

Standard Operating Procedure

Title: Determination of Heat Resistance of Spore Forming Organisms

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3. Spore Stain - Schaeffer & Fulton
 - 3.1. Flood the slide with 2% aq. Malachite Green.
 - 3.2. Heat the slide to steaming for 5 minutes over a small beaker of water.
 - 3.3. Remove from heat and leave the Malachite Green solution to act for 10-15 minutes.
 - 3.4. Rinse under running water.
 - 3.5. Counterstain with 0.5% aq. Safranin for 30 seconds or Carbol Fuschin for 15-20 seconds.
 - 3.6. Rinse with water and drain or blot to dry.

Bacterial bodies stain Red; Spores stain Green.
 4. If sporulation has taken place in 50% or more of the population proceed to step 5. If sporulation is <50%, re-incubate the culture at 37°C (-0.5 to +1.5°C) and examine daily for sporulation. The culture can be incubated for up to 1 week at 37°C (-0.5 to +1.5°C). If sufficient sporulation is not achieved after this time, return to Step 1.
 5. Wash the growth from the slope with 5-10mL sterile Distilled Water into a sterile McCartney Bottle. Spin down the culture at 4000rpm for 10 minutes. Remove the supernatant and resuspend the pellet in 10mL sterile Distilled Water. Repeat this step twice.
 6. Heat the suspension using an Attest Biological Incubator at 56°C (±1°C) for 30 minutes to kill any vegetative organisms that may be present.
 7. Transfer 3mL of the spore suspension to a 5mL glass ampoule. Heatseal the opening without heating the suspension by using tongs and a Bunsen burner. This ampoule will be used in Step 8 and the remaining spore suspension can be left in the McCartney bottle for use in Step 9.
 8. **Calculation of Final Number of Spores Present per mL**
 - 8.1. Using a pair of tongs, submerge the 5mL ampoule for 2 to 5 minutes in the Constant Temperature Bath set at 121°C (±1°C).
 - 8.2. Remove the ampoule and plunge it into a beaker of iced water.
 - 8.3. Perform serial dilutions to 10⁻² using 9mL volumes of peptone water in autoclaved McCartney bottles.
 - 8.4. Using the Plate Count Method, obtain a final spore count on the cooled suspension. Perform spread plates on Nutrient Agar using 0.1mL of the 10⁰, 10⁻¹ and 10⁻² dilutions and incubate at 32°C (±1.5°C) for 24-48 hours.
 - 8.5. Select the plate that yields between 20-200 colonies and calculate the number of spores remaining in **cfu/mL**. This value is used as part b of the D-value calculation in Step 10.
 9. **Calculation of Initial Number of Spores Present per mL**
 - 9.1. Using the Plate Count Method, obtain an initial spore count on the remaining spore suspension from Step 7. Perform serial dilutions to 10⁻⁶ using 9mL volumes of peptone water in autoclaved McCartney bottles. Store the remaining spore suspension at 4°C (±1°C) until the D-value determination of the organism is complete.
 - 9.2. Perform spread plates on Nutrient Agar using 0.1mL of the 10⁻³ to 10⁻⁶ dilutions and incubate at 32°C (±1.5°C) for 24-48 hours.
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