Whitepaper

Lab equipment

Thermo Scientific CO_2 incubators with 140°C dry heat sterilization cycle achieve proven sterilization

Authors

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Abstract

A Thermo Scientific™ Forma™ Steri-Cult™ CO_2 incubator features an on demand, convenient and automated 140°C dry heat sterilization procedure. The cycle design has been independently tested and proven to achieve a 12-log sterility assurance level (SAL), according to the "overkill" approach, using approved biological indicators. This was demonstrated by elimination of more than 1 million *Bacillus atrophaeus* bacterial spores in a truncated 1-hour cycle at 140°C. Therefore, the complete cycle with 2+ hours at 140°C meets the 12-log SAL guidance in the United States Pharmacopeia. We discuss the methods and results from independent test laboratories.

Introduction

Steri-Cult CO_2 incubators are the largest available stacked CO_2 incubators from Thermo Fisher Scientific. There are two sizes available: model 3307/3308 is 232 L (8.2 ft^3) and model 3310/3311 is 323 L (11.4 ft^3). They feature an external active humidification system which is popular for use in manufacturing facilities, and an in-chamber HEPA filtration system that provides ISO Class 5 conditions in five minutes following every door opening. The active airflow design ensures fast recovery from a door opening and outstanding uniformity even in these large chambers. Such excellent culturing conditions are necessary for reproducibly producing sensitive cells for therapy. Steri-Cult incubators are now available as part of the Thermo Scientific™ CTS™ series laboratory equipment portfolio[1], offering additional features for GMP processes including a fully electropolished chamber to increase chemical resistance and reduce microbial attachment, and a complete documentation package to ease regulatory compliance and qualification. Steri-Cult incubators also feature a 140°C sterilization cycle which has been independently tested and proven effective on a different Thermo Scientific CO_2 incubator model.[2] Here we provide further proof with independent testing of the two different Steri-Cult model sizes to demonstrate that the sterilization design and efficacy meet the requirements for sterilization even in these larger chambers.

For every sterilization program design, it is paramount to strictly follow validation requirements as well as to provide the proof of effectiveness, as per directive in the European Union Pharmacopeia (EUP)[3] and the United States Pharmacopeia (USP).[4] The process of sterilization should technically deactivate any types of microorganisms in the tested product, but since an absolute state of sterility can only be defined in terms of probability, the only way to provide proof is by following a precisely defined validation method.

To prove dry heat sterilization, the pharmacopeias require proof of elimination of a prescribed biological indicator microorganism, *Bacillus atrophaeus* heat resistant spores; it is critical to properly handle these bacterial endospores to help ensure that they are not in the vegetative state, which would be much less heat resistant and easier to kill. To this end, testing by an experienced third-party commercial lab is imperative. In addition, uniformity of the heat distribution during the process should be demonstrated. The first condition requires a proof of a 6-log (1×10^{-6}) reduction of heat resistant spores, which is equal to achieving a Sterility Assurance Level (SAL) of 10^{-6}. Proof of a complete elimination of the bioindicator during the sterilization process can only be achieved by parallel recovery of at least one million (10^6) bacterial colony forming units (CFU) from the positive controls which are treated exactly the same as the test indicators with the exception of the sterilization program itself. The second condition requires extensive temperature mapping to prove that all areas reach the specified temperature, and according to the pharmacopeias, such uniformity can be only achieved by applying a forced air circulation using a fan or blower situated in the CO_2 incubation chamber. Since dry heat sterilization is a function of the time at a specified temperature, the temperature map requirement demonstrates that no cold areas exist where microorganisms might survive the heat treatment.

The Steri-Cult CO_2 incubator design was tested following the requirements in the USP for statistical proof using the "overkill" method. The verification of the overkill method is demonstrated here using proof of elimination of at least 1×10^6 heat resistant bacterial endospores in half the lethality (half the standard sterilization time) at 140°C. This approach means that a complete standard 140°C cycle provides an additional 6-log reduction, equaling a total 12-log SAL. The standard cycle holds at 140°C for 2 hours, but the unit tested for microbiological elimination was interrupted after one hour at 140°C (half of the time), such that it held the temperature at 140°C for only 1 hour. The cycle time for heating to 140°C and cooling back down were not impacted by this interruption.

In order to comply with pharmacopeia guidelines and to show that the 140°C dry heat sterilization cycle on the Steri-Cult CO_2 incubators achieve true sterilization, each of the Steri-Cult models (232 L and 323 L) were tested separately by different independent laboratories.[5,6,7] Additionally, the temperatures throughout the complete 140°C dry cycle were measured at fifty-one locations.

The materials, methods and results of the testing carried out are presented here. The 140°C dry heat sterilization cycle was evaluated by each laboratory by testing the complete cycle as well as three truncated cycles, to verify the ability to "overkill" the selected microorganisms and biological indicators.[5,6,7]

Materials and methods

Microbial species

Following the current guidance of the USP and the EUP for prescribed biological indicators for dry heat sterilization, *Bacillus atrophaeus* heat resistant endospores were tested alongside other actively growing forms of microorganisms as follows:

- B*acillus atrophaeus*, a.k.a. *Bacillus subtilis*. This is the biological indicator prescribed for dry heat sterilization in the USP and the EUP, due to the endospores' resistance to heat and desiccation. Two lots of commercially available spore strips for dry heat sterilization (I - Simicon: Bio-Indicator DH/HL; II - BioCheck: BI-HE-1) as well as a separate *B. atrophaeus* spore suspension (ATCC 6633) were tested.[5,7]
- Additionally, the vegetative (actively growing) form of *B. atrophaeus* (ATCC 6633) was tested separately, by propagating it on Tryptic Soy Agar with 5% Sheep Blood (SBA). SBA plates were incubated aerobically at 30-35°C for 24 ± 2 hours. After incubation, an isolated colony was transferred to Tryptic Soy Broth (TSB) and incubated at 30-35°C for 24 ± 2 hours. The bacterial suspension was directly applied to stainless steel carries for testing.[6]
- *Pseudomonas aeruginosa* (ATCC 15442 and ATCC 9027) emerged as an important multidrug resistant pathogen as well as a common cell culture contaminant in the last two decades. *P. aeruginosa* (ATCC 15442) was propagated on Tryptic Soy Agar with 5% Sheep Blood (SBA). SBA plates were incubated aerobically at 30-35°C for 24 ± 2 hours. After incubation, an isolated colony was transferred to Tryptic Soy Broth (TSB) and incubated at 30-35°C for 24 ± 2 hours.[5,6]
- *Escherichia coli* (ATCC 8739). *E. coli* are commonly used in cell and molecular biology laboratories and can be a contamination concern. The suspensions of *Escherichia coli* cells were prepared, and CFU/mL were determined for every tested cycle before applying on stainless-steel carriers.[5]
- *Aspergillus brasiliensis* (ATCC 16404) is a black fungal mold, usually living in soil but is also a common type of contaminant in cell culture laboratories. *A. brasiliensis* was propagated on Potato Dextrose Agar (PDA) in a tissue culture flask at 20-25°C for 5 to 10 days. After the incubation period, the tissue culture flask was rinsed twice with 20 mL of 0.9% saline containing 0.05% Tween 80. Each rinse was centrifuged at 4,500 rotations per minute (RPM) for 10 minutes, decanted, reconstituted with 5 mL of phosphate buffered saline (PBS), and filtered through sterile gauze to obtain a spore suspension for inoculation. Mold concentration was estimated on a wet mount prepared with a hemocytometer following reconstitution.[5,6]

Preparation of test carriers

10 µL of each microbial suspension was applied to a round stainless-steel sterile coupon and spread uniformly. After inoculation, the carriers were allowed to dry for 1 hour at 37 ± 1°C. After 1 hour, the carriers were visually inspected to ensure the test culture suspension was uniformly dried before testing was initiated.

Procedure for each sterilization test

The prepared carriers were placed into the Steri-Cult CO_2 Incubator in each of the following four locations (See Figure 1):

- Middle shelf (of 3)
- Bottom shelf (of 3)
- Back wall
- Glass door

For the glass door and back wall, the carriers were fixed with adhesive magnets, inoculum side out.

In a second series of tests, the carriers and *B. atrophaeus* spore strips were secured with aluminum tape, in each of the following nine locations:

- Glass door
- Left side wall
- Right side wall
- Back wall
- Bottom wall
- All 3 shelves
- The chamber ceiling, where HEPA filter is normally placed

The 140°C sterilization cycle was initiated near the end of the workday, and according to the manufacturer's instructions. Each microorganism was evaluated using the complete cycle and additionally 3 separate truncated cycles to verify the heat cycle's ability to overkill.

- For the complete cycle, the 140°C dry heat cycle was initiated, then the incubator heated from 37°C to 140°C in approximately 3.5 hours. The 140°C sterilization cycle ran overnight for 14 hours, holding at 140°C for about 2 hours. The messaging center display indicates the transition from HEATING to STERILIZING, allowing determination of the phase switch to be used for a truncated cycle.
- For the 3 independent truncated cycles, the 140°C dry heat cycle was initiated then the incubator heated from 37°C to 140°C in approximately 3.5-4 hours on average. One hour into the 140°C phase the cycle was cancelled. Normally this 140°C phase would last two hours, but this was modified in order to test the effectiveness at half the designed lethality.

Figure 1: Interior of the Steri-Cult 3310 CO_2 Incubator. This image indicates the locations of test carriers on middle shelf, bottom shelf, back wall, and the inside of the glass door.

For both the complete and truncated cycles, after the 140°C phase, the incubator followed its automatic cool-down to the set temperature of 37°C, over approximately 8.5 hours.

Controls

For each of the test microorganisms, three positive and negative controls were conducted for each test run. The positive controls were prepared as for the test carriers, but not placed into the incubator. For the negative controls, uninoculated sterile carriers were assayed. Additionally, for each microbe used in the procedure, positive and negative media quality controls were included.

Microbial analysis

Following the 140°C dry heat cycle, the carriers were aseptically collected and processed as follows:

- The carriers inoculated with *B. atrophaeus* and *P. aeruginosa* were transferred to individual separate tubes that contained 10 mL of Tryptic Soy Broth (TSB), mixed using a vortex mixer for 2 minutes to dislodge remaining microorganisms, then incubated at 30-35°C for 48 hours ± 2 hours.
- The carriers inoculated with *A. brasiliensis* were transferred to individual separate tubes that contained 10 mL of Sabouraud Dextrose Broth with Letheen (SDB/L). Each tube was mixed using a vortex mixer for 2 minutes, then incubated at 20-25°C for 5 to 7 days.
- The carriers and spore strips that were fixed with aluminum tape were transferred into 5 mL nutrient broth (TSB), mixed using a vortex mixer for 1 minute, then incubated at 37°C for 7 days.

In all cases the cultures were observed for growth by turbidimetric observation.

In case the 140°C sterilization cycle was unable to eliminate viable microorganisms, the recovery procedure would be confirmed by preparing ten-fold serial dilutions in PBS followed by a pour plate technique to evaluate number of colonies (CFU/plate).

The results of both the complete and truncated sterilization cycles showed a "total kill" from the test carriers at all specified locations, therefore no further recovery procedures were performed. Thus, only a qualitative test was performed, along with positive controls.

The positive controls were handled with slight differences in the recovery procedures. For the 232 L model studies, the positive carriers were placed into separate tubes with the appropriate broth and vortexed for 2 minutes. Ten-fold serial dilutions were made in PBS and each carrier was plated and incubated as follows:

- *B. atrophaeus* and *P. aeruginosa*: Each PBS dilution was plated into duplicate sterile Petri dishes, tempered Microbial Content Test (MCT) agar was added, mixed thoroughly and allowed to solidify. Plates then were incubated at 30-35°C for 48 ± 2 hours.
- *A. brasiliensis*: Each PBS dilution was plated into triplicate sterile Petri dishes, 12-15 mL of tempered SDA/L was added, mixed thoroughly and allowed to solidify. Plates then were incubated at 20-25°C for 5 to 7 days.
- All the positive carriers recovered for the 232 L model were moved straight into 5 mL PBS, then ten-fold serial dilutions were performed, followed by incubation on TSBA plates at 27°C for 48 hours for bacteria and at 25°C for 120 hours for fungi.

All positive controls showed microbial growth, indicating a positive result. The colonies were counted after the incubation period and raw data was recorded as CFU per each positive control. All the negative controls showed negative growth.

Mathematical determination of the effectiveness of the 140°C dry heat cycle

In keeping with the USP and the EUP, the effectiveness of the 140°C 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 140°C cycle

To confirm that all areas of the incubator reached 140°C for a minimum of 2 hours during the 140°C cycle, 51 temperature probes were used to monitor the Steri-Cult CO_2 Incubator interior chamber, exterior surfaces, ambient room conditions, and incubator heater.

Fifty-one electronically calibrated nickel-chromium/nickel thermocouples with an accuracy of ± 0.1°C were distributed in the chamber including the left, right and rear walls, ceiling, floor, glass door, and three shelves. The technical area and air duct, where the external humidity system is located, also were tested with five different temperature probes, recording temperatures between 140°C and 143.5°C. The probes were touching the surface in all areas except on the shelves, where they were fixed with a minimum 15 mm distance 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 complete and the truncated 140°C sterilization cycles are shown in Table 1. In each case, no growth was found in any of the samples from the stainless-steel carriers following any of the 140°C dry heat cycles, proving total elimination. All truncated runs on different days show consistency of the results, with zero growth. The negative controls showed no growth (results not shown), demonstrating that there was no contamination of the samples by the technicians. The positive controls were treated the same as the test samples except that they were not put into the incubator. Since all the sterilization cycles were able to eliminate all microorganisms including the prescribed biological indicator spores for dry heat sterilization, the recovery procedures were only used for positive controls. The results presented in Table 2 for the 323 L model clearly indicate that all positive controls recovered had a minimum of 10^6 CFU/carrier, thus all the acceptance criteria were met successfully. The results presented in Table 3 for the 232 L model demonstrate a minimum of 10^6 CFU/carrier recovered for the most important biological indicator, the *Bacillus atrophaeus* heat resistant spore strips. These results prove that the 140°C sterilization procedure achieves a minimum of 6-7 Log_{10} reduction of *B. atrophaeus* heat resistant spores, meeting the required standard of sterilization for dry heat sterilization as per the EUP and USP.

Additionally, the elimination of other types of dangerous microbes, for example black fungal mold (*A. brasiliensis*), a common contaminant that is difficult to eradicate from cultures, or highly drug resistant pathogens such as *P. aeruginosa*, provide a further proof of high efficacy of the 140°C dry heat cycle and additional level of assurance. Elimination of 6-7 logs of these highly resistant microorganisms was also achieved in half the standard sterilization time at 140°C, equaling a total 12 log reduction when the full 2 hours time is employed.

Table 1: Results of microbiological tests for complete and truncated sterilization cycles. Approved biological indicators are indicated in red. No growth was recovered for any species following exposure to the complete and truncated sterilization cycles.

Model	Sterilization cycle	Microorganism	Locations	Replicates	Results
323 L	Complete (2 hours at 140°C)	*B. atrophaeus* (ATCC 6633) – vegetative form	4	1	NG
		B. atrophaeus (ATCC 6633) – spore suspension	4	1	NG
		P. aeruginosa (ATCC 15442)	4	1	NG
		A. brasiliensis (ATCC 16404)	4	1	NG
	Truncated (1 hour at 140°C)	*B. atrophaeus* (ATCC 6633) – vegetative form	4	3	NG
		B. atrophaeus (ATCC 6633) – spore suspension	4	3	NG
		P. aeruginosa (ATCC 15442)	4	3	NG
		A. brasiliensis (ATCC 16404)	4	3	NG
232 L	Complete (2 hours at 140°C)	*E. coli* (ATCC 8739)	9	1	NG
		P. aeruginosa (ATCC 9027)	9	1	NG
		A. brasiliensis (ATCC 16404)	9	1	NG
		B. atrophaeus - spore strips I	9	1	NG
		B. atrophaeus - spore strips II	9	1	NG
	Truncated (1 hour at 140°C)	*E. coli* (ATCC 8739)	9	3	NG
		P. aeruginosa (ATCC 9027)	9	3	NG
		A. brasiliensis (ATCC 16404)	9	3	NG
		B. atrophaeus - spore strips I	9	3	NG
		B. atrophaeus - spore strips II	9	3	NG

Table 2: Results of positive control complete and truncated sterilization cycles reported in CFU/carrier recovered, percent and log_{10} Reduction, for the 323 L model. Approved biological indicator results are highlighted in red. A greater than 6-log reduction of these bioindicators in the truncated cycle equals a 12-log SAL for the complete cycle, meeting the "overkill" requirement in the USP.

Sterilization cycle	Microorganism	Positive controls (CFU/carrier)	Percent reduction	Log reduction
Complete (2 hours at 140°C)	*B. atrophaeus* (ATCC 6633) - vegetative form	5,300,000	99.9	6.72
	B. atrophaeus (ATCC 6633) - spore suspension	3,800,000	99.9	6.58
	P. aeruginosa (ATCC 15442)	1,200,000	99.9	7.08
	A. brasiliensis (ATCC 16404)	3,400,000	99.9	6.53
Truncated 1 (1 hour at 140°C)	*B. atrophaeus* (ATCC 6633) - vegetative form	2,600,000	99.9	6.41
	B. atrophaeus (ATCC 6633) - spore suspension	3,700,000	99.9	6.57
	P. aeruginosa (ATCC 15442)	8,000,000	99.9	6.90
	A. brasiliensis (ATCC 16404)	2,100,000	99.9	6.32
Truncated 2 (1 hour at 140°C)	*B. atrophaeus* (ATCC 6633) - vegetative form	5,600,000	99.9	6.75
	B. atrophaeus (ATCC 6633) - spore suspension	4,800,000	99.9	6.68
	P. aeruginosa (ATCC 15442)	9,800,000	99.9	6.99
	A. brasiliensis (ATCC 16404)	3,800,000	99.9	6.58
Truncated 3 (1 hour at 140°C)	*B. atrophaeus* (ATCC 6633) - vegetative form	6,600,000	99.9	6.82
	B. atrophaeus (ATCC 6633) - spore suspension	4,400,000	99.9	6.64
	P. aeruginosa (ATCC 15442)	7,900,000	99.9	6.90
	A. brasiliensis (ATCC 16404)	3,500,000	99.9	6.54

Table 3: Results of Positive Control Complete and Truncated Sterilization Cycles Reported in CFU/Carrier recovered, Percent and Log_{10} Reduction, for the 232 L model. Approved biological indicator results are highlighted in red. A greater than 6-log reduction of these bioindicators in the truncated cycle equals a 12-log SAL for the complete cycle, meeting the "overkill" requirement in the USP.

Sterilization cycle	Microorganism	Positive controls (CFU/carrier)	Percent reduction	Log reduction
Complete (2 hours at 140°C)	*E. coli* (ATCC 8739)	6,100	99.9	3.78
	P. aeruginosa (ATCC 9027)	8,200	99.9	3.91
	A. brasiliensis (ATCC 16404)	810,000	99.9	5.91
	B. atrophaeus - spore strips I	910,000	99.9	5.96
	B. atrophaeus - spore strips II	1,200,000	99.9	6.08
Truncated 1 (1 hour at 140°C)	*E. coli* (ATCC 8739)	2,600	99.9	3.41
	P. aeruginosa (ATCC 9027)	7,300	99.9	3.86
	A. brasiliensis (ATCC 16404)	760,000	99.9	5.88
	B. atrophaeus - spore strips I	1,200,000	99.9	6.08
	B. atrophaeus - spore strips II	1,300,000	99.9	6.11
Truncated 2 (1 hour at 140°C)	*E. coli* (ATCC 8739)	4,500	99.9	3.65
	P. aeruginosa (ATCC 9027)	5,600	99.9	3.75
	A. brasiliensis (ATCC 16404)	730,000	99.9	5.86
	B. atrophaeus - spore strips I	940,000	99.9	5.97
	B. atrophaeus - spore strips II	1,400,000	99.9	6.15
Truncated 3 (1 hour at 140°C)	*E. coli* (ATCC 8739)	3,400	99.9	3.53
	P. aeruginosa (ATCC 9027)	6,100	99.9	3.78
	A. brasiliensis (ATCC 16404)	840,000	99.9	5.92
	B. atrophaeus - spore strips I	1,100,000	99.9	6.04
	B. atrophaeus - spore strips II	1,400,000	99.9	6.15

Temperature mapping

The parameters critical to success of dry heat sterilization are temperature and time. As per the EUP, the reference conditions for dry heat sterilization are 160°C for 2 hours, but different combinations of the time and temperature can also be used, as long as the process is demonstrated to deliver an adequate and reproduceable level of lethality. In validating sterilization, it is important to not only reach a temperature that is known to eliminate microorganisms, but to provide evidence that all areas 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.

The Steri-Cult CO_2 Incubators were mapped for temperature in fifty-one locations throughout the sterilization cycle, and the average results are shown in Figure 2. At time zero, the 140°C sterilization cycle was initiated from 37°C, with the temperature rising to 140°C over approximately 3.5 hours. As shown, some areas reached 140°C faster than others, but all tested areas reach a minimum of 135.8°C for minimum two hours. Only one position did not reach 140°C, and this was on the inside of the glass door. This position remains near this temperature for over four hours. Since the glass door was one of the locations tested using the *B. atrophaeus* spores, this test demonstrates that this location is sterilized even though it did not quite reach the specified 140°C temperature. One other spot only reached 132.7°C, but this location was inside the outer steel door and was not in the incubator chamber, so is not required to be sterilized. Lower temperatures were recorded on external incubator surfaces, and demonstrate that these areas will not be dangerously hot to the touch.

Conclusions

Independent studies on both Steri-Cult CO_2 Incubator models, the 323 L and the 232 L sizes, proved that a Steri-Cult CO_2 incubator featuring the 140°C dry heat automated high temperature sterilization cycle achieved true sterilization, completely eliminating more than 6 logs (>I x 10^6) of approved biological indicator organisms in half the normal sterilization time. Thus, the standard 140°C cycle, which holds at 140°C for 2 hours, is designed to achieve a true 12 log SAL, meeting the international pharmacopeia requirements for the "overkill" method to prove sterilization.

The tests of the modified 140°C cycle showed complete elimination of heat resistant bacterial endospores (*B. atrophaeus*), the biological indicator required by both EU and US Pharmacopeias for the validation of a dry heat sterilization process. This was demonstrated for both model sizes, using a prepared spore suspension as well as commercially available heat resistant spore strips. The extensive temperature mapping showed that all areas are designed to hold lethal temperatures as per the design specification.

Experienced cell culturists understand that battling these common contaminants is an ongoing challenge, and the 140°C dry heat sterilization cycle offers a great tool in the battle for quality cell culture results. A Steri-Cult CO_2 Incubator featuring the 140°C cycle assures total, uniform sterilization of all chamber surfaces,

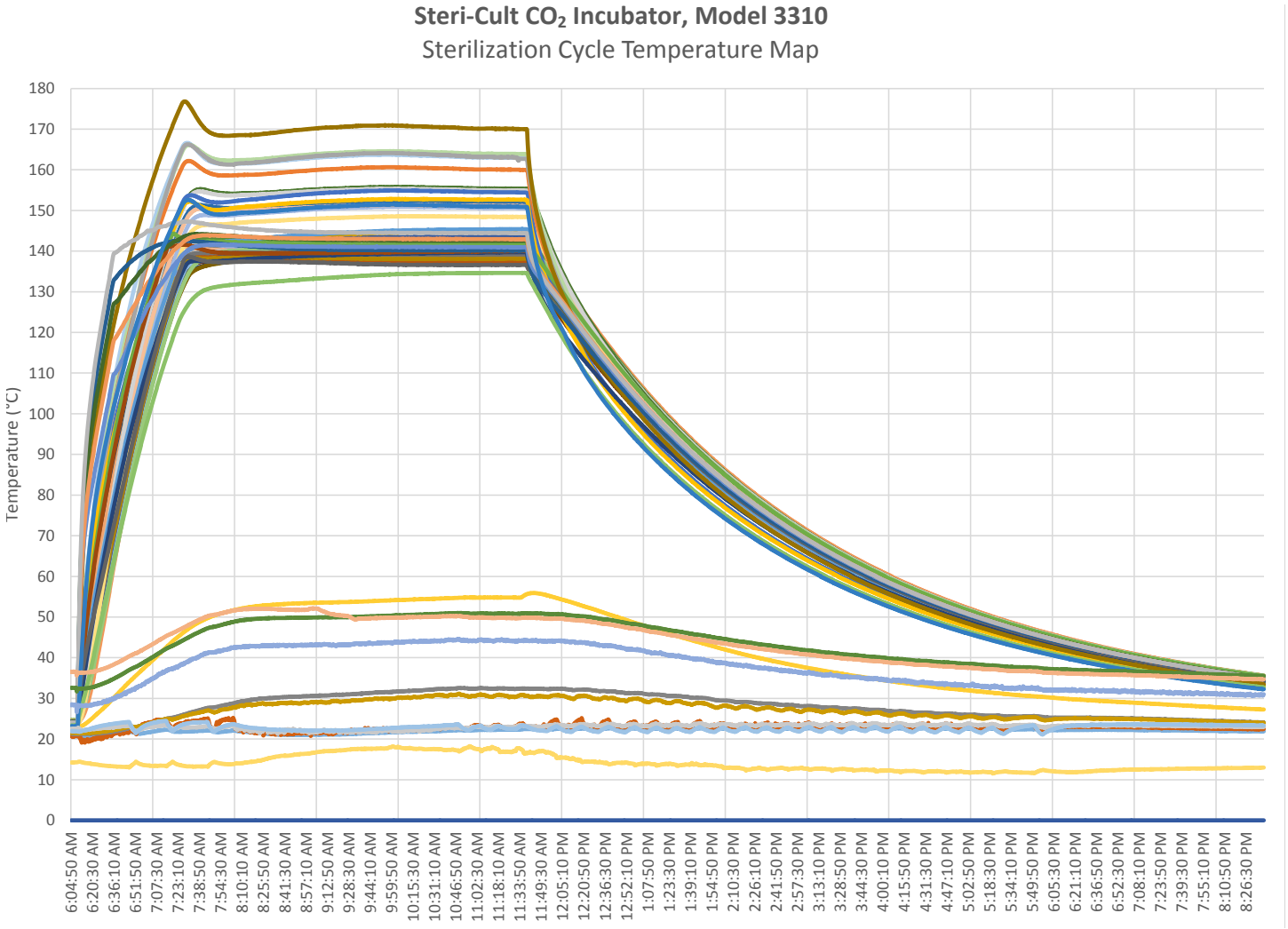

Figure 2: Typical temperature map for full 140°C dry heat cycle. Thermocouples were place in 51 locations in the incubator, including the walls, floor, glass door, water reservoir cover and floor, and 3 shelves. All internal areas reached a minimum of 135.8°C for at least 4 hours during the complete cycle. Lower temperatures were probes placed on external incubator surfaces.

including the Steri-Cult CO_2 Incubator technical area where the external humidity system is located. 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 also reduces the need for potentially toxic germicides which could pose a risk for sensitive cultures.

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