

Population Assay Instructions

Spore Suspensions

I. List of Components:

Mesa Labs, Bozeman Manufacturing Facility sells components for performing population assays. These include:

PAK-G includes: four 19.5 x 145 mm, sterilized, flat bottom glass tubes with four 6 mm beads and cap; twelve 16 x 125 mm, sterilized, borosilicate dilution blank tubes; two 10 mL pipettes; two 5 mL pipettes; eight 2 mL pipettes; eight 1 mL pipettes

PAK-M includes: one 250 mL Wheaton bottle containing 240 mL of sterile Difco brand growth medium

Items required are: growth medium, sterilized flat-bottom tubes with four 6mm beads, sterilized blank tubes for dilution, pipettes, 160 mL purified sterile water*, a pre-heated (according to Table 1) heat-shock bath and incubator, an instrument used for holding the melted growth medium at 45- 50°C, a timing device, a vortex machine, an ice bath, and 15 x 100 mm petri plates. An ultrasonic cleaner (45-60 kHz) is required when assaying spore suspensions. A calibrated pipette will be needed for spore suspension assays.

*Throughout this procedure when sterile purified water is referenced this includes; Sterile distilled, DI or RO water. WFI, phosphate buffers or physiological saline solutions are not recommended.

II. Preparing the Growth Medium for use:

NOTE: If you have purchased growth medium from Mesa Labs, the medium was prepared according to Good Manufacturing Practices (GMP), and has been tested for sterility and its growth promotion ability (see Certificate of Performance).

1. The growth medium must be completely melted prior to use. This can be accomplished by using a microwave oven. CAUTION: Melting agar presents a significant risk of explosion if not performed properly. It is important to loosen the screw cap on the bottle prior to placing into the oven. This will prevent pressurization of the bottle. Recommended power setting and operating time will vary depending on the oven type; however, the oven should ONLY be operated at LOW POWER SETTINGS.
2. When completely melted, the agar should be tempered at 45 to 50°C until ready for use.
3. A control plate should be poured with each assay. The purpose of the control plate is to verify the sterility of the growth medium. The control plate should be prepared upon completion of the assay and it consists of pouring the remaining growth medium into a sterile Petri plate. The control plate should be incubated with the plates from the assay and should result in no growth

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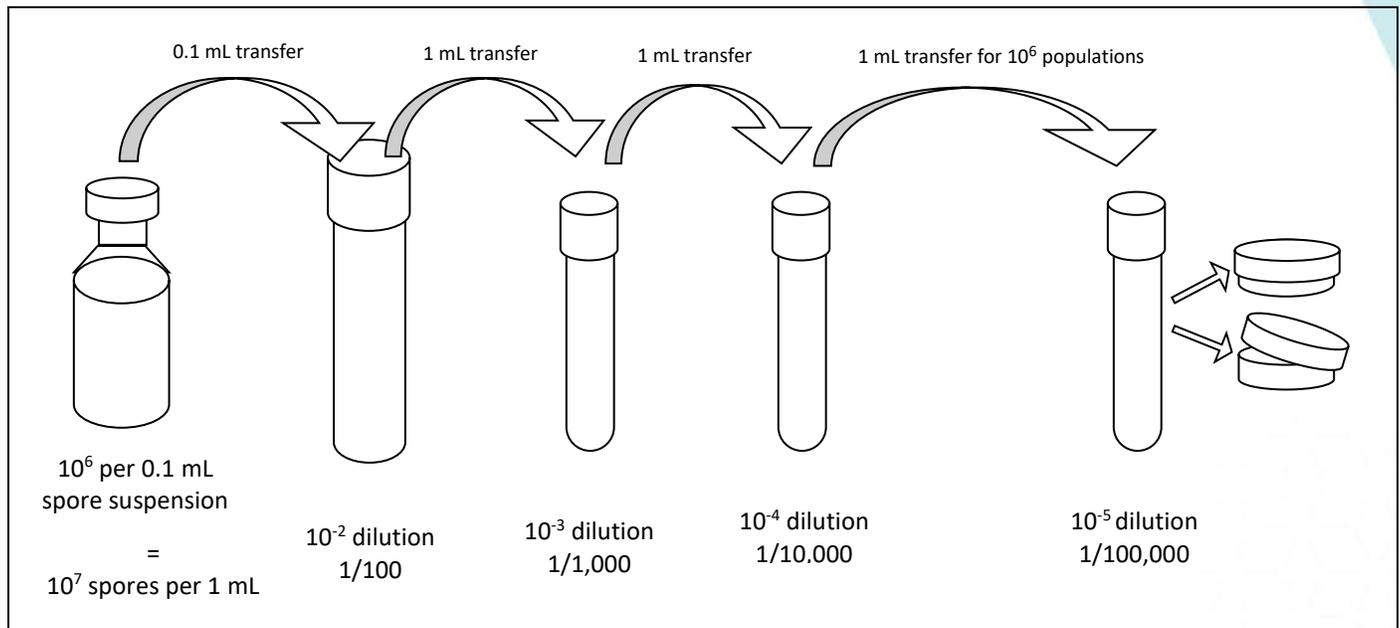
III. Spore Suspension Population Assay (method for assaying 10^6 spores per 0.1 mL):

1. Remove the beads from one 19.5 x 145 mm flat bottom tube. Use a 10 mL pipette to transfer 9.9 mL of sterile purified water into the 19.5 x 145 mm flat bottom tube.
2. Use a 10 mL pipette to transfer 9 mL of sterile purified water into six of the 16 x 125 mm dilution blank tubes.
3. Vortex the spore suspension vial for not less than one minute.
4. Microscopically check the suspension for clumping.
 - 4.1 If no clumping of spores appears proceed to step five.
 - 4.2 If clumping of spores does appear, sonicate the suspension for up to three minutes.
 - 4.3 It may be necessary to perform steps 4 and 4.2 several times until the suspension is free of clumps.
5. When the suspension appears to be homogeneous (microscopically), vortex for at least 10 seconds.
6. Immediately use a calibrated pipette to extract and transfer 0.1 mL from the spore suspension vial and add it to the 19.5 x 145 mm flat bottom tube containing the 9.9 mL sterile purified water. This is the 10⁻² dilution. Vortex this tube for 30 seconds.
7. In a pre-heated bath, heat-shock this tube according to the test organism (see Table 1) starting the timing immediately upon insertion of sample into the preheated bath.
8. Remove tube and cool rapidly in an ice bath.
9. Dilution series for a 10^6 population (modify as appropriate for suspensions with higher/lower concentrations of spores per 0.1 mL).

Two dilution series will be made from the heat-shocked tube. NOTE: It is extremely important to make each serial transfer immediately after vortexing. Vortex the heat-shocked tube for at least 10 seconds. Using a 1 mL pipette, transfer a 1 mL aliquot to a dilution blank containing 9 mL of sterile purified water. Vortex the dilution tube for at least 10 seconds. Use a 1 mL pipette to transfer 1 mL to a second dilution blank containing 9 mL of sterile purified water. **Repeat this step one more time with a 1 mL pipette for a 10^6 per 0.1-mL population.** Vortex this tube for at least 10 seconds. From this dilution tube, use the 2 mL pipette to withdraw 2 mL. Pipette 1 mL per plate into two 15 x 100 mm Petri plates. Pour approximately 20 mL of melted growth medium cooled to 45 to 50°C into the Petri plates. Swirl to ensure adequate mixing and allow the agar to solidify. Do not use agar that has been melted and held longer than eight hours. From the heat-shocked tube, repeat the above dilution sequence one additional time.

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10. Pour control plate
11. Allow to solidify then invert and incubate plates according to test organism (see Table 1).
12. After 48 hours of incubation, remove the plates from the incubator and count the colony forming units (CFU) on each plate. Preferably plates with counts between 30 and 300 CFU should be used, per ISO and USP.
13. Average the counts and then multiply by the dilution factor. The result is the concentration of the spores per 1 mL. Divide this by 10 to obtain the concentration of spores per 0.1 mL for comparison to the label claim.
14. Document all information.

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Table 1. Heat-shock and Incubation Temperatures for Mesa Labs, Bozeman Manufacturing Facility Biological Indicator Test Organisms

Test Organism	Heat shock**	Incubation
<i>G. stearothermophilus</i>	95 - 100°C for 15 minutes	55 - 60°C for 48 hours*
<i>B. atrophaeus</i>	80 - 85°C for 10 minutes	30 - 35°C for 48 hours
<i>B. subtilis</i> '5230'	80 - 85°C for 10 minutes	30 - 35°C for 48 hours
<i>B. subtilis</i> subsp. <i>spizizenii</i> '6633'	80 - 85°C for 10 minutes	30 - 35°C for 48 hours
<i>B. smithii</i>	95 - 100°C for 15 minutes	48 - 52°C for 48 hours*
<i>B. pumilus</i>	65 - 70°C for 15 minutes	30 - 35°C for 48 hours

* Bag plates to avoid dehydration of media at this temperature.

** Start timing immediately upon insertion of sample into preheated bath.

REFERENCE DOCUMENT:

LP-305 Population Assay of Biological Indicators (Based on)