STANDARD OPERATING PROCEDURE				
Title: Antimicrobial Effectiveness Testing				
SOP No.:		**Department:**	Microbiology	
		Effective Date:		
Revision No.:	00	**Revision Date:**		
Supersede Revision No.:	Nil	**Page No.:**	1 of 16	

1.0 OBJECTIVE

To describe the procedure to demonstrate the efficacy of the antimicrobial agent used in a final dosage form, when challenged with a prescribed inoculums of suitable microorganism and storing the inoculated preparation at a prescribed temperature.

2.0 SCOPE

This SOP is applicable for testing of effectiveness of antimicrobial preservatives in various products at microbiology laboratory.

3.0 RESPONSIBILITY

Prepared by - Executive Microbiology

Checked by - Assistant Manager

Approved by - Head QA/QC

4.0 PROCEDURE

4.1 General

4.1.1 The antimicrobial activity of the preparation in its final container is investigated over the period of validity to ensure that such activity has not been impaired by storage.

4.1.2 This test consists of challenging the preparation, wherever possible in its final container with a prescribed inoculum of suitable microorganisms.

4.1.3 Store the inoculated preparation at a prescribed temperature.

4.1.4 Samples are withdrawn at specific intervals of time and counting of organisms is carried out to determine the viable count.

4.1.5 This test is carried out for multiple dose injections, tropical and oral dosage form and other dosage form such as ophthalmic, otic, nasal, irrigation product and dialysis fluid.

4.1.6 The test is not intended to be used for routine control purposes.

4.2 Culture medium and Culture organisms

4.2.1 Following culture organisms is to be used for the test.

Test Organisms	ATCC No.
Candida albicans	ATCC 10231 or equivalent
Aspergillus niger	ATCC 16404 or equivalent
Pseudomonas aeruginosa	ATCC 9027 or equivalent
Staphylococcus aureus	ATCC 6538 or equivalent
Escherichia coli	ATCC 8739 or equivalent
Culture Media	
Soya bean Casein digest agar	
Sabourauds Dextrose agar	

4.2.2 Prepare all the culture media as per the SOP.

4.3 Preparation of Inoculum

4.3.1 From a recently grown stock culture of each of the test organisms, subculture on the surface of respective media slants (2 each) as given below.

Test Organisms	Suitable Culture Medium	Incubation Temperature	Inoculum Incubation Time	Microbial Recovery Incubation Time
S. aureus ATCC 6538	SCDA	32.5 ± 2.5°C	18 - 24 hrs	3 - 5 days
E. coli ATCC 8739	SCDA	32.5 ± 2.5°C	18 - 24 hrs	3 - 5 days
P.aeruginosa ATCC 9027	SCDA	32.5 ± 2.5°C	18 - 24 hrs	3 - 5 days
C. albicans ATCC 10231	SDA	22.5 ± 2.5°C	44 - 52 hrs	3 - 5 days
A. niger ATCC 16404	SDA	22.5 ± 2.5°C	6 - 10 days	3 - 7 days

4.3.2 Use about 9-10 ml of sterile saline TS (0.9 % w/v) for harvesting of bacterial cultures and *Candida albicans*.

4.3.3 Add sufficient sterile saline to obtain the microbial count to about $1x10^8$ cfu/ml.

4.3.4 Use about 9-10 ml of sterile saline TS (0.9 % w/v) with 0.05 % polysorbate-80 for harvesting of *Aspergillus niger* culture.

4.3.5 Add sufficient sterile saline to obtain the microbial count to about $1x10^8$ cfu/ml.

4.3.6 Refrigerate the culture suspension if it is not used with in 2 hours.

4.3.7 Determine the number of cfu / ml in each suspension by carrying out the viable count.

4.3.8 This value serves to determine the inoculum and the baseline to use in the test.

4.3.9 The bacterial suspensions are to be used within 24 hours of harvesting but the fungal preparation may be stored under refrigeration for up to seven days.

4.4 Total Viable Count

4.4.1 Prepare ten fold serial dilutions using sterile saline TS (0.9 % w/v) for bacterial cultures and *Candida albicans*.

4.4.2 Prepare ten fold serial dilutions using sterile saline TS (0.9 % w/v) with 0.05 % polysorbate-80 for *Aspergillus niger*.

4.4.3 Using the pour plate technique, plate out, in duplicate the last five dilutions using the SCDA medium in case of bacteria and SDA medium in case of yeast and molds.

4.4.4 Gently rotate the plate for proper mixing of culture suspension and culture medium and then allow solidifying the plates.

4.4.5 Invert the above medium plates and incubate the plates for bacterial culture at 32.5 ± 2.5 ^{O}C for 3 - 5 days and the plates for yeast and molds culture at 22.5 ± 2.5 ^{O}C for 3 - 7 days respectively.

4.4.6 After completion of incubation count the no. of cfu / ml.

4.4.7 Record all the result of total viable count in the annexure - I.

4.5 Testing of Product

4.5.1 The test can be carried out either in the five original containers, if sufficient volume of product is available in each container.

4.5.2 The product container can be entered aseptically (i.e. using a needle & syringe through an elastomeric rubber stopper), or in sterile, capped bacteriological containers of suitable size into which a sufficient volume (e.g. 10 - 20 ml) of product has been transferred.

4.5.3 Inoculate each original product container or sterile capped bacteriological tube with one of the standardized microbial suspensions using a ratio equivalent to 0.1 ml to 0.2 ml of inoculum suspension to 20 ml of product.

4.5.4 Mix thoroughly to ensure homogeneous distribution.

4.5.5 The final concentration of test organisms should be between 1×10^5 to 1×10^6 microorganisms per ml of product (for those of category 1, 2, & 3).

4.5.6 The final concentration of test organisms should be between 1 x 10^3 to 1 x 10^6 microorganisms per ml of product (for those of category 4).

4.5.7 The initial concentration of viable microorganisms in each test preparation is calculated on the basis of the count of microorganisms determined in the inoculum as determine by plate count method.

4.5.8 Incubate the inoculated containers / tubes at 22.5 ± 2.5 ^{O}C up to the end of the test.

4.5.9 Withdrawn the sample from each inoculated after appropriate time interval.

4.5.10 Record any changes observed in appearance at these interval.

4.5.11 Prepare serial dilutions using sterile saline TS (0.9 % w/v) for bacterial cultures and *Candida albicans*.

4.5.12 Prepare ten fold serial dilutions using sterile saline TS (0.9 % w/v) with 0.05 % polysorbate-80 for *Aspergillus niger* culture.

4.5.13 Prepare serial dilutions up to 10^{-6}.

4.5.14 Using the pour plate technique, plate out, in duplicate the dilutions using the SCDA medium in case of bacteria and SDA medium in case of yeast and molds.

4.5.15 Gently rotate the plate for proper mixing of culture suspension and culture medium and then allow solidifying the plates.

4.5.16 Invert the above medium plates and incubate the plates for bacterial culture at 32.5 ± 2.5 ^{O}C for 3 - 5 days and the plates for yeast and molds culture at 22.5 ± 2.5 ^{O}C for 3 - 7 days respectively.

4.5.17 Calculate the initial concentration of the inoculum in product (at zero day - Initial).

4.5.18 Carry out the control test along with the product test.

4.5.19 Take 10-20ml of sterile 0.1 % peptone in screw-capped bacteriological tubes, and inoculate with volumes of the microbial culture suspensions separately as used in the product.

4.5.20 Perform the step 4.5.11 to 4.5.17 for control test.

4.5.21 The viable count observe in control test will serve as the base line to determine the number of organisms used for challenge.

4.5.22 Draw suitable samples at different time intervals and determine the viable count as follows.

- For Category 1 Product - 7th day, 14th day and 28th day.
- For Category 2,,and 4 Product - 14th day and 28th day.

4.5.23 Tabulate the initial count and the counts obtained at different time intervals in the annexure - I to assess the antimicrobial effectiveness.

4.6 Product Category

The product categories are as given below.

Category	Product Description
1.	Injection, other parenterals including emulsions, otic products, sterile nasal products, and ophthalmic products made with aqueous base or vehicles
2.	Tropically used products made with aqueous base or vehicles, non sterile nasal products and emulsions, including those applied to mucous membranes
3.	Oral products other than antacids, made aqueous base or vehicles
4.	Antacids made with an aqueous base

4.7 Interpretation of Result and Acceptance Criteria

4.7.1 There should be required reduction in count of viable microorganisms at different time intervals.

4.7.2 Using the calculated concentration of Cfu/ml present at the start of the test, calculate the change in log value of the concentration of cfu/ml for each microorganism at the applicable test intervals, and express the change in term of log reduction.

4.7.3 Acceptance criteria are given below -

Test Organisms	Acceptance Criteria
For Category 1 Products	
Bacteria	Not less than 1.0 log reduction from the initial calculated count at 7^{th} day. Not less than 3.0 log reduction from the initial calculated count at 14^{th} day. No increase from the 14^{th} day count at 28^{th} day.
Yeast & Molds	No increase from the initial calculated count at 7^{th}, 14^{th} and 28^{th} day.
For Category 2 Products	
Bacteria	Not less than 2.0 log reduction from the initial calculated count at 14^{th} day and no increase from the 14^{th} day count at 28^{th} day.

Yeast & Molds	No increase from the initial calculated count at 14th and 28th day.
For Category 3 Products	
Bacteria	Not less than 1.0 log reduction from the initial count at 14th day and no increase from the 14th day count at 28th day.
Yeast & Molds	No increase from the initial calculated count at 14th and 28th day.
For Category 4 Products	
Bacteria, Yeast & Molds	No increase from the initial calculated count at 14th and 28th day.

5.0 SAFETY & PRECAUTIONS

After completion of analysis discard the inoculated product containers and media plates as per the SOP.

6.0 REVISION HISTORY

Revision No.	Reason for Revision	Superseded from & date
00	New	--------

7.0 REFERENCES

SOP and United State Pharmacopoeia - 30 Volume 1.

8.0 ABBREVIATIONS

SOP : Standard Operating Procedure

No. : Number

QC : Quality Control

SCDA : Soyabean casein Digest Agar

SDA : Sabourauds Dextrose Agar

QA	:	Quality Assurance
ATCC	:	American Type Culture Collection
°C	:	Degree Centigrade
hrs	:	Hours
%	:	Percentage
ml:	:	Milliliter
cfu	:	Colony forming unit

9.0 ANNEXURES

Annexure - I : Record Sheet for Antimicrobial Effectiveness Testing.

ANNEXURE – I

RECORD SHEET FOR ANTIMICROBIAL EFFECTIVENESS TESTING

Name of the Product / Material: _____Batch No.: _____A. R. No.: _____

PREPARATORY TEST:

Detail of Sub Culturing and Harvesting:

Test Organisms	ATCC No.	Sub culturing					Harvesting	
		Working culture passage no.	Sub culturing done by / on	Media used for sub culturing	Incubation temperature / time	Observation done by/ on	Media used for harvesting	Harvesting done by / on
C.albicans	ATCC 10231 or equivalent							
A. niger	ATCC 16404 or equivalent							
P.aeruginosa	ATCC 9027 or equivalent							
S. aureus	ATCC 6538 or equivalent							
E.coli	ATCC 8739 or equivalent							

Determination of Initial CFU/ mL of Harvested Test Organisms (Stock):

Test Organisms	ATCC No.	Plate No.	Dilution of Stock										No. of cells / mL of stock	Dilution to be used for test (Inoculums)
			10^{-1}	10^{-2}	10^{-3}	10^{-4}	10^{-5}	10^{-6}	10^{-7}	10^{-8}	10^{-9}	10^{-10}		
C.albicans	ATCC 10231 or equivalent	I st plate												
		II nd plate												
		Average												
A. niger	ATCC 16404 or equivalent	I st plate												
		II nd plate												
		Average												

Name of the Product / Material: _____Batch No.: _____A. R. No.: _____

PREPARATORY TEST:

Determination of Initial CFU/ mL of Harvested Test Organisms (Stock):

Test Organisms	ATCC No.	Plate No.	Dilution of Stock										No. of cells / mL of stock	Dilution to be used for test (Inoculums)
			10^{-1}	10^{-2}	10^{-3}	10^{-4}	10^{-5}	10^{-6}	10^{-7}	10^{-8}	10^{-9}	10^{-10}		
P.aeruginosa	ATCC 9027 or equivalent	I st plate												
		II nd plate												
		Average												
S. aureus	ATCC 6538 or equivalent	I st plate												
		II nd plate												
		Average												
E.coli	ATCC 8739 or equivalent	I st plate												
		II nd plate												
		Average												

Media Details:

Name of the Media Used For Sub culturing	Sterilized Media Lot No.	Name of the Media Used For Harvesting and Suspension / Dilution Preparation	Sterilized Media Lot No.
Soyabean Casein Digest Agar		Sterile saline (0.9% w/v)	
		Sterile saline (0.9% w/v) + Polysorbate 80 (0.05% w/v)	
Sabourauds Dextrose Agar		Soyabean Casein Digest Agar	
		Sabourauds Dextrose Agar	

Name of the Product / Material: _____Batch No.: _____A. R. No.: _____

PREPARATORY TEST:

Calculation for the addition of inoculum in the product:

Container No.	Test Organisms	ATCC No.	Quantity of inoculum added	Concentration of inoculum	Volume of the product	Final concentration of inoculum (CFU/mL of the product)
1.	C.albicans	ATCC 10231 or equivalent				
Control Test					0.1 % Peptone	
2.	A. niger	ATCC 16404 or equivalent				
Control Test					0.1 % Peptone	
3.	P.aeruginosa	ATCC 9027 or equivalent				
Control Test					0.1 % Peptone	
4.	S. aureus	ATCC 6538 or equivalent				
Control Test					0.1 % Peptone	
5.	E.coli	ATCC 8739 or equivalent				
Control Test					0.1 % Peptone	

Note: Add between 0.5 to 1.0 % inoculum suspension of the volume of the product to yield a concentration of $1x10^5$ to $1x10^6$ CFU/ml of the product as per the below mentioned formulae.

$$\text{CFU / ml of the product} = \frac{\text{\_\_\_\_\_ml of inoculum suspension taken x CFU / ml of the inoculum}}{\text{Volume of the product}}$$

Analysis done by: _____ Observation done by: _____ Checked by: _____
(Sign & Date) (Sign & Date) (Sign & Date)

PHARMA DEVILS

PRODUCTION DEPARTMENT

STANDARD OPERATING PROCEDURE

Title: Antimicrobial Effectiveness Testing

SOP No.:		**Department:**	Microbiology
		Effective Date:	
Revision No.:	00	**Revision Date:**	
Supersede Revision No.:	Nil	**Page No.:**	11 of 16

Name of the Product / Material: _____Batch No.: _____A. R. No.: _____

TEST:

Tested on / by	0 Day	7th Day	14th Day	28th Day

Storage condition of inoculated product: 22.5°C ± 2.5 °C for 28 days.

Total Viable Count of Test Organisms in Inoculated Product (CFU/mL)

Culture Organisms		Candida albicans					Aspergillus niger					Pseudomonas aeruginosa				
		Control Test	Initial (0 Day)	7th Day	14th Day	28th Day	Control Test	Initial (0 Day)	7th Day	14th Day	28th Day	Control Test	Initial (0 Day)	7th Day	14th Day	28th Day
Physical appearance →																
Dilution Factor	Plate No.															
10^{-1}	I st plate															
	II nd plate															
	Average															
10^{-2}	I st plate															
	II nd plate															
	Average															
10^{-3}	I st plate															
	II nd plate															
	Average															

Name of the Product / Material: _____Batch No.: _____A. R. No.: _____

TEST:

Total Viable Count of Test Organisms in Inoculated Product (CFU/mL)																
Culture Organisms		**Candida albicans**					**Aspergillus niger**					**Pseudomonas aeruginosa**				
		Control Test	**Initial (0 Day)**	**7th Day**	**14th Day**	**28th Day**	**Control Test**	**Initial (0 Day)**	**7th Day**	**14th Day**	**28th Day**	**Control Test**	**Initial (0 Day)**	**7th Day**	**14th Day**	**28th Day**
Physical appearance →																
Dilution Factor	Plate No.															
10^{-4}	I st plate															
	II nd plate															
	Average															
10^{-5}	I st plate															
	II nd plate															
	Average															
10^{-6}	I st plate															
	II nd plate															
	Average															
Final Concentration (CFU/mL)																
Observation done by / on																

Name of the Product / Material: _____Batch No.: _____A. R. No.: _____

TEST:

Tested on / by	0 Day	7th Day	14th Day	28th Day

Storage condition of inoculated product: 22.5°C ± 2.5 °C for 28 days.

Total Viable Count of Test Organisms in Inoculated Product (CFU/mL)

Culture Organisms		Staphylococcus aureus					Escherichia coli				
		Control Test	Initial (0 Day)	7th Day	14th Day	28th Day	Control Test	Initial (0 Day)	7th Day	14th Day	28th Day
Physical appearance →											
Dilution Factor	Plate No.										
10^{-1}	I st plate										
	II nd plate										
	Average										
10^{-2}	I st plate										
	II nd plate										
	Average										
10^{-3}	I st plate										
	II nd plate										
	Average										

Name of the Product / Material: _____Batch No.: _____A. R. No.: _____

TEST:

Total Viable Count of Test Organisms in Inoculated Product (CFU/mL)										
Culture Organisms		**Staphylococcus aureus**					**Escherichia coli**				
		Control Test	**Initial (0 Day)**	**7th Day**	**14th Day**	**28th Day**	**Control Test**	**Initial (0 Day)**	**7th Day**	**14th Day**	**28th Day**
Physical appearance →											
Dilution Factor	Plate No.										
10^{-4}	I st plate										
	II nd plate										
	Average										
10^{-5}	I st plate										
	II nd plate										
	Average										
10^{-6}	I st plate										
	II nd plate										
	Average										
Final Concentration (CFU/mL)											
Observation done by / on											

Name of the Product / Material: _____Batch No.: _____A. R. No.: ___ _____

MEDIA DETAIL:

Name of the Media	Sterilized Media Lot No.				
	Control Test	Initial (0 Day)	7th Day	14th Day	28th Day
Sterile saline (0.9% w/v)					
Sterile saline (0.9% w/v) + Polysorbate 80 (0.05% w/v)					
0.1 % Peptone		Not Applicable			
Soyabean Casein Digest Agar					
Sabourauds Dextrose Agar					

INSTRUMENT DETAIL:

Incubator ID Temperature range - 20 - 25ºC		Colony Counter ID	
Incubator ID Temperature range - 30 - 35ºC		LAF ID	

Checked by: _____
(Sign & Date)

Name of the Product / Material: _____Batch No.: _____A. R. No.: _____

LOG REDUCTION CALCULATION:

Note: Calculate the log reduction as per the below mentioned formulae.

Log Reduction = [Log of Final Concentration {CFU/ml at 0 day (Initial)} - Log of Final Concentration {CFU/ml at 7th / 14th / 28th day}]

Container No.	Test Organisms	Final Concentration in Product									Log Reduction		
		Control Test	Initial (0 Day)		7th Day		14th Day		28th Day		7th Day	14th Day	28th Day
		CFU/mL	CFU/mL	Log value	CFU/mL	Log value	CFU/mL	Log value	CFU/mL	Log value			
1.	C.albicans												
2.	A. niger												
3.	P.aeruginosa												
4.	S. aureus												
5.	E.coli												

ACCEPTANCE CRITERIA:

BACTERIA	Not less than 1.0 log reduction from the initial calculated count at 7 days, Not less than 3.0 log reduction from the initial count at 14 days, Not increase from the 14 days' count at 28 days.	YEAST AND MOLDS	Not increase from the initial calculated count at 7, 14 & 28 days.

RESULT: The product complies / does not comply as per the laid down specification.

Observation done by: _____ Checked by: _____

(Sign & Date) (Sign & Date)