

Standard Operating Procedure

Title: Determination of Heat Resistance of Spore Forming Organisms

- 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 re-suspend 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. Heat-seal 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.