Hot air sterilization at 180 °C – an internationally recognized and validated method to eliminate microbial contamination in CO_2-incubators

A comparison of different decontamination concepts for CO_2-incubators – from a user perspective

Daniela Maurer, Dipl. Biochem., Scientific Product Management
BINDER GmbH, Tuttlingen

Introduction

Microbial contamination, caused by bacteria, bacterial spores, viruses, mycetozoa, yeast or other microorganisms, frequently presents a major risk in cell culture experiments. Since this contamination does not necessarily occur together with the overgrowth of the cultivated cell type, it is often detected too late. More subtle effects, such as the deprivation of essential nutrients and segregation of microbial metabolites are caused by slight changes in the pH, which for human and mammalian cells has to be kept within a range of 7.4-7.6; the resulting hyperacidity of the culture medium then slows down the growth rate and decreases confluence. Changes in host cell morphology and even genetic changes such as chromosomal aberration and translocation, can, for instance, be caused by mycoplasma infection. In extreme cases, a single germ can destroy the work of weeks or months of intricate research effort.

Causes for the introduction of germs or dispersion of contamination can be innumerable. Use of cell lines, media, serum, or other reagents with hidden contamination, airborne bacteria, or laboratory equipment that was not properly disinfected or sterilized, or contamination which is accidentally introduced by lab technicians.

Since checks for the presence of germs frequently involves complicated and tedious procedures, measures to for contamination control must be initiated.

The importance of contamination control when working with cell lines and primary cultures

In view of the significant progress in the area of sensitive cell culture applications, such as tissue engineering or regenerative cell and tissue therapy, the requirements for CO_2-incubators have changed.

Highest standards are thus applied to the perfection and reliability of the entire process chain, in which the CO_2-incubator occupies a key position, since it must replicate the natural in vivo conditions for optimal cell growth as accurately as possible. For all cell-based therapeutics, e.g. a cell suspension of autologous chrondrocytes for reimplantation in a patient, the problem lies in that the end product itself cannot be sterilized, unlike certain other pharmaceutics, for instance. For this reason, guidelines such as the Good Manufacturing Practice (GMP)[1], the draft guideline for Good cell culture practice (GCCP)[2] as well as the European Human Tissue Directive[3], amongst other things, recommend the use of sterile disposable articles and/or equipment which can be sterilized for processing of human cells and tissue. Sterile conditions must be guaranteed for in vitro cell cultures throughout the entire cultivation period, since in addition to the risk of spreading contamination, the life-threatening danger of infecting patients is ever present.

Clarification of terms: disinfection, sterilization, decontamination

Let us briefly look at the concepts of sterilization, decontamination and disinfection. Sterilization stands for the complete elimination and/or absence of viable microorganisms; disinfection is understood as the elimination or inactivation of all pathogens present, which frequently only represent a partial quantity of all the present contaminants, however. The term decontamination, on the other hand, can be used in various ways such as for the removal of biological or chemical or radioactive contamination, but it often does not allow any precise quantifiable conclusions to be made with respect to its effectiveness.

Concerning the mechanisms and verification of the effectiveness of disinfection and sterilization methods, a multitude of different guidelines and standards exists worldwide, particularly for use in the pharmaceutical industry and in the clinical sector. The pharmacopeias basically specify autoclave sterilization, hot air sterilization and the use of ethylene oxide and sterile filtration as sterilization methods. The suitability of a specific method for a specific application must therefore be carefully scrutinized and the sterilization process used requires validation with defined test organisms.

For effective sterilization, the various national pharmacopeias[4] have agreed upon using a 6-log reduction of viable microorganisms, which is equivalent to one viable microorganism in a million, i.e. 1:1,000,000 units. This corresponds to a reduction of 99.9999% min. in the number of test organisms that were initially used.

The development of decontamination concepts for CO_2-incubators

The various manufacturers of CO_2-incubators have developed some very different concepts for the prevention and control of contamination; recently, the focus has increasingly been on process safety, effectiveness and cost awareness.

In this context, the requirement for sterility of a cell culture inside a CO_2-incubator has posed significant technical challenges.

In selecting a suitable decontamination method, the following critical aspects must be taken into account:

- that the inner chamber of the incubator is suitable for periodic spray/wipe disinfection, which is the standard process to reduce the bio burden, i.e. the microbiological load of the CO_2-incubator system. Easy to clean metal and glass surfaces (incubator interior, and the glass door which closes off the test space) that have no welding seams, and wherever possible should have no screw connections and/or elements which must be dismantled before disinfection (fan impellers, covers of air duct elements) to allow prompt cleaning and uniform wetting of all interior surfaces with disinfectant. Reducing the number of interior fittings such as sliding rack systems, humidification systems, etc. to the absolute minimum technical essentials in order to minimize potential contamination of inner surfaces right from outset.
- prevention of condensation which could serve as breeding ground for germs in the incubator interior
- secure elimination of potential contamination by means of verifiable, effective sterilization processes

In addition, the cell culture system used should prevent the introduction of airborne germs, some of which are present even under clean room conditions. Cell culture bottles with a 0.2 µm bacterial filter were found to be suitable for this purpose.

The following decontamination processes can presently be found on the market:

- Hot air disinfection at temperatures between 120 °C and 140 °C, used at different contact and cycle times (sometimes combined with HEPA filter systems), which do not represent hot air sterilization in accordance with the pharmacopeias, (see *Fig. 1*) For a process using dry heat at 140 °C, a 6-log reduction was indicated for *B. subtilis* var. Niger spores ATCC #9372[5].
- Disinfection with wet steam at 90° which has shown that more thermally resistant spores may not be safely eliminated[6].
- A combination of wet steam 95 °C / 145 °C hot air decontamination procedures in combination with HEPA filters, for which no studies regarding its effectiveness are available, and in which filters must be regularly replaced after the decontamination procedure.
- HEPA filter systems with different pore sizes, e.g. 0.3 µm, which achieve particle reduction within the incubator atmosphere[7], but which also need regular maintenance
- Inner chambers made of copper to release bactericidal copper ions through oxidation, which act as cytotoxins on the respiratory chain of bacterial metabolisms. This effect has been known for hundreds of years and has been scientifically substantiated. However, this method is not suitable for all types of bacteria species, or bacteria and fungus spores and also not for viruses, and it thus offers only limited protection. Moreover, the released copper ions are also toxic to humans. This process furthermore also discolors all copper surfaces in the incubator. The effectiveness of copper/stainless steel alloys and/or copper enriched stainless steel on test organisms as demonstrated in a series of experiments[8] usually amounts to 99.847% to max. 99,998%, which therefore does not fulfill the sterility requirements (see above); strictly speaking, for disinfection, a 99.999% reduction in the number of initial germs must be proven.
- UV treatment by application of non-ozonogenic UVC radiation with a wavelength of 253.7 nm. The mutagenic effect of UV radiation has been proven, its effectiveness however depends directly on direct irradiation, since it has only limited penetration and is thus only suitable for the treatment of surfaces. The use of UV radiation sources for water disinfection is particularly well known. The effectiveness of treating water in humidification systems in CO_2-incubators has been described[8]; however, it appears that additional UV treatment is not necessary, if the water in the water pan is regularly replaced with sterile, distilled water (manufacturer's recommendation are generally once to twice weekly). In addition, for airborne germs the germicidal effect seems neglect able since the dwelling time in the area of direct irradiation is marginal. Wallhäußer et. al.[4] note a decreasing effect of UV radiation at ambient humidity values of larger than 80% R.H.
- Hot-air sterilization at temperatures ≥160 °C, i.e. with dry heat, at the exposure times defined in the pharmacopeias (see *Fig. 1*). Evidence of successful sterilization of test germs pursuant to USP has been proven for single hot air sterilization programs[6].

International standards concerning hot-air sterilization

Standard	Temperature	Sterilization period
US Pharmacopoeia	170 °C	120 min
European Pharmacopoeia	160 °C minimum	120 min minimum
American Dental Association	160 °C	120 min
ANSI/AAMI ST63-D	≥160 °C	Not defined
ANSI/AAMI ST50	160 °C	120 min
DIN 58947	180 °C	30 min
Pharmacopoeia Nordica	180 °C	30 min
Hygiene Directive Robert Koch Institute	160 °C 180 °C	200 min or 30 min
Japanese Pharmacopoeia	160 °C - 170 °C or 170 °C - 180 °C or 180 °C - 190 °C	120 min or 60 min or 30 min
British Pharmacopoeia	160 °C minimum	60 min minimum

Fig. 1 International standards for hot-air sterilization

A basic requirement is that the contact period for the goods to be treated, i.e., the inner surfaces in the case of CO_2-incubators, is appropriate. The cycle time can be calculated by allowing for the additional times needed to heat and cool-down the system.
As a rule of thumb, the higher the sterilization temperature applied, the shorter the sterilization period required.

In current publications, the focus has been on the total time of the decontamination procedure and the need for continuous contamination; any critical review should also consider the actual process time required as well as the costs for retrofitting high-maintenance components, such as replacement of HEPA filters and UV lamps, as well as any costs that are associated with the recommended subsequent, manual spray/wipe disinfection. At this point, special attention should be given to the fact that the above-mentioned processes, except for the hot air sterilization at temperatures of ≥160 °C with the necessary exposure time, do not constitute processes that comply with the standards in terms of the pharmacopeias mentioned and are therefore not regarded as approved sterilization methods.

Simplicity in the application of different decontamination concepts, such as process safety, effectiveness, and cost awareness

In the following, the suitability of these processes for standards-compliant sterilization and their process safety, effectiveness and cost awareness, will be compared.

- Disinfection through use of wet steam as 90 °C wet heat: in this case, the advantage is that a large volume of germicidal steam can be generated with a relatively small amount of water; however, this process is not comparable to the effectiveness of autoclave sterilization with live steam at 121 °C. It has been proven that the effectiveness on temperature resistant spores of species *Bacillus subtilis* and *Bacillus stearothermophilus* is unsatisfactory[6,9].
  At times, this required a cycle time of at least 25 hours, followed by subsequent recalibration of the CO_2 sensor system. The condensate produced by the cooling of the wet steam involves potential risk of re-contamination of the treated stainless steel inner surfaces. The manufacturer recommends subsequent spray/wipe disinfection with a suitable disinfectant, including the use of sterile cloths.

- The use of HEPA filters to reduce particle concentration in clean rooms and clean benches is a recognized and verifiably effective process. However, the application of HEPA filters to control contamination in CO_2-incubators is contingent upon the following conditions:

    - The incubator air is sucked into a HEPA filter with defined removal efficiency through suction that is generated with the fan impeller; with suitable filters, a reduction in the particle load of the incubator air can be achieved.
    - In some CO_2-incubators, this forced air recirculation is also required for homogenous temperature distribution and/or homogenization of the CO_2 concentration within the incubator.
    - However, HEPA filters still contain viable germs, which require regular filter replacement. Extended autoclaving is recommended prior to disposing of the filters.
    - Cycles with dry or wet steam disinfection run in CO2-incubators with HEPA filter require routine filter replacement i.e. filters elements should be replaced prior to starting the decontamination procedure. If this decontamination procedure is carried out regularly, this can amount to considerable costs.
    - When open culture flasks are handled, e.g. when moving between culture flasks, working in a low particle environment, such as a clean bench, is imperative in order to keep germs out of cultures.
    - While a reduction of particles within an incubator atmosphere can minimize the contamination risk in open culture systems, such as when working with Petri dishes, it should be noted that high-quality cell culture containers with integrated bacterial filters in the screw cap are available in order to block the entry of germs from the incubator atmosphere and to securely prevent any cross contamination between the individual cell culture containers.
    - Sterile conditions are absolutely essential inside cell culture containers. In this case, a reduction of particles in the atmosphere of a CO_2-incubator would thus not be required.

- The application of UV irradiation in combination with a copper/stainless steel alloy was described previously[8]. For intensive treatment of all interior surfaces, the manufacturer recommend 24-hour direct UV irradiation, if necessary, which requires prior dismantling of the sliding rack system along with the air plenum components, including the fan impeller. At the same time, all interior fittings must also be autoclaved. Following UV cycle, all interior surfaces must once again be disinfected with 70% isopropyl alcohol and a sterile cloth. For routine application, which might be necessary at any time, this process seems relatively expensive and labor-intensive, compared to hot air sterilization using an overnight cycle. In spite of the relatively long service life of UV lighting systems (1000 hours, according to manufacturer's specifications), the replacement of lamps would be a rather expensive item in regular application.

The BINDER concept to minimize surface contamination and effectively eliminate contamination

The BINDER's series CB and C 150 CO_2-incubators are designed for easy spray/wipe disinfection and routine auto-sterilization. This customized design facilitates application and does not require replacement of expensive parts, such as filters, UV lamps, etc. It contains the following components:

- easy to clean seamless, deep-drawn inner chamber with 27% percent less surface for potential contamination, and an integrated shelf mounting system to minimize surface contamination

- absence of condensation, even when working under conditions of highly saturated air humidity, and mechanically polished stainless steel surfaces without welding seams to prevent nesting of airborne germs

- Verifiable, effective automatic hot air sterilization at 180 °C in compliance with standards, which can be performed conveniently overnight and meets international guideline requirements for hot air sterilization.

The above narrative is an attempt to compare different decontamination concepts for CO_2-incubators from a user's point of view. Cell culture specialists can thus select among the various options offered by the manufacturers of CO_2-incubators to find a concept which will meet their requirements.

International guideline references:

- US Pharmacopoeia www.usp.com
- Pharmcopeia Europea 1997 5.1.1
- American Dental Association www.ada.org
- Association for the Advancement of Medical Instrumentation (AAMI) www.aami.org
- German Institute for Standardization (DIN) www2.din.de
- Pharmacopoeia Nordica Online Reference via www.dekker.com
- Federal Law Gazette 22, No. 10, performing sterilization, Appendix to sub-paragraph 7.1 of the Guideline for the identification, prevention and control of infection in hospitals, Ordinance for the prevention of infetious disease (Hygiene Directive)], Robert-Koch-Institut, 2003
- Japanese Pharmacopoeia www.jpdb.nihs.go.jp/jp14e
- British Pharmacopoeia Commission Methods of Sterilization. London, UK: Appendix X VIII, 2003

---

[1] http://www.fda.gov/cdrh/comp/gmp.html

[2] S. Coecke et. al. Guidance on Good Cell Culture Practice, A Report of the Second ECVAM Task Force on Good Cell Culture (GCCP), ATLA 33, 261-287, 2005

[3] European Human Tissue Directive, 2006/17/EC implementing Directive 2004/23/EC of the European Parliament and of the Council as regards certain technical requirements for the donation, procurement and testing of human tissue and cells

[4] K.H. Wallhäußer, Practice of Sterilization, disinfection – Conservation, Germ Identification, 5. edition, 1995

[5] J.Dalamasso, APEX Laboratories, Effective Heat Sterilization in CO_2-incubators , Vol. 4, No. 3, Thermo Electron Corporation's Heat Sterilization White Paper, 2003

[6] P. Distler, 180 °C Hot air sterilization: a safe method against microbiological contamination in CO_2-incubators Lab Asia, November 2003, p. 11

[7] A. Campbell, D. Figel, Importance of Class 100 Air in a CO_2-incubator, Vol. 4, No. 1, Thermo Electron Corporation's Class 100 Air White Paper, 2003

[8] H. Basujima, D. Mistry, Technical Development Report, Sanyo Electric Biomedical Co., Ltd. A Comparative Analysis of Ultra-violet Light Decontamination versus High-Heat Sterilization in the Cell Culture CO_2-incubator, with the Use of Copper-Enriched Steel Construction to Achieve Background Contamination Control™, 2007

[9] Biosafety Investigation Unit, CAMR, Efficacy of a CO_2-incubator heat disinfection cycle on dried microbes, 1998