days
6.	*In-house Isolate*	NA	Soyabean Casein Digest Agar	30-35^{0}C for 3-5 Days

6.2.2 Harvest the culture by using 9 ml of 0.9% Sterile Saline Solution except to the one that contains *Aspergillus brasiliensis*, in this add Sterile Saline Solution with tween 80 (0.05%) and re-suspend the biomass of each culture.

6.2.3 Microbial suspension shall be adjusted to obtain a microbial count of about 1X10^8 cfu/ml.

6.2.4 Carry out the serial dilutions of each suspension up to 10^{-9} by taking 1 ml of suspension to a tube containing 9 ml of sterile saline solution (0.9% for bacteria, *C. albicans* and for *A. brasiliensis* sterile solution containing 0.05% Polysorbate 80).

6.2.5 Store the culture suspensions at 2 to 8°C for 7 days.

6.2.6 After Incubation, Count the cfu observed in plates with Colony Counter and select the plates that have between 25 to 250 cfu/plate for bacterial and Yeast culture and 8 to 80 cfu/plate for *A. brasiliensis.*

6.2.7 This value serves to determine the size of inoculum to be used in the test. If the standardized suspensions are not used promptly, before use or with test monitor the suspensions by the plate-count method to determine any loss of viability.

6.3 GROWTH PROMOTION AND SUITABILITY OF THE RECOVERY METHOD:

6.3.1 The ability of the producer to detect challenged microorganisms in the presence of a suitable neutralized product to be tested shall be established.

6.3.2 Prepare the Normal Saline, Soybean-Casein Digest Agar and Sabouraud Dextrose Agar/Sabouraud Chloramphenicol Agar media.

6.3.3 Perform the Growth promotion test of prepared media and count obtained must be at least 50% of the calculated of a standardized inoculums.

6.3.4 Product dilution of **10^{-1}, 10^{-2}** and **10^{-3}** shall be prepared in normal saline and to each dilution tube add an appropriate number of challenged organisms (*E.coli* ATCC No. 8739, *Pseudomonas aeruginosa* ATCC No. 9027, *Staphylococcus aureus* ATCC No. 6538, *Candida albicans* ATCC No. 10231 and *Aspergillus brasiliensis* ATCC No. 16404 and Environment Isolate), So that final concentration of organisms should be NMT 250 CFU/ml of Bacteria & Yeast and NMT 80 CFU/ml of *Aspergillus brasiliensis.*

6.3.5 Mix the product with each challenged inoculums dilution and transfer 1 ml from each dilution of the challenged organisms into sterilized Petri plate in duplicate.

6.3.6 For the positive control mix the same culture suspension into saline and transfer 1 ml into sterile Petri plates and add 20-25 ml of Soybean Casein Digest Agar for bacterial culture and Sabouraud Dextrose ager /Sabouraud Chloramphenicol Agar for fungal culture and incubate bacterial culture at 30-35°C for 3-5days and fungal culture at 20-25°C for 3-7 days.

6.3.7 Perform the negative control by transferring the 1ml of saline into sterile Petri plate in duplicate and add 20-25 ml Soybean Casein Digest agar medium for bacterial and Sabouraud Dextrose agar /Sabouraud Chloramphenicol Agar for fungal culture and incubate for bacteria at 30-35°C for 3-5 days and for fungal at 20-25°C for 3-7 days.

6.3.8 If the diluted product exhibits antimicrobial properties it shall be neutralized by incorporating neutralizers into diluents or the recovery media.

6.3.9 Bisulfate, Dilution, Glycine, Lecithin Mg^{+2} or Ca^{+2} ions, Polysorbate, Thioglycollate and Thiosulfate are the neutralizers that can be used to neutralize the antibacterial properties of products.

6.3.10 The ability of the procedure to measure preservative efficacy may be compromised if the method suitability requires significant dilution (10^{-2} or 10^{-3}) as this will affect the measured recovery (e.g., it may be difficult to measure a 3 log unit reduction for a 10^{5}- 10^{6} inoculum).

6.3.11 If no suitable neutralizing agent or method is found and method suitability requires significant dilution, a higher level of inoculum (e.g., 10^{7}-10^{8}) may be used so that a 3 log unit reduction can be measured.

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6.4 TESTING OF PRODUCTS:

6.4.1 For the purpose of testing Preservative efficacy test is divided into four categories of formulation. The criteria of antimicrobial effectiveness for these categories area a function of the route of administration. It is expected that formulations containing preservatives will meet minimal efficacy standards, whether packaged as multidoses or unit doses. Following are the Product categories:

Table-1

Categories	Product description
1	**Injection:** other parenterals including emulsions, otic products, sterile nasal products and ophthalmic products made with aqueous bases or vehicles.
2	Topically used products made with aqueous bases or vehicle; non sterile nasal products and emulsions, including those applied to mucous membranes.
3	Oral products other than antacids, made with aqueous bases or vehicles.
4	Antacids made with an aqueous base.

6.4.2 The test can be conducted either in 6 original containers/tubes if a sufficient volume (minimum 10 ml) of product is available in each container and can be entered aseptically (e.g., needle and syringe through a elastomeric rubber stopper) or in 6 sterile capped bacteriological containers of suitable size into which a sufficient volume of product can be transferred.

6.4.3 Mark the each container with Name of product date of testing and date of completion and name of the challenged organism and other relevant information if required.

6.4.4 Each container shall be inoculated with one of the prepared and standardized inoculum and mix.

6.4.5 The volume of suspension inoculum used in between 0.5% and 1.0% of the volume of the product to minimize the potential effects on the product.

6.4.6 The concentration of microorganisms that is added to the product (Categories 1, 2 or 3) is such that the final concentration of the test preparation after inoculation is between 1X 10^5 and 1X10^6 cfu/ml of the product.

6.4.7 The initial concentration of viable microorganisms in each test preparation is estimated based on the concentration of microorganisms in each of the standardized inoculum as determined by the plate-count method.

6.4.8 Incubate the inoculated container at 20-25°C; take sample at the appropriate intervals as specified in below table.

Table-2

For Category 1 Products	
Bacteria	NLT 1.0 log reduction from the initial calculated count at 7 days, NLT 3.0 log reduction from the initial count at 14 days, and no increase from the 14 days' count at 28 days.
Yeast and Molds	No increase from the initial calculated count at 7, 14 and 28 days.
For Category 2 Products	
Bacteria	NLT 2.0 log reduction from the initial calculated count at 14 days, and no increase from the 14 days' count at 28 days.
Yeast and Molds	No increase from the initial calculated count at 14 and 28 days.
For Category 3 Products	
Bacteria	NLT 1.0 log reduction from the initial calculated count at 14 days, and no increase from the 14 days' count at 28 days.
Yeast and Molds	No increase from the initial calculated count at 14 and 28 days.
For Category 4 Products	
Bacteria, Yeast and Molds	No increase from the initial calculated count at 14 and 28 days.

6.4.9 Reduction in count shall be determined by Plate count method or membrane filtration methods at each interval.

6.4.10 Plate count shall be done by transferring the 1 ml sample from each container into sterile Petri plate in duplicate separately. In case of membrane filter; duplicate membrane filter shall be used for each estimate.

6.4.11 Using the calculated concentration of cfu/ml present at the start of the test, calculate the changes in log 10 values of the concentration of cfu/ml for each microorganism at the applicable test intervals, and express the changes in concentration in terms of the log reductions.

6.4.12 The log reduction is defined as the difference between the log 10 unit value of the starting concentration of cfu/ml in the suspension and the log 10 unit value of cfu/ml of the survivors at that time point.

6.4.13 Mix the contents with medium & allow solidifying and incubating the plates for bacterial count at 30 to 35°C for 3 to 5 days and for fungal count at 20 - 25°C for 3 to 7 days.

6.4.14 Determine the Viable Count at 0, 7, 14, 28 days subsequent to inoculation.

6.4.15 Record the results in **Annexure-I**, Titled **"Antimicrobial Preservative Test Report"**.

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6.5 ACCEPTANCE CRITERIA:

6.5.1 The requirements for the antimicrobial effectiveness are met if the criteria specified in **Table-2** are met.

6.5.2 **"No increase"** in counts is defined as NMT 0.5 $\log_{10}$ unit more than the value to which it is compared.

7.0 ANNEXURES:

ANNEXURE No.	TITLE OF ANNEXURE	FORMAT No.
Annexure – I	Antimicrobial Preservative Test Report	

ENCLOSURES: SOP Training Record

8.0 DISTRIBUTION:

- Controlled Copy No.01 — Quality Assurance
- Controlled Copy No.02 — Microbiology
- Master Copy — Quality Assurance

9.0 REFERENCES:

- United State Pharmacopeia (USP)

10.0 REVISION HISTORY:

CHANGE HISTORY LOG

Revision No.	Change Control No.	Details of Changes	Reason for Change	Effective Date	Updated By

PHARMA DEVILS

MICROBIOLOGY DEPARTMENT

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ANNEXURE-I
ANTIMICROBIAL PRESERVATIVE TEST REPORT

Name of product		**Batch No.**	
Qty. sampled		**Name of preservative**	
Mfg. date		**Exp. Date**	
Incubator I.D (20 – 25°C)		**Incubator I.D (30 – 35°C)**	
Product category		**Date of completion**	

Inoculums Preparation Details:

Date of Preparation		**Prepared by Sign & Date**	

Name of culture	Media Used	Reference No.	cfu / ml observed with respect to dilution									Observed by Sign & Date	Checked by Sign & Date
			10^{-1}	10^{-2}	10^{-3}	10^{-4}	10^{-5}	10^{-6}	10^{-7}	10^{-8}	10^{-9}		
E. coli ATCC 8739													
S. aureus ATCC 6538													
P. aeruginosa ATCC 9027													
C. albicans ATCC 10231													
A.brasiliensis ATCC 16404													
In-house Isolate													

Product Inoculation and Observation Details

Date of Inoculation		**Inoculated by Sign & Date**	

Name of Culture	Inoculums cfu/ml	Product qty. (ml)	Inoculum Used (in ml)	OBSERVATION						
				0 Day	7 Days		14 Days		28 Days	
					Cfu/ml	Log Reduction	Cfu/ml	Log Reduction	Cfu/ml	Log Reduction
E. coli ATCC 8739										
S. aureus ATCC6538										

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P. aeruginosa ATCC 9027										
C. albicans ATCC 10231										
A.brasiliensis ATCC 16404										
In-house Isolate										

Analysis details of '0' day

Date of Analysis		**Analyzed By Sign & Date**	

Name of culture	**Media Used**	**Cfu/ml observed with respect to dilution**							**Observed by Sign & Date**	**Checked by Sign & Date**
		10^{-2}	**10^{-3}**	**10^{-4}**	**10^{-5}**	**10^{-6}**	**10^{-7}**	**10^{-8}**		
E. coli ATCC 8739										
S. aureus ATCC 6538										
P. aeruginosa ATCC 9027										
C. albicans ATCC 10231										
A. brasiliensis ATCC 16404										
In-house Isolate										

Analysis details of '7' days

Date of Analysis		**Analyzed By Sign & Date**	

Name of culture	**Media Used**	**Cfu/ml observed with respect to dilution**							**Observed by Sign & Date**	**Checked by Sign & Date**
		10^{-2}	**10^{-3}**	**10^{-4}**	**10^{-5}**	**10^{-6}**	**10^{-7}**	**10^{-8}**		
E. coli ATCC 8739										
S. aureus ATCC6538										
P. aeruginosa ATCC 9027										
C. albicans ATCC 10231										

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A. brasiliensis ATCC 16404										
In-house Isolate										

Analysis details of '14' days:

Date of Analysis		**Analyzed By Sign & Date**	

Name of culture	**Media Used**	**Cfu/ml observed with respect to dilution**							**Observed by Sign & Date**	**Checked by Sign & Date**
		10^{-2}	**10^{-3}**	**10^{-4}**	**10^{-5}**	**10^{-6}**	**10^{-7}**	**10^{-8}**		
E. coli ATCC 8739										
S. aureus ATCC6538										
P. aeruginosa ATCC 9027										
C. albicans ATCC 10231										
A. brasiliensis ATCC 16404										
In-house Isolate										

Analysis details of '28' days:

Date of Analysis		**Analyzed By Sign & Date**	

Name of culture	**Media Used**	**Cfu/ml observed with respect to dilution**							**Observed by Sign & Date**	**Checked by Sign & Date**
		10^{-2}	**10^{-3}**	**10^{-4}**	**10^{-5}**	**10^{-6}**	**10^{-7}**	**10^{-8}**		
E. coli ATCC 8739										
S. aureus ATCC6538										
P. aeruginosa ATCC 9027										
C. albicans ATCC 10231										
A. brasiliensis ATCC 16404										
In-house Isolate										

PHARMA DEVILS

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Acceptance Criteria

Categories	Product description	Organism	Acceptance Criteria
1	**Injection:** other parenteral including emulsions, otic products, sterile nasal products and ophthalmic products made with aqueous bases or vehicles.	Bacteria	NLT 1.0 log reduction from the initial calculated count at 7 days, NLT 3.0 log reduction from the initial count at 14 days, and no increase from the 14 days' count at 28 days.
		Yeast and Molds	No increase from the initial calculated count at 7, 14 and 28 days.
2	Topically used products made with aqueous bases or vehicle; non sterile nasal products and emulsions, including those applied to mucous membranes.	Bacteria	NLT 2.0 log reduction from the initial calculated count at 14 days, and no increase from the 14 days' count at 28 days.
		Yeast and Molds	No increase from the initial calculated count at 14 and 28 days.
3	Oral products other than antacids, made with aqueous bases or vehicles.	Bacteria	NLT 1.0 log reduction from the initial calculated count at 14 days, and no increase from the 14 days' count at 28 days.
		Yeast and Molds	No increase from the initial calculated count at 14 and 28 days.
4	Antacids made with an aqueous base.	Bacteria Yeast and Molds	No increase from the initial calculated count at 14 and 28 days.

Remarks: The above sample complies / does not comply as per acceptance criteria.

Done By:
Date

Reviewed By:
Date