STANDARD OPERATING PROCEDURE	
Department: Microbiology	**SOP No.:**
Title: Maintenance of Microbial Cultures & Microbial Culture Dilution	**Effective Date:**
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1.0 OBJECTIVE:

To lay down the procedure for Maintenance of Microbial cultures and Microbial Culture Dilution.

2.0 SCOPE:

This SOP is applicable to maintain microbial culture and Culture Dilution in Microbiology Department.

- To maintain the authenticity, viability and purity of test cultures, which are used as positive controls in different microbiological testing.
- To minimize the risk of phenotype changes, which may occur due to prolonged storage of culture & thereby resulting in failure of organism to grow in relevant medium.

3.0 RESPONSIBILITY:

Microbiologist- Quality Control

Head- Quality Control

4.0 PROCEDURE:

4.1 Microbial Maintenance:

Source:

4.1.1 The microbial cultures should be procured from recognized institute every six months and procurement frequency may be changed according to passage of culture received **for example:** If fourth passage culture is received then it is used for only one month and new culture procure after one month.

4.1.2 In case of **Bioballs** (ready to use lyophilized cultures) cultures should be procured from recognized institute as per requirement.

4.1.3 Procure the culture one month prior to due date. In case any organism is not available, then continue the work with the working culture kept separately as stand-by till the availability of the required organism. However this can be done for a short period based on the

performance of the Stand-by culture or for One Month.

4.1.4 In case any working culture is lost its activity due to improper handling or storage of culture tubes, do the culture maintenance/transfer using the previous master culture kept separately at 2°C-8°C.

4.1.5 Preserve the mother culture slant after the preparation of master culture slants up to three months till the new mother culture is received.

4.1.6 Use the culture up to five passages only.

4.2 Source for Microbial cultures:

- NCIM, Pune, India.
- MTCC, Chandigarh, India
- Micro biologics, USA
- Biomerieux Australia

4.3 Mode of supply:

Lyophilized Stick, Lyophilized Pellet or the microbial strains on slants and lyophilized vials in case of Bioballs (ready to use cultures).

4.4 Reviving Lyophilized cultures:

4.4.1 Procedure for Pouch Stick:

4.4.1.1 Tear open pouch at notch and remove swab stick.

4.4.1.2 Disinfect the culture ampoule surface with sterile 70% v/v isopropyl alcohol.

4.4.1.3 Tear off pull-tab portion.

4.4.1.4 Pinch (only once) the middle of the ampoule in the cap to release the hydrating fluid.

4.4.1.5 Tear off pull-tab portion

4.4.1.6 Hold vertically and tap to fluid through shaft into bottom of unit containing pellet.

4.4.1.7 Crush the pellet and mix in fluid using a pinching action.

4.4.1.8 Immediately saturated swab in hydrating fluid.

4.4.1.9 Stick the loopful on preincubated slants.

4.4.1.10 Incubate the slants of aerobic Bacteria at 30ºC to 35º C for 24 hours, anaerobic bacteria at 30ºC to 35º C for 72 hours and Fungi at 20ºC to 25º C for 120 hours.

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4.4.1.11 This would consider as a Mother Culture.

4.4.2 Procedure for Lyophilized Pellet:

4.4.2.1 Remove the unopened vial from storage and allow equilibrating to room temperature.

4.4.2.2 Remove one pellet with sterile forcep .Do not remove desiccant. Immediately recap vial and return to 2-8ºC.

4.4.2.3 Crush the pellet with the swab and evenly saturated the same swab in the hydrating fluid.

4.4.2.4 Stick the loopful on preincubated slants.

4.4.2.5 Incubate the slants of aerobic Bacteria at 30ºC to 35º C for 24 hours, anaerobic bacteria at 30ºC to 35º C for 72 hours and Fungi at 20ºC to 25º C for 120 hours.

4.4.2.6 This would consider as a Mother Culture

4.5 Master/Working culture preparation:

4.5.1 Prepare the media specific for different microbial strains. Prepare the required media (slants or tubes) as per reference SOP and pre incubate them at 30°C to 35°C for 48 hours before streaking.

4.5.2 Prepare one master culture (MC) and three working culture Slants (Wna, Wnb & Wnc) by streaking from the mother culture onto the respective media slants and incubate at specified temperature. This would be the first passage from original one. Sub culturing is done only from the previous master culture. Where n stands for passage number, alphabet a, b, c stands for number of slants prepared.

4.5.3 For example M3 is the first subculture from mother culture & kept for second sub culturing activity, W3a is the first working passage used for first fortnightly culture dilution activity and W3b for second fortnightly activity for a particular month of each organisms used for culture dilution, W1c is the stand by working culture kept in case of improper culture response of the W1a or W1b or breakage or spillage or contamination of the said culture.

4.5.4 Follow the incubation conditions as specified in Annexure – I.

4.5.5 Label the slants as per Annexure – II.

4.5.6 Preserve the grown culture in refrigerator at 2° to 8°C.

4.5.7 Prepare M4 (Master 4, fourth generation), W4a, W4b, W4c and so on till M5, W5a, W5b & W5c in the same way from point No. 4.5.1 to 4.5.3

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Prepare working cultures every month from the master culture and use it After 15 days for the culture dilution activity. Perform the sub culturing using the previous master culture only and the remaining Wnc will be discarded after completion of the month if any.

4.5.8 Record the details of sub culturing as per Annexure-III

4.5.9 For all culture work, follow the Chart / Tree diagram as per 4.7.

4.5.10 Check the purity of mother culture at the time of receipt before sub culturing and record all the details as per annexure-IV. Master culture purity is also checked monthly before sub culturing activity to Prepare Wna, Wnb & Wnc in the same manner by colony morphology and Gram reaction. For Example: Purity of the Master-3 (M3) is checked before to sub culturing to master culture-4 (M4), W4a, W4b, W4c and so on.

4.5.11 Record the details of sub culturing as per Annexure-III

4.5.12 For all culture work, follow the Chart / Tree diagram given below.

4.5.13 Record the details of Purity check and identification in annexure-IV & in. annexure - V for in house isolate.

4.5.14 Master culture-3 (M3) is discarded as per the SOP after two months of its preparation, i.e. when the M5 is prepared and growth is observed on it. Rational for keeping M3 for two months is, in case if there is a problem or additional requirement of the working culture of the same passage shall be made available.

4.5.15 After the preparation of last working culture of the month, previous working cultures shall be discarded.

4.6 Precautions:

4.6.1 Intact culture glassware and culture packets/culture vials (bioballs) shall be transferred after U.V exposure through dynamic pass box for not less than 15 minutes and mop the outside surface with filtered 70 % v/v IPA under laminar air flow to minimize the cross contamination.

4.6.2 Culture handling activity includes sub culturing, growth promotion test, biological indicator test ,antibiotic assay and culture dilution activity shall be performed only after routine microbiological activities such as raw materials, finish products, water analysis, bacterial

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endotoxin test and plate preparation.

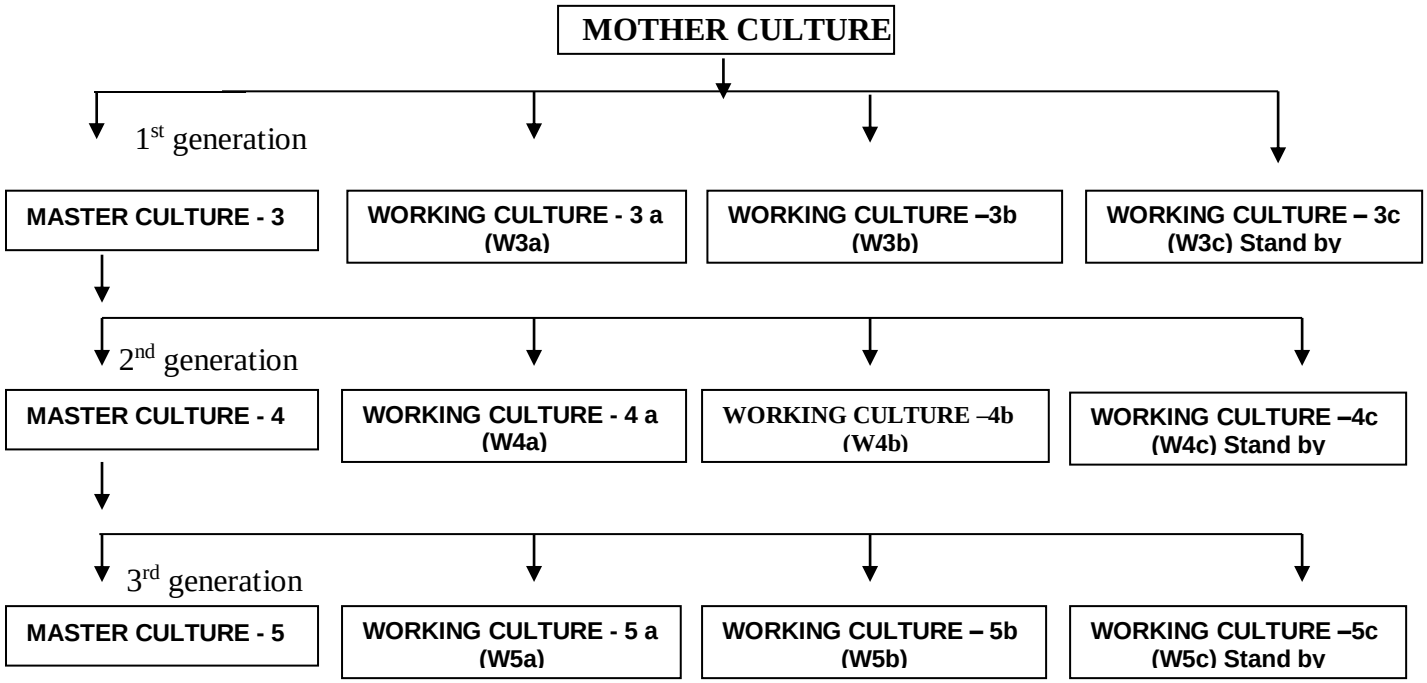
4.6.3 If positive cultures handle in microbiology testing area before routine analysis, clean area with routine disinfectant than only proceed further routine analysis.

4.6.4 After completion of culture handling activity remove sterile gown in Change Room-I and packed in bag and wash separately these garments in garment washing machine located in microbiology washing area.

4.6.5 Open only one culture at a time under Laminar air flow and closed immediately after use.

4.6.5 Culture handling activity shall be followed by cleaning of microbiology testing area as per reference SOP.

4.7 **Frequency:** Monthly i.e. 30 days



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B) Culture Dilution:

4.8 Serial dilution:

4.8.1 For the purpose of Fortnightly Culture Suspension inoculate a loopful culture from working culture slant of appropriate microorganism into 10 ml of SCDM for bacteria & 10ml of SBDB for fungi & also 10ml of COMM for *Clostridium sporogenes ATCC 19404.*

4.8.2 Incubate the bacterial suspension at 30°C-35°C for 24 hours & fungal suspension at 20°C-25°C for 48 hours.

4.8.3 Serially dilute the enriched suspension in 0.9% sterile saline solution.

4.8.4 Except for A. brasiliensis ATCC-16404 add 0.1% of Tween-80 in 0.9% sterile saline solution.

4.8.5 Prepare 1:10 Dilution ratio and same proportion should be added for other volumes by transferring & thoroughly mixing each dilution as shown in Table-1 to yield counts NMT 100 cfu/ml up to 10^8.

4.8.6 Check the number of cfu/ml from the above-prepared dilutions by transferring 1ml of the inoculum in presterilized petriplates. Cool the Media approximately 40ºC-45ºC (check with infra red thermometer before use) and add sterile molten soybean casein digest agar for Bacteria and Sabouraud dextrose agar plate for Fungi by pour plate method in duplicate.

4.8.7 Plate out last four dilutions in duplicate plates for Bacteria and Fungi except for A. brasiliensis selects last five dilutions in duplicate plates.

4.8.8 Incubate the plates at specified temperature as mentioned in table – 2.

4.8.9 On observation of visible microbial growth on each plate, count the number of colonies from various dilutions and record the average in each respective dilution column in " Preparation of viable count of test organisms'' as per Annexure –VI.

4.8.10 For bacteria count total cfu NMT 100 cfu/ml and for fungi NMT 50 cfu/ml.

4.8.11 Calculate the amount of inoculum or the appropriate dilution containing colony-forming units NMT 100 CFU/ ml and note down the same in the Column No. of organisms in Annexure-VI.

4.8.12 Labels the Dilution as per Annexure- VII.

4.9 Storage & usage:

4.9.1 Store the appropriate dilution of NMT 100 cfu / ml of culture suspensions in refrigerator at 2°c- 8°c, use it for fifteen days & discard (from date of preparation) as per Media Destruction

SOP.

4.9.2 Take appropriate volume of above prepared culture suspension containing NMT 100 CFU/ML for positive control (For Example –environmental monitoring, sterility testing, and growth promotion test, Microbial Limit Test.).

4.9.3 **FREQUENCY: Fortnightly**

TEST DESCRIPTION

TABLE-1 (Details of Serial Dilution)

Tube number	Sample	Diluent (Saline solution)	Dilution
A	1ml of stock	9.0 ml*	1×10^{-1}
B	1.0 ml of A	9.0ml*	1×10^{-2}
C	1.0 ml of B	9.0ml*	1×10^{-3}
D	1.0ml of C	9.0ml*	1×10^{-4}
E	1.0ml of D	9.0ml*	1×10^{-5}
F	1.0ml of E	9.0ml*	1×10^{-6}
G	1.0ml of F	9.0ml*	1×10^{-7}
H	1.0ml of H	9.0ml*	1×10^{-8}

*Volume of culture dilutions shall be prepared as per requirement.

TABLE-2: Temperature and Incubation period for different microorganisms.

Micro-organism	Growth Medium	Growth Temperature	Incubation Period (hours)	Condition
Staphylococcus aureus ATCC 6538 P	SCDA	30°C -35°C	24	Aerobic
Candida albicans ATCC10231	SBDA/PODA	20°C -25°C	48	Aerobic
Aspergillus brasiliensis ATCC 16404	SBDA/PODA	20°C -25°C	120	Aerobic
Bacillus subtilis ATCC 6633	SCDA	30°C -35°C	24	Aerobic
Escherichia coli ATCC 8739	SCDA	30°C -35°C	24	Aerobic
Pseudomonas aeruginosa ATCC 9027	SCDA	30°C -35°C	24	Aerobic
Clostridium sporogenes ATCC 19404	CLOA	30°C -35°C	72	Anaerobic
Salmonella abony NCTC 6017	SCDA	30°C -35°C	24	Aerobic
Shigella boydii ATCC-9207	SCDA	30°C -35°C	24	Aerobic
Common Isolates-I	SCDA	30°C -35°C	24	Aerobic
Common Isolates-II	SCDA	30°C -35°C	24	Aerobic

4.9.4 Procedure For Ready to Use Culture Dilutions(Bioballs):

4.9.4.1 **Purity Check:** Check the purity of each strain of microbial cultures at the time of receipt and record the results as per Annexure-IV, ensure that cultures purity test should be completed before use of microbial strains.

Frequency: At the time of receiving of new consignment

4.9.4.2 **Inoculum Preparation:** Disinfect the culture vial and rehydration fluid vial surface with sterile 70% v/v isopropyl alcohol. Open the cap of culture vial and rehydration fluid vial and aseptically transfer lyophilized culture ball to a

rehydration fluid vial (reconstitution fluid supplied by manufacturer and rehydration fluid volume to be used as per manufacturer instruction). Maintain the Bioballs stock, issuance and usage as per Annexure-VIII.

4.9.4.3 Replace the cap of re hydration fluid vial and wait for 30 seconds for proper mixing of bioball culture and reconstitution solution.

4.9.4.4 Vortex for 5 minutes and culture suspension now ready to use.

(i) **For Solid Media:** Pour 100 µl aliquot into the petriplates and follow procedure for different type of microbiological culture media as per procedure mention below:

Pour Plate Method: Pour 15-20 ml specific culture media for pour plate method used for growth promotion test of SCDA or any other general microbiological culture media. Incubate plates at specified temperature.

Spread Plate Method: Spread culture dilution on solid agar media with the help of sterilized spreader and air dried culture plate aseptically under laminar air flow for selective media and incubate at specified temperature.

(ii) **For Liquid Media:** Pour 100 µl aliquot on to plate/tubes/flask and incubate the glassware at specified temperature (refer table-02).

4.9.4.5 Storage condition of Bioballs strains, Lyophilized culture and reconstitution fluid. (Refer table -03)

TABLE-3: Temperature and Incubation period for Bioball Strains and Reconstitution Fluid:

Micro-organism	Storage Condition (For Lyophilized Cultures)	Storage Condition (For Reconstituted Cultures
Bacillus subtilis ATCC 6633	-18 to -33 ºC	02 to 08 ºC
Staphylococcus aureus ATCC 6538	-18 to -33 ºC	02 to 08 ºC
Pseudomonas aeruginosa ATCC 9027	-18 to -33 ºC	02 to 08 ºC
Escherichia coli ATCC 8739	-18 to -33 ºC	02 to 08 ºC
Salmonella abony NCTC 6017	-18 to -33 ºC	02 to 08 ºC
Clostridium sporogenes ATCC 11437	-18 to -33 ºC	02 to 08 ºC
Candida albicans ATCC10231	-18 to -33 ºC	02 to 08 ºC

5.0 ANNEXURE (S):

Annexure-I: List of Microorganisms and incubation conditions (temperature and duration) required for Sub culturing.

Annexure- II: Specimen for Labels of Cultures.

Annexure-III: Culture Maintenance Record

Annexure-IV: Record Sheet For Purity and Identification of Mother/Master Cultures

Annexure-V: Record Sheet For Identifications of In House Isolates.

Annexure-VI: Preparation of viable count of Test Organism

Annexure-VII: Specimen for Label for Culture dilution

Annexure-VIII: Bioballs Stock, Issuance and Usage Record

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6.0 REFERENCE (S):

Pharmacopoeia: As per current versions of Indian Pharmacopoeia, British Pharmacopoeia, Unites States Pharmacopoeia and European pharmacopoeia.

SOP: Procedure for preparation of media

SOP: Disposal of microbial culture media and cleaning of glassware used for culture media

SOP: Preparation of Procedure for preparation of disinfectant and cleaning of microbiology laboratory.

SOP: Preparation, Approval, Distribution control, revision and Destruction of Standard operating Procedure (SOP).

7.0 ABBREVIATION (S) / DEFINITION (S):

NCIM: National Collection of Industrial Micro organisms

MTCC: Microbial Type Culture Collection.

ATCC: American Type Culture Collection

N: number of passage.

CFU: Colony Forming Unit.

PODA: Potato Dextrose Agar

SCDA: - Soyabean Casein Digest Agar

SBDB: Sabouraud dextrose Broth.

NMT: Not More Than.

NLT: Not Less Than.

COMM: Cooked meat medium

SBDA: Sabouraud dextrose agar

v/v : volume by volume

QCM: Quality Control Microbiology

QC: Quality Control

SOP : Standard operating procedure

I.R.Thermometer : Infra Red Thermometer

v/v : volume by volume

PHARMA DEVILS

MICROBIOLOGY DEPARTMENT

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REVISION CARD

S.No.	REVISION No.	REVISION DATE	DETAILS OF REVISION	REASON (S) FOR REVISION	REFERENCE CHANGE CONTROL No.
01	00	---	---	New SOP	---