Thermo Scientific Steri-Run Sterilization Cycle Proves Total Sterilization

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 10⁶) 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 x 10⁶ 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 x 10⁶ 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
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 containing 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 $37 \, ^\circ C \left(\pm 2 \, ^\circ C\right)$ overnight and any colonies counted.

- **E. coli**: The PBS suspension was further serially diluted in PBS + 0.1mL. 0.1 mL of $10^{-1}$ and $10^{-2}$ dilutions was spread onto duplicate TSA plates and incubated at $37 \, ^\circ C \left(\pm 2 \, ^\circ C\right)$ 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 $30 \, ^\circ C \left(\pm 2 \, ^\circ C\right)$ for 3 days and any colonies counted.

- **G. stearothermophilus**: 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 $60 \, ^\circ C \left(\pm 2 \, ^\circ C\right)$ 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 $37 \, ^\circ C \left(\pm 2 \, ^\circ C\right)$ 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. stearothermophilus* were transferred to individual universal containers containing 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 $37 \, ^\circ C \left(\pm 2 \, ^\circ C\right)$ 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).

**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:

$$\text{Log Reduction} = \frac{\text{Average total CFU of positive control}}{\text{Average total CFU per sample}} \times \log_{10}$$

**Measurement of temperature during the Steri-Run cycle**

To confirm that all areas of the incubator reached $180 \, ^\circ C$ 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 $\pm 1 \, ^\circ C$ 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.
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.

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.
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 Association, Centers for Disease Control and the World Health Organization, 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 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.

References