Dry Heat Sterilization Effectiveness of Esco CelCulture® with High Heat Sterilization CO_2 Incubator

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Summary

Esco CelCulture® with High Heat Sterilization CO_2 Incubator is equipped with a 180°C dry heat sterilization feature. This feature has been evaluated for its effectiveness against bacteria, spores, and fungi. The bacteria tested were *Enterococcus faecalis*, *Escherichia coli*, *Pseudomonas aeruginosa*, *Staphylococcus aureus*, *Geobacillus stearothermophillus*, *Bacillus subtilis* spores, and *Aspergillus brasiliensis* fungal spore. All tested microorganisms were placed on the top-right front of the incubator which has the lowest temperature. The study result shows that 6 logs of vegetative cells and bacterial spores also 5 logs of fungi are totally killed after a half-cycle of sterilization phase.

Key Words: CelCulture® CO_2 Incubators, High Heat Sterilization, Log Reduction

Introduction

CO_2 Incubator is an essential device for maintaining a constant and suitable environment for growth and cultivation of cells. These cells must be clear from any contaminant such as bacteria, fungi, and viruses which are detrimental to the development of the cells. Some microbial contaminations can cause deprivation of essential nutrients. Moreover, the excretion of metabolites from microbial contaminants have been found to cause pH changes which compromise cell proliferation. Since contamination has become a primary issue in cell culture in the last decade, CO_2 incubators are equipped with various contamination control features including automated disinfection or sterilization cycles to help eliminate contamination without much effort from the lab operators[2].

Disinfection includes the usual aseptic techniques required in a health care environment. Objects are usually disinfected by liquid chemical or by wet pasteurization. This method eliminates mycobacteria, vegetative bacteria cells, viruses, and most fungi, but it is not effective against bacterial spores[1]. Another method to remove contamination is through sterilization. Sterilization is described as the elimination process of all form of microbial life physically or by method such as steam under pressure, dry heat, EtO gas, Hydrogen peroxide (H_2O_2), and many other liquid chemicals[1]. One of the sterilization methods commonly used in CO_2 incubators is the dry heat sterilization. This method is nontoxic, noncorrosive, convenient, and environment friendly. Dry heat sterilization uses only hot air sterilization at temperatures more than 160°C and it is the only method that conform to the medical device sterilization standards.

Esco CelCulture® CO_2 Incubator with High Heat Sterilization adopts a 180°C dry heat sterilization feature. The factory setting for sterilization temperature is 180°C for approximately 1 hour. To verify the effectiveness of sterilization procedures, a SAL (Sterility Assurance Level) of 10^{-6} is required, by calculating the reduction of 12-log microorganisms. This experiment used the half-cycle method with half of the sterilization period. When the 6-log of tested microorganisms are reduced, it can be stated that when applying the full cycle, the reduction of 12-log microorganisms might be achieved[11].

Materials and Method

Materials used in this experiment were:

1. Esco CelCulture® with High Heat Sterilization 170L CO_2 Incubator SN: 2020-149877 (unit tested)
2. Biosafety Cabinet Class II (Esco Airstream®)
3. Incubator (Esco CelCulture®)
4. Datalogger (Yokogawa)
5. Thermocouple 42 channels
6. Vortex (Labnet)
7. Pipette volume 10-100µL (Santorius)
8. Pipette volume 100-1000µL (LABMATE+)
9. Pipette tips
10. Tryptone Soya Broth medium (Neogen)
11. Tryptone Soya Agar medium (Neogen)
12. Sterile DI water
13. Sterile 0.9% NaCl solution (produced in-house and sterilized)
14. 10mm stainless steel disc (produced in-house and sterilized)

Microbial species

In this study, the following test strain on the recommendation of cell culture specialists due to their resistant natures as shown below:

1. *Bacillus subtilis* (ATCC 6633) (BS), is a typical gram-positive bacterium. *B. subtilis* is found in the soil and the gastrointestinal tract of ruminants and humans. This is the indicator for dry heat sterilization in the U.S. Pharmacopeia and the EU Pharmacopeia[3].
2. *Enterococcus faecalis* (ATCC 19433) (EF), is a typical gram-positive bacterium. *Enterococcus* bacteria are present in the intestinal flora of humans and animals. Also, they can be found in plants, soil, and water. Some *Enterocccus spp* can survive temperatures of 60°C for short periods and can grow in high salt concentration[4].
3. *Escherichia coli* (ATCC 8739) (EC), is a typical gram-negative bacterium. Most strain of *E. coli* are harmless and an important part of a healthy intestinal tract[5].
4. *Pseudomonas aeruginosa* (ATCC 9027) (PA), is a common gram-negative. It lives primarily in water, soil, and vegetation. These bacteria are able to remove antibiotics from the cell, so the bacteria have good resistance to antibiotics and common disinfection[6].
5. *Staphylococcus aureus* (ATCC 6538) (SA), is a typical gram-positive bacterium. *S. aureus* is carried by people and even a superbug, on their skin and the mucous membrane has antibiotic-resistant variant such as methicillin / Multi resistant *Staphylococcus aureus* (MRSA)[7].
6. *Geobacillus stearothermophillus* (ATCC 12980) (GS), is a typical gram-positive bacterium. *Geobacillus* bacteria are utilized in the biotechnology industry as a source of thermo-stable enzymes. These bacteria have a self-preservation technique – they can form endospores and can remain in this dormant stage for long periods. *G. stearothermophillus* is used to verify decontamination of many laboratory processes as a biological indicator[8].
7. *Aspergillus brasiliensis* (ATCC 16404) (AB) is a spore-producing fungi that produce black conidia (spores) that are readily dispersed in the environment[9]. Usually living in soil but a common type of contaminant in cell culture laboratories.

Preparation of suspension tested

The microorganisms (vegetative cells of bacteria, bacteria, and fungi spore) were inoculated from slant agar to 10 mL 0.9% (NaCl) sodium chloride solution. Enumeration was done with the Total Plate Count method on Tryptone Soya Agar (TSA). The number of bacteria should reach 10^8 CFU/ml and 10^7 CFU/ml for spore fungi.

Preparation of test carrier

Stainless steel disc carriers (d=10mm) were prepared from the same material as the Esco CelCulture® CO_2 Incubator chamber to imitate the contaminated incubator chamber surface. The discs were inoculated with 10 µL of bacteria inoculums and spore suspension then let it dry in the BSC. From each microorganism (inoculated to 5 carriers for each microorganism), 2 discs were prepared for positive control and enumerate the initial number of population.

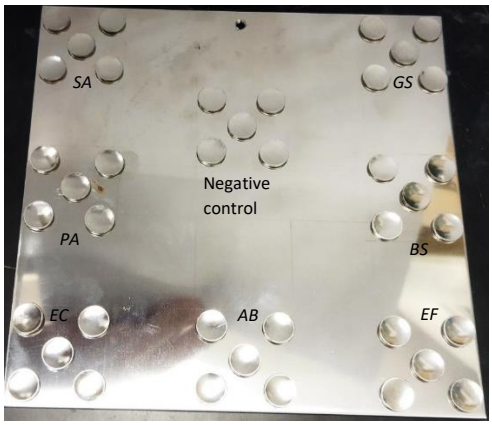


Figure 1. Disc Carrier Arrangement

Positive control discs were put into a TSB medium and the initial number of discs were aseptically transferred into 0.9% NaCl solution and then enumerated.

Temperature mapping

Temperature mapping was performed in an Esco CelCulture® CO_2 Incubator with High Heat Sterilization during three repetitions of the sterilization cycle. To record the temperature at each point during the sterilization cycle, 42 thermocouples were connected to the surface of the incubator chamber, tray, and glass door.

Based on the temperatures recorder, the corner area on the top-right front of the incubator had the lowest temperature during the sterilization cycle.

Figure 2. Area with the lowest temperature in the chamber (Top-Right Front) of the Esco CelCulture® CO_2 Incubator with High Heat Sterilization® CO_2 Incubator

Procedure for sterilization test

Prepared carriers for the test were placed into the Esco CelCulture® CO_2 Incubator at the lowest temperature point (Figure 2). In this test, we use a half cycle of the sterilization phase (for about 30 minutes) and skip the cooling phase. Test for establishing a sterilization cycle was conducted using growth/no growth criteria as evidence of complete kill of inoculated carriers.

Test Bacteria	Type of Bacteria	Inoculated Population (positive control)
Bacillus subtilis	Bacterial spore	2.93×10^6
Enterococcus faecalis	Vegetative bacteria	6.70×10^6
Pseudomonas aeruginosa	Vegetative bacteria	7.60×10^6
Staphylococcus aureus	Vegetative bacteria	1.17×10^6
Escherichia coli	Vegetative bacteria	5.47×10^6
Geobacillus stearothermophillus	Bacterial spore	2.98×10^6
Aspergillus brasiliensis	Fungal spore	1.37×10^5

Result and Discussion

The temperature of Esco CelCulture® CO_2 Incubator with High Heat Sterilization cycle was initiated from 30°C, with a temperature rising to 180°C within 2 hours, followed by sterilization phase at 180°C for averaging 60 minutes, and a final cooling phase of approximately 9 hours (Figure 3). The lowest temperature where the sample was placed is 160°C, and the sterilization time is 30 minutes (half-cycle method). Since the time of this sterilization cycle is lower than the reference condition of Pharmacopoeia European (for temperature 160°C for at least 2 hours), physical and biological validation should be provided to verify an SAL of $\leq 10^{-6}$ [2].

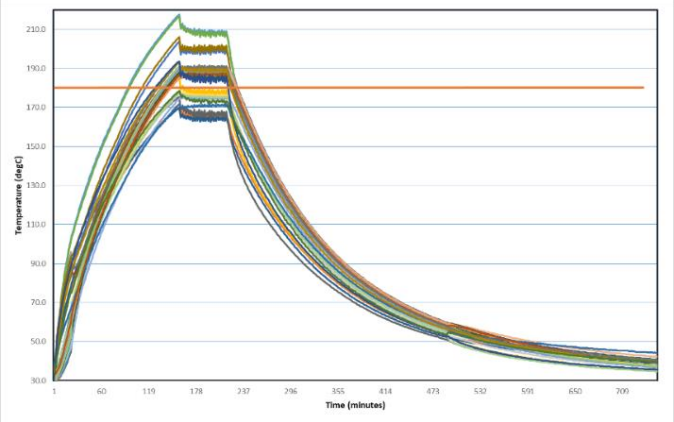
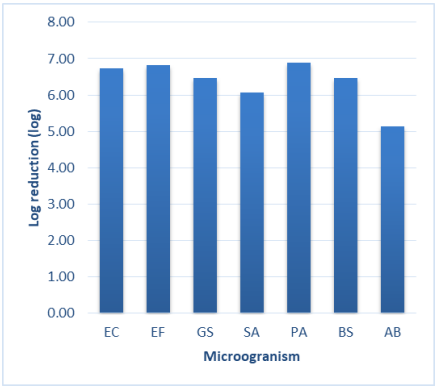


Figure 3. Graph of Temperature during Full Cycle Sterilization

Verification of the sterilization cycle in the Esco CelCulture® CO_2 Incubator, both physical and biological validation achieve the specified conditions, including temperature, time, and lethality. For the biological validation, the D value of the tested microorganisms should be determined and should not be less than 2.5 minutes[10].

Microbiological tests

The results of the microbiological tests for Esco CelCulture® CO_2 Incubator with High Heat Sterilization Cycle after 7 days incubation period obtained immediately after half-time of sterilization, all broth cultured exhibited no growth result, and the negative control broth remained clear while the positive control broth became turbid. This result confirms that after exposure to a half cycle of the sterilization, all tested microorganisms were totally killed. Log reduction of all tested bacteria exceed 6-log, and 5-log for fungal spore *Aspergillus brasiliensis*.



EC: *Escherichia coli*

EF: *Enterococcus faecalis*

GS: *Geobacillus stearothermophillus*

SA: *Staphylococcus aureus*

PA: *Pseudomonas aeruginosa*

BS: *Bacillus subtilis*

AB: *Aspergillus brasiliensis*

Figure 4. Log reduction of tested microorganisms after half cycle of sterilization phase

The D-value is the total time to reduce a quantity by 1 log factor or 90%. To determine the D value, the holding time of sterilization is divided by the difference between logs of the initial population and the final population (log reduction). The D value at 160°C (30 minutes) for *Enterococcus faecalis*, *Escherichia coli*, *Pseudomonas aeruginosa*, *Staphylococcus aureus*, *Geobacillus stearothermophillus*, and *Bacillus subtilis* is approximately 5 minutes, also *Aspergillus brasiliensis* have D value 6 minutes.

Conclusion

The Esco CelCulture® CO_2 Incubator with High Heat Sterilization is able to eliminate contaminants inside the entire chamber, leaving it clean, cool, and dry at the end of the cycle. The Esco CelCulture® CO_2 Incubator with High Heat Sterilization cycle achieved half-cycle sterilization, completely eliminating more than 6 logs of bacteria and more than 5 logs of fungal spores, and the D-value is 5 minutes (bacteria) and 6 minutes (fungi) which meets the standard of European pharmacopoeia for D-value is not less than 2.5 minutes.

References

1. Rutala, W.A, D.J. Weber, and Healthcare Infection Control Practices Advisory Committee (HICPAC). 2008. CDC: Guideline for Disinfection and Sterilization in Healthcare Facilities
2. European Medicine Agency. 2019. Guideline on the Sterilization of the Medicinal Product, Active Substance, Excipient, and Primary Container
3. Wickham Laboratories Contract Analytical Service. Fact Sheet *Bacillus subtilis*. [Accessed on April 2022]. Available at www.wickhamlabs.co.uk
4. Wickham Laboratories Contract Analytical Service. Fact Sheet *Enterococcus spp*. [Accessed on April 2022]. Available at www.wickhamlabs.co.uk
5. Wickham Laboratories Contract Analytical Service. Fact Sheet *Escherichia coli*. [Accessed on April 2022]. Available at www.wickhamlabs.co.uk
6. Wickham Laboratories Contract Analytical Service. Fact Sheet *Pseudomonas aeruginosa*. [Accessed on April 2022]. Available at www.wickhamlabs.co.uk
7. Wickham Laboratories Contract Analytical Service. Fact Sheet *Staphylococcus aureus*. [Accessed on April 2022]. Available at www.wickhamlabs.co.uk
8. Wickham Laboratories Contract Analytical Service. Fact Sheet *Geobacillus stearothermophillus*. [Accessed on April 2022]. Available at www.wickhamlabs.co.uk
9. Wickham Laboratories Contract Analytical Service. Fact Sheet *Aspergillus brasiliensis*. [Accessed on April 2022]. Available at www.wickhamlabs.co.uk
10. European Pharmacopoeia 9.0. 2011. Chapter 5.1.2 'Biological Indicators of Sterilisation'
11. Woedtke, Thomas von, & Axel Kramer. 2008. The Limits of Sterility Assurance. GMS German Medical Science. Vol3 (3): 1-10.
12. European Pharmacopoeia 9.0. 2011. Chapter 5.1.1 'Method of Preparation of Sterile Product'
13. Sastri, Vinnt R. 2022. Section 4.2.2 'Dry Heat Sterilization' in Material Requirements for Plastic Used in Medical Devices. [Accessed on July, 8th 2022]. Available at https://www.sciencedirect.com/topics/engineering/heat-sterilisation