

# Exploring CO<sub>2</sub> Incubator Technologies Designed for Cleanroom Cell Culturing

**The boom in cell biology is expected to reignite through 2022 and 2023, and with it comes a greater need for dedicated incubators that are both effective at replicating *in vivo* conditions, and contaminant free**

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Advances in cell biology are enabling new therapy approaches for an increasing range of medical conditions, including cancer, autoimmune disorders, and infectious diseases. While growth of the global cell and gene therapy market slowed in 2020 due to the COVID-19 pandemic, it is expected to recover and reach \$13 billion in 2023 at a compound annual growth rate of 24.1% (1).

Novel cell-based therapies use highly sensitive stem cells and primary cells that require carefully controlled growth conditions to reproducibly deliver the desired therapeutic effect. While long-established, immortalised cell cultures can be grown in standard CO<sub>2</sub> incubators, stem cells and primary cells, owing to their sensitivity, must be cultivated in advanced laboratory incubators that closely replicate and maintain *in vivo* conditions, including temperature, carbon dioxide, humidity, and sometimes oxygen.

At the same time, regulatory authorities demand measures to reduce contaminating particulates and microorganisms in the production suite that might pose a risk to patient safety in the final product. Like procedures, personnel, training and record-keeping, production equipment must comply with GMP guidelines. CO<sub>2</sub> incubators are no exception and should offer independently proven methods for preventing cell culture contamination to ensure compatibility with a cleanroom environment.

Some laboratory equipment manufacturers are now responding to the needs of the growing cell therapy market by developing novel, dedicated CO<sub>2</sub> incubators designed for therapeutic applications in a cleanroom setting. So, what are the critical parameters that cell therapy developers should be looking for in a cleanroom-compliant CO<sub>2</sub> incubator?

## Providing an Ideal Growth Environment

First, CO<sub>2</sub> incubators used in cell therapy production must ensure that sensitive stem and primary cells always remain in their ideal growth environment, enabling consistent growth and proper gene expression while limiting stress responses (2).

As the opening of incubator doors cannot be avoided, cell culture conditions should recover quickly – ideally, in 10 minutes or less following a 30-second door opening (see **Figure 1**). Incubators should also be equipped with sensors located inside the culture chamber (rather than in an exterior compartment). When combined with gentle air circulation and continuous monitoring of the entire chamber, such sensors can react immediately to correct for changes in the environment, protecting the cells in the culture vessels (3).

Critical incubation parameters include:

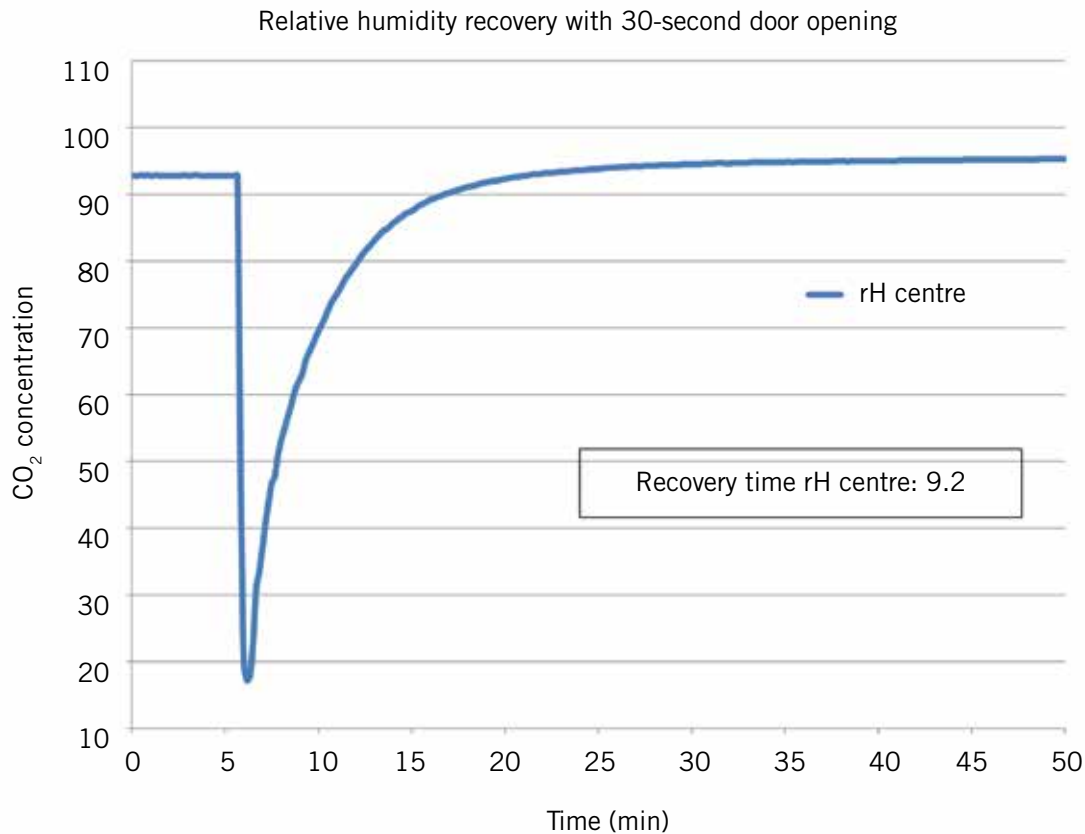


Figure 1: Documented recovery of relative humidity (to 95% of starting value) after 30-second door opening from a cleanroom-compliant CO<sub>2</sub> incubator currently on the market

- **Temperature:** Mammalian cells are endangered and stressed by temperatures even one degree above 37°C. At lower temperatures, cell growth is slower, and genetic expression profiles might be affected
- **Carbon dioxide concentration:** Maintaining a neutral pH, emulating the bloodstream, is critical for proper growth and cell morphology
- **Humidity levels:** Water evaporation from growth media may result in a toxic concentration increase of trace nutrients leading to cell damage or death. Stable and consistent humidity levels limit evaporation, yet too much humidity will condense into water droplets, which provide a breeding ground for microbial contaminants
- **Uniformity:** Even slight zonal variation can lead to differences in cell growth and cellular responses. Gentle and indirect airflow eliminates the stratification of temperature and gases and prevents desiccation

**Critical Considerations on the Route to Cleanroom Compatibility**

Beyond providing optimised growth conditions, cleanroom-compatible CO<sub>2</sub> incubators must be designed to ensure that contaminants – both microbial and particulate – are effectively and continuously removed, and that all surfaces are compatible with appropriate cleaning and disinfection procedures.

**Certificates and Documentation**

A cleanroom-certified CO<sub>2</sub> incubator should be tested according to ISO 14644-1 requirements and be proven

- ✓ Technical specifications
- ✓ Replaceable parts list and dates of last replacement
- ✓ Preventive maintenance checklists and evidence
- ✓ Equipment drawings
- ✓ EC, UL, REACH, and ISO 13485 certificates
- ✓ Material certification
- ✓ Certificates of quality
- ✓ Factory Acceptance Testing reports
- ✓ Sensor certifications
- ✓ Accessory validation protocols

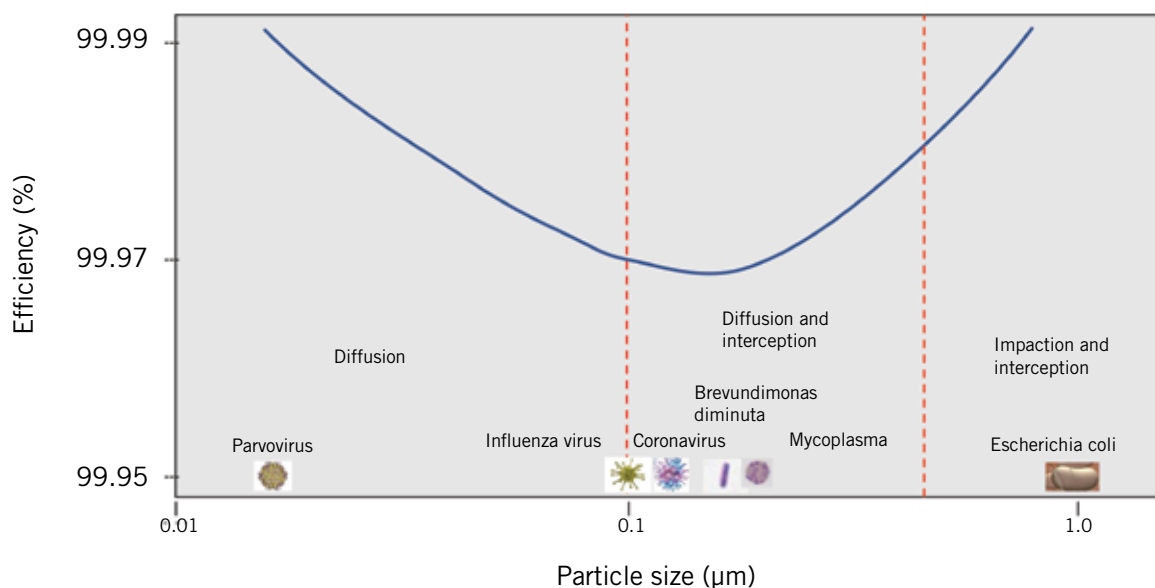


Figure 2: Efficiency of particle removal by HEPA filters relative to particle size

to adhere to air quality limits within Grade A/B cleanroom standards (4). For unbiased data, the CO<sub>2</sub> incubator evaluation should be performed by an independent third party that is an established industry specialist with a proven record of trust.

Manufacturers should provide all documentation needed to meet regulatory requirements and smoothly facilitate the audit process (see **Table 1**). The documentation should not only cover specifications and materials, but also go further; it should precisely indicate equipment performance and provide clear guidance for cleaning, disinfection, routine maintenance, and the replacement of parts and accessories. Such documentation should also enable users to properly maintain and control the CO<sub>2</sub> incubator, with operation easily understandable even for new users. Ultimately, users should be able to easily monitor all conditions at a glance, and onboard data logging should be expected.

### Limiting Particulate Emission

Non-viable contaminants, or particulates, can pose significant health hazards to the patient, from unwanted immune responses to emboli and infarction (5). The risk is increased for patients suffering from severe health conditions and for therapies administered intravenously – both hallmarks of advanced cell therapy scenarios (5). Even though cell therapy manufacturing processes usually take place in a cleanroom, 22% of FDA recalls of sterile injectables between 2008 to 2012 were due to non-viable particulates, representing the second leading cause of product recalls between 2009 to 2019 (5, 6). Particulates

discovered in final products increase the risk for product recall, potentially delaying clinical trials and affecting maintenance of a commercial inventory (7).

In addition, leachables and extractables from these particles can alter the pH of the environment or contain compounds that are toxic to the cells, affecting both the manufacturing process and product stability (7). The most commonly found non-viable particles are bits of metal, glass, plastic, hair, rubber, cell debris, and fabric, with particle sizes ranging from 0.1µm to about 5µm (4, 7). While approximately 70% of particulates can be attributed to cleanroom personnel, about 15% come from the equipment used to grow and process the cell cultures (7).

Hence, equipment manufacturers are increasingly considering cleanroom-compatible designs that limit the number of particulates released. Qualified industry institutes have developed clearly documented procedures, such as those outlined in ISO 14644-14, specifying the methodology to assess the suitability of equipment for use in cleanrooms and associated controlled environments (4, 8). For a cleanroom-certified incubator, consider one with the entire external casing sealed to limit the release of particulates to the cleanroom. A high-efficiency particulate air (HEPA) filter system should be used to capture airborne particulates from the incubator itself, and the incoming air needed to cool the electronics should also be filtered.

### Eliminating Microbial Contamination

Contamination with microorganisms is a major concern, as they can severely impact the cell therapy manufacturing

process and product safety. Mycoplasma species, in particular, were discovered in 15-35% of all cell cultures in 2015 (9). To combat these risks, a cleanroom compliant CO<sub>2</sub> incubator should help prevent microbial contamination in the chamber.

For example, a dry heat sterilisation cycle built into a CO<sub>2</sub> incubator can eliminate resident microorganisms, but efficacy must be proven. For dry heat sterilisation, the pharmacopoeias of the US and the EU require proof of sterilisation, demonstrated by the elimination of one million specific, heat-resistant bacterial endospores (10,11). To achieve such highly effective elimination, hot air must be continuously circulated using a fan. In addition, the entire incubation chamber should be temperature mapped to confirm that the specified temperature is reached in all areas, ensuring there are no cold spots where microbes could survive and grow.

While sterilisation methods eliminate microbial contamination between production runs, in-chamber HEPA filtration systems should be used to constantly protect the cell cultures inside the incubator from airborne microorganisms. This is important because each time the incubator door is opened, surrounding air may carry airborne microbes into the interior of the incubation chamber. More specifically, following a 30-second door opening, ISO Class 5 cleanroom conditions should be re-established within 5 minutes. This can be effectively achieved by a CO<sub>2</sub> incubator design featuring an H13-rated HEPA filter combined with active airflow to capture all microorganisms, regardless of size. This is important, because medium-sized particles of ~0.3µm are the hardest to catch and are used as rating criteria for HEPA filters (see **Figure 3**, page 26). Ideally, the entire chamber air volume should be filtered every 60 seconds, approaching zero particulates circulating over time.

### Cleaning and Disinfection Compatibility

Cleaning and disinfection are usually performed using chemicals, such as hydrogen peroxide (H<sub>2</sub>O<sub>2</sub>). H<sub>2</sub>O<sub>2</sub> can be used in liquid form, typically in low concentrations of 1-6%, which are compatible with paint, stainless steel, glass, and plastics. But with regular use of aggressive disinfectants, there is a risk of build-up of chemical residues that must be removed, as the resulting fumes may harm cultured cells and elicit stress responses, and the residues can cause corrosion of the surfaces over time (12,13). Surfaces of a cleanroom-compatible incubator should, therefore, be compatible with regular cleaning using a 70% ethanol (EtOH) or 70% isopropanol (IPA) solution to remove those residues and protect the cultured cells.

H<sub>2</sub>O<sub>2</sub> is also used in vaporised form (vaporised hydrogen peroxide [VHP]) in concentrations as high as 35% to sterilise cleanrooms and equipment by fumigation. Several variants of VHP, with different hydrogen peroxide concentrations and added chemicals, such as peracetic acid, are commercially available. Because condensation of high concentration VHP chemicals can damage incubators and cause peeling of painted steel surfaces, the process should be carefully controlled. Proof of sterilisation and chemical neutralisation must be provided to prevent potential harm to equipment, cultured cells, and personnel.

For CO<sub>2</sub> incubators used in the cleanroom, brushed 304 stainless-steel exteriors are recommended, as they are more resistant to both chemical disinfectants and VHP. Electropolished stainless-steel incubator chambers and components should be used to reduce microscopic structures, allowing for easier cleaning and reducing the areas available for microbial growth. Ingress





Figure 3: Features of a CO<sub>2</sub> incubator certified for cleanroom use

protection 54 (IP54)-rated electronics and a silicone-sealed touchscreen display should be sought, as they protect from dirt and splashed liquids, further increasing compatibility with cleaning processes.

### Certified Equipment for the Growing Cell Therapy Market

Producing therapeutic cultures in a CO<sub>2</sub> incubator that is certified to control particulate emission, withstand stringent cleaning protocols, and provide conditions for sensitive cells to promote consistent cell growth and gene expression is a cornerstone for the success of cell therapy. It also helps mitigate the health risk for the patient, and facilitates regulatory approval.

The first cleanroom-compatible incubators are now entering the market, offering valuable tools to further promote the development and production of effective and safe cell-based therapies.

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