Application Note:
ANCO2THERMDIS 0809

Total Thermal Disinfection of CO_2 incubators

Fully automated, Residue-Free and Completely Safe

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Key words:
Thermal Disinfection Procedures,
CO_2 incubators, 180°C Hot Air Disinfection,
Moist Heat Disinfection

Summary

Thermal disinfection procedures are utilized wherever the highest level of hygiene and demonstrable safety are required. In cell culture laboratories, the CO_2 incubator must meet such requirements. In order to test the disinfection routines for Thermo Scientific Heracell® incubators, the reduction or elimination of various types of test germs, artificially applied to the interior walls was examined. This examination employed both vegetative, relatively, sensitive species of bacteria, as well as spore suspensions, known to be heat-resistant.

Both the 180°C hot air disinfection over three hours using dry heat (Thermo Scientific Cytoperm® 2, Thermo Scientific Heraeus BBD 6220) as well as the ContraCon disinfection routine employed by the Heracell® and operating at 90°C for nine hours with moist heat resulted in the secure elimination of all applied test germs and spore suspensions.

While alternate disinfection procedures were also tested, leading to a drastic reduction in the number of test germs, none of these resulted in the complete elimination of the germs. A routine operating at a disinfection temperature of 90°C and utilizing dry heat was found to be inadequate, even when the unit was held at the disinfection temperature for 15 hours. When employing moist heat at 90°C, disinfection times of less than nine hours were also found to be inadequate for a complete disinfection of the incubators.

Introduction

When working with cell cultures, aseptic procedures and higher levels of hygiene have absolute priority in the entire laboratory area. Microbial contamination results not only in frustration, but may also lead to a fatal distortion of the results. In addition, contamination often leads to the loss of cell cultures, leading to wasted time and effort.

Next to the humidification water, humans undoubtedly represent the primary source of contamination. Germs can be transferred both in the air, bound to particles (So-called “air-borne germs”), as well as by means of direct human contact (so-called “smear infections”). Such germs are found on, and inside, a wide variety of laboratory equipment, including the CO_2 incubator. Because incubators provide the best possible growth environment for not only the cell cultures, but also for any intruding germs, these devices must receive special attention. Germ infection and the lacking or inadequate decontamination of the incubator represent possible sources for the contamination of cell cultures. Thermo Scientific CO_2 incubators have fully automated decontamination procedures for their equipment in order to prevent and eliminate contamination sources in the incubator. The efficiency of these procedures was examined in numerous experiments employing defined, artificial contamination with a variety of test bacteria and spores.

Thermal Disinfection Procedures

Where thermal disinfection procedures are concerned, a differentiation is made between two different types:

1) Procedures employing dry heat, e.g., hot air disinfection;
2) Procedures employing moist heat, e.g., steam disinfection

The results of, and time required for, these disinfection procedures depend to a great extent on the following factors (1):

- The type, functional state, and initial number of the contaminants present
- The disinfection temperature
- The relative humidity during disinfection, and
- The treatment period

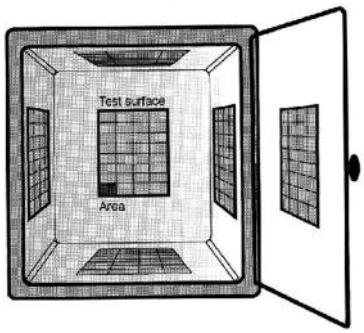

Figure 1: Incubator sample chamber and glass door
Distribution of the test surfaces and individual areas for artificial contamination with various germ species

Three different disinfection procedures were examined:

180°C hot air disinfection:

The entire incubator sample chamber, including any built-in components and sensors, is heated to 180°C. Once the disinfection temperature has been reached, the unit is held in this state for three hours, after which it cools off to the predefined desired operating temperature. This disinfection procedure is employed by Cytoperm 2 and BBD 6220 incubators.

90°C hot air disinfection:

This procedure was tested as an alternative to the 180°C hot air disinfection routine.
The entire incubator sample chamber, including any built in components and sensors, is heated to 90°C. The unit is held at this temperature for 15 hours, after which it cools off to the predefined desired operating temperature.

90°C moist heat disinfection:

Prior to the start of the disinfection routine, a defined amount of water (distilled) is placed in the unit. Once the routine is started, the entire incubator sample chamber, including any built in components and sensors, is heated to 90°C, while a relative humidity of > 80% develops. In various tests, this disinfecting atmosphere was maintained for 15 hours, nine hours (ContraCon disinfection routine), or four and one-half hours. At the end of the routine, the unit cools off to the predefined desired operating temperature.

Test Germs	Primary Culture L+S AG, Germany	Type of Germ	Occurrence/Properties
Bacillus subtilis varietas niger	ATCC 3972	spore suspension	ubiquitous, heat resistant
Bacillus subtilis	ATCC 6633	spore suspension	ubiquitous, heat resistant
Bacillus stearothermophilus	ATCC 7953	spore suspension	ubiquitous, heat resistant
Enterococcus faecalis	ATCC 29212	vegetative bacteria	humans, intestinal flora
Escherichia coli	ATCC 8739	vegetative bacteria	humans, potential pathogenic/hygienic relevance
Pseudomonas aeruginosa	ATCC 9027	vegetative bacteria	water, potential pathogenic/hygienic relevance
Staphylococcus epidermidis	ATCC 12228	vegetative bacteria	human, skin and mucous membranes
Corynebacterium xerosis	ATCC 7711	vegetative bacteria	human, skin and mucous membranes
Aspergillus niger	ATCC 16404	spore suspension	ubiquitous

Table 1: Test Germs

Testgerms / Area	*B. subtilis*	*B. stearothermophilus*	*Ec. faecalis*	*E. coli*	*P. aeruginosa*	*S. epidermidis*	*C. xerosis*	*A. niger*
Area 1	Control surface for the quantitative determination of the actually applied germ counts **prior** to decontamination (Method): Surface procedure; detection limit: 10^5 CFU/area to 100 CFU/area							
Area 2	For the quantitative determination of the germ count **after** decontamination (Method): Surface procedure; detection limit: 10^5 CFU/area to 100 CFU/area							
Area 3	For the quantitative determination of the germ count **after** decontamination (Method): Membrane filtration; detection limit: 300 CFU/area to 1 CFU/area							
Area 4	For the qualitative determination of sublethally damaged germs **after** decontamination (Method): Specific enrichment procedures*, this permits the determination of even sublethally damaged bacteria.							

* Enrichment in species-specific media over 48 hours: Molds at 23°C, Bacteria at 36°C, Thermophilic Bacteria species at 55°C.

Table 2: Subdivision of the various test surfaces into areas, and procedures to determine the germ count.
CFU = Colony Forming Unit

Test setup

Re-Isolation of the Test Germs
Templates were used to outline test areas on all six interior walls of the incubators (right side wall, left side wall, rear wall, top, bottom, glass door) (Fig.1). Each test surface was divided into 4x4 cm test areas, to which a defined number of bacteria from the test contaminants described below were applied (Table 1).

To verify that surface contamination had, in fact, occurred, the control surfaces were re-isolated prior to the start of the disinfection (Table 2, Area 1). Both quantitative and qualitative methods of bacterial confirmation were employed to examine and determine the efficiency of the disinfection procedures.

All tests were performed several times.

The selection of the test bacteria included both ambient bacteria as well as species with pathogenic/hygienic relevance. All germs were examined for identitiy and purity by means of a biochemical profile (ATB system, BioMérieux, Nürtingen, Germany).

In addition to the test contaminants described in Tab. 1, commercial bioindicators normally employed to test the effectiveness of certain sterilization procedures and required for the validation of "official procedures" were also tested.

(Stericon plus indicators, Merck, Darmstadt, Germany; Number of germs: *Bacillus stearotherophilus* 5 x 10^5 until 1 x 10^7 CFU/ampoule;

BioStrips, BAG Biologische Analysesysteme GmbH, Lich, Germany Number of germ: *Bacillus stearothermophilus* 1,8 x 10^5, Number of germs: *Bacillus subtilis* 2,1 x 10^6).

Contamination of the test surfaces and germ re-isolation

Each test germ was applied to four areas of every test surface. 0.1 ml of the germ suspension were applied to the marked areas by means of pipetting and/or swabbing to provide a bacterial count of approx. 10^5 CFU/area. The disinfection routine was started approximately one hour after the application of the test germs.

Germ re-isolation prior to the decontamination

One hour after the application of the test germ, the actually applied germ count in Area 1 was determined for each type of bacteria (Table 2). A moistened swab and the conventional wipe technique were employed. The swab was placed in 10 ml of a sterile, NaCI/peptone buffer solution (2), and the germ count was determined by means of the surface procedure with the aid of a spirometer. The decontamination process was then started.

Germ re-isolation after decontamination

At the end of each individual decontamination cycle, the areas were examined for the presence of surviving germs. In order to be able to precisely quantify different germ reduction, three different re-isolation techniques were employed for each test germ and each test surface (Table 2, Area 2-4). This resulted in a detection limit of one germ per area. The possible complete elimination of the bacteria was also verified by means of species-specific enrichment methods.

Results

The results of the germ re-isolation from the control surfaces before decontamination (area 1 of each test surface) show an almost identical germ density of approx. 10^5 CFU/area in all cases. This documents the validity of the contamination and re-isolation procedures.

Efficiency of the procedures

180°C Hot Air Disinfection employed in the cytoperm 2 and BBD 6220

While, depending on their type and functional state, vegetative forms of contaminants (bacteria, molds) are destroyed within a matter of minutes at temperatures above approx. 80°C, higher temperatures or longer exposure periods are generally necessary to destroy known heat resistant forms (bacterial spores) (1). For this reason, only the test germ, *Bacillus subtilis varietas niger*, was employed here. This bacterium is recommended for official sterilization procedures by the *European Pharmacopoeia, 1997, official German edition* (Ph. Eur. 97, 5.1.2) and the *United States Pharmacopoeia*, USP 23 (3).

After multiple, independent tests, no germs were re-isolated from any of the test surfaces. Even contaminations with spores of *B. subtilis varietas niger* at a density of 8-9 x 10^5 CFU/area were completely eliminated (Table 3).

This is a clear validation of the efficiency of the 180°C hot air disinfection for sterilization. The parameters for time and temperature (3 h/180°C) specified in the program are suitable for the routine sterilization of incubators.
The selected test procedure also confirmed that this process also results in the sterilization of the entire sample chamber, including all surfaces and sensors.

90°C hot air disinfection

As the result indicate, 90°C of dry heat (even over a period of 15 hours) is inadequate to completely destroy, in particular, the employed, heat-resistant bacillus spores (Table 3). Although the germ density on the test surfaces was, in some cases, drastically reduced, bacillus species were quantitatively detected, as were representatives of vegetative germ species after enrichment.

90°C moist heat disinfection

Heracell ContraCon Disinfection

In numerous tests, germ applied directly to the interior walls at a density of 10^5 CFU/area were totally eliminated after a holding time of at least 9 hours (Table 3). Even spore suspensions prepared from laboratory host cultures of *B. subtilis* and *B. stearothermophilus*, as recommended by the Ph. Eur., 1997, and the USP 23 for validation of sterilization procedures with moist heat, were eliminated (Fig. 2). The same applies to a spore suspension of *B. stearothermophilus* taken from a Sterikon ampoule and applied directly to the test areas. This bacterial strain, a commercial

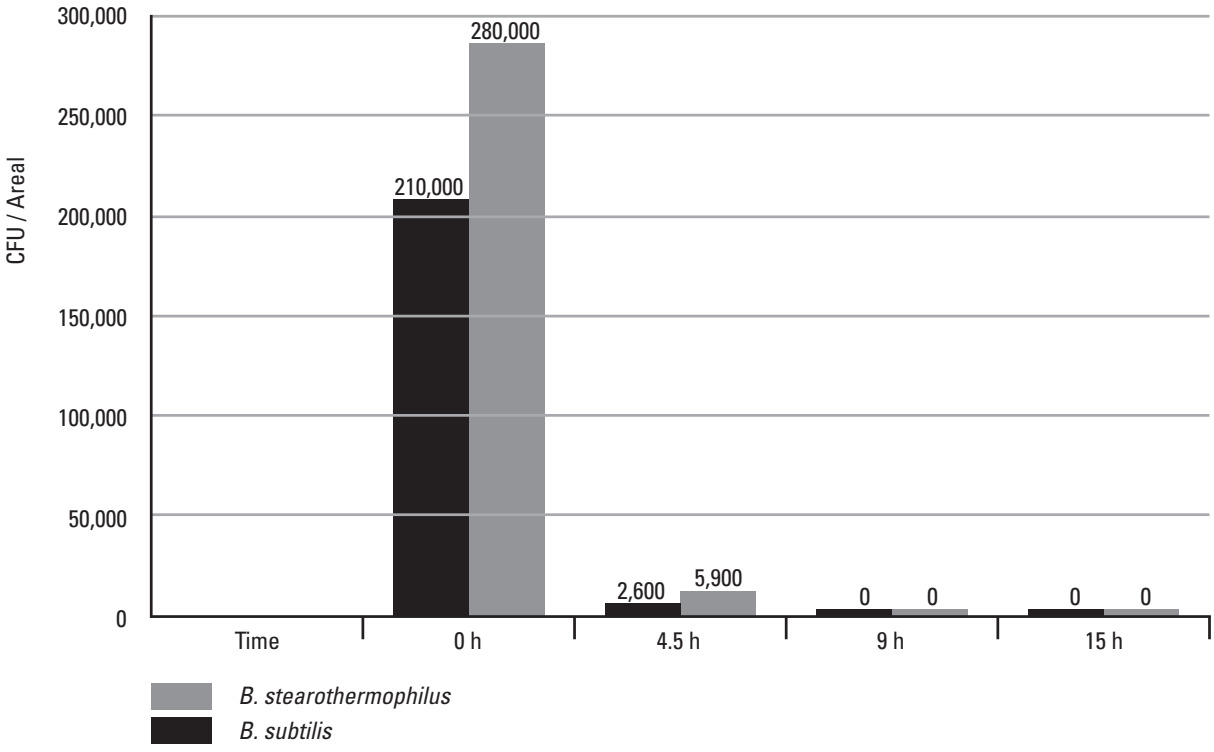

Figure 2: Time dependent kinetics of germ reduction. Bacillus spores at 90°C and moist heat.

Disinfection procedures	Dry heat		Moist heat		
Disinfection temperature	**180°C**	**90°C**		**90°C**	
Holding time	**3 h**	**15 h**	**4.5 h**	**9 h ContraCon**	**15 h**
*Bacillus subtilis**	-	(+)	(+)	-	-
*Bacillus stearothermophilus**	-	(+)	(+)	-	-
Enterococcus faecalis	-	(+)		-	-
Escherichia coli	-	(+)		-	-
Pseudomonas aeruginosa	-	(+)		-	-
Staphylococcus epidermidis	-	(+)		-	-
Corynebacterium xerosis	-	-		-	-
*Aspergillus niger**	-	(+)		-	-

+ Evidence of the test germs in the primary culture
(+) Reduction in the germ count
- Elimination of the germs
* Spore suspension

Table 3: Comparison of the efficiency of thermal disinfection procedures.

bioindicator with a D_{121} value of 1.5 +/- 0.5 minutes, exhibits an extremely high heat resistance. With the goal of shortening the time required to perform this disinfection routine, a treatment period of four and one-half hours at 90°C and moist heat was also tested. While this shortened treatment time led to a reduction in the tested bacillus spores by a factor of 100, it did not eliminate them (Fig. 2, Table 3).

Therefore, only the selected parameters (9h / moist heat / 90°C) and the programmed process employed in the Heracell ContraCon disinfection routine are suitable for totally destroying all contaminants on every surface of the sample chamber. The complete elimination of all germs is assured, even at germ densities of approx. 10^5 CFU/area (4). This applies to vegetative bacterial forms as well as spores – even those of *B. stearothermophilus*, that are known to possess high thermal resistance.

Conclusion

With the established 180°C hot air disinfection employed by the Cytoperm and BBD 6220, and the new ContraCon disinfection routine employed by the Heracell unit, you have two different processes for the total thermal disinfection of CO_2 incubators.

Periodic application of the fully automated disinfection routine prevents the formation and spread of contamination sources, even in those areas of the incubator that are difficult to access during manual cleaning.

The thermal disinfection routines employed by the incubators leave no residue, are environmentally friendly, fully automatic, and require little in the way of preparatory or subsequent work. Since all built-in components, fans, and sensors remain inside the unit during disinfection, an additional autoclave is not required. In addition, maintenance and replacement of, for example, UV-lamps and sterile filters are eliminated.

Since the efficiency of thermal procedures can be tested in a manner similar to that for chemical disinfection, the manufacturer feels that it is his responsibility to make these test reports available to the public.

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