

MICROBIOLOGICAL PERFORMANCE QUALIFICATION METHODS A AND B: LABORATORY TESTING REQUIREMENTS

TECHNICAL TIP #17

The international standard ANSI/AAMI/ISO 11135¹, titled "Medical devices – Validation and routine control of ethylene oxide sterilization," describes three methods that may be used for microbiological performance qualification:

1. Method A, the survivor curve method;
2. Method B, the fraction-negative method; and
3. Method C, the half cycle method. Method C is addressed in another Technical Tip and will not be presented in this document.

The 11135 document supplies detailed instructions for the performance of each method.

Acceptable laboratory test methods that will be used in the execution of the microbiological performance qualifications can be found in references such as the United States Pharmacopeia.² Any test method that has been adequately validated by the testing laboratory is acceptable for the performance of the microbiological testing.

Method A: survivor curve construction

The survivor curve construction method of microbiological performance qualification consists of exposing inoculated process challenge devices (PCDs) to graded EO exposure times, removing the biological indicators (BIs) from the PCDs, and enumerating or counting the number of surviving organisms on each BI. This data generates a survivor count that can then be used in developing a survivor curve. The D-value (the time it takes to reduce the population by 90 percent or 1 log) can then be estimated from the survivor curve. Following is a detailed example of the survivor curve construction method for microbiological performance qualification.

Prepare the PCDs that will be used in the study. When preparing the PCDs, the BI must be placed in the most difficult-to-sterilize location of the PCD. The selection of the most-difficult-to-sterilize location of the PCD should be determined by either a sterilization specialist or by the performance of resistance studies. At least five PCDs should be used in each qualification study. The same number of PCDs should be utilized in each Method A study.

At least five studies should be conducted at graded EO exposure times (e.g., 0, 4, 8, 12, 16 minutes). These studies may require reduced temperature, reduced EO concentrations, and/or other cycle modifications to reduce the lethality of the cycle. Once the sublethal cycle parameters are selected, all of the fractional

studies should be performed using the same cycle parameters, with the exception of EO exposure time. At least three of the EO exposure times must result in BIs with countable populations.

The starting population (N_0) should be used as the first point (Y intercept) of the survivor curve line, followed by four (graded) EO exposure times. The starting population should be determined using BIs that have been exposed to all stages of the process prior to sterilant injection.

At the completion of the sterilization cycle, remove the PCDs from the load after the minimum aeration time limit is met. Some countries set minimum aeration time limits that must be met before a worker can remove samples from or work on a load; 6 hours minimum aeration is usually used. PCDs should be refrigerated until shipment and should be forwarded to the testing laboratory in a cooler with a cooling media.

The testing laboratory will then perform a population enumeration on each BI after it is removed from the PCD. A population enumeration may typically be performed as follows:

1. Aseptically transfer each BI, removed from the protective glassine envelope (if it still remains), into a separate sterile dilution tube. A dilution tube is a laboratory tube, usually 20 mL total, that has a screw cap with a Teflon® (DuPont) liner.
2. Add 4 to 6 sterile glass beads and 10 mL of sterile water to the dilution tube. This is your starting (10-1) dilution tube.
3. Allow the BI to soften for 10 to 15 minutes or up to 2 hours if refrigerated.
4. Using a vortex, macerate (separate into small fibers by mechanical means) the BI for approximately 1 to 2 minutes. The BIs should be macerated into single fibers and until no clumps remain. The use of a blender for maceration is not recommended because of the additional kinetic energy that it imparts upon the BIs.
5. Add an extra 5 mL of sterile water to the tube to dilute out the BI fibers and to avoid clogging during the serial dilutions. Using a vortex or manually shaking, mix the dilution tube well.
6. Perform five serial dilutions by transferring with a pipette 1 mL from the current dilution tube into a new dilution tube containing 9 mL of sterile water. This will create the 10^{-2} , 10^{-3} , 10^{-4} , 10^{-5} , and 10^{-6} dilutions.

7. Heat shock any dilutions that will be tested. Heat shocking is performed by placing the dilution tubes into a hot water bath that has been equilibrated at 80° to 85°C (176° to 185°F). The tubes should be left in the hot water bath for 10 minutes longer than it requires for a thermometer placed into a control dilution tube containing 10 mL of the same sterile water used in the dilution process to heat up to 80°C.
8. Immediately remove the dilution tubes from the hot water bath and place into a chilled water bath.
9. Create duplicate pour plates for each dilution level. A pour plate is created by transferring 1.5 mL of the dilution (well mixed) into a petri dish, pouring TSA agar into the petri dish until it forms a continuous layer, swirling the petri dish 15 to 20 times to disperse any organisms evenly throughout the plate, and allowing the agar to harden.
10. Incubate the pour plate inverted for 48 hours at 30° to 35°C (86° to 95°F).
11. Count any colonies that appear and calculate the population of organisms that were present on the BIs at each exposure period.

Once the population of each BI is determined, a survivor curve is constructed using correlation techniques to plot the linear regression of the survivors vs. the EO exposure time. The slope of the regression line is then used to estimate the D value of the microbial population of the PCD.

The D value multiplied by six (for a 6-log reduction) should be the minimum amount of EO exposure time required for a successful half cycle. The minimum EO exposure time required for a successful full cycle should be the D value multiplied by twelve (for a 12-log reduction). In actual practice, when using a D value study to calculate the half or full cycle EO exposure time, an additional safety factor is added to the exposure time.

Method B: fraction-negative

The fraction-negative method of microbiological performance qualification consists of exposing inoculated process challenge devices (PCDs) to graded EO exposure times, removing the biological indicators (BIs) from the PCDs, immersing the BIs into growth media, checking the growth media for signs of growth, and rating the BI sterility tests as growth/no growth of the BI. The proportion of BIs with no growth compared to those with growth and the corresponding time intervals are utilized to determine the D value. Following is a detailed example of the fraction-negative method for microbiological performance qualification.

The PCDs that will be used in the study should be prepared in the same manner as those in the survivor curve method. At least 10 PCDs should be utilized in each qualification study at each exposure time. The same number of PCDs should be utilized in each Method B study. At least five fractional studies should be conducted at graded EO exposure times (e.g., 0, 4, 8, 12, 16, 20 minutes), if possible. These studies may require reduced

temperature, reduced EO concentrations, and/or other cycle modifications to reduce the lethality of the cycle. Once the sublethal cycle parameters are selected, all of the fractional studies should be performed using the same cycle parameters, with the exception of EO exposure time.

At least one study must result in growth of all of the PCD BIs. At least two studies must result in fractional growth of the PCD BIs. At least two studies must result in the complete inactivation or no growth of all of the PCD BIs. Additional studies may be required if the above results are not achieved.

At the completion of the sterilization cycle, remove the PCDs from the load after the minimum aeration time limit is met. Some countries set minimum aeration time limits that must be met before a worker can remove samples from or work on a load; 6 hours minimum aeration is usually used. PCDs should be refrigerated until shipment and should be forwarded to the testing laboratory in a cooler with a cooling media.

Upon receipt, the testing laboratory will then perform a biological indicator sterility test on each BI after it is removed from the PCD. A biological indicator sterility test may be performed as follows:

1. Aseptically transfer each BI, removed from the protective glassine envelop (if it still remains), into a sterile tube containing 30 mL of SCD/TSB.
2. Incubate the tubes for 7 days at 30° to 35°C (86° to 95°F).
3. At the end of 7 days, check the tubes for any signs of growth.
4. Record the number of growths and no-growths for each study.

If the interval between the EO exposure times and the number of replicates at each exposure is constant, then the Spearman-Kärber Equation should be utilized to calculate the D value. If the interval between the EO exposure times or the number of replicates at each exposure is not constant, then the Stumbo, Murphy, Cochran Equation should be used to calculate the D value. The aforementioned equations can be found in the ANSI/AAMI/ISO 11135 standard or in *Disinfection, Sterilization, and Preservation*,³ edited by Seymour S. Block.

The calculated D value multiplied by six (for a 6-log reduction) should be the minimum amount of EO exposure time required for a successful half cycle. The minimum EO exposure time required for a successful full cycle should be the calculated D value multiplied by twelve (for a 12-log reduction). In actual practice, when using a D value study to calculate the half or full cycle EO exposure time, an additional safety factor is usually added to the exposure time.

Method A and B: comparison

The fraction-negative method is the preferred method of microbiological performance qualification due to both its simplicity as well as the complexity of the survivor curve method. When expense is an issue, the fraction-negative method is again the method of choice, unless an in-house laboratory can be utilized.

References

1. ANSI/AAMI/ISO 11135 – 2007, Medical devices – Validation and routine control of ethylene oxide sterilization.
2. The United States Pharmacopeia, USP 31st edition, 2008.
3. Anthony N. Parisi and William E. Young. Sterilization with Ethylene Oxide and Other Gases. As: Disinfection, Sterilization, and Preservation fourth edition. Edited by Seymour S. Block. Lea & Febiger. Pages 580-595.

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