TESTING METHODOLOGY OF BIOLOGICAL INDICATORS

PURPOSE:
This document describes the testing methodology of Biological indicators. It also explains use of biological indicators in monitoring/validation of equipment such as autoclave, dry heat sterilizer, holding tanks, pipelines, FFS machines during SIP.

RESPONSIBILITY:
Microbiology Section Head and Microbiologist.

1.0 EQUIPMENTS:

1.1 Serological water bath

1.2 Laminar airflow bench

1.3 Colony counter

1.4 Mechanical blender

1.5 Incubator

2.0 GENERAL CONSIDERATIONS:

2.1 User department should give the requirements in advance to Microbiology Department. The BI requirement should specify numbers, Type, Manufacturer's name to Microbiology Department. Indent the Biological indicators as per requirement. In indent mention ATCC number, name of the organisms, carrier form and quantity.

2.2 On receipt of the biological indicators check as per 2.3. Check the Lot no. and Exp. date on container. Enter in the log of Biological Indicator.

2.3 Ensure that the Certificate of analysis is received along with the lot of biological indicators and check the certificate for the following information.

2.3.1 : The name of the organism along with the ATCC number from which the spores are derived and the batch or lot number. .

2.3.2 : The total viable spore count or mean population per unit of biological indicator.

2.3.3 : 'D' value and the method used to determine 'D' value

2.3.4 : 'Z' value

2.3.5 : Manufacturing date

2.3.6 : Expiry date

2.3.7 : Storage specifications

2.3.8 : Disposal procedure

2.3.9 : Bacteriological medium, which will meet the requirement for growth promoting ability.

2.3.10 : Conditions under which reproducible resistance characteristics are obtained.

2.3.11 : Survival time and kill time under specified conditions.

2.4 In case D values of a particular biological indicator are not available in the certificate of the supplier, supplier to be requested to specify the D value of the original spore suspension used to make the biological indicator preparation. Carry out the procedure as given in point 8.0.

2.5 The Batch No. / Lot No. of the biological indicator used in any study / validation should be recorded in the report.

2.6 Store the BI as per manufacturer's instructions. The Retest of the BI for Total Viable Count should be done after one year. The Retest date should not exceed the Expiry date of the BI.

2.7 Place the BI in the autoclave, FFS machine, Pipelines as per the loading pattern in SOP.

2.8 After steam sterilization, retrieve the BI from the load when the cycle is complete and the load is cool and dry. After steam sterilization, the contents of Ampoule or Self contained BI are hot and under pressure. Always allow it to cool. Failure to do so may cause the glass ampoule to burst and may result in injury from hot liquid or flying debris.

2.9 The self contained BI (e.g. EZTest from SGM Biotech) should have a label, which turns from blue to black when steam sterilized. This is to differentiate between units undergone sterilization and those not sterilized.

3.0 BIOLOGICAL INDICATORS FOR STEAM STERILIZATION:

3.1 AMPOULES: A defined preparation of viable spores made from culture derived from Geobacillus stearothermophilus (Bacillus stearothermophilous) ATCC 7953 or 12980 in a suitable media sealed in ampoule readily penetrable by steam, and characterized for predictable resistance to steam sterilization.

3.2 PAPER CARRIER: A defined preparation of viable spores made from a culture derived from a specified strain of Geobacillus stearothermophilus (Bacillus stearothermophilous) ATCC 7953 or 12980, on a suitable grade of paper carrier individually packaged in a suitable container readily penetrable by steam, and characterized for predictable resistance to steam sterilization.

3.3 SELF CONTAINED: A paper carrier strain of Geobacillus stearothermophilus (Bacillus stearothermophilous) ATCC 7953 or 12980 individually packaged in a suitable container readily penetrable by steam and designed to hold an appropriate bacteriological culture medium, so as to enable the packaged carrier, after subjection to saturated steam sterilization conditions, to be incubated in the supplied medium in a self - contained system.

4.0 DETERMINATION OF TOTAL VIABLE SPORE COUNT AMPOULES (STEAM STERILIZATION):

4.1.1 Randomly pick 3 ampoules from each Batch received. Under aseptic conditions, cut open these ampoules with ampoule cutter. Pool the contents in a sterile 250 ml conical flask

4.1.2 Add chilled sterilised purified water to make 100-ml and mix for 3 to 5 minutes to achieve a homogeneous suspension.

4.1.3 Transfer a 10-ml aliquot to a sterile screw cap tube (16 x 125-mm) and place the tube in a water bath at 95°C to 100°C for 15 minutes. Cool rapidly in an ice water bath (0°C to 4°C).

4.1.4 Transfer 1-ml aliquot to suitable tubes, in duplicate and prepare ten-fold serial dilution in sterile purified water to yield preferably 30 to 300 colonies but not less than 6 when plated. The serial dilutions are prepared as per the label claim.

4.1.5 Place 1 ml of each selected dilution into 4 sterile petri-plates. Within 20 minutes, add to each plate 20-ml of Soyabean Casein Digest Agar Medium (prepared as given in MM05), which has been melted and cooled to 45°C to 50° C. Mix well, and allow them to solidify. Keep media negative control.

4.1.6 Incubate 2 plates in an inverted position at 55°C to 60°C for 48 hours. The other 2 plates are to be incubated at 30°C to 35°C. The plates incubated at 30 to 35°C should not show any evidence of growth. If growth occurred in the plate incubated at 30 to 35°C, then the lot or the batch of BI should be rejected and returned to manufacturer.

4.1.7 Count the number of colonies after 24 and 48 hours and use the number of colonies after 48 hours to calculate the final results (The test is valid if the log number of spores per ampoules after 48 hours is equal to or greater than the log number of spores after 24 hours).

4.1.8 The requirements of the test are met if the log average number of viable spores per container is not less than 0.3 log labelled spore count per container and does not exceed the log labelled spore count per container by 0.48. Record the results in record sheet.

Example for calculation of Total viable spore count

COA claims: Spore population 2.0×10^6.

Lower Limit: (Log 2.0×10^6) – 0.3 = 6.0010

Antilog 6.0010 = 1.0×10^6. i.e. –50%

Upper Limit: (Log 2.0×10^6) + 0.48 = 6.7810

Antilog 6.7810 = 6.0×10^6 i.e. +300%

LOWER LIMIT	CLAIMED VALUE	UPPER LIMIT
1.0×10^6 (-50%)	2.0×10^6.	6.0×10^6 (+300%)

4.1.9 Observe the colony characteristics on the petri-plates and record the same. Perform the Gram staining of the isolated colonies obtained as per MM06. Carry out the biochemical characterisation test using a validated identification kit such as API / BBL system up to genus level.

4.1.10 Retest for total viable spore count of ampoules should be carried out after one year.

4.2 USE OF AMPOULES FOR MONITORING/VALIDATION OF AUTOCLAVE, FFS MACHINES, PIPELINES USED FOR SIP:

4.2.1 For Microbiology laboratory, allocate A.R.No. for monitoring to be carried out once a month and carry out the validation of the autoclave as per validation protocol once in six months. Place the BI as per the loading pattern.

4.2.2 For other user departments, place specified biological indicator ampoules at different positions within the autoclave chamber, FFS machines or pipelines and carry out the sterilisation cycle, as per equipment operating procedure or SIP procedure. The user department should raise the TI sheet and the processed BI should be sent to Microbiology. The Microbiology laboratory should start processing of BI within two hrs after sterilization.

4.2.3 Incubate the ampoules of BI between 55°C to 60°C for 7 days along with an unexposed container (positive control). It is best to read the routinely results every day.

4.2.4 Record the observations in the record sheet (Annexure).

5.0 DETERMINATION OF TOTAL VIABLE SPORE COUNT: PAPER CARRIER (STEAM STERILIZATION):

5.1.1 Randomly pick 3 strips from each batch received. Under aseptic conditions, remove the strips from the individual envelops, and pulp the paper into component fibres by placing them in a sterile 250 ml cup of a suitable blender containing 100 ml of chilled sterilized purified water and blend for 3 to 5 minutes to achieve a homogeneous suspension.

OR

5.1.2 Randomly pick 3 strips from each Batch received. Under aseptic conditions, remove the strips from the individual envelope. Place these strips in 250 ml sterile conical flask containing 100.0 ml of chilled sterilized purified water and sterile glass beads. Vortex until the paper carrier is macerated to achieve a homogeneous suspension.

5.1.3 Transfer a 10-ml aliquot to a sterile screw cap tube (16 x 125-mm) and place the tube in a water bath at 95°C to 100°C for 15 minutes. Cool rapidly in an ice water bath (0°C to 4°C).

5.1.4 Transfer 1-ml aliquot to suitable tubes, in duplicate and prepare ten-fold serial dilution in sterile purified water to yield preferably 30 to 300 colonies, but not less than 6 when plated. The serial dilutions are prepared as per the label claim.

5.1.5 Place 1.0 ml of each selected dilution into 4 sterile petri-plates. Within 20 minutes, add to each plate 20-ml of Soyabean Casein Digest Agar Medium which has been melted and cooled to 45°C to 50°C. Mix well and allow to solidify. Keep media negative control.

5.1.6 Incubate two plates in an inverted position at 55°C to 60°C for 48 hours. The other two plates to incubate at 30 to 35°C. The plates incubated at 30 to 35°C should not show any evidence of growth. If growth occurred in the plate incubated at 30 to 35°C, then the lot or the batch no. of BI should be rejected and returned to manufacturer.

5.1.7 Count the number of colonies after 24 and 48 hours, using the number of colonies after 48 hours to calculate the final results (The test is valid if the log number of spores per strip after 48 hours is equal to or greater than the log number of spores after 24 hours).

5.1.8 The requirements of the test are met if the log average number of viable spores per container is not less than 0.3 log labelled spore count per container and does not exceed the log labelled spore count per container by 0.48.

5.1.9 Calculate as given in point 4.1.8

5.1.10 Record the observations in the record sheet.

5.1.11 Retest for total viable spore count should be carried out after one year.

5.2 USE OF PAPER CARRIER FOR MONITORING/VALIDATION OF STEAM STERILISER FFS MACHINE, PIPELINES DURING SIP:

5.2.1 For Microbiology laboratory, allocate A.R.No. for monitoring to be carried out once a month and carry out the validation of the autoclave as per validation protocol once in six months. Place the BI as per the loading pattern.

5.2.2 For other user departments, place specified biological indicator strips at different positions within the autoclave chamber, FFS machines or pipelines and carry out the sterilisation cycle, as per equipment operating procedure or SIP procedure. The user department should raise the TI sheet and the processed BI should send to Microbiology. The BI should be processed within two hours after sterilization.

5.2.3 Transfer the biological indicator strips in sterile 10-ml Nutrient broth or Soyabean Casein Digest Medium with the help of a sterile forceps, under laminar airflow. Incubate the tubes at 55°C to 60°C for 7 days along with an unexposed indicator strips (positive control). It is best to read the routinely results every 24 hrs.

5.2.4 Record the observations in the record sheet (Annexure).

6.0 DETERMINATION OF TOTAL VIABLE SPORE COUNT SELF CONTAINED BI (STEAM STERILIZATION):

6.1.1 Randomly select three self contained BI from the lot to be assayed. Extract the spore strips from the plastic tubes. Under aseptic conditions, transfer the strips and pulp the paper into component fibres by placing them in a sterile 250 ml cup of a suitable blender containing 100 ml of chilled sterilized purified water and blend for 3 to 5 minutes to achieve a homogeneous suspension.

OR

6.1.2 Randomly select three self contained BI from the lot to be assayed. Extract the spore strips from the plastic tubes. Under aseptic condition transfer the strips in 250-ml sterile conical flask containing 100 ml of chilled sterilized purified water and sterile glass beads. Vortex until the paper carrier is macerated to achieve a homogeneous suspension.

6.1.3 Transfer a 10-ml aliquot to a sterile screw cap tube (16 x 125-mm) and place the tube in a water bath at 95°C to 100°C for 15 minutes. Cool rapidly in an ice water bath (0°C to 4°C).

6.1.4 Transfer 1-ml aliquot to suitable tubes, in duplicate and prepare ten-fold serial dilution in sterile purified water to yield 30 to 300 colonies but not less than 6 when plated. The serial dilutions are prepared as per the label claim.

6.1.5 Place 1.0 ml of each selected dilution into 4 sterile petri-plates within 20 minutes, add to each plate 20-ml of Soyabean Casein Digest Agar Medium (prepared as given in MM05) which has been melted and cooled to 45°C to 50°C. Mix well and allow to solidify. Keep media negative control.

6.1.6 Incubate the plates in an inverted position at 55°C to 60°C for 48 hours. The other two plates to incubate at 30 to 35°C. The plates incubated at 30 to 35°C should not show any evidence of growth. If growth occurred in the plate incubated at 30 to 35°C, then the lot or the batch should be rejected and returned to manufacturer.

6.1.7 Count the number of colonies after 24 and 48 hours, using the number of colonies after 48 hours to calculate the final results (The test is valid if the log number of spores per strip after 48 hours is equal to or greater than the log number of spores after 24 hours).

6.1.8 The requirements of the test are met if the log average number of viable spores per container is not less than 0.3 log labelled spore count per container and does not exceed the log labelled spore count per container by 0.48.

6.1.9 Calculate as given in point 4.1.8.

6.1.10 Record the observations in the record sheet (Annexure).

6.1.11 Retest for total viable spore count should be carried out after one year.

6.2 MEDIUM SUITABILITY:

After receiving the lot / batch of self contained BI, the following test is to be carried out. If the following test does not comply, the units of Self-contained BI should be rejected and return to manufacturer.

6.2.1 **Sterility:** Incubate 10 self-contained BI at 55 to 60°C, or at the optimal recovery temperature specified by the manufacturer, for 48 hrs. making sure that there is no contact between individual spore strips and supplied medium. Examine the incubated medium visually (for change in colour indicator or turbidity) and microscopically (for absence of microbial growth). Record the results in record sheet. (Annexure)

6.2.2 **Growth promotion of medium prior to sterilization treatment:** Submerge10 self contained BI units in a water bath maintained at 95 to 100°C for 15 minutes. Start timing when the temperature of the container contents reaches 95°C. Cool rapidly in ice-water bath (0 to 4°C). Remove the units from ice water bath, submerge each spore strip with self contained medium, and incubate 55 to 60°C or at the optimal recovery temperature specified by the manufacturer, for 48 hrs. Examine the incubated medium visually (for change in colour indicator or turbidity) and microscopically (for absence of microbial growth). All the specimens under study should show growth. If one or more of the specimens do not show growth, repeat the test with 20 additional units. The additional units all should grow. Record the results in record sheet.

6.2.3 **Growth promotion of the medium after exposure to sterilization conditions:** Expose the specified number of units (5 each) for both the Survival time and Kill time as stated in labelling. Incubate the spore strips submerged in the self contained medium according to the instructions of the manufacturer. At the end of the incubation medium confirm the existence of growth in each of the specimens that were exposed for each survival time and the absence of growth in each of the specimens that were exposed for each kill time by visually (for change in colour indicator or turbidity) and microscopically of each specimen and confirm, where applicable, correspondence of the labelled colour to the appearance of growth in the supplied medium. Record the results (Annexure).

6.3 USE OF SELF CONTAINED BI FOR MONITORING/VALIDATION OF STEAM STERILISER FFS MACHINE, PIPELINES DURING SIP:

6.3.1 For Microbiology laboratory, allocate A.R.No. for monitoring to be carried out once a month and carry out the validation of the autoclave as per validation protocol once in six months. Place the BI as per the loading pattern.

6.3.2 For user department, place specified self contained BI at different positions within the autoclave chamber, FFS machines or pipelines and carry out the sterilisation cycle, as per equipment operating procedure or SIP procedure. The user department should raise the TI sheet and the processed BI should be sent to Microbiology. The BI should be processed within two hrs after sterilization.

6.3.3 Activate the self contained BI by compressing the plastic vial to break the glass ampoule. This will allow the growth media to come in contact with the spore strip. Use the crushing device provided by supplier or other mechanical crushing device. Incubate the plastic tubes at 55°C to 60°C for 7 days along with an unexposed indicator strips (positive control). It is best to read the routinely results every 24 hrs.

6.3.4 Record the observations in the record sheet (Annexure).

7.0 BIOLOGICAL INDICATOR FOR DRY HEAT STERILIZATION:

7.1 PAPER CARRIER:

A defined preparation of viable spores made from a specified strain of Bacillus atropheus (Bacillus subtilis subspecies niger) ATCC 9372, on a suitable grade of paper carrier, individually packaged in a container readily penetrable by dry heat, and characterised by predictable resistance to dry heat sterilization.

7.2 DETERMINATION OF TOTAL VIABLE SPORE COUNT:

7.2.1 Randomly pick 3strips from each batch received. Under aseptic conditions, remove the strips from the individual envelops, and pulp the paper into component fibres by placing them in a sterile 250 ml cup of a suitable blender containing 100 ml of chilled sterilized purified water and blend for 3 to 5 minutes to achieve a homogeneous suspension.

OR

Randomly pick 3 strips from each Batch received. Under aseptic conditions, remove the strips from the individual envelope. Place these strips in 250-ml sterile conical flask containing 100 ml of chilled sterilized purified water and sterile glass beads. Vortex until the paper carrier is macerated to achieve a homogeneous suspension.

7.2.2 Transfer a 10-ml aliquot to a sterile screw cap tube (16 x 125-mm) and place the tube in a water bath at 80°C to 85°C for 10 minutes. Cool rapidly in an ice water bath (0°C to 4°C).

7.2.3 Transfer 1-ml aliquot to suitable tubes, in duplicate and prepare ten-fold serial dilution in sterile purified water to yield 30 to 300 colonies when plated. The serial dilutions are prepared as per the label claim.

7.2.4 Place 1.0 ml of each selected dilution into 2 sterile petri-plates. Within 20 minutes, add to each plate 20-ml of Soyabean Casein Digest Agar Medium (prepared as given in MM05) which has been melted and cooled to 45°C to 50°C. Mix well and allow to solidify. Keep media negative control.

7.2.5 Incubate the plates in an inverted position at 30°C to 35°C for 48 hours. Examine the plates for evidence of contamination with other microorganisms.

7.2.6 Count the number of colonies after 24 and 48 hours, using the number of colonies after 48 hours to calculate the final results (The test is valid if the log number of spores per strip after 48 hours is equal to or greater than the log number of spores after 24 hours).

7.2.7 The requirements of the test are met if the log average number of viable spores per container is not less than 0.3 log labelled spore count per container and does not exceed the log labelled spore count per container by 0.48.

7.2.8 Calculate as given in point 4.1.8

7.2.9 Record the observations in the record sheet (Annexure).

7.2.10 Retest for total viable spore count should be carried out after one year

7.2.11 Observe the colony characteristics on the petri-plates and record the same. Perform the Gram staining of the isolated colonies obtained. Carry out the biochemical characterisation test using a validated rapid identification kit (e.g. API or BBL system) up to genus level.

7.2.12 Retest for the total viable spore count should be carried out after one year.

7.3 USE OF PAPER CARRIERS FOR MONITORING/VALIDATION OF DRY HEAT STERILIZER:

7.3.1 For Microbiology laboratory, allocate A.R.No. for monitoring to be carried out once a month and carry out the validation of the dry heat steriliser as per validation protocol once in six months. Place the BI as per the loading pattern.

7.3.2 For User department, place specified biological indicator ampoules at different positions within the dry heat sterilizer as per SOP. The user department should raise the TI sheet and the processed BI should send to Microbiology.

7.3.3 Transfer the biological indicator strips in sterile 10-ml Nutrient broth or Soyabean Casein Digest Medium with the help of a sterile forceps, under laminar airflow. Incubate the tubes at 30°C to 35°C for 7 days along with an unexposed indicator strips (positive control). It is best to read the routinely results every 24 hours.

7.3.4 Record the observations in the record sheet (Annexure).

8.0 PROCEDURE TO BE CARRIED OUT IN CASE D VALUE OF A PARTICULAR BIOLOGICAL INDICATOR IS NOT AVAILABLE (STEAM STERILIZATION):

8.1 Expose the biological indicator to steam sterilization at 121±1°C for 5 minutes, check for the presence of the revivable spores. In case of spores dried on to a solid surface transfer the biological indicator strips in sterile 10-ml Nutrient broth or Soyabean Casein Digest Medium with the help of a sterile forceps, under laminar airflow. Incubate the tubes at 55°C to 60°C for 7 days. In case of spores in liquid culture incubate the spore solution at 55°C to 60°C for 7 days check for growth as reflected by colour change. The same procedure to carry out at 10 minutes of autoclave time.

8.2 Expose the biological indicator to steam sterilization at 121± 1°C for 15 minutes check that there is not growth. In case of spores dried on to a solid surface transfer the biological indicator strips in sterile 10-ml Nutrient broth or Soyabean Casein Digest Medium with the help of a sterile forceps, under laminar airflow. Incubate the tubes at 55°C to 60°C for 7 days along with an unexposed indicator strips (positive control) In case of spores in liquid culture incubate the spore solution at 55°C to 60°C for 7 days check for growth as reflected by colour change.

8.3 The readings of cfu of 5, 10 minutes will give the range of survival minutes. At 15 minutes of autoclaving there should be no survivors. Record the results.

9.0 USAGE AND ACCOUNTABILITY OF BI:

The usage of Biological Indicators should be logged. The purpose, numbers of BI, Lot number, date should be mentioned in Log. (Annexure). Microbiology Section Head is responsible for keeping the record of usage of BI.

10.0 DISPOSAL OF USED BI:

10.1 Sterilise the used biological indicator in the autoclave at 121°C for 30 minutes before disposal.

ABBREVIATIONS:

BI – Biological indicator
SIP – Steam In Place
TI – Technical Information
FFS – Form Fill Seal
AR – Analytical Reference
ATCC – American Type Culture Collection
SOP – Standard Operating Procedure

ANNEXURES:

A1 - Record sheet for total viable spore count - ampoules (Steam Sterilization).
A2 - Record sheet for autoclave sterilisation check using ampoule BI.
A3 - Record sheet for total viable spore count - paper carrier (Steam Sterilization).
A4 - Record sheet for autoclave sterilisation check using paper carrier BI.
A5 - Record sheet for total viable spore count – self-contained BI (Steam Sterilization).
A6 - Record sheet for sterility, growth promotion prior to sterilization and growth promotion after sterilization of media, in self-contained.
A7 - Record sheet for autoclave sterilisation check using self-contained BI.
A8 - Record sheet for total viable spore count – paper carrier for dry heat Sterilization.
A9 - Record sheet for Dry Heat Sterilisation check.
A10 - Biological Indicator Stock and Usage record.

REFERENCES:

SOP- Preparation/Disposal of microbial culture media.

Biological Indicator for Dry Heat Sterilization, Paper Carrier, USP 27, 245, 2004.

Biological Indicator for Steam Sterilization, Paper Carrier, USP 27, 246, 2004.

Biological Indicator for Steam Sterilization, Self contained, USP 27, 247, 2004.

Biological Indicators – Resistance Performance tests, USP 27, <55>, 2150, 2004.

Biological Indicators for sterilization, Ph Eur 4.3, 5.1.2, 2002.

Appendix: Methods of Sterilization (Methods of Preparation Sterile Products), Annex 1, Biological Indicators of Sterilization, BP, 2002.