



Cell Cultivation without Contamination

Hot air sterilization and other means of contamination control in
CO₂ incubators – a comparison of concepts from the user perspective

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Summary

No other problem in cell culturing is as universal as microbial contamination. In order to avoid it, good sterile techniques and thorough culture handling are essential. Beyond that, the CO₂ incubator plays a key role because it provides optimal growth conditions not only for cell cultures but also for various unwanted microbes. Taking that into account, every high-quality incubator exhibits several features for contamination avoidance. However, a sensible decision for purchasing one or the other incubator takes more than just the addition of technical details. In fact, the complete systems and especially the anti-contamination concepts need to be compared and evaluated. It turns out that system complexity does not per se lead to higher safety. The incubator should rather enable optimal contamination avoidance without extensive hands-on time while keeping running costs low.

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Significance of contamination control for working with cell cultures

Microbial contaminations as caused by bacteria, fungi or viruses represent a major risk in cell culturing. Since contaminations do not necessarily overgrow the cultivated cells they may remain undetected for a long time. More subtle effects such as the deprivation of essential nutrients and the excretion of microbial metabolites may cause a pH shift which eventually compromises cell proliferation. Most dreaded mycoplasma infections may alter host cell morphology or even cause chromosomal aberrations. In extreme cases, a single germ may turn the research work of weeks or months worthless.

There are countless paths for the introduction of contaminations: the use of cell lines, media, serum, or other reagents with undetected contaminations, airborne spores or improperly disinfected lab equipment, or accidentally introduced contamination by lab technicians. Since proof for the absence of germs involves complicated and tedious procedures, measures for contamination control must be established.

Considering the significant progress in the area of sensitive cell culture applications, such as tissue engineering and regenerative cell and tissue therapy, the hygiene requirements for CO₂ incubators have risen. Highest standards are thus applied to the perfection and reliability of the entire process chain with the CO₂ incubator playing a key role. The inherent problem of all cell-based therapeutics, e.g. cell suspensions of autologous chondrocytes for re-implantation into the patient, is that the end product cannot be sterilized. 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 Directive³, recommend the use of sterile disposables and/or sterilizable equipment for processing human cells and tissues. Sterile conditions must be guaranteed for those in vitro cell cultures throughout the entire cultivation period not only to reduce the risk of spreading contamination but, more importantly, to avoid life-threatening infections of patients.

Terminology of fighting germs: decontamination, disinfection, sterilization

The term **decontamination** is not clearly defined. It describes the removal of hazardous materials such as biological, chemical or radioactive contamination and does not imply any quantification of its effectiveness.

Disinfection plays a prominent role in aseptic techniques in health care. In defined test scenarios it provides a reduction of certain test germs by five orders of magnitude. i.e. 1 out of 100,000 test germs may survive disinfection.

Sterilization stands for the complete elimination or inactivation of viable microorganisms. Since a 100 % security cannot be practically obtained, various national pharmacopeias consistently allow a remaining contamination risk of 1 to 1,000,000, i.e. one viable microorganism in a million sterilized units.

Concerning the mechanisms and verification of the effectiveness of disinfection and sterilization methods⁴, 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, ethylene oxide fumigation and sterile filtration as sterilization methods. The suitability of a specific method depends on the application and requires validation with defined test organisms.

Measures to avoid incubator-caused contaminations of cell cultures

The requirement of sterile conditions around living cell cultures inside the CO₂ incubator represents a major technical challenge because the optimal growth conditions for cell cultures also favor unwanted microorganisms.

The following crucial aspects need to be considered for a coherent concept for contamination control:

- Suitability of the incubator chamber for frequent spray/wipe disinfections, which is the standard process for reducing the microbiological load of the CO₂ incubator system.
- Complete inactivation of potential contaminants, to be performed regularly or on demand, by means of a straight-forward, true sterilization processes.
- Avoidance of interior fittings such as rack systems, fans or air ducts which may provide hiding space for contaminants and requires tedious disassembly for cleaning and disinfection.
- Condensation management to avoid wet corners which could serve as breeding ground for germs in the incubator interior.
- Prevention of the transfer of airborne germs which are omnipresent to a certain extent

Measures to avoid incubator-caused contaminations of cell cultures

The manufacturers of CO₂ incubators have developed or adopted a variety of features for contamination control with more or less complex process flows. We need to differentiate between decontamination processes to be run regularly or on demand with the incubator put out of operation and features which continuously reduce the risk of contamination in the operating incubator. Table 1 lists the most common methods.

Decontamination on demand	Continuous contamination control
Dry heat at 160 – 180 °C	Minimized, seamless surfaces
Dry heat at 120 – 140 °C	Humidity limit control
Damp heat at 90 – 95 °C	Bactericidal surface properties
Hydrogen peroxide vapor gassing	HEPA air filtering
UV-C irradiation	UV-C irradiation

Tab. 1: Measures to minimize the contamination risk

Hot-air sterilization at temperatures of 160 – 180 °C is the only of the above listed methods which is compliant with the standards for sterilizing medical devices (see Table 2). The incubator's sterilization program consists of three phases: I. heat up to maximum temperature, II. expose at maximum temperature and III. cool down to incubation temperature, for instance 37 °C. Evidence of successful inactivation of test germs pursuant to USP has been proven for hot air sterilization programs⁵.

Different national standards and pharmacopoeias define sterilization temperatures of 160 – 180 °C with exposure times of 30 minutes to two hours. Accordingly, all standards are fulfilled with 180 °C for two hours.

Measures to avoid incubator-caused contaminations of cell cultures

Standard	Temperature	Exposure Time
British Pharmacopoeia	160 °C	60 min
European Pharmacopoeia	160 °C	120 min
Japanese Pharmacopoeia	160 – 170 °C	120 min
	170 – 180 °C	60 min
	180 – 190 °C	30 min
Pharmacopoeia Nordica	180 °C	30 min
US Pharmacopoeia	170 °C	120 min
American Dental Association	160 °C	120 min
ANSI/AAMI ST50	160 °C	120 min
DIN EN 556 (Sterilization of medical devices)	160 °C	120 min
	180 °C	30 min

Tab. 2: International standards for the dry heat sterilization process

Hot air disinfection at temperatures between 120 °C and 140 °C does not represent a true sterilization in accordance with the pharmacopeias but reduces germs significantly. For a dry heat process at 140 °C, a 6-log reduction was reported⁶ for *B. subtilis* var. *Niger* spores, ATCC #93725.

Disinfection with **damp heat at 90 °C** is not comparable to the effectiveness of a true autoclave steam sterilization at 121 °C. It has been shown that the effectiveness on temperature resistant spores of species *Bacillus subtilis* and *Bacillus stearothermophilus* is unsatisfactory^{5,10}.

Hydrogen peroxide (H₂O₂) vapor treatment is a standard decontamination procedure for clean rooms⁷. The CO₂ incubator-adapted method requires a safe and complete inactivation of the corrosive and cytotoxic H₂O₂, e.g. by UV irradiation.

Measures to avoid incubator-caused contaminations of cell cultures

UV treatment by application of non-ozonogenic UV-C radiation with a wavelength of 253.7 nm. The mutagenic effect of UV radiation has been proven⁸, its effectiveness however depends directly on direct irradiation, since it has only limited penetration and is thus only suitable for the treatment of surfaces. Wallhäußer et. al.⁴ note a decreasing effect of UV radiation at ambient humidity values of larger than 80 % r.h. Nevertheless, the effectiveness of treating water in humidification systems of CO₂ incubators under certain conditions has been described⁸.

The use of **HEPA filters** (High Efficiency Particulate Airfilter) to reduce particle concentration in clean rooms and clean benches is a recognized and verifiably effective process. In the CO₂ incubator, a fan in the inner chamber draws air through the HEPA filter to deposit airborne contaminants of a certain size effectively⁹.

Inner chambers surfaces made of **copper** release bactericidal copper ions through oxidation. However, this method is not effective for several bacteria species, fungus spores and viruses. The effectiveness of copper/stainless steel alloys on test organisms was demonstrated in a series of experiments⁸ but lower content copper alloys exhibit a reduced bactericidal effect¹².

Humidity limit control keeps the relative humidity at high levels (~95 %) to avoid media evaporation but below the dew point of the inner wall to avoid uncontrolled condensation. Microbes are not able to grow on dry surfaces.

Plain design: Cleaning efforts and contamination risk increase with the surface area and complexity of fixtures. Accordingly, small surface area and low complexity can be considered as a continuous contamination control measure.

Process safety, effectiveness, and costs of different decontamination concepts

When it comes to contamination management, the end user's focus is clearly on process safety, effectiveness and cost awareness. The respective suitability of the described processes and features, combined to market-typical concepts (see Table 3) of common CO₂ incubators, will be compared in the following.

	Decontamination on demand		Continuous decontamination	Contamination risk caused by		
				Fan	Air duct	Shelf rack
Concept 1	dr 180 °C	10 – 12 h	—	no	no	no
Concept 2	da 90 °C	25 h	—	yes	no	yes
Concept 3	dr 140 °C	12 – 14 h	HEPA Filter	yes	yes	yes
Concept 4	H ₂ O ₂	3 h	UV irradiation, Cu	yes	yes	yes

Tab. 3: Contamination control concepts (dr = dry heat, da = damp heat)

Concept 1 is the only concept which features a true sterilization process. After running the automatic sterilization routine (~10 h), the incubator is essentially clear of any microorganisms. Further technical features for decontamination have been omitted by design, instead the contamination risk is being further reduced by minimizing contamination-prone surface area and hiding spots. A fan has also been avoided, leading to low air movement and making the HEPA air filter redundant. The complete concept does not comprise any consumables which keeps the running costs low.

Process safety, effectiveness, and costs of different decontamination concepts

Concept 2: Disinfection by damp heat at 90 – 95 °C is far less effective than the true autoclave sterilization. The process requires a cycle time of more than 24 hours, followed by recalibration of the CO₂ sensor system. The condensate generated during the cooling down phase represents a potential risk of re-contamination of the treated inner stainless steel surfaces. Therefore, the manufacturer recommends a subsequent spray/wipe disinfection. Overall the process is not sufficient for a complete wipe-out while the handling is cumbersome and time-consuming.

Concept 3: The core of this concept is a particle filter which intercepts airborne contaminants. However, germs and spores just get “collected” and must be removed from the incubator by replacing the expensive HEPA filter. Furthermore, HEPA filters are not designed for high humidity conditions making its effectiveness questionable. Operating costs increase with the diligence of the operator. The filtering technology requires an airflow generated by a fan and air ducts which are contamination-prone per se. The accompanying 140 °C disinfection is not a true sterilization.

Process safety, effectiveness, and costs of different decontamination concepts

In **Concept 4**, two well-accepted procedures for cleanroom decontamination have been combined: H_2O_2 disinfection and UV-C radiation. Hydrogen peroxide disinfection is a fast decontamination method because it does not include a high-temperature process with the required heating-up and cooling-down times. It should, however, be carried out by trained personnel to avoid endangerment of staff and cultured cells. UV light irradiation is applied to inactivate the corrosive and cytotoxic H_2O_2 . In addition, UV is used for periodic decontamination of the air stream. The systems require a fan promoting airborne contamination spreading. Racks and air ducts represent hiding spaces for unwanted microbes.

H_2O_2 plus UV-C irradiation in combination with a copper/stainless steel alloy surfaces was described previously⁸. 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. The air stream disinfection by UV light appears to be unnecessary, if the air movement is slow anyway and if the water pan gets regularly cleaned and filled with sterile, distilled water (manufacturer's recommendation is once to twice per week).

Overall, this is the most sophisticated concept available with the shortest down-time for the decontamination routine. The system's complexity makes it generally prone to failure or user error and involves significant operating costs and hands-on time.

The BINDER concept for minimizing the contamination risk

The BINDER CO₂ incubators offer a conclusive concept for contamination avoidance. It simplifies routine spray/wipe disinfection and it enables automatic auto-sterilization. The plain design results in minimum effort and (almost) no extra cost for each disinfection. The convincing BINDER concept contains the following main elements:

- **Simplified routine disinfection:** The seamless deep-drawn inner chamber without any sharp corners or fixtures is well-suited for easy and convenient spray/wipe disinfections.
- **Uncompromised sterilization:** the well-proven automatic hot air sterilization at 180 °C complies with international standards for medical products. Even the CO₂ sensor in IR technology remains in the chamber (new CB series) during sterilization.
- **Minimized surface area:** Contamination-prone surface area in the inner chamber is minimized by omitting surplus fixtures like racks, air ducts, fans, filters or UV lamps.
- **Condensation management:** The patented double-pan humidification system generates high relative humidity and limits its maximum to 95 % by means of a defined cold spot. The resulting dry inner walls prevent airborne germs from nesting.

Conclusions

In recent publications¹¹, the attention has been drawn to the duration of the decontamination routine and to the need for continuous contamination. Any critical review should also consider the hands-on time required and the costs for replacing high-maintenance components such as expensive HEPA filters and UV lamps.

This whitepaper is an attempt to compare different contamination control concepts for CO₂ incubators from the user's perspective. Contaminations cannot be completely avoided but cell culture equipment supports the user more or less in keeping a good cell culture practice. Most incubators do their job when they are new but the device must be robust and should offer the same performance and safety over many years. For achieving this in the long term, a conclusive concept for contamination control is key.

Imprint

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Dr. Jens Thielmann is a biologist and Product Manager Growth & Storage at BINDER GmbH. He is responsible for various incubators used in medicine, science and pharmaceutical research for the incubation of bacteria or mammalian cell cultures, as well as for ultra low temperature freezers for long-term stable storage of sensitive samples.

| Company profile

BINDER is the world's largest specialist in simulation chambers for the scientific and industrial laboratory. With its technical solutions, the company contributes significantly to improving the health and safety of people. Our range of products is suitable for routine applications, highly specialized work in research and development, production and quality assurance. With round about 400 employees worldwide and an export quote of 80 %, BINDER 2013 sales were more than 60 million euros.

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