PHARMA DEVILS

MICROBIOLOGY DEPARTMENT

STANDARD OPERATING PROCEDURE

Title: Microbial Culture Suspension Preparation, Cell Enumeration, Use, Storage and Destruction

SOP No.:		**Department:**	Microbiology	
		Effective Date:		
Revision No.:	00	**Revision Date:**		
Supersede Revision No.:	Nil	**Page No.:**	1 of 21	

1. **Purpose:** The purpose of this SOP is to describe the procedure for the preparation of culture suspension and enumeration of cells.

2. **Scope:** This SOP is applicable to Bacterial and Fungal cultures used for Growth promotion test.

3. **References, Attachments & Annexures:**
   - 3.1 **References:**
     - 3.1.1 BP
     - 3.1.2 USP
     - 3.1.3 SOP No.: Procedure for procurement, Transfer,Preservation and Disposal of Microbiological Culture.
   - 3.2 **Attachments:**
     - 3.2.1 Attachment–1:Flow chart for the preparation of stock culture suspensions and cell enumeration
     - 3.2.2 Attachment–2:Template for the preparation of microbial culture suspensions and cell enumeration
     - 3.2.3 Attachment–3: Culture suspension validation template
   - 3.3 **Annexures:** None

4. **Responsibilities:**
   - 4.1 **Microbiologist:**
     - 4.1.1 To perform the activity as per SOP
     - 4.1.2 To maintain all the records as per SOP
   - 4.2 **QC Head or designee:**
     - 4.2.1 To check the SOP.
     - 4.2.2 To give training to all concerned persons before implementation of SOP
   - 4.3 **Quality Assurance:**
     - 4.3.1 To check the SOP.

4.3.2 To ensure the implementation of system as per SOP.

4.4 **Regulatory Affairs, Quality Head , Plant Head:**

4.4.1 To review and approve the SOP.

5. **Distribution:**

5.1 Quality Assurance

5.2 Quality Control (Microbiology section)

6. **Abbreviations & Definition of Terms :**

6.1 **Abbreviations:**

6.1.1 ATCC : American type culture collection

6.1.2 NCTC : National culture type collection

6.1.3 Approx. : Approximately

6.2 **Definition of Terms**

6.2.1 **SOP:** Standard Operating Procedure

6.2.2 **Growth promotion test:** Also refered to as fertility or nutritive properties test, which is performed on the media used during different tests like sterility test, microbial limit test, preservative efficacy test etc to demonstrate that it is capable of supporting the growth of micro- organisms.

6.2.3 **Colony forming unit (CFU):** Visible outcome of growth of micro-organisms arising from a single or multiple cells.

7. **Procedure:**

7.1 **Preparation of culture suspension, dilutions, enumeration of cells and record**

7.1.1 Follow the procedure described in Microbial Culture Suspension Preparation, and Cell enumeration as per attachment-1.

7.2 **Preparation of stock culture suspension & it's usage:**

7.2.1 Use appropriate quantity and dilution for the preparation of stock culture suspension.

7.2.2 Use the appropriate quantity of the stock culture suspension as per the requirement of the test.

7.2.3 In case of intermittent use of the culture dilutions, remove them from refrigerator at least half an hour before use.

7.2.4 Transfer back the culture dilutions in refrigerator within two hours

7.3 **Storage of stock culture suspensions:**

7.3.1 Store the culture suspension in refrigerator up to one month from the date of preparation .

7.4 **Validation of the hold time of stock culture suspension:**

7.4.1 Validate the hold (storage) time by enumerating the count in culture suspension at 30th day and compare count with initial count as per attachment-3.

7.4.2 Acceptance criteria: Count of the stored culture suspension should be within 50-200% (factor 2) of the initial count.

7.5 **Destruction of culture suspensions.:**

7.5.1 Follow the procedure defined in Procurement,transfer,preservation and disposal of microbiological culture SOP and maintain the record .

Attachment -1

Flow chart for the preparation of stock culture suspensions and cell enumeration

Flow chart for the preparation of stock culture suspensions and cell enumeration:

Transfer microbial culture from working culture tubes to broth or agar media and incubate.

Observe the growth and harvest the cells (incase of bacterial cultures.use 24 hr. Broth cultures for preparation of further dilutions)

Serial dilutions of microbial cultures.

Cell count of last 4-5 dilutions of culture suspension by pour plate method for identification of dilution containing
<250 cfu per ml
<50 cfu per ml

Select previous dilution of above dilution for stock culture preparation

Confirmation of the count in stock culture suspension and usage

Storage of the stock culture suspension and in refrigerator when not in use.

Attachment -2

Template for the preparation of microbial culture suspensions and cell enumeratio

S.No.	Activity			Sign./ Dt.
1.0	**Bacterial culture suspension preparation, dilution and cell density enumeration.**			
1.1	**Bacterial culture suspension preparation.**			
1.1.1	**Organisms to be used**	**Working culture ID**	**Organisms used**	
	1. *Pseudomonas aeruginosa* ATCC 9027			
	2. *Staphylococcus aureus* ATCC 6538			
	3. *Escherichia coli* ATCC 8739			
	4. *Salmonella spp*. NCTC 6017			
	5. *B. subtilis* ATCC 6633			
1.1.2	Identify five containers having ____(10 ml) Soybean Casein Digest Broth as, PAB : Broth culture for ***P. aeruginosa.*** SAB : Broth culture for ***S. aureus.*** ECB : Broth culture for ***E. Coli.*** SLB : Broth culture for ***Salmonella spp.*** BSB : Broth culture for ***B. subtilis.***			
1.1.3	Inoculate a loopful of the respective bacterial strains separately in above broth media containers. Incubate at at _____(30-35°C) for ____(18-24) hours.			

Attachment -2

Template for the preparation of microbial culture suspensions and cell enumeratio

S.No.	Activity						Sign./ Dt.
1.1.4	Observe the containers for growth (Turbidity). Record the details in below table.						
	Culture suspension preparation details	***Ps. aeruginosa***	***S. aureus***	***E. coli***	***Salmonella spp.***	***B. subtilis***	
	Identification of media containers						
	Lot no. of SCDM broth used.						
	Incubation in date						
	Incubator no.						
	Incubation out date						
	Growth observed /not observed						
	Note- After growth, if culture is not used within 2 hrs, store upto 24 hrs at 2-8°C						
1.2	**Preparation of serial dilutions of the bacterial cultures.**						
1.2.1	Aseptically transfer1 ml of (NMT 24 hr age) broth culture into 9 ml of n-saline. Vortex test tubes for about 30 seconds to homogenate contents.						
1.2.2	Make serial dilution upto 10^{-8} using sterile n-saline. Homogenate the contents by vortexing the test tubes for about 30 second while preparing each dilution.						

Attachment -2

Template for the preparation of microbial culture suspensions and cell enumeration

S.No.	Activity						Sign./ Dt.
	Identify dilutions as follows :						
	Dilution	**Identification of culture dilutions**					
		P. aeruginosa	***S. aureus***	***E. coli***	***Salmonella spp.***	***B. subtilis***	
	10^{-1}	PA-1	SA-1	EC-1	SL-1	BS-1	
	10^{-2}	PA-2	SA-2	EC-2	SL-2	BS-2	
	10^{-3}	PA-3	SA-3	EC-3	SL-3	BS-3	
	10^{-4}	PA-4	SA-4	EC-4	SL-4	BS-4	
	10^{-5}	PA-5	SA-5	EC-5	SL-5	BS-5	
	10^{-6}	PA-6	SA-6	EC-6	SL-6	BS-6	
	10^{-7}	PA-7	SA-7	EC-7	SL-7	BS-7	
	10^{-8}	PA-8	SA-8	EC-8	SL-8	BS-8	
	Record the details in the below table:						

Sr.No.	**Activity**					**Sign./ Dt.**
	Dilution	**Broth culture /Earlier dilution used for further dilution**	**Qty. of Broth culture/ earlier dilution used.**	**Qty. of n-saline used.**	**Dilution identified as**	
	10^{-1}					
	10^{-2}					
	10^{-3}					
	10^{-4}					
	10^{-5}					
	10^{-6}					
	10^{-7}					
	10^{-8}					
	Culture *: S. aureus* (ATCC 6538)					
	10^{-}					
	10^{-2}					
	10^{-3}					
	10^{-4}					
	10^{-5}					
	10^{-6}					
	10^{-7}					
	10^{-8}					

Attachment -2

Template for the preparation of microbial culture suspensions and cell enumeratio

S.No.	Activity					Sign./ Dt.
	Dilution	**Broth culture /Earlier dilution used for further dilution**	**Qty. of Broth culture/ earlier dilution used.**	**Qty. of n-saline used.**	**Dilution identified as**	
	Culture : *E. coli* (ATCC 8739)					
	10^{-1}					
	10^{-2}					
	10^{-3}					
	10^{-4}					
	10^{-5}					
	10^{-6}					
	10^{-7}					
	10^{-8}					
	Culture : *Salmonella spp.* (NCTC 6017)					
	10^{-1}					
	10^{-2}					
	10^{-3}					
	10^{-4}					
	10^{-5}					
	10^{-6}					
	10^{-7}					
	10^{-8}					

STANDARD OPERATING PROCEDURE

Title: Microbial Culture Suspension Preparation, Cell Enumeration, Use, Storage and Destruction

SOP No.:		**Department:**	Microbiology
		Effective Date:	
Revision No.:	00	**Revision Date:**	
Supersede Revision No.:	Nil	**Page No.:**	10 of 21

S.No.	Activity					Sign. /Dt.
	Dilution	**Broth culture /Earlier dilution used for further dilution**	**Qty. of Broth culture/ earlier dilution used.**	**Qty. of n-saline used.**	**Dilution identified as**	
	Culture : B. subtilis (ATCC 6633)					
	10^{-1}					
	10^{-2}					
	10^{-3}					
	10^{-4}					
	10^{-5}					
	10^{-6}					
	10^{-7}					
	10^{-8}					

1.3	**Enumeration of culture suspension:** To identify the dilution containing <250 cfu/ml.
1.3.1	Transfer aseptically ____(1 ml) of 10^{-5} to 10^{-8} dilution of ***P. aeruginosa*** into empty sterile petri plates in duplicate.
1.3.2	Add about 15 to 20 ml of previously melted and cooled to approximately 45°C Soybean Casein Digest Agar medium, mix the culture suspension with the agar and allow it to solidify at room temperature. Repeat above step for remaining dilutions and four organisms i.e ***S. aureus, E. coli, Salmonella spp and B. subtilis.*** Immediately store the culture suspensions in freeze.
1.3.3	Incubate the plates in invert position at _____(30-35°C) for _____(18 to 24) hrs. observe and report number of cfu/plate and record in below table

	Culture : *P. aeruginosa* (ATCC 9027)							
Dilution No./ID	**Qty. of dilution**	**Lot no. of SCDA**	**Incubation in date**	**Incubator no.**	**Incubation out date**	**No of CFU/plate**		**Mean CFU/ml**
						1	**2**	
10^{-4}								
10^{-5}								
10^{-6}								
10^{-7}								
10^{-8}								

	Culture : *S. aureus* (ATCC 6538)							
10^{-4}								
10^{-5}								
10^{-6}								
10^{-7}								
10^{-8}								

S.No.	**Activity**							**Sign./Dt.**
Dilution No./ID	**Qty. of dilution**	**Lot no. of SCDA**	**Incubation in date**	**Incubator no.**	**Incubation out date**	**No of CFU/plate**	**Mean CFU/ml**	
	Culture : *E. coli* (ATCC 8739)							
10^{-4}								
10^{-5}								
10^{-6}								
10^{-7}								
10^{-8}								
	Culture : *B. subtilis* (ATCC 6633)							
10^{-4}								
10^{-5}								
10^{-6}								
10-7								
10^{-8}								
	Culture : *Salmonella spp.* (NCTC 6017)							
10^{-4}								
10^{-5}								
10^{-6}								
10^{-7}								
10^{-8}								

S.No.	Activity	Sign./Dt.
1.4	**Stock culture suspension preparation and confirmation of count per ml:**	
1.4.1	Select the previous dilution of the identified dilution (having <250 cfu/ml) of culture suspension and dilute it further to have sufficient amount of the suspensions.	
1.4.2	Take _____(5 ml) of selected dilution and add into _____(45 ml) of n-saline.	
1.4.3	Transfer aseptically_____ 1 ml of stock culture suspension of ***P. aeruginosa*** into empty sterile petriplates in duplicate. Add about 15 to 20 ml of previously melted and cooled to approximately 45°C Soybean Casein Digest Agar medium, mix the culture suspension with the agar and allow it to solidify at room temperature. Repeat above procedure for remaining four organism i.e. *S. aureus, e.coli, Salmonella spp*. and *B. subtillis*	
1.4.4	Immediately store the stock culture suspensions in freeze. Incubate the plates in invert position at ____(30-35°C) for ____(18 to 24hrs).	
1.4.5	Observe the plates and report number of cfu/plate. Record in below table.	

S.No.	Activity								Sign./Dt.
	Culture :. *P. aeruginosa* (ATCC 9027)								
Dilution used	**Qty. of dilution used.**	**Qty. of n-saline**	**Lot no. of SCDA**	**Incubation in date**	**Incubator no.**	**Incubation out date**	**No of CFU/plate**		**Mean (CFU/ml in stock culture suspension)**
							1	**2**	
	Culture : *S. aureus* (ATCC 6538)								
	Culture : *E. coli* (ATCC 8739)								

Culture *: B. subtilis* (ATCC 6633)

Culture : Salmonella spp (NCTC 6017)

2.0	**Fungal culture suspension preparation, dilution and cell density enumeration.**		
2.1	**Fungal culture suspension preparation.**		
2.1.1	Organisms to be used	Working culture ID	Organisms used
	1. *C. albicans* ATCC 10231		
	2. *A. niger* ATCC 16404		
2.1.2	Identify two slants of Sabouraud Dextrose Agar (SDA) slants as, CAC : Culture of ***C. albicans*** , ANC : Culture of ***A. braciliances.***		
2.1.3	Streak a loopful of the recently grown working culture of fungal strains separately on the above slants.		
2.1.4	Incubate the slants of ***C. albicans*** at_____(20-25°C) for _____(44-52) hours. and ***A. braciliances*** at _____(20-25°C) for _____(6-7 days).		
2.1.5	Observe the slants for growth. Record the details in below table.		

	Culture suspension preparation details.	***C. albicans***	***A. braciliances***
	Identification of cultures tubes.		
	Lot no. of SDA medium used.		
	Incubation in date.		

	Incubator no.			
	Incubation out date.			
	Growth observed /not observed.			

S.No.	Activity			Sign./Dt.
2.2	**Preparation of serial dilutions of the fungal cultures.**			
2.2.1	Take a loopful of the slant culture and transfer into ____(9 ml) of sterile n-saline aseptically.			
2.2.2	Use n-saline containing 0.05% polysorbate 80 for making dilutions of fungal culture. Vortex test tubes for about 30 seconds to homogenate the contents.			
2.2.3	Make serial dilution upto 10^{-8} using sterile n-saline and homogenate contents by vortexing the test tubes for about 30 second while preparing each dilution.			
2.2.5	**Identify dilutions as follows.**			
	Dilution	**Identification of culture dilutions**		
		C. albicans	***A. braciliances***	
	10^{-1}	CA-1	AN-1	
	10^{-2}	CA-2	AN-2	
	10^{-3}	CA-3	AN-3	
	10^{-4}	CA-4	AN-4	
	10^{-5}	CA-5	AN-5	
	10^{-6}	CA-6	AN-6	
	10^{-7}	CA-7	AN-7	
	10^{-8}	CA-8	AN-8	

S.No.	Activity					Sign./Dt.
2.2.6	Record the details in the below table.					
	Culture : *C. albicans* (ATCC 10231)					
	Dilution	**Broth culture /Earlier dilution used for further dilution**	**Qty. of Broth culture/earlier dilution used.**	**Qty. of n-saline used.**	**Dilution identified as**	
	10^{-1}					
	10^{-2}					
	10^{-3}					
	10^{-4}					
	10^{-5}					
	10^{-6}					
	10^{-7}					
	10^{-8}					
	Culture : *A. braciliances* (ATCC 16404)					
	10^{-1}					
	10^{-2}					
	10^{-3}					
	10^{-4}					
	10^{-5}					
	10^{-6}					
	10^{-7}					

	10^{-8}					
2.3	**Enumeration of culture suspension.**					
2.3.1	Transfer,aseptically_____(1 ml) of 10^{-4} to 10^{-8} dilution of *C. albicans* into empty sterile petri plates in duplicate. Add about 15 to 20 ml of previously					
	melted and cooled to approx.45°C Sabouraud Dextrose Agar medium, mix the culture suspension with the agar and allow it to solidify at room temperature.					
2.3.2	Repeat above step for remaining four dilutions and for dilutions of *A. braciliances.*					
2.3.4	Incubate plates of *C. albicans* at____(20-25°C) for _____(44 to 52 hrs.) and of *A. niger* at ____(20-25°C) for____(6 to7) days and observe the plates and report number of cfu/plate. Record the details in below table.					

Dilution Id	**Qty. of dilution**	**Lot no. of SDA**	**Incubation date**	**Incubator no.**	**Incubation out date**	**No of CFU/plate**		**Mean**	**CFU/ml in the final selected dilution for stock preparation**
						1	2		
Culture : *C. albicans* (ATCC 10231)									
10^{-4}									
10^{-5}									
10^{-6}									
10^{-7}									
10^{-8}									
Culture :. *A. braciliances* (ATCC 16404)									
10^{-4}									
10^{-5}									
10^{-6}									
10^{-7}									
10^{-8}									

2.4	**Stock culture suspension preparation and confirmation of count per ml:**
2.4.1	Select the previous dilution of identified dilution (having <50 cfu/ml) of culture suspension and dilute it further to have sufficient amount of suspensions. Take ___(5 ml) of selected dilution and add into_____ (45 ml) of n-saline.
2.4.2	Transfer aseptically 1 ml of stock culture suspension of ***C. albicans*** **(ATCC 10231)** into empty sterile petri plates in duplicate. Add about 15 to 20 ml of previously melted and cooled to approximately 45°C Sabarouds Dextrose
	Agar medium, mix the culture suspension with the agar and allow it to solidify at room temperature. Repeat above procedure for ***A. braciliances*** **(ATCC 16404)**
2.4.3	Immediately store the stock culture suspensions in freeze at 2-8 °C. not more than 24 hours.
2.4.4	Incubate the plates of ***C. albicans*** at _____(20-25°C) for ____(44 to 52 hrs) and for ***A. braciliances*** at _____(6 to 7 days) in invert position .
2.4.5	Observe plates and report number of cfu/plate.Record details in below table.

Dilution used	**Qty. of dilution used.**	**Qty. of sterile n-saline**	**Lot no. of SDA**	**Incubation in date**	**Incubator no.**	**Incubation out date**	**No of CFU/plate**		**Mean (CFU/ml in the stock culture suspension)**
							1	2	
	Culture : *C. albicans* (ATCC 10231)								
	Culture : *A. braciliances* (ATCC 16404)								

Analyzed by :	**Checked By :**
Date :	**Date :**

STANDARD OPERATING PROCEDURE

Title: Microbial Culture Suspension Preparation, Cell Enumeration, Use, Storage and Destruction

SOP No.:		**Department:**	Microbiology
		Effective Date:	
Revision No.:	00	**Revision Date:**	
Supersede Revision No.:	Nil	**Page No.:**	20 of 21

Attachment -3
Culture suspension validation template

Date of initial counts observation : Bacterial _____ Fungal _____
Date of final counts observation : Bacterial _____ Fungal _____

Test organism	Initial counts	Final counts

Done by	Checked by

8. **History:**

Version No.		Effective Date	