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1.0 PROTOCOL – APPROVAL:

This is a protocol to demonstrate the hold time study of cell suspension at ……... The protocol has been prepared, reviewed and approved for execution by personnel from the following departments:

Microbiologist

PREPARED BY	SIGNATURE	DATE

Department Head

REVIEWED BY	SIGNATURE	DATE

Head Quality Assurance

APPROVED BY	SIGNATURE	DATE

Quality Head

APPROVED BY	SIGNATURE	DATE

2.0 PURPOSE:

Purpose of this protocol is to provide documented evidence through the scientific data to establish and verify the stability of prepared microbial suspension upon holding for a period of time used in microbial testing.

3.0 SCOPE:

The scope of this protocol is to evaluate the hold time of prepared inoculum suspension (cfu/ml) upon holding upto 21 days. This protocol shall also define the storage conditions effectiveness of inoculum stored at 2-8 °C at Microbiology Department of ……..

4.0 RESPONSIBILITY:

4.0 **Microbiology Executive/Designee-** Preparation of validation protocol, Execution of the validation studies and Completion of the validation report.

4.1 **Head Q.C./Designee** – Responsible for review of the protocol and its summary report for execution of experimental validation study and arranging resources for the validation program and review of validation results and summary report.

4.2 **Head Q.A./R.A. or Designee** – Responsible for review of the protocol and summary report, after completion of qualification summary report shall be checked and reviewed.

4.3 **Head Quality**: Responsible for the final approval of the protocol and summary report, after completion of qualification summary report shall be checked, reviewed and approved.

5.0 REFERENCES:

5.1 SOP “Preparation, Sterilization and Qualification of the Media”.

5.2 SOP “Growth Promotion Test and Inhibition Test of Media”.

5.3 SOP “Preparation of Microbial Culture Suspension”.

5.4 USP Chapter 1227 “Validation of Microbial Recovery from pharmaceutical articles”.

6.0 SAFETY CONSIDERATIONS:

6.1 All the procedures should be carried out aseptically.

6.2 All the glassware and materials that are to be used should be sterile.

6.3 All the glassware and materials used for handling the microorganisms should be decontaminated.

7.0 TEST PRE REQUISITES

- Sterilized glassware
- Prepared inoculum suspension.
- Culture Medium
- Calibrated micro pipette and sterilized pipette tips
- Incubators (for 22.5 $\pm$ 2.5°C, 32.5 $\pm$ 2.5°C and 43 $\pm$ 1°C)
- Pre validated autoclave
- Colony Counter
- Pre validated LAF Units
- Calibrated Analytical balance
- Sterilized inoculation loops

8.0 PROCEDURE FOR HOLD TIME STUDY:

8.1 Prepared the fresh slant of SCDA and SDA. After the solidification of the media slants, transfer the SCDA slants, normal saline solution tubes and peptone solution tubes to the incubator for incubation at 30 to 35ºC for 24-48 hours and SDA slants at 20 to 25ºC for 48 hours for checking of any contamination.

8.2 Streak the surface of the SCDA slant with the bacterial culture and SDA slant with fungal culture.

8.3 Incubate the SCDA slants at 30 to 35ºC for24-48 hours and SDA slants at 20 to 25ºC for 48 hours for *Candida albicans* and *Aspergillus niger* culture for 3- 5 days.

8.4 Prepare culture suspension by washing and scraping the surface of the slant by means of sterile inoculating loop in 10 ml of 0.9% saline for Bacterial, and buffered sodium chloride peptone solution containing 0.5% Tween 80 solution for yeast and fungal culture.

8.5 Transfer the culture suspension in a sterile test tube.

8.6 Vortex the culture suspension to obtain a uniform suspension.

8.7 Carry out serial dilution so as to obtain a culture suspension of 10-100 cfu/ml by following the steps given below.

8.8 Vortex every dilution carefully for 20-30 seconds before Pipetting into next tube.

8.8.1 Transfer 1 ml of the suspension to 9 ml sterile normal saline solution – 10^{-1} Dilution.

8.8.2 1 ml of 10^{-1} Dilution to 9 ml sterile normal saline solution – 10^{-2} Dilution.

8.8.3 1 ml of 10^{-2} Dilution to 9 ml sterile normal saline solution – 10^{-3} Dilution.

8.8.4 1 ml of 10^{-3} Dilution to 9 ml sterile normal saline solution – 10^{-4} Dilution.

8.8.5 1 ml of 10^{-4} Dilution to 9 ml sterile normal saline solution – 10^{-5} Dilution.

8.8.6 1 ml of 10^{-5} Dilution to 9 ml sterile normal saline solution – 10^{-6} Dilution.

8.8.7 1 ml of 10^{-6} Dilution to 9 ml sterile normal saline solution – 10^{-7} Dilution.

8.8.8 1 ml of 10^{-7} Dilution to 9 ml sterile normal saline solution – 10^{-8} Dilution.

8.9 Pipette out 1 ml of the each microbial inoculum from last three dilution tubes into sterile petriplates in duplicate (for bacteria & Yeast)

8.10 Pipette out 1 ml of the each microbial inoculum from last three dilution (10^{-3}, 10^{-4} & 10^{-5}) tubes into sterile petriplates in duplicate (for Mold).

8.11 Incubate the SCDA plates at 30 to 35ºC for 24-48 hours for bacterial cultures and SDA plates at 20 to 25ºC for 48 hours for *Candida albicans* and for 3-5 days for *Aspergillus niger* culture.

8.12 Till the observation of the microbial counts preserve all the dilution tubes at 2 to 8ºC.

8.13 After incubation count the colonies and note the microbial count and label the dilution indicating count/ml, date and store at 2-8 °C.

8.14 Perform the testing for working culture suspension at initial and there after every 3rd day upto 21 days storage, During the study culture shall be stored at 2-8 °C upto hold time study. When holiday falls on testing day, perform the test on next working day.

9.0 ACCEPTANCE CRITERIA

9.1 No growth should be observed in negative control plate of media.

9.2 The average number of cfu recovered from the challenge is not less than 70% of that recovered from the inoculum control.

10.0 SUMMARY OF DEVIATIONS:

10.1 Any deviation(s) from the protocol while performing the methodology shall be investigated and documented in the report.

11.0 ABBREVIATIONS:

11.1 °C - Degree Celsius

11.2 QC - Quality Control

11.3 QA - Quality Assurance

11.4 SCDA - Soyabean Casein Digest Agar

11.5 SDA - Sabouraud Dextrose Agar

11.6 CFU - Colony Forming Units

11.7 ml - Milliliter

11.8 % - Percentage

12.0 DOCUMENTATION AND ARCHIVAL

12.1 **Report :** At the end of the study a report shall be prepared.

12.2 **Archival :** The original and executed document shall be hand it over to QA for archival.

13.0 ANNEXURES:

13.1 **Annexure-1** : Count observation

13.2 **Annexure-2 :** Summary Sheet of Microbial Cell Suspension

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Annexure -1

COUNT OBSERVATION SHEET OF MICROBIAL CELL SUSPENSION
(…….)

Protocol No.: …….

Analysis Start on		**Inoculum Prepared on**	
Name of Media		**Lot No./Batch No. of Media**	
Incubator ID		**Incubator ID**	
Incubation Temperature	32.5±2.5ºC	**Incubation Temperature**	22.5±2.5ºC
Storage Condition of Cell suspension	2-8°C	**Cooling Chamber ID**	

Name of Organisms	Initial Count	Count observed (cfu/ml)			% Recovery	Observed on	Observed by
		Plate-1	Plate-2	Average			
- Ve Control					-		

Remarks:

Checked by:
(Sign/Date)

Annexure -2

SUMMARY SHEET OF MICROBIAL CELL SUSPENSION

Protocol No.:……..

SUMMARY:

Recorded by:
Sign/Date

Reviewed by:
Sign/Date