This Technical Bulletin outlines the procedure recommended for use in verifying the certified population of True Indicating product configurations containing Spore Discs of filter paper or stainless steel.

1. Obtain one sterile 20 mm x 150 mm screw cap test tube (or equivalent) containing approximately 1" or 2.54 cm of sterile 3 mm glass beads for each Spore Disc to be evaluated.

   Use of a minimum of 4 Spore Discs is outlined in ISO 11138-1 Sterilization of health care products – Biological indicators – Part 1: General requirements.

2. Remove the inoculated carrier from the packaging material, if applicable, and add to the sterile test tube with glass beads. Add 10 mL of Purified Water (PW), Water For Injection (WFI) or Sterile Deionized Water (SDI).

Maceration or Dislodging of Spores

3. For filter paper carriers, macerate the carrier through agitation using either a vortex, manually shaking the test tube or a combination of the two until a homogeneous pulp is obtained. This process will take approximately 10-15 minutes; longer if the tube is only vortexed.

   For stainless steel disc carriers, ensure the disc is surrounded by glass beads. Sonicate the tube for a minimum of 10 minutes. Periodically pause the sonication process and manually shake the tube to ensure that the cupped side of the disc is not stuck facing the glass tube wall and is submerged in the extraction diluent. Vortex for a minimum of 3 minutes to dislodge the spores from the carrier. During the vortex period, frequently shake the tube to ensure the disc remains suspended within the glass beads and is submerged in the extraction diluent. No visible evidence of the spores should remain on the steel disc. Spores will appear as a white residue on the carrier. If spores remain on the carrier, continue to vortex and manually shake until the steel disc shows no evidence of spores.

Heat Shock

4. For population levels except for 10^3/carrier, transfer 1 mL of the fluid to 9 mL of PW, WFI or SDI (this is the 10^{-2} dilution). For population levels of 10^3/carrier, retain all 10 mL in the original test tube.
5. Prepare a "blank" test tube containing 10 mL of the diluent only (PW, WFI or SDI). Place a thermometer in the "blank" test tube. For population levels of 10^3/carrier, the "blank" should also include glass beads.
6. Place the test tube(s) and the "blank" into a water bath.
7. 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		
Geobacillus stearothermophilus	95ºC to 100ºC	15 minutes

8. At the end of the heat shock period, transfer the tubes from the water bath to an ice bath and allow to cool at 0º - 4ºC.

Dilution and Plating

9. Based on the target population level of the carrier, perform 1:10 dilutions (1 mL into 9 mL diluent) or 1:100 dilutions (0.1 mL into 9.9 mL diluent) until the dilution corresponding to the theoretical population of 30 to 300 spores per mL is reached. Refer to the following table for guidance on dilution required.

Population of Carrier	Dilution Required	Volume to Be Plated
10^3	10^{-1}	1 mL
10^4	10^{-2}	
10^5	10^{-3}	
10^6	10^{-4}	

10. Obtain 100 mm x 15 mm petri dishes. Label each petri dish with Inoculated Carrier number, the dilution factor and the plate number. A total of 3 transfers to petri dishes or three plates per carrier is recommended.

11. Transfer a 1 mL aliquot from the final dilution tube of each carrier into separate dishes as per labeled above.

12. 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.

13. Allow the agar to solidify.

Incubate and Enumerate

14. 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	
Geobacillus stearothermophilus	55ºC to 65ºC

15. After incubation, enumerate the colonies on each plate and calculate the overall mean count based on the average of the results for each carrier.

16. Based on the dilution factor, calculate the total viable spore count. See example below for guidance:

    Example: 10^{-4} Dilution

Spore Disc/Carrier 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: 1.7×10^6/disc

Acceptance Criteria

17. 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 Discs with a certified population of 1.7×10^6/disc, would be acceptable if the verified average population was in the range of 8.5×10^5 to 5.1×10^6/disc.