This Technical Bulletin outlines the procedure recommended for use in verifying the certified population of True Indicating Spore Suspensions. This bulletin applies to Spore Suspensions of *Bacillus atrophaeus, Bacillus pumilus, Bacillus subtilis* and *Geobacillus stearothermophilus* with populations of 10^2/0.1 mL through 10^8/0.1 mL.

1. Obtain sterile test tubes of sufficient size to hold a minimum of 25 mL in the appropriate quantity to accommodate the required dilution series, see Appendix 1 for guidance of determining the dilution series required for the specific population level of the Spore Suspension.
2. Fill test tubes with 9 or 9.9 mL of Sterile Deionized Water (SDI) or Water For Injection (WFI) to meet requirements of the determined dilution series.
3. Vortex or manually shake the test Spore Suspension to ensure the spores are evenly dispersed throughout the vial. Transfer 0.1 mL of suspension to 9.9 mL of diluent to achieve a 10^{-2} dilution for all population levels except for 10^3 where a 1:10 dilution is required to achieve a10^{-2} dilution. Transfer 1 mL into 9 mL of 10^2/0.1 mL (10^3/mL) Suspension to achieve 10^{-1} dilution.

Heat Shock

4. Prepare a "blank" test tube containing 10 mL of the diluent only (WFI or SDI). Place a thermometer in the "blank" test tube.
5. Place the test tube(s) containing the initial dilution (10^{-1} for 10^2/0.1 mL (10^3/mL) or 10^{-2} for all other population levels) and the "blank" into a water bath.
6. Start timing the length of the heat shock period when the thermometer reaches the organism's minimum heat shock temperature as outlined in the table below.

   Continue the heat shock period for the time specified.

Organism	Heat Shock Temperature	Length of Heat Shock Period
Bacillus atrophaeus	80ºC to 85ºC	10 minutes
Bacillus pumilus		
Bacillus subtilis		
Geobacillus stearothermophilus	95ºC to 100ºC	15 minutes

Dilution and Plating

7. Perform dilutions (1:10 = 1 mL into 9 mL diluent or 1:100 = 0.1 mL into 9.9 mL diluent) until the dilution corresponding to the theoretical population of 30 to 300 spores per mL is achieved.
8. Obtain 100 mm x 15 mm petri dishes. Label each petri dish with Vial and plate number. A total of 2 transfers to petri dishes or two plates per Vial is recommended at a minimum.
9. Transfer a 0.5 mL or 1 mL aliquot based on the dilution series created from the final dilution tube of each vial into separate dishes as per labeled above.
10. Within 20 minutes, add approximately 20 mL of molten Soybean Casein Digest Agar (SCDA)/Tryptic Soy Agar (TSA) to each dish and mix by gently swirling. The temperature of the media is a critical factor as media which has not been properly tempered will damage and/or kill the spores thus reducing the recovery. Ensure media is approximately 45ºC when poured into the petri dishes.
11. Allow the agar to solidify.

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Incubate

12. Invert the petri dishes and incubate for a minimum of 48 hours at the appropriate growth temperature for the organism, as outlined below:

Organism	Incubation Temperature
Bacillus atrophaeus	30ºC to 40ºC
Bacillus pumilus	
Bacillus subtilis	
Geobacillus stearothermophilus	55ºC to 65ºC

Enumerate

13. After incubation, enumerate the colonies on each plate and calculate the overall mean count based on the average of the results for each vial.
14. Based on the dilution factor of 10^{-5} plated at **0.5 mL**, calculate the total viable spore count. See example below for guidance:

Vial No.	Plate 1	Plate 2	Plate 3	Average	Multiply x 2
1	76	70	82	76	152
2	90	70	96	85	170
3	86	97	76	86	172
4	72	91	98	87	174

Overall Mean: 167
Total Viable Spore Count: 1.7 x 10^6/0.1 mL or 1.7 x 10^7/mL

Based on the dilution factor of 10^{-5} plated at 1 mL, calculate the total viable spore count. See example below for guidance:

Vial No.	Plate 1	Plate 2	Plate 3	Average
1	152	140	165	152
2	180	141	191	171
3	172	193	153	173
4	144	182	196	174

Overall Mean: 167.5 = 168
Total Viable Spore Count: 1.7 x 10^6/0.1 mL or 1.7 x 10^7/mL

Acceptance Criteria

15. Per ISO 11138-1, the population should be within 50% to 300% of the certified population (manufacturer's label claim) to be considered acceptable. A Lot of Spore Suspension with a certified population of 1.7 x 10^6/0.1 mL, would be acceptable if the verified average population was in the range of 8.5 x10^5 to 5.1x 10^6/0.1 mL.

Appendix 1 – Dilution Series

Population		Dilution to be Plated	Volume to be Plated
Per 0.1 mL	Per mL		
10^2	10^3	10^{-1}	1 mL
			0.5 mL*
10^3	10^4	10^{-2}	1 mL
			0.5 mL*
10^4	10^5	10^{-2}	0.1 mL
		10^{-3}	1 mL
			0.5 mL*
10^5	10^6	10^{-3}	0.1 mL
		10^{-4}	1 mL
			0.5 mL*
10^6	10^7	10^{-4}	0.1 mL
		10^{-5}	1 mL
			0.5 mL*
10^7	10^8	10^{-5}	0.1 mL
		10^{-6}	1 mL
			0.5 mL*
10^8	10^9	10^{-6}	0.1 mL
		10^{-7}	1 mL
			0.5 mL*

*Where 0.5 mL is plated, multiply counts by two (2) to obtain mean plate count.

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