

Protocol

February 25, 2003

**DETERMINATION OF D-VALUE OF *Geobacillus stearothermophilus* SPORES
SUSPENDED IN A LIQUID PRODUCT**

I. Objective:

The objective of this study is to determine the D-value of *G. stearothermophilus* spores suspended in a liquid product.

II. Anticipated Results:

The results of this study will consist of a D-value of *G. stearothermophilus* spores in the product and WFI at the specified sterilization temperature.

III. Equipment and Materials:

- 1) *G. stearothermophilus* spore suspension
- 2) Liquid pharmaceutical product (100 ml) supplied by client
- 3) Cozzoli ampoule filling machine
- 4) Glass ampoules (7 mm X 30 mm) USP type 1 treated
- 5) Difco Brand Tryptic Soy Agar (TSA)
- 6) Joslyn Sterilizer Corp. Steam B.I.E.R.
- 7) Incubator set at 55-60°C
- 8) Filtering apparatus with appropriate filters
- 9) Water For Injection (WFI)
- 10) Sonicator (ultrasonic cleaner) set at 47 –57 kHz
- 11) Vortex machine
- 12) Laminar flow clean bench or Biosafety cabinet
- 13) General lab supplies

The client will also supply the following documentation for the product being tested:

- Material Safety Data Sheet (MSDS)
- Any special filtration methods used to perform sterility testing including filter type, neutralizing agents, rinse fluids, etc.
- Other properties or characteristics not mentioned in the MSDS that may influence testing (e.g. preservatives, viscosity).

IV. Procedure:

A. Sample preparation:

Approximately forty (40) glass ampoules containing the product with a fill level of 0.3 ml and a concentration of 10^6 spores/ampoule will be manufactured. An additional lot of ampoules containing WFI with a similar concentration of spores will also be manufactured. Ampoules will be filled and sealed on the Cozzoli ampoule-filling machine.

The pH of the product will be measured.

B. Effects of the Liquid Product on the Recovery and Growth of *G. stearothermophilus* Spores:

Population assays will be performed on the ampoules initially and upon completion of the study. The purpose of these assays is to verify that the spore concentration in the ampoules is stable for the duration of the study.

C. Preliminary Exposures:

In order to identify a suitable range of exposures for this study and to determine what effect the product has on the resistance of the spores, preliminary exposures will be performed on both sets of ampoules (one set containing the product and one set containing WFI). These exposures will be performed as follows:

Two (2) exposures will be made in the Joslyn steam B.I.E.R. at the specified temperature. When possible, the exposure times will be estimated from data previously collected on these spores. Each exposure will consist of a minimum of four (4) ampoules containing the product plus a minimum of four (4) ampoules containing water. From each exposure, each type of ampoule will be combined and a population assay performed. The data obtained from these samples will be used to establish the exposure times necessary to achieve valid survivor curve data.

D. D-value Determination:

The D-value will be determined as follows:

Using the remaining ampoules manufactured in step IV. A., a series of exposures will be made in the Joslyn steam B.I.E.R. as described in part C. The exposure times will be determined from the results of part C.

The D-value of the spores in both WFI and the liquid product will be determined by spore count reduction according to ISO 11138-1:1994(E), Annex B section B.5 (Attachment 2).

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SGM Biotech, Inc.

Date

Approved by:

Date

Attachment 1

Population Assay Procedure

1. Place ampoules into a sterile container and aseptically fracture glass.
2. Add appropriate volume of dilution fluid.
3. Sonicate, using an ultrasonic cleaner at 47-57 kHz, for three minutes.
4. Vortex sample for approximately one minute.
5. Filter the sample with a Nalgene cellulose nitrate filter, maximum pore size of 0.2 μm (or other appropriate filter), which will allow for separation of the spores from the pharmaceutical product.
6. Rinse the filter with 100 ml of rinse fluid to remove any additional product from it.
7. Aseptically transfer the filter to a sterile 100 ml Pyrex bottle with a stir bar and add 50 ml of sterile 0.1% Tween 80.
8. Sonicate, using an ultrasonic cleaner at 47-57 kHz, for three minutes.
9. Let the filter stir on a stir plate for 1 hour.
10. Vortex the sample for approximately 1 minute.
11. Perform serial dilutions, vortexing each intermediate dilution for approximately ten seconds.
12. Pipette desired aliquots into Petri dishes.
13. Pour 20-25 ml melted Difco Tryptic Soy Agar at 45-50°C into each plate, swirl, and allow to solidify at room temperature.
14. Incubate inverted plates at 55-60°C for 48 hours.
15. Count Colony Forming Units (CFU) on plates and multiply the counts by the dilution factor to calculate the population.

Attachment 2

ANNEX B ISO 11138-1:1994(E)
(normative)

Survivor curve method

NOTE 10: The ideal survivor curve is linear over the full range of inactivation. In practice deviations from this ideal occur but linearity must be maintained within acceptable limits. Construction of a survivor curve by direct enumeration establishes the resistance for surviving populations greater than 5×10^1 whereas the MPN method establishes the resistance for surviving populations below 5×10^0 . Good correlation of the D values obtained by the two methods can therefore be used to establish that there are no serious deviations from a linear survivor curve.

B.1 Test samples shall be subjected at graded exposures to the defined exposure conditions. The range of exposures shall be stated. Each exposure period or dose shall differ from the previous exposure period or dose by a constant interval.

NOTE 11 Details of the performance requirements for exposure apparatus are given in ISO 11138-2 and ISO 11138-3, as appropriate.

B.2 A minimum of five exposures shall be used and shall include:

- a. one exposure in which the sample is not subjected to the sterilant (The sterilant may be absent, or replaced by a non-lethal gas.);
- b. reduction of the viable population to not more than 0.01% of the original inoculum with at least one exposure employed.

B.3 Not less than four inoculated carriers shall be used for each exposure in each determination. The same number of replicates shall be used for each exposure.

B.4 Within 2 h after each exposure, the test samples shall be treated to remove the test organisms from the carrier and a viable count assay performed using the specified culture conditions and methods stated by the manufacturer.

B.5 Using all the data obtained, plot the \log_{10} of the surviving population against time in minutes or dose level and determine the best-fit rectilinear curve by regression analysis using the method of least squares. Survivor data points within 0.5-logarithm of the initial population shall not be included in the regression analysis. Calculate the negative reciprocal of the slope of the line obtained, which is equal to the D value in minutes or absorbed irradiation dose.

B.6 The value obtained for the correlation coefficient for the linearity of the survivor curve shall be not less than 0.8.