

Thermo Scientific Steri-Run Sterilization Cycle Proves Total Sterilization

Key Words

CO₂ Incubators, Sterilization, Decontamination, Cell Culture

Introduction

A new Thermo Scientific™ CO₂ incubator design incorporates the Thermo Scientific™ Steri-Run™ sterilization cycle. The Steri-Run cycle includes a 2 hour heating phase, followed by a 1.5 hour sterilization phase at 180 °C, and a final cool-down phase of approximately 8 hours. At the completion of the cycle, the incubator is returned to the set incubation temperature.

Technically, something is sterile when there is an absence of life. Since it is not possible to prove that no microorganisms exist in or on a given article, we can define *sterile* by using proof of probability. *Sterilization* is a carefully defined term in pharmacopeias from both the European Union¹ and the United States². Each defines sterilization as proof that there is less than a one in one million (10⁶) chance that any microorganism survived the process. Each requires proof of a 6-log (1 x 10⁻⁶) reduction of microorganisms. Also, during dry heat sterilization, the air must be kept continuously circulating using a fan or blower. In order to be assured that all areas reach the specified temperature, detailed temperature mapping should be provided. This is important because areas that do not heat to high enough temperature for a long enough time could allow some microorganisms to survive.

In an empty chamber such as an incubator, products are not sterilized which can later be tested for the presence of microorganisms. Therefore, Thermo Scientific CO₂ incubators are tested following the requirements in the U.S. Pharmacopeia (USP) for statistical proof using the *overkill* method. This approach requires that you prove elimination of at least 1 x 10⁶ heat resistant bacterial endospores, and then double the treatment for an additional 6-log reduction, providing a total 12-log Sterility Assurance Level (SAL).

In order to comply with these requirements and show that Thermo Scientific CO₂ incubators with the Steri-Run sterilization cycle achieve total sterilization, a CO₂ incubator with the Steri-Run cycle incorporated was

tested by the Biosafety Investigation Unit of the Public Health England Institute at Porton Down, UK (PHE). The materials, methods and results of that testing³ are provided here. Normally the Steri-Run cycle holds at 180 °C for 90 minutes, but the unit tested for microbiological elimination was modified to hold at 180 °C for only 45 minutes to meet the overkill requirements by administering half the lethality of the full cycle.

As part of internal specification measurements for IQ/OQ requirements, temperatures throughout the Steri-Run cycle were measured twice each in four units at 47 locations. Temperature mapping proves that all areas reach and hold at the specified temperature. Those methods and results are also presented.

Materials and Methods

Microbial species

Based on recommendations of the U.S. Pharmacopeia² and the EU Pharmacopoeia¹ (EUP), the following microorganisms were tested by PHE:

- *Bacillus atrophaeus* (ATCC 51189, NCTC10073), a.k.a. *Bacillus subtilis*. This is the indicator for dry heat sterilization in the U.S. Pharmacopeia and the EU Pharmacopoeia, among others, due to the resistance of the endospores to heat and desiccation. A PHE stock batch of endospore suspension was diluted in sterile distilled water to a concentration of 5.00 x 10⁹ colony forming units per mL (CFU/mL).
- *Escherichia coli* (ATCC 25922, NCTC 12241). *E. coli* are commonly used in cell and molecular biology laboratories and can be a contamination concern. A new vial was obtained from the National Collection of

Type Cultures (NCTC) and stock plates made. A full loop of *E. coli* was added to 10 mL of Nutrient Broth in a universal glass bottle and incubated 18-24 hours at 37 °C (+/- 2 °C). The suspension was then assayed to determine the concentration of the bacteria. A 1:10 dilution was made and 0.1 mL deposited onto duplicate Tryptone Soya Agar (TSA) plates and incubated 18-24 hours at 37 °C (+/- 2 °C). The colonies were counted to determine CFU/mL.

- *Aspergillus brasiliensis* (ATCC 16404). This is a black fungal mold, usually living in soil but a common type of contaminant in cell culture laboratories. Ten Malt Extract Agar (MEA) plates carrying *A. brasiliensis* conidiospores were grown to confluency over 5 days at 30 °C (+/- 2 °C). Five mL of a suspension of 0.1% Tween in sterile distilled water was pipetted onto each plate to remove the spores. The spore-containing suspension was recovered from the plate and transferred to a sterile universal container. 0.1 mL of this suspension was deposited onto duplicate TSA plates. These were incubated 3-5 days at 30 °C (+/- 2 °C) and counted to determine the concentration of 1.00×10^6 CFU/mL.
- *Geobacillus stearothermophilus* (ATCC 12980, NCTC 10339). Due to their superior resistance to heat and desiccation, endospores of *G. stearothermophilus* are the indicator for autoclave sterilization in the U.S. Pharmacopeia and EU Pharmacopoeia, among others. Coupons containing *G. stearothermophilus* endospores at a concentration of 2.80×10^6 CFU/mL were purchased from APEX Laboratories, Inc. The coupons were stored at 4 °C before use, and were removed from the packaging immediately before they were placed into the incubator.

- *Mycoplasma pneumoniae* (ATCC 15531, NCTC 10119). This is one of approximately 100 mycoplasma species (estimates vary). Mycoplasma are technically bacteria, but do not have a cell wall and are therefore immune to common antibiotics. As human pathogens and normal flora, mycoplasma are common cell culture contaminants. PHE obtained a liquid suspension of *M. pneumoniae* from NCTC. The storage medium was removed by centrifugation at 4,400 rotations per minute (rpm) for 10 minutes and the supernatant removed. The pellet containing the bacteria was resuspended in 1 mL of Mycoplasma Horse Serum Broth (MHSB) and stored at 4 °C (+/- 2°C) until use.

Preparation of test coupons

For each test, a 10 µL preparation of each microorganism was deposited by pipette onto a 1 cm diameter round, sterile, stainless steel coupon. The coupons were dried at 37 °C (+/- 2 °C) for 1 hour. For each test microorganism, 7 coupons were prepared for each of the 3 sterilization tests.

Procedure for each sterilization test

Prepared coupons for each of the 5 test microorganisms were placed into the Thermo Scientific CO₂ incubator with the Steri-Run cycle. One coupon for each microorganism was placed in each of the following locations (Figure 1):

- Middle shelf (of 3)
- Bottom shelf
- Back wall next to the plenum
- Glass door

For the glass door and back wall, the coupons were hung from hooks.

The Steri-Run cycle was initiated near the end of the work day and according to the manufacturer's instructions. The incubator heated from 37 °C to 180 °C over approximately 2 hours, then held at 180 °C for 45 minutes. Normally this 180 °C phase in the Steri-Run cycle lasts for 90 minutes, but this unit was modified in order to test effectiveness at half the designed lethality. Following the 180 °C phase, the incubator followed its automatic cool-down to the set temperature of 37 °C, over approximately 7.5 hours.

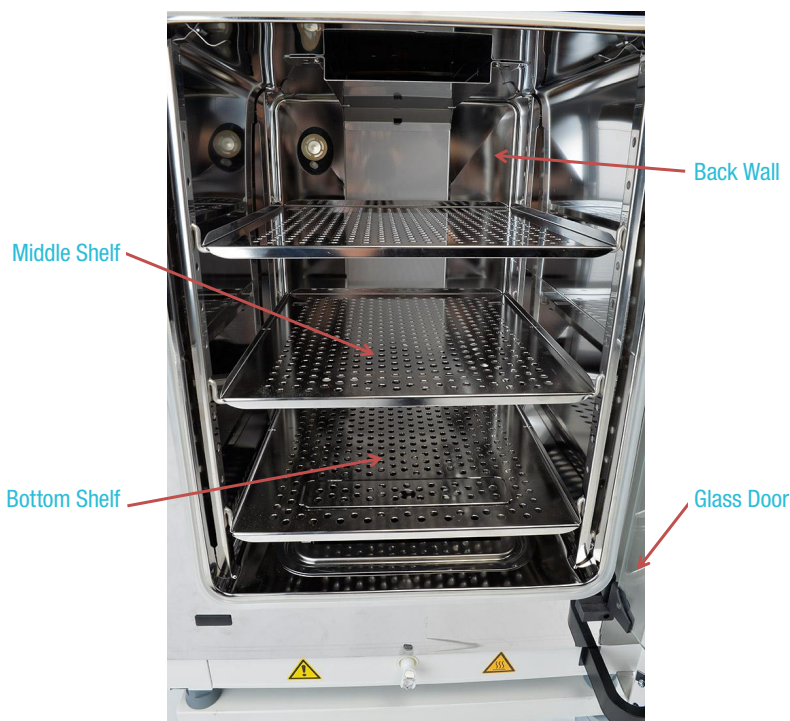
Controls

For each of the tested microorganisms, 3 positive and negative controls were conducted for each test run. The positive controls were prepared in the same manner as the test coupons, but not placed into the incubator during the sterilization cycle. For the negative controls, uninoculated sterile coupons were assayed.

Microbial analysis

The coupons were collected following the Steri-Run cycle. For the first test run, the coupons inoculated with *B. atrophaeus*, *E. coli*, *A. brasiliensis*, and *G. stearothermophilus* were transferred to individual

Figure 1. Interior of the Thermo Scientific CO₂ incubator with Steri-Run cycle, indicating the locations of coupons containing test microorganisms.

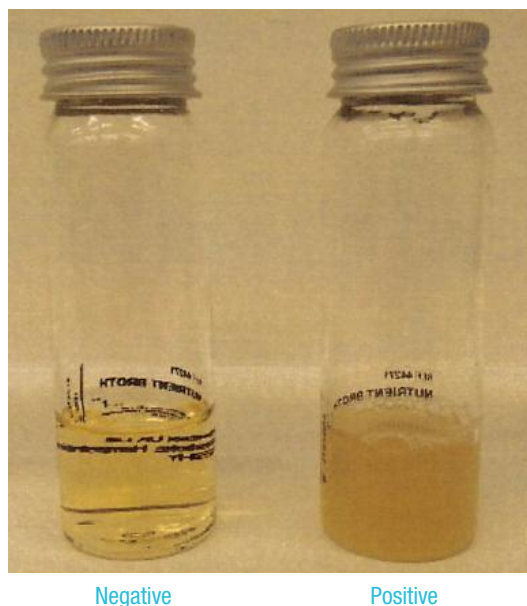


universal containers that contained 10 mL of Phosphate Buffered Saline (PBS). The coupons that were inoculated with *M. pneumoniae* were transferred to individual universal containers that contained 10 mL MHSB. Each was mixed using a vortex mixer for 10 minutes to dislodge remaining microorganisms. 0.1 mL of the suspension was spread onto duplicate agar plates to give a quantitative Total Viable Count (TVC). All were assayed using TSA plates except *M. pneumoniae*, which was assayed using Eaton's Agar plates.

- *B. atrophaeus*: The PBS suspension was further serially diluted in PBS. 0.1 mL of 10^{-2} , 10^{-3} and 10^{-4} dilutions was spread onto duplicate TSA plates and incubated at 37 °C (+/- 2 °C) overnight and any colonies counted.
- *E. coli*: The PBS suspension was further serially diluted in PBS + 0.1 mL. 0.1 mL of 10^{-1} and 10^{-2} dilutions was spread onto duplicate TSA plates and incubated at 37 °C (+/- 2 °C) for 48 hours and any colonies counted.
- *A. brasiliensis*: The PBS suspension was further serially diluted in PBS. 0.1 mL of 10^{-1} and 10^{-2} dilutions was spread onto duplicate TSA plates and incubated at 30 °C (+/- 2 °C) for 3 days and any colonies counted.
- *G. stearothersophilus*: The PBS suspension was further serially diluted in PBS. 0.1 mL of 10^{-2} , 10^{-3} , and 10^{-4} dilutions was spread onto duplicate TSA plates and incubated at 60 °C (+/- 2 °C) overnight and any colonies counted.
- *M. pneumoniae*: The MHSB suspension was further serially diluted in PBS. 0.1 mL of 10^{-1} and 10^{-2} dilutions was spread onto duplicate Eaton's Agar plates and incubated anaerobically at 37 °C (+/- 2 °C) for a minimum of 14 days and any colonies counted.

The results of the first sterilization run showed a total kill, so PHE elected to do a qualitative test for the second and third runs. Here, the coupons for *B. atrophaeus*, *E. coli*, *A. brasiliensis*, and *G. stearothersophilus* were transferred to individual universal containers containing

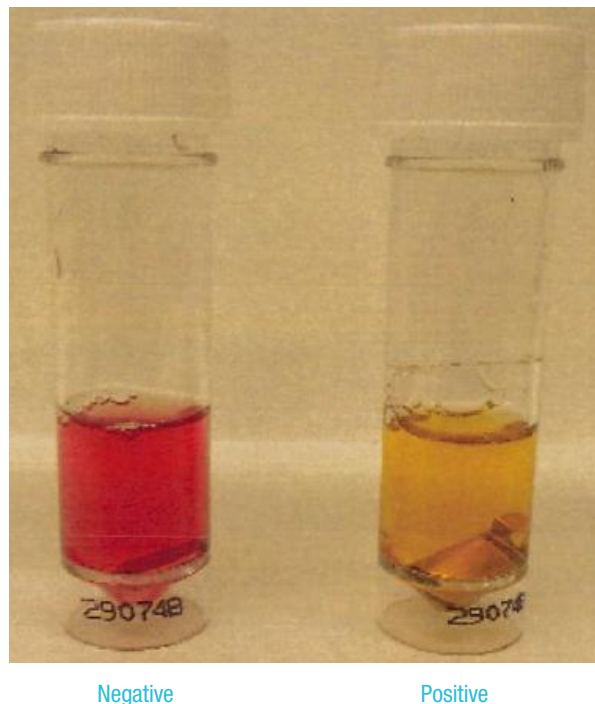
Figure 2. Example of no growth/negative control or growth/positive control of microorganisms in Nutrient Broth.



10 mL of nutrient broth. All were incubated at the appropriate temperature (see above) for 7 days. During this time, any cloudiness in the growth medium signified microbial growth and a positive result, indicating survival during the Steri-Run cycle (Figure 2).

Following all 3 Steri-Run cycle test runs, all the coupons inoculated with *M. pneumoniae* and transferred to universal containers containing 10 mL of MHSB were incubated at 37 °C (+/- 2 °C) for 14 days. During this time, any color change from red to orange-yellow signified a positive result, indicating survival during the Steri-Run cycle (Figure 3).

Figure 3: Example of no growth/negative control (red) or growth/positive control (orange-yellow) of *M. pneumoniae* in MHSB.



Mathematical determination of the effectiveness of the Steri-Run cycle

In keeping with the USP and the EUP, the effectiveness of the Steri-Run cycle is given in terms of log reduction of the test microorganisms. Log reduction is determined as follows:

$$\text{Log Reduction} = \left(\frac{\text{Average total CFU of positive control}}{\text{Average total CFU per sample}} \right) \text{Log}_{10}$$

Measurement of temperature during the Steri-Run cycle

To confirm that all areas of the incubator reached 180 °C for a minimum of 90 minutes during the Steri-Run cycle, 4 different units were each tested twice, once with a standard glass door and once with a segmented glass door.

Forty-seven electronically calibrated nickel-chromium/nickel thermocouples with an accuracy of +/- 1 °C were distributed in the chamber including the left, right and rear walls, ceiling, water reservoir floor, water reservoir cover, glass door, and 3 shelves. The probes were touching the surface in all areas except on the shelves,

Tables 1-5: Results of microbiological tests. Each test was performed on a different day. Positive controls were treated the same as samples exposed to the Steri-Run cycle. "NG" = No Growth. Log reduction = the log amount of microorganisms eliminated, calculated as indicated in the text above.

Table 1. Recovery of *Aspergillus brasiliensis* (ATCC 16404)

Location Tested	Cells Recovered			Minimum Log Reduction		
	Test 1	Test 2	Test 3	Test 1	Test 2	Test 3
Positive Control	2.63 x 10 ⁴	3.28 x 10 ⁴	3.03 x 10 ⁴	0	0	0
Glass Door	NG	NG	NG	> 4.4	> 4.5	> 4.5
Back Wall	NG	NG	NG	> 4.4	> 4.5	> 4.5
Middle Shelf	NG	NG	NG	> 4.4	> 4.5	> 4.5
Bottom Shelf	NG	NG	NG	> 4.4	> 4.5	> 4.5

Table 2. Recovery of *Escherichia coli* (ATCC 25922)

Location Tested	Cells Recovered			Minimum Log Reduction		
	Test 1	Test 2	Test 3	Test 1	Test 2	Test 3
Positive Control	2.20 x 10 ⁴	2.16 x 10 ⁴	2.30 x 10 ⁴	0	0	0
Glass Door	NG	NG	NG	> 4.3	> 4.3	> 4.4
Back Wall	NG	NG	NG	> 4.3	> 4.3	> 4.4
Middle Shelf	NG	NG	NG	> 4.3	> 4.3	> 4.4
Bottom Shelf	NG	NG	NG	> 4.3	> 4.3	> 4.4

Table 3. Recovery of *Mycoplasma pneumoniae* (ATCC 15531)

Location Tested	Cells Recovered			Minimum Log Reduction		
	Test 1	Test 2	Test 3	Test 1	Test 2	Test 3
Positive Control	1.18 x 10 ⁶	1.25 x 10 ⁶	1.33 x 10 ⁶	0	0	0
Glass Door	NG	NG	NG	> 6.1	> 6.1	> 6.1
Back Wall	NG	NG	NG	> 6.1	> 6.1	> 6.1
Middle Shelf	NG	NG	NG	> 6.1	> 6.1	> 6.1
Bottom Shelf	NG	NG	NG	> 6.1	> 6.1	> 6.1

Table 4. Recovery of *Bacillus atrophaeus* endospores (ATCC 51189)

Location Tested	Cells Recovered			Minimum Log Reduction		
	Test 1	Test 2	Test 3	Test 1	Test 2	Test 3
Positive Control	2.48 x 10 ⁶	6.05 x 10 ⁶	1.95 x 10 ⁶	0	0	0
Glass Door	NG	NG	NG	> 6.4	> 7.8	> 6.3
Back Wall	NG	NG	NG	> 6.4	> 7.8	> 6.3
Middle Shelf	NG	NG	NG	> 6.4	> 7.8	> 6.3
Bottom Shelf	NG	NG	NG	> 6.4	> 7.8	> 6.3

Table 5. Recovery of *Geobacillus stearothermophilus* endospores (ATCC 12980)

Location Tested	Cells Recovered			Minimum Log Reduction		
	Test 1	Test 2	Test 3	Test 1	Test 2	Test 3
Positive Control	2.66 x 10 ⁶	8.58 x 10 ⁶	3.18 x 10 ⁶	0	0	0
Glass Door	NG	NG	NG	> 6.4	> 6.9	> 6.5
Back Wall	NG	NG	NG	> 6.4	> 6.9	> 6.5
Middle Shelf	NG	NG	NG	> 6.4	> 6.9	> 6.5
Bottom Shelf	NG	NG	NG	> 6.4	> 6.9	> 6.5

where they were fixed a minimum of 15 mm from the surface. The ambient room temperature was recorded and drafts, direct sunlight and heat from neighboring equipment were eliminated. Each thermocouple took a measurement every 10 seconds.

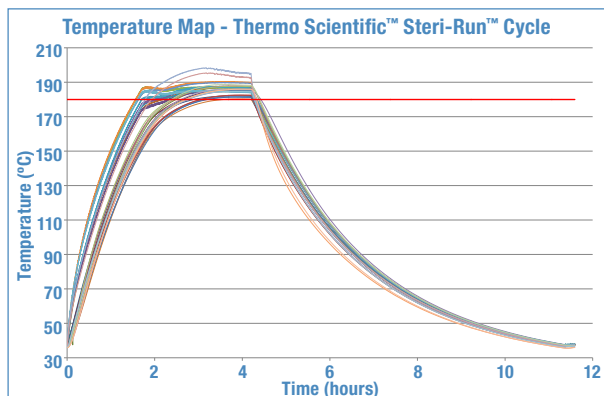
Results and Discussion

Microbiological tests

The results of the microbiological tests for the modified Steri-Run cycle, which held at 180 °C for 45 minutes instead of the standard 90 minutes, are shown in Tables 1-5. In each case, no growth was found in any of the samples from the stainless steel coupons following the Steri-Run cycle, proving total elimination. The positive controls were treated the same as the samples except that they were not put into the incubator. The negative controls showed no growth (results not shown), demonstrating that there was no contamination of the samples by the technicians. Three independent runs on different days demonstrated consistency of the results.

Representative species of black fungal mold (*A. brasiliensis*), bacteria (*E. coli*) and mycoplasma, a common contaminant that is difficult to eradicate from cultures (*M. pneumoniae*), all provide proof of broad efficacy of the Steri-Run cycle. More importantly, total elimination of *B. atrophaeus* shows that the Steri-Run cycle meets the standard of sterilization for dry heat. Further, the total elimination of the highly heat-resistant *G. stearothermophilus*, the biological indicator for autoclave sterilization, provides an additional level of assurance. Elimination of 6-7 logs of these highly resistant microorganisms meets the minimum standards for sterilization, but this was achieved in half the normal holding time of 90 minutes at 180 °C, meeting the U.S. Pharmacopeia requirements for a total 12-log reduction when the full 90 minute cycle is employed.

Figure 4: Typical temperature map for full Steri-Run cycle. Thermocouples were placed in 47 locations in the chamber, including the walls, glass door, water reservoir cover and floor, and 3 shelves. All areas reached a minimum of 180 °C. N = 2 x 4 units.



Temperature mapping

In validating sterilization, it is critical to not only reach a temperature that is known to eliminate microorganisms, but to provide evidence that all areas in multiple units achieve and hold this temperature. This is because if some areas are not heated to a high enough temperature, some resistant microorganisms in those areas could survive.

Eight tests with 47-point temperature mapping were performed. The average results are shown in Figure 4. At time zero, the standard Steri-Run cycle was initiated from 37 °C, with the temperature rising to 180 °C over approximately 2 hours. As shown, some areas reached 180 °C faster than others, but all 47 locations held at 180 °C for a minimum of 90 minutes. Many areas showed temperatures considerably higher than 180 °C. Of interest, the entire chamber was at 160 °C or above for greater than 3 hours. Some sources, including the America Dental Association⁴, Centers for Disease Control⁵ and the World Health Organization⁶, have recommended dry heat sterilization at 160 °C for 2-3 hours. This extensive temperature mapping of multiple units (8 total tests) provides clear proof that the Steri-Run cycle reaches and holds at 180 °C in all areas.

Conclusions

Three separate tests at PHE proved that a Thermo Scientific CO₂ incubator with the Steri-Run automated high temperature sterilization cycle achieved total sterilization, completely eliminating more than 6 logs (>1 x 10⁶) of approved biological indicator organisms in half the normal sterilization time. Thus, the standard

Steri-Run cycle, which holds at 180 °C for 90 minutes (instead of the 45 minutes tested here), is designed to achieve a true 12-log SAL, meeting the standards for the overkill method to prove sterilization.

The PHE tests of the modified Steri-Run cycle showed elimination of common cell culture contaminants (*A. brasiliensis*, *E. coli*, and *M. pneumoniae*), and even heat-resistant bacterial endospores (*B. atrophaeus* and *G. stearothermophilus*) used for validation of sterilization in dry heat sterilizers and autoclaves (respectively). The extensive temperature mapping showed that all areas are designed to hold lethal temperatures for double the time required to eliminate microorganisms in the PHE tests.

Experienced cell culturists understand that battling these common contaminants is an ongoing challenge. The Steri-Run cycle offers a new tool in the battle for quality cell culture results. A Thermo Scientific CO₂ incubator featuring the Steri-Run cycle assures total, uniform sterilization of all chamber surfaces. With the push of a button, the simple overnight routine provides fast, easy elimination of microbial contaminants and eliminates the need for separate autoclaving of parts and use of potentially toxic germicides.

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