

Water is life – even in CO₂ incubators

Technical solutions for maintaining an optimally water vapour saturated atmosphere without uncontrolled condensation



Contents

| | | |
|--------|---|----|
| 1. | Introduction | 3 |
| 2. | What is a CO ₂ incubator used for? | 3 |
| 2.1. | Controllable parameters in the CO ₂ incubator | 3 |
| 2.2. | The importance of continuous CO ₂ supply in the CO ₂ incubator | 4 |
| 2.3. | The importance of homogeneous temperature and humidity distribution in the CO ₂ incubator for the water balance of the cells | 4 |
| 2.4. | Why is it so important to avoid condensation in the work space? | 6 |
| 3. | Controlled atmosphere in the Memmert ICOvermed CO ₂ incubator | 7 |
| 3.1. | Different humidification system | 8 |
| 3.1.1. | Passive humidity limitation | 8 |
| 3.1.2. | Active humidity control | 9 |
| 3.1.3. | Temperature constancy and stability | 11 |
| 3.1.4. | Decontamination control | 14 |

1. Introduction

There are a variety of CO₂ incubators on the market. Every manufacturer claims to create optimal and reproducible conditions for the cultivation of living organisms in its device. In everyday laboratory work, however, the lack of reliable moisture management repeatedly leads to falsification of the test results or even to total losses due to contaminated interiors. This white paper is dedicated to the presentation of the physical and constructive interactions in a CO₂ incubator. It shows that all technical components in a CO₂ incubator must work together to maintain the sensitive system of temperature, humidity, carbon dioxide and oxygen.

2. What is a CO₂ incubator used for?

Experiments with which the processes in living organisms can be simulated in a controlled environment are called in vitro. In vitro – in glass – means the cultivation of animal or plant cell populations in a nutrient medium outside the organism.

CO₂ incubators are specially designed to create and maintain this artificial and as natural an atmosphere as possible.

2.1. Controllable parameters in the CO₂ incubator

An essential requirement for scientific experiments and processes is reproducibility under the same experimental conditions. All environmental parameters vital for cell cultures must therefore be regulated, controlled and documented in a CO₂ incubator. The greatest opponents of the incubator are the doors being opened, which is unavoidable in everyday laboratory use and cause the atmosphere in the incubator to fluctuate significantly. Even if the door is open for 30 seconds, the relative humidity in the interior drops from more than 90% to below 20%, the CO₂ concentration from 5% to below 1%. Depending on the size of the incubator and the design, the recovery times for the individual parameters are plus/minus 10 minutes.

Temperature: In the vast majority of cases, the incubation temperature in a CO₂ incubator corresponds to the human body temperature (37°C). For insect cells it is usually 27°C.

Relative humidity: To prevent the cultures from drying out, the relative humidity should ideally be at least 95%. If, for example, the influence of infections on the water balance in the cells is also to be investigated, the relative humidity must be actively controllable.

CO₂ concentration: Buffer solutions are added to most nutrient media. The simultaneous aeration with CO₂ keeps the pH value in the medium, which is important for biochemical reactions in the cell, as neutral as possible. Depending on the culture medium, the CO₂ content is between 5 and 10%.

Oxygen concentration: In various applications, the oxygen content must be adjustable below the normal oxygen supply of 21% (normoxia) ; for example, certain cells prefer the CO₂ partial pressure of their body tissue. Recent research results show that hypoxia/oxygen deficiency is a relevant factor for cell cycle, programmed cell death or energy metabolism, especially in stem and cancer cell research.

2.2. The importance of continuous CO₂ supply in the CO₂ incubator

Even the smallest fluctuations in the parameters that can be set in a CO₂ incubator can be fatal; be it that the test results are falsified and not reproducible, be it that the growth of cell or tissue cultures is impaired or that they even die.

The continuous CO₂ supply is the most important factor within the six notes of cell culture, nutrient medium, temperature, relative humidity, carbon dioxide and oxygen. Maintaining the optimal physiological pH value (for mammalian or human cells 7.2 - 7.4; for insect cells 6.2) is necessary for a variety of processes in the body and in the cells. Cell cultures are closed systems in which the metabolic products in the unbuffered state would rapidly shift the pH value in the medium into (unphysiological) acid. In order to keep it in the physiological range for as long as possible and thus postpone the change of medium, a buffer capacity is built up in the nutrient medium by adding a buffer solution. In cell cultures, this is often hydrogen carbonate (HCO₃⁻/CO₂), the main buffer system in the blood and cells. Depending on the culture medium, the buffer system requires a CO₂ content of the surrounding atmosphere of between 5 and 10%.

If the CO₂ supply fails even for a short time, the pH value slips into the alkaline. The cells are severely stressed by the unphysiological pH value and can die.

Important purchase criterion:



Is the CO₂ supply ensured in the event of a power failure?

2.3. The importance of homogeneous temperature and humidity distribution in the CO₂ incubator for the water balance of the cells

The water in the cells - up to 95% of which is contained in blood plasma, for example - serves to transport oxygen, nutrients and metabolic products. Preventing the dehydration of cells in a cell culture is therefore one of the most urgent tasks of a CO₂ incubator. If liquid evaporates from the culture medium, the osmotic concentration changes. The relative lack of water causes a stress reaction of the cell, which in turn can lead to the loss of the culture. In the experiment itself, cells stressed in this way may produce a false result, as they may overlay the desired effects and experiments may have to be repeated several times to gain clarity. Conversely, however, the air must not be supersaturated with moisture and uncontrolled condensation into the cell culture vessels must be avoided, as this also changes the nutrient concentration in the medium.

With cell-based assays, the dreaded edge effect must be avoided. It describes the phenomenon that in well plates the liquid in the outer wells evaporates faster than in the inner wells. This also changes the nutrient concentration in the medium, so that the external wells can no longer be used for comparisons above a certain degree of evaporation. In the CO₂ incubator, this effect is counteracted by constantly keeping the air in an almost saturated state via a humidification system. In addition, a homogeneous temperature distribution and temperature constancy over the entire interior is decisive. Cold spots, i.e. places where the temperature is so low that humidity could condense out there, form with poor temperature distribution and temperature gradients result from this primarily at corners, walls or door seals. Many manufacturers heat the incubator door, and internal glass doors are usually standard fittings. Heating on all six sides also contributes considerably to homogeneous temperature distribution.



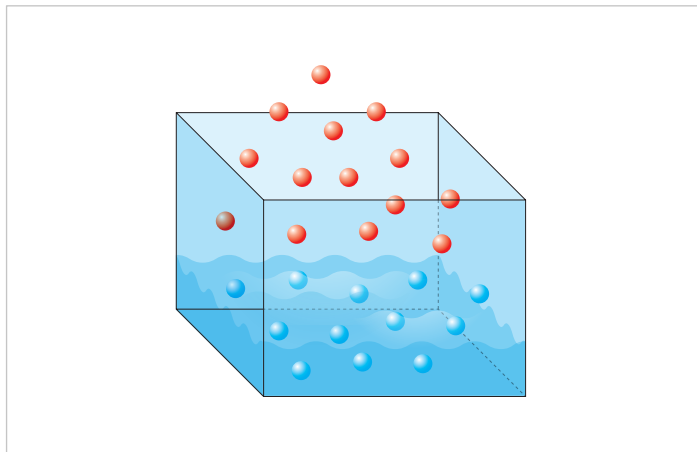
Excursus into physics

Relative humidity rh: This value indicates the water vapour content in the gas mixture of the air. It describes the ratio between the moisture currently present in the air and the maximum possible absolute moisture (degree of saturation 100%) in per cent. The relative humidity depends on the air pressure and the ambient temperature. The warmer the air, the more water it can absorb.

The zeroth law of thermodynamics: A warmed body cools down in a colder environment without external influence until a thermal equilibrium has been achieved. It therefore has the same temperature as its surroundings and vice versa.

Second law of thermodynamics: Energy that passes from one thermodynamic system (e.g. water, ice, etc.) to another is called heat if the energy transport is caused by a temperature difference with the environment. Heat always flows naturally from the warm to the cold body.

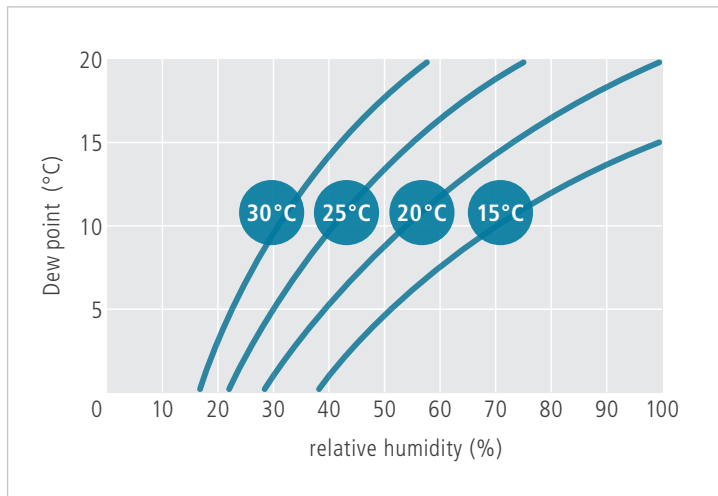
Evaporation: Evaporation, i.e. the conversion from liquid to gaseous, occurs when water molecules with a high kinetic energy (e.g. due to the influence of heat) overcome the binding energy (attraction forces) of molecules with lower kinetic energy and can leave the liquid. If the particles remaining in the cooled liquid continue to draw energy from the environment, the liquid evaporates completely in the end.



Evaporation of liquid in a temperature-controlled environment, such as a temperature control cabinet

Condensation: If the air is 100% saturated with moisture, the excess water vapour condenses in the form of fine water droplets. The water changes from a gaseous to a liquid state. The temperature at which this condition occurs is called the dew point temperature. It corresponds to 35.7°C at 37°C and 93% rh in the environment and a maximum water content of 40.7 g/m³ in the air. At 10°C ambient temperature and identical humidity, the dew point is already reached at 8.9°C and a water vapour content of 8.7 g/m³. A typical example of a dew point effect is suddenly fogged up spectacle lenses when you move from the cold into a warm room in winter. Since the room temperature is higher than the temperature of the glass, the air cools down at the point where it comes into contact with the glass surface and moisture condenses.

Temperature gradient: If heat or cold is supplied to a body with a homogeneous temperature, the outer layers adjust their temperature more quickly than the inside of the body. The temperature gradient describes the temperature changes along the energy transport.



The dew point as a function of relative humidity for four different air temperatures.

2.4. Why is it so important to avoid condensation in the work space?

In addition to the change in concentration in the nutrient medium, uncontrolled condensation in the work space of a CO₂ incubator also poses a considerable risk of contamination. The humid-hot conditions in a CO₂ incubator are also ideal for the growth of harmful microorganisms. If the temperature at any point in the incubator falls below the dew point, water precipitates there and provides a breeding ground for bacteria, fungi, viruses and mycoplasmas.

Neglect during sterile work often leads to bacteria and other contaminants being able to penetrating the CO₂ incubator. Hands, clothing or saliva, cross-contamination, germs in the refrigerator, in the water bath or on the microscope, contaminated primary cultures - the sources of contamination are numerous.

Walls and doors in particular are possible points of condensation due to temperature gradients and thus also possible sources of surface contamination in the incubator.

Important purchase criterion:



1. Does the CO₂ incubator have functions for complete decontamination?
2. Does it offer reliable humidity management?
3. How are temperature homogeneity and uniform temperature distribution achieved constructively?

3. Controlled atmosphere in the Memmert ICOfed CO₂ incubator

Memmert has been developing and producing temperature control appliances for science and research since the end of the 1940s. ICOfed is the latest generation Memmert CO₂ incubator. Heating and humidity systems are coordinated with each other in order to keep the humidity content of the air constantly close to saturation and to exclude uncontrolled condensation in the interior as far as possible.

The key data:

Temperature range up to +50°C

4 model sizes (56 to 241 litres interior volume)

Fail-safe FDA-compliant data logging

Battery buffered ControlCOCKPIT: Operating display as well as measurement and logging of all parameters and control for CO₂ and O₂ fully functional for up to one hour even in the event of a power failure

Optical and acoustic alarm and mobile phone alarm message to the if individually adjustable corridors for temperature, CO₂, O₂, humidity are exceeded

Humidity limitation control or active humidity control

Interior including all built-in components and all sensors can be sterilised in a 60-minute programme at +180°C

Fast reaction direct heating (no water jacket, no air jacket)

Inner glass doors heated and divided

Classified as Class IIa medical device for in vitro fertilisation and biosynthesis

3.1. Different humidification systems

In general, a distinction is made between two systems for humidifying the interior of a CO₂ incubator: passive humidification and active humidity control.

3.1.1. Passive humidity limitation

The air in the interior is humidified by evaporation from a water-filled tank. At a set-point temperature of 37°C, the relative humidity is between 85% and 95%. If the moisture content approaches the saturation limit of more than 90%, condensation is practically unavoidable. There must therefore be constructive solutions to control them.

Advantages: inexpensive and uncomplicated

Disadvantages: The moisture content is directly related to the ambient temperature and cannot be set separately to a target value. The humidity rise times and recovery times after opening the door are considerably longer than with active humidity control. In particular, frequent door openings and changing environmental conditions in the laboratory make the operation of a CO₂ incubator without knowledge of the actual moisture content in the interior and the possibility of intervention sometimes like flying blind.

Important purchase criterion:



1. Does the CO₂ incubator have a humidity sensor for measuring the actual state of the interior?
2. Does the CO₂ incubator work with passive moisture limitation with a targeted cold trap? Can the condensate condense out uncontrolled?

In addition, the more or less large water surface in the evaporation tank represents three possible sources of danger: uncontrolled condensation on the inner walls, uncontrolled bedewing of the cell culture and a breeding ground for microorganisms.

Some manufacturers prevent contamination by a decontamination routine with UV light or a cover over the water container. In both cases, however, the first phenomenon, condensation after a drop in the dew point, is not prevented. This is achieved in some appliances by the targeted creation of the dew point in or under the water tray. Care must be taken to ensure that no condensation can form on the sides or below the water tray.

Passive humidity limitation plus humidity sensor: The humidity is limited to 93% rh (\pm 2.5% rh) via a Peltier moisture trap in the rear wall of the appliance by keeping the temperature of the moisture trap minimally below the dew point temperature. Excess moisture only condenses at this point. The droplets flow back via a sealing lip exclusively into the stainless steel water tray, which is placed exactly in front of the cold trap.



The sealing lip of the evaporation tank is pulled magnetically to the rear wall and directs the water condensing at the moisture trap back into the water tray.

Passive humidity limitation plus humidity sensor: The passive humidity limiter with humidity sensor is available as an option. This enables the actual moisture to be displayed on the control display and an alarm to be sounded if the moisture content drops, e.g. if the water tray is empty.

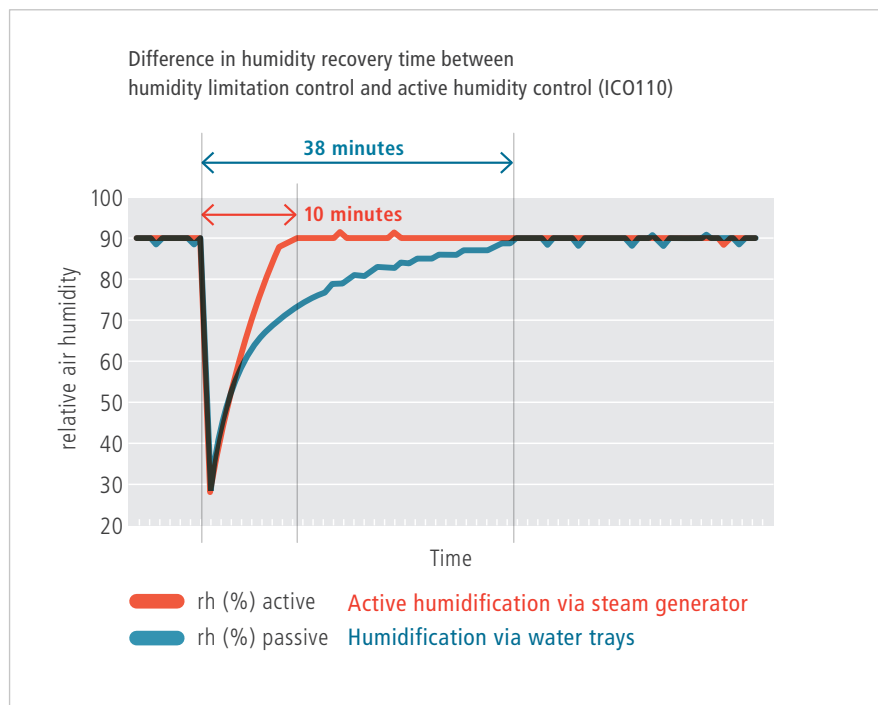
3.1.2. Active humidity control

In CO₂ incubators with active humidity control, the moisture content in the interior is controlled by the permanent comparison of setpoint and sensor-based actual values. Moisture can be supplied via various systems. Ultrasonic systems atomise water with the help of sound waves and convert it into a fine mist. Other appliances introduce sterile superheated steam from outside, which is mixed with the air in the interior. This is the most effective way to sterilise the moisture supplied from outside.

Advantages: The moisture content can be set separately to a target value via a digital control. Permanent control and feedback is carried out via the measurement data of an integrated humidity sensor. The humidity rise times and recovery times after opening the door are considerably shorter than with passive humidity limitation.

Active humidity control in the Memmert ICOver: The interior is humidified via a hot steam generator at the rear of the unit, with controlled evaporation of water from a canister. The sterile superheated steam is fed into the interior above the fan and mixed with the air flow.

When heating up, the speed the humidity approaches the humidity setpoint is dynamically controlled as a function of the dew point of the interior temperature in the setting range between 40 and 97% rh. The interior is dehumidified by a metered supply of fresh air via a sterile filter. A capacitive humidity sensor is integrated for humidity measurement.



Excursus: Experiment on the advantages of active humidity control

A Memmert INCO108med CO₂ incubator (humidity control identical in construction to model ICOMed) was evaluated at the Chair of Bioprocess Engineering at the University of Erlangen in order to exclude device-related influences on test results with multiwell plates. The door was opened frequently because the incubator was used by different employees.

The moisture and CO₂ content was documented over a period of 10 days. In order to determine the viability of the cells, a clear saline solution (PBS) was stained with trypan blue and 600 µl of it was added to each well. The corrugated plate was deliberately not enclosed with parafilm. The speed of reaction of the incubator to the constant changes in the interior gas mixture humidified with 95% rh was demonstrated after the 10-day test. Due to the frequent opening of the door, the outer rows of corrugations had lost about 40% of the liquid, some of the inner rows only 10%. **On non-working days, on the other hand, volume constancy and liquid increase were observed.**

For shorter experiments, there were no significant limitations regarding the incubator or the choice of well. Even if the wells could still be evaluated after the 14-days of test phase, the researcher responsible saw that the limit for valid studies under the above-mentioned test conditions with constant opening of the incubator door had been reached during this period. For longer-term experiments, he recommended limiting it to the inner wells.

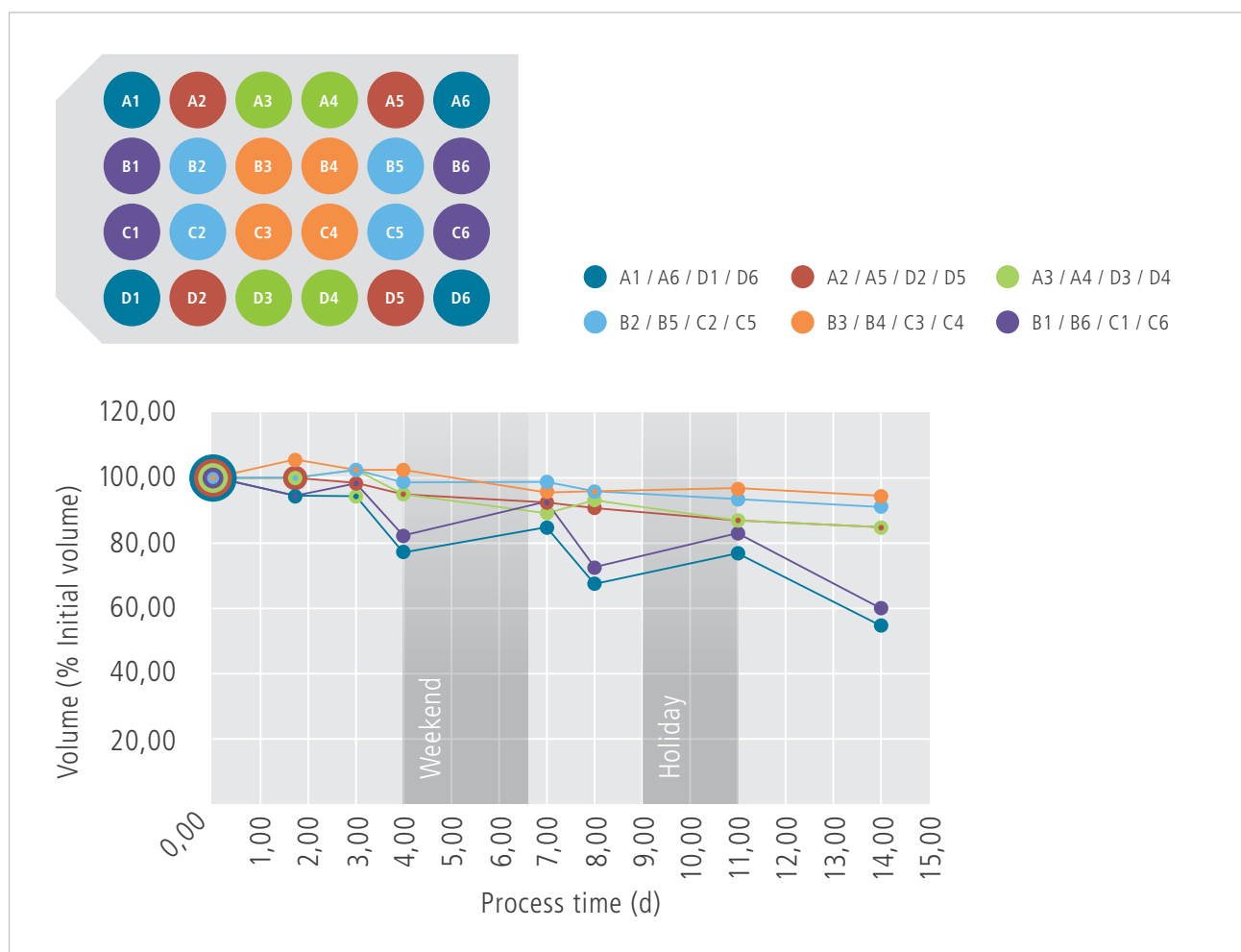


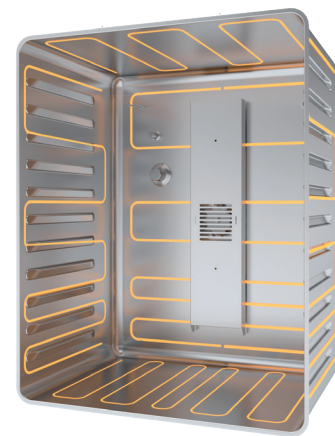
Figure Cell viability after 10 days in the Memmert CO₂ incubator

3.1.3. Temperature constancy and stability

When the door is opened in daily laboratory operation, there is a continual drop in the moisture content and temperature in the interior and thus also a fluctuation in the ideal conditions of 37°C and more than 93% rh. The interaction of active humidity control and heating control is therefore essential for avoiding cold spots and thus unwanted evaporation in a CO₂ incubator. The active humidity control guarantees that the required humidity is reached quickly after the door has been opened and closed. The heating system ideally supports this by heating up quickly and minimising time and spatial temperature deviations.

There are various constructive solutions for heating. Many CO₂ incubators are surrounded by a heated air jacket, and more rarely by a water jacket. This should maintain the temperature for a certain period after a power failure.

In the Memmert ICO, the interior is heated directly over five sides and additionally surrounded by an insulating jacket. In addition, the door and the door seals are heated in order to prevent condensation from forming even in these areas, which are more difficult to insulate. The dense arrangement of the heating elements is the basis for the measured values of ± 0.3 K for temperature homogeneity and ± 0.1 K for temperature constancy (measured according to DIN 12880:2007-05). In addition, they enable fast heating and short recovery times after door opening.

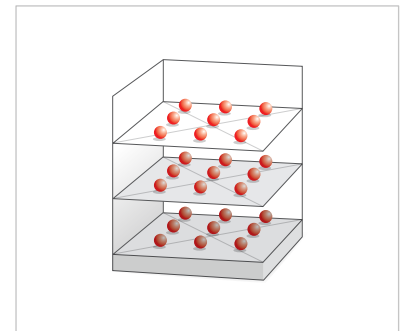


Direct heating in the Memmert ICOfed

Excursus DIN 12880:2007-05

In Germany, DIN 12880:2007-05 applies to the testing of ovens and incubators. Here the measuring setup for the determination of the parameters temperature homogeneity and temperature constancy, for the determination of heating-up times and cooling times as well as for the determination of recovery times after opening the door is laid down.

The **temperature constancy** results from the temperature course of the measuring point which shows the largest temporal temperature deviation. The **temperature homogeneity**, i.e. the spatial temperature deviation, results from the difference between the two measuring points with the largest and smallest measured temperature value (measured at the same time).



Measuring setup with 27 measuring points according to DIN 12880:2007-05 for appliances with more than 50 litres interior volume

Technical data ICO/ICOfed with evaporation tray

| Größe / size | | 50 | 105 | 150 | 240 |
|---|------|-----|-----|-----|-----|
| Räumliche Temperaturabweichung / temperature uniformity in chamber [± K] *1 | 37°C | 0,3 | 0,3 | 0,3 | 0,3 |
| Zeitliche Temperschwankung / temperature variation in timer [± K] | | 0,1 | 0,1 | 0,1 | 0,1 |
| Aufheizzeit / heating up time timer [Minuten / minutes] *2 | | 20 | 20 | 25 | 30 |
| Erholzeit / recovery time [Minuten / minutes] *3 | | 3 | 4 | 5 | 6 |

Technical data ICO/ICOMed with active humidity control

| Größe / size | | 50 | 105 | 150 | 240 |
|--|-----------------|-----|-----|-----|-----|
| Räumliche Temperaturabweichung / temperature uniformity in chamber [+/- K] *1 | 37°C | 0,3 | 0,3 | 0,3 | 0,3 |
| Zeitliche Temperschwankung / temperature variation in timer [+/- K] | | 0,1 | 0,1 | 0,1 | 0,1 |
| Aufheizzeit / heating up time timer [Minuten / minutes] *2 | | 20 | 20 | 25 | 30 |
| Erholzeit / recovery time [Minuten / minutes] *3 | | 3 | 4 | 5 | 6 |
| Räumliche Feuchteabweichung / humidity uniformity in chamber [+/- %rh] | 37°C / 93%rh | 1,5 | 1,5 | 1,5 | 1,5 |
| Zeitliche Feuchteschwankung / humidity variation in time [+/- %rh] | | 1,0 | 1,0 | 1,0 | 1,0 |
| Erholzeit Feuchte / recovery time humidity [Minuten / minutes] *3 | | 8 | 10 | 12 | 15 |

3.1.4. Decontamination control

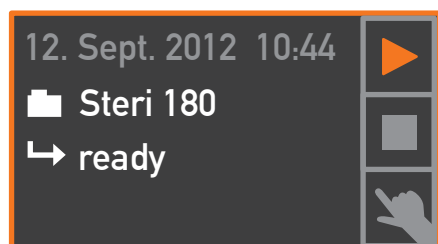
Hot air sterilisation: If germs and other microorganisms have been detected in the interior, the incubator must be thoroughly cleaned and decontaminated immediately. How often the cleaning should be carried out routinely depends on the use. In the Memmert ICOMed CO₂ incubator, the interior is completely sterilised with dry heat within 60 minutes of reaching the sterilisation temperature of 180°C, including all fittings and sensors, in accordance with standards. The German Pharmacopoeia 10 (DAB 10), a collection of recognised pharmaceutical rules on the quality, testing, storage, dispensing and designation of pharmaceutical products and the legally binding substances used in their manufacture, provides the following guideline values for the sterilisation period:

Hot air 180°C: at least 30 minutes

Hot air 170°C: at least 1 hour

Hot air 160°C: at least 2 hours

According to the Robert Koch Institute and the Japanese and Nordic Pharmacopoeias, an exposure time of 30 minutes at 180°C hot air is also sufficient. To be on the safe side, the Memmert ICOMed keeps the sterilisation time twice as long.



Sources:

¹ Kasper C., Egger D.: "Hypoxie – Wenn weniger (Sauerstoff) mehr (Stammzellen) macht", Biospektrum, Edition 4/2017, S. 404 - 407.

² The ideal CO2 incubator for cell culture, interview with Prof. Dr. Gerhard Unteregger,
<https://www.atmosafe.net/themen/aktuelle-themen/der-ideale-brutschrank-fuer-die-zellkultur.html> (abgerufen im August 2019)

³ Lundholt B., Scudder K., Pagliaro L.: "A Simple Technique for Reducing Edge Effect in Cell-Based Assays", Journal of Biomolecular Screening 2003:566-570
<https://journals.sagepub.com/doi/pdf/10.1177/1087057103256465>

⁴ Active humidification - a must when cultivating cells in a CO2 incubator, an experiment at the Chair of Bioprocess Engineering at the University of Erlangen
<https://www.atmosafe.net/bebrueten-und-zuechten/zellkultivierung.html> (abgerufen im August 2019)

Now in its third generation, Memmert has been developing and producing heating and drying ovens, incubators, climate chambers as well as water- and oilbaths at two locations in southern Germany (Schwabach and Buechenbach) for a very wide range of applications. Around 450 employees from about 30 nations are involved in the success of our company. In over 190 countries all over the world, hundreds of thousands of Memmert products have been permanently in use for decades. Therefore Memmert is one of the most innovative and leading manufacturers of temperature control devices worldwide.