Application Solutions

Mettler-Toledo GmbH
Process Analytics

Pharmaceutical
Industry

CO_2 Theory
and Best Practice

METTLER TOLEDO

CO_2 Theory
and Best Practices

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1 Introduction

This booklet provides an in-depth overview on the theory and practice of dissolved carbon dioxide (CO_2) measurement, in particular, as it relates to the biopharmaceutical industry. The booklet is also a best practice guide to the operation of METTLER TOLEDO's InPro™ 5000i dissolved CO_2 sensor. This sensor's measuring principle is based on the Severinghaus electrode (Chapter 3) and is designed to meet all relevant requirements in bioprocessing. Dissolved CO_2 measurement based on the Severinghaus electrode is very common in the biopharmaceutical industry as the technology is relied upon in prevalent off-line equipment such as blood gas analyzers. However, attempts to integrate this measurement technique into in-line sensors had previously fallen short due to limitations in pH sensor technology. METTLER TOLEDO's competencies in glass pH electrode design has meant that this constraint has been overcome.

1.1 Application importance

A bioprocess can be briefly described as a process that uses living cells in combination with a nutrient medium to obtain one or more desired end products. To maximize yield and maintain quality attributes and minimize production time on each occasion a bioprocess is run, control of certain parameters must be maintained. pH and dissolved oxygen are the two major process analytical parameters controlled in most bioreactors, together with physical parameters including pressure, temperature and aeration rate. For several fermentation models or cell culture applications, dCO_2 is another important analytical parameter, as it plays a vital role for most biological processes. The dCO_2 concentration and its unwanted accumulation rate can significantly impact cell growth, quality attributes and productivity rates, as it affects the flux in key metabolic pathways as well as extracellular and intracellular pH. Various reasons for measuring dissolved CO_2 in bioprocessing are shown in Table 1.

Why Measure CO_2? Process understanding, control, and optimization metabolic indicator	
Metabolic Indicator	CO_2 levels impact metabolic fluxes that are responsible for driving specific productivities and specific growth rates.
	CO_2 can be used to design strategies for process optimization (e.g., feeding, induction, temperature shift).
	CO_2 can be used to determine specific CO_2 production or consumption rates.
pH Control	CO_2 impacts extracellular and intracellular pH, both have productivity and product quality implications.
	CO_2 concentration impacts the buffer system of bicarbonate-buffered media, threatening pH stability.
Osmolality	The dissolved CO_2 concentration can impact extracellular and intracellular osmolality.
Product Quality	CO_2 concentration can directly and indirectly impact glycosylation patterns and molecule stability.

Table 1 Reasons why measuring CO_2 can help in the understanding, control and productivity optimization of biological processes.

With the exception of some autotrophic and autologous established fermentation models with microorganisms and several animal cells, dCO_2 has been neglected as a source influencing the physiology of a cell population in a bioreactor.

Although the partial pressure of CO_2 in the headspace of a fermenter can remain constant due to the characteristics of aeration, agitation and the size of the fermenter, the carbonated forms of dCO_2 present in the medium will vary depending on the pH control and in particular the buffer strategy used to avoid a level of $dCO_2$2 which could become cytotoxic.

Historically, for the production of baker's yeast, fermentation to manufacture several amino acids (glutamic acid, histidine and arginine) and antibiotics (penicillin, streptomycin), dCO_2 can play an inhibitory role that is important to control. More recently, it has been recognized that the same is true for the production of monoclonal antibodies and different recombinant proteins in the context of vaccine production.

Dissolved CO_2 has been often reported as a factor of inhibition affecting growth rate and inducing unwanted cell morphology properties. This is due to its free passage as a dissolved gas through cell membranes, and its uncontrolled rate in media affects glucose uptake rates at various stages of a fermentation. OUR (Oxygen Uptake Rate) and CER (Carbon Evolution rate) are both separately and together interesting to manipulate to estimate viable cell density and physiological changes of a defined cell population. However, and specifically for animal cells, due to the lowest oxygen demand of animal cells in comparison to aerobic microbial strains and high accuracy required to measure the oxygen content in a gas phase, using an OUR dynamic mass balance calculation alone has limited the application and possibility to better monitor a cell culture.

Regarding animal cells, insect and mammalian cells are well-established models for the production of recombinant proteins and monoclonal antibodies. It has been often estimated that more than 60 % of all biologics, including viral vaccines, gene therapy vectors and monoclonal antibodies are manufactured using mammalian cells. In addition, as it is in-vivo, in respect of dCO_2 and possible CER calculation, it is becoming essential at any scale to better characterize the impact of dCO_2 and correlate fluctuation and rate of dCO_2 accumulation to the physiological state of the cells. Such characterization can lead to an optimized gassing strategy and nutrient supply. CER is often described as difficult or challenging to determine with mammalian cells due to buffering systems to avoid pH fluctuations and the fact that CO_2 as a gas is used to lower the pH. So it is often seen as complex or problematic to estimate dCO_2 from cellular respiration and dCO_2 generated as a result of pH control and bicarbonate buffers equilibrium in culture media. Nevertheless, the measurement of dCO_2 will remain widely used in order to study its impact on cellular metabolism with animal cells.

1.1.1 CO_2 as a metabolic indicator

Dissolved CO_2 is an important metabolite produced and consumed in numerous metabolic reactions. Dissolved CO_2 levels can significantly influence overall metabolic reaction fluxes key for high growth and productivity.

All cells, anaerobic or aerobic, produce and consume CO_2 within several metabolic networks. Many of these reactions, which occur in series, are reversible and diffusion driven. If the CO_2 concentration is too high (cytotoxic) or too low (reaction limiting), the CO_2 producing or consuming reactions negatively impact the overall metabolic reaction flux. For example, Figure 1 shows an aerobic central carbon metabolic network consisting of glycolysis and the tricarboxylic acid (TCA) cycle (also known as the citric acid cycle or Krebs cycle). It is well documented that the preferred pathway (highlighted in yellow) of converting glucose to pyruvate for utilization in the TCA cycle, is the most energy efficient. This pathway is highly active and plays a critical role in governing specific cell growth and productivity rates. Within this pathway, CO_2 is produced and consumed at multiple reversible and irreversible steps that occur in series. Sub-optimal CO_2 concentrations would reduce the overall reaction flux of the preferred pathway.

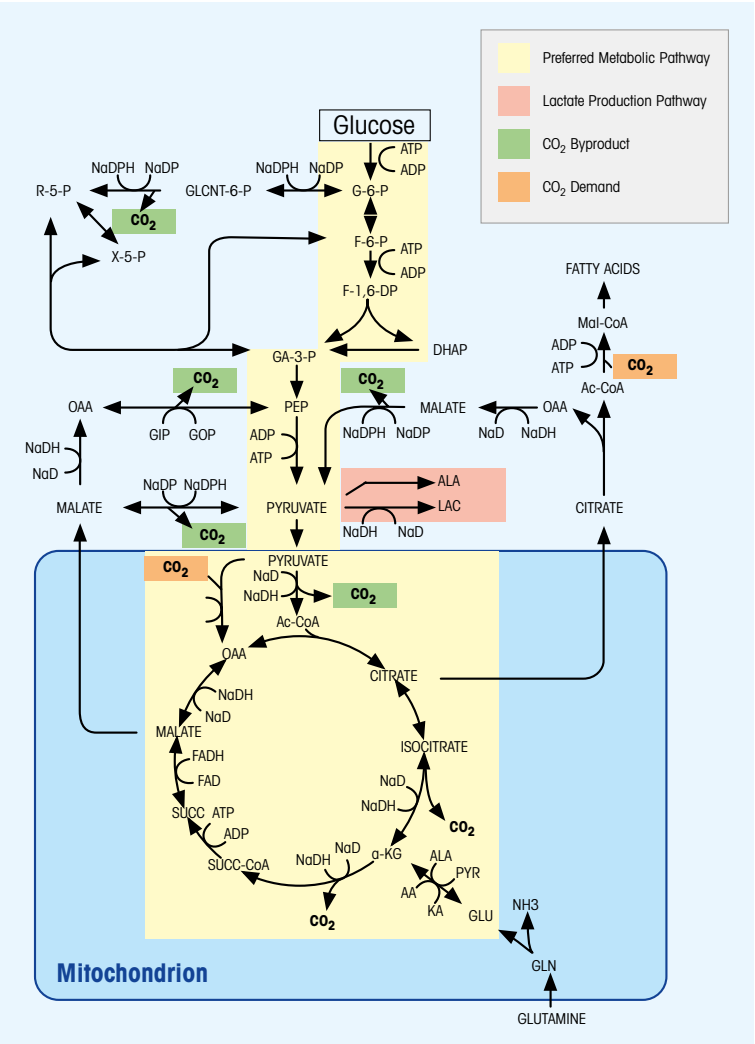


Figure 1 Chinese hamster ovary (CHO) metabolic network for glycolysis and the tricarboxylic acid cycle. This central carbon pathway plays a critical role in governing specific cell growth and productivity rates. Even though there is a net production of CO_2, many of the reactions produce and consume CO_2. These reactions are highly influenced by the available CO_2 concentrations. Optimal levels are at the "sweet spot" – neither too high nor too low.

1.1.2 Impact of CO_2 on extracellular and intracellular pH

Dissolved CO_2 reacts with water to form carbonic acid which dissociates to produce a hydrogen ion resulting in a reduction in pH (Equation 1). Intracellularly, this reaction can also be modulated enzymatically.

$$CO_2 + H_2O \rightleftharpoons H^+ + HCO_3^-$$

Equation 1 Dissociation reaction of CO_2 in water.

Intracellular and extracellular pH has a significant bearing on physical and chemical reactions (e.g., metabolism, molecule stability), as well as on the types of processes a cell expends energy on performing.

The lipid bilayer of a cell wall generally modulates the transport of polar or charged molecules (e.g., ions, sugars) across it; however, some uncharged polar molecules such as dCO_2 are able to permeate freely through the membrane. The ease for dCO_2 to enter a cell, and once inside alter the intracellular pH, emphasizes the importance of the extracellular dissolved CO_2 concentration. Inside the cell, dCO_2 reacts with water to produce a hydrogen ion. This bypasses the cell's mechanism used to control hydrogen ion concentration (dCO_2 can act as a biological Trojan horse!). The intracellular pH change caused by undesired dCO_2 levels results in the cell expending more energy towards pH control and reducing the resources used for cell growth and production of the desired protein, as shown in Figure 2. Controlling extracellular dissolved CO_2 is one of the few control levers available for influencing intracellular pH.

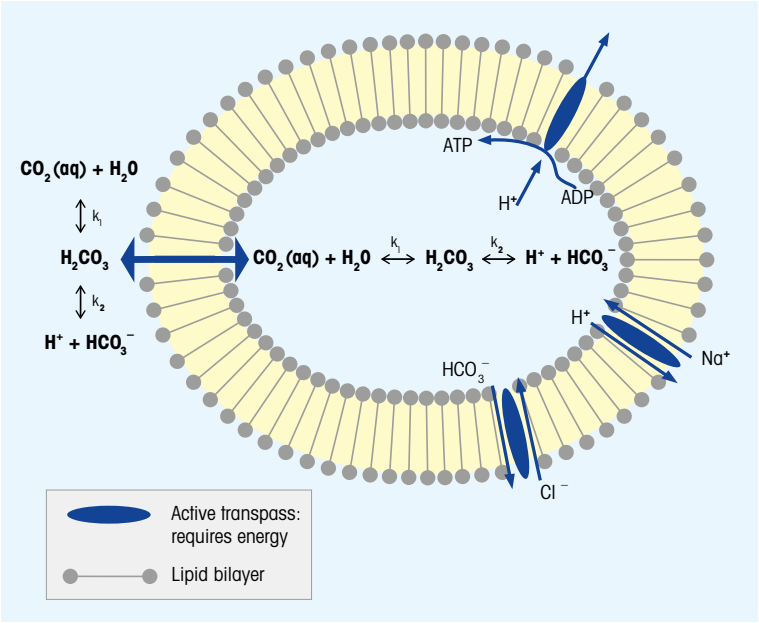


Figure 2 dCO_2 is able to pass freely through a cell membrane/wall. This transport is primarily driven by concentration gradients between the internal and external dCO_2 concentrations. Since CO_2 reacts with water to produce pH-reducing species, this can put an energy toll on a cell trying to control intracellular pH.

1.2 CO_2 measurement and control

The optimal operating range for dCO_2 concentration for CHO cell cultures has commonly been reported as 5 – 20 %, but the true optimal range depends on the specific process and recombinant cell line being used. Dissolved CO_2 control can be particularly important for monitoring a scale up to reduce productivity gaps. Despite the criticality of dCO_2 levels in growth and feed culture media, this measurement drew little attention in the past. One reason for this has been the lack of reliable sensors and measuring methods. Many legacy processes use off-line or at-line analyzers to measure dCO_2 because in-line sensors that remain stable after sterilization cycles have not been available. At-line

and off-line measurements are not efficient for the measurement of dCO_2 (see Table 2); therefore, many processes had to be designed with loose CO_2 control leading to high variability and sub-optimal productivity.

Method	Disadvantages
Off-line Grab Samples	**High sampling error:** Off-gassing, sample contamination during handling and prone to error if sample is diluted.
	Sample dynamics: Temperature change, CO_2 production or consumption leading to potential evolution of the sample if not analyzed immediately or stored at +4 °C.
	Reliance on compensation: The sample is applied to the off-line analyzer outside of the process. This measurement must be compensated back to bioreactor conditions. Many analyzers do not allow input of compensation curves based on media properties (e.g., blood gas analyzers perform temperature compensating based on blood and not media).
At-line Off-gas Analysis	**Not a direct measure in the liquid phase:** Off-gas analysis measures CO_2 in the gas phase. This method is insufficient because it is unable to account for changes in dissolved CO_2 content due to solution dynamics such as KLa and solubility.
	Expensive (e.g., cost of mass spectrometer)
	Complex, high maintenance of analyzers

Table 2 Disadvantages of common methods used for measuring dissolved CO_2.

Measuring dCO_2 in-line eliminates many of the disadvantages associated with the at-line and off-line methods discussed in Table 2. METTLER TOLEDO's InPro 5000i dCO_2 sensor employs a potentiometric carbon dioxide electrode (Severinghaus electrode), that measures exactly the same dissolved dCO_2 partial pressure as the cells encounter; so errors from sampling are eliminated and metabolic changes in dCO_2 concentration affecting system dynamics will be reflected in the measurement.

Control of dCO_2 can be complex because of the many ways dCO_2 interacts within a biological model (see Table 3 for common ways that dCO_2 concentrations can be managed in bioprocessing).

Increased CO_2 Concentration	Reduced CO_2 Concentration
Excretion by cells (e.g., aerobic metabolism)	Consumption by cells (e.g., autotrophs)
CO_2 sparge or overlay	Air, O_2, N_2 sparge or overlay (stripping)
Supplemental feeds (e.g., bicarbonate)	Supplemental feeds (e.g., CO_2 scavengers)

Table 3 Common ways to increase and decrease dissolved CO_2 concentration.

It is therefore important, from the early discovery pre-studies and all pre-clinical phases (even at the ½-liter scale), to characterize the impact of dissolved CO_2 at low, medium and high cell densities. Such dCO_2 monitoring will make it possible to quickly characterize the impact of dissolved CO_2 on the pH, the cells and the targeted expression during process development. It will also affect more quickly the adoption of CO_2 monitoring during the commercial phase, even if the CO_2 measurement is not used as an analytical measurement for control. Finally, in the context of a technology transfer, knowledge of dCO_2 monitoring and control is a valuable tool, not only to ensure the success of a technology transfer, but also when used to solve discrepancies between batches during process qualification before moving on to first clinical batch manufacturing.

2 Physical background of CO_2 measurement

Carbon dioxide typically is encountered as a gas. Therefore, the in-line and on-line measurement of carbon dioxide in processes is a measurement of the pressure which the carbon dioxide applies either in the gas or in the liquid in which it is dissolved.

There are two particular physicochemical laws concerning pressure that are central to understanding CO_2 behavior:

- Dalton's law of partial pressures
- Henry's law on gas dissolved in liquids

2.1 Dalton's law

Dalton's law states that in ideal gases the total pressure of a gas mixture is equal to the sum of the partial pressures of the individual gases, as shown in Equation 2.

$$P_{Total} = p_1 + p_2 + \ldots + p_n$$

Equation 2 Dalton's law of partial pressures. P_{Total} is the total pressure of the gas mixture; p_i represents partial pressures of individual gases within the gas mixture.

The following is an example of Dalton's law for dry air (partial pressure of water p_w=0). Dry air consists of nitrogen, oxygen, carbon dioxide, argon, and other trace gases; the volume contribution is shown in Figure 3. According to Dalton's law, the pressure of air is the sum of the partial pressures of the individual gases, as shown in Equation 3.

$$P_{Total\ air} = p_{nitrogen} + p_{oxygen} + p_{carbon\ dioxide} + p_{argon} + p_{trace\ gases}$$

Equation 3 Dalton's law for the composition of dry air (p_w=0).

Figure 3 shows the gaseous composition of dry air in volume percent. Carbon dioxide contributes about 0.038 % to the total pressure of a given volume of air. At an atmospheric pressure of 1013 mbar (hPa), 0.038 % of this pressure is contributed by carbon dioxide, the so-called partial pressure of carbon dioxide (p_{CO_2}). In dry air it is 0.38 mbar according to Dalton's law.

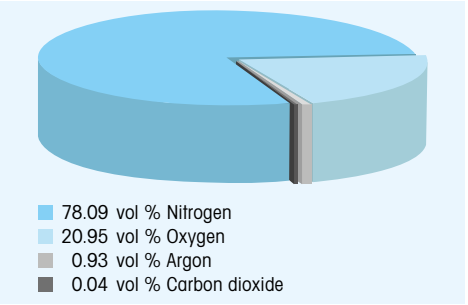


Figure 3 Volumetric contribution of gases within air.

Due to its low volume contribution, air is not suitable for calibration of a CO_2 sensor. Therefore, calibration gases with defined carbon dioxide contents are used for calibration of carbon dioxide sensors, like, for example, a gas mixture with 5 % CO_2, 20 % O_2, 75 % N_2. According to Dalton's law, this would result in the following partial pressures under normal conditions (1013 hPa and 25 °C) for dry gases: carbon dioxide 50.7 mbar, oxygen 202.6 mbar and nitrogen 759.8 mbar.

2.2 Henry's law for gases dissolved in liquids

A gas is said to be dissolved in a liquid when it is "surrounded" by liquid molecules or distributed evenly throughout the liquid molecules. Gas bubbles are not dissolved because they are a cluster of gas molecules surrounded by each other. The propensity for gas molecules to dissolve in liquid at a given temperature and pressure is known as the solubility (mg/L or mmol/L).

When working with process liquids, the carbon dioxide to be determined is normally dissolved in these liquids. This dissolved carbon dioxide is in a steady-state or equilibrium with the carbon dioxide in the ambient gas phase.

The physical relationship is described by Henry's law. It states that the partial pressure of a gas above a liquid is directly proportional to the concentration of that gas dissolved in the liquid (Equation 4). This means that the concentration of dissolved oxygen can be determined by measuring the partial pressure of carbon dioxide and multiplying it with the Henry constant. The Henry constant is dependent on the nature of the liquid (polar, non-polar), in which the gas is dissolved, its salinity, as well as on the temperature. These factors influence the solubility of the gas (see Section 2.2.2).

$$C_{CO_2} = a \cdot p_{CO_2}$$

C_{CO_2} = carbon dioxide concentration in the solution
a = Henry constant
p_{CO_2} = partial pressure of carbon dioxide

Equation 4 Henry's law of gases dissolved in liquids.

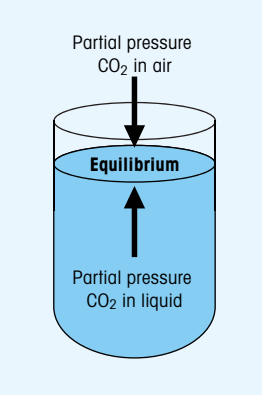


Figure 4 Henry's law – the concentration of carbon dioxide in the liquid is proportional to the partial pressure of the carbon dioxide in the gas phase – the partial pressures are the same.

With this relationship, the absolute dissolved CO_2 concentration can be determined by knowing the partial pressure of CO_2 gas above the liquid, as long as Henry's constant is known. Without Henry's constant, only relative dissolved CO_2 content can be determined (these measurements are relative to the saturation conditions set during calibration).

2.2.1 Influence of vapor pressure

The carbon dioxide sensor (see Chapters 3 and 4) normally is calibrated by a process calibration with a calibration gas of known partial pressure. Since the dry calibration gas is in equilibrium with the process medium, it is saturated with water. Therefore, the partial pressure of water has to be subtracted from the total pressure to get the right calibration partial pressures of the gas.

The partial pressure of water is not dependent on the total pressure, meaning if total pressure is doubled at constant temperature, it remains the same (Claudius-Clapeyron). It is only a function of temperature. Table 4 gives an overview of the vapor pressure of water at different temperatures.

Temperature [°C]	Vapor pressure of water [mbar/HPa]
5	8.72
10	12.27
15	12.79
20	17.54
25	23.76
30	31.82
35	42.18
37	47.07
40	55.32
50	95.51
60	149.38

Table 4 Vapor pressure of water at different temperatures

Table 5 and Figure 5 show an example of how doubling the process pressure more than doubles the partial pressure of the different gases in the calibration gas mixture, if dealing with a humid environment in a closed system. The vapor pressure of water does not depend on the process pressure, but only on the temperature. To get the right partial pressures of the calibration gas in a humid environment, the vapor pressure has to be subtracted from the process pressure.

Vessel A and Vessel B are pressurized with the same calibration gas mixture consisting of 5 % carbon dioxide, 20 % oxygen and 75 % nitrogen. They are both at 30 °C. Vessel B has double the process pressure of Vessel A.

The partial pressures in Vessel B are more than double that of Vessel A. This is due to the diminishing fractional contribution of the vapor pressure as the process pressure increases.

Composition of calibration gas	Percentage	Dry gas: Partial pressure in dry calibration gas at 1000 mbar, 30 °C [mbar]	Vessel A: Partial pressure with calibration gas saturated with water at 1000 mbar, 30 °C [mbar]	Vessel B: Partial pressure with calibration gas saturated with water at 2000 mbar, 30 °C [mbar]
Carbon dioxide	5 %	50	48.4	98.4
Oxygen	20 %	200	193.6	393.6
Nitrogen	75 %	750	726.2	1476.2

Table 5 Partial pressure of gases in a gas mixture as dry gas, and under different pressure conditions with gas in equilibrium with the process medium at constant temperature.

Vessel A

Total pressure p_{total}: 1000 mbar

Vapor pressure of water p_w at 30 °C: 31.8 mbar

$p_{total} - p_w$ = 1000 mbar – 31.82 mbar = 968.2 mbar

Vessel B

Total pressure p_{total}: 2000 mbar

Vapor pressure of water p_w at 30 °C: 31.8 mbar

$p_{total} - p_w$ = 2000 mbar – 31.82 mbar = 1968.2 mbar

The % CO_2 saturation remains constant between the two vessels because this is a measure relative to the total pressure of the system (Figure 5).

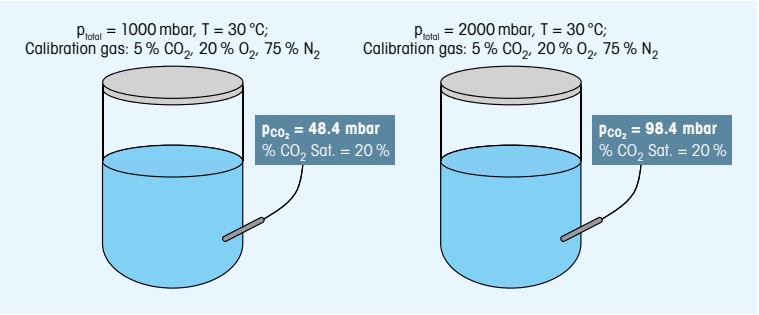


Figure 5 Doubling the calibration gas pressure more than doubles the partial pressure of CO_2.

When calibrating the InPro 5000i at pressures different than atmospheric, care must be taken regarding this effect in order to calculate and input the correct calibration CO_2 partial pressure. This is done by subtracting the vapor pressure of water from total pressure and then calculating the percentages.

On the other hand, for determining % CO_2 saturation it is important to input the correct process pressure.

2.2.2 Solubility of gases

The solubility is also expressed as the saturation concentration or the maximum dissolved gas carrying capacity of the liquid.

Gases are soluble in liquid to varying degrees. Solubility of a gas in a liquid is a function of:

- The temperature – the higher the temperature the lower the solubility
- The salinity – increasing salinity leads to reduced solubility of gases
- The solution itself.

The solubility of carbon dioxide also highly depends on the pH, due to Equation 1. This solubility, expressed as mg/L or a mole fraction, is proportional to the partial pressure of the gas over the liquid (Henry's law).

	Saturation concentration in mg/L at 1013 hPa gas pressure		
Temperature [°C]	Oxygen	Nitrogen	Carbon dioxide
0	69.4	29.4	3350
20	43.4	19.0	1690
30	35.6	16.2	1260
40	30.8	13.9	970

Table 6 Solubility (saturation concentration) in mg/L of oxygen, nitrogen and carbon dioxide in water at 1013 mbar/hPa pressure of the pure gas and 100 % saturation.

According to Henry's law, the carbon dioxide concentration is proportional to its partial pressure (p_{CO_2} in Equation 4). If "a" is constant, the CO_2 partial pressure can be determined using a sensor. This means that in media with constant temperature and constant solutions, the absolute carbon dioxide concentration can be determined. If the nature of a solution changes – as occurs during fermentation processes – a new calibration to this solution has to be done, since the Henry constant also changes.

Therefore, determination of the absolute carbon dioxide concentration is only possible with a constant and known solubility factor "a".

2.3 Units of measurement: CO_2

Depending on the kind of application and also on the habits of a certain industry, the dissolved CO_2 content is expressed in different units. For molecules that at equilibrium favor the gas phase under standard conditions, such as CO_2 and oxygen, it is common for the raw or reference readout to be based on the gas phase in the units of pressure, independent of the measurement occurring in the gas or liquid phase.

Common units of pressure and conversions are shown in Table 7.

	Pascal	**Bar**	**Technical Atmosphere**	**Physical Atmosphere**	**Torr**
	N/m^2	$Mdyn/cm^2$	kp/cm^2	p_{STP}	mmHg
1 Pa	1	$1.0000 \cdot 10^{-5}$	$1.0197 \cdot 10^{-5}$	$9.8692 \cdot 10^{-6}$	$7.5006 \cdot 10^{-3}$
1 bar	$1.0000 \cdot 10^{5}$	1	1.0197	$9.8692 \cdot 10^{-1}$	$7.5006 \cdot 10^{2}$
1 at	$9.8067 \cdot 10^{4}$	$9.8067 \cdot 10^{-1}$	1	$9.6784 \cdot 10^{-1}$	$7.3556 \cdot 10^{2}$
1 atm	$1.0133 \cdot 10^{5}$	1.0133	1.0332	1	$7.6000 \cdot 10^{2}$
1 Torr	$1.3332 \cdot 10^{2}$	$1.3332 \cdot 10^{-3}$	$1.3595 \cdot 10^{-3}$	$1.3158 \cdot 10^{-3}$	1
1 psi	$6.8948 \cdot 10^{3}$	$6.8948 \cdot 10^{-2}$	$7.0307 \cdot 10^{-2}$	$6.8046 \cdot 10^{-2}$	$5.1715 \cdot 10^{1}$

Table 7 Common units and conversion factors for pressure units.

2.3.1 Gas phase measurement units

Gas phase measurements are typically based on the volume that a particular gas occupies in the gas phase. Common units used in gas phase measurements are shown in Table 8.

Equations	
Partial pressure CO_2 (P_{CO_2}) [Pa, Bar, ATM, Torr, psi]	$= p_{CO_2} \cdot (p_{total} - p_w)$; see also Equation 2
% Volume CO_2 (% CO_2(g)) [%]	$= \frac{p_{CO_2} \cdot (p_{total} - p_w)}{p_{total}} \cdot 100 = \frac{p_{CO_2}}{p_{total}} \cdot 100$
ppm_v ($ppm_{CO_2(g)}$)	$= \frac{p_{CO_2}}{p_{total}} \cdot 10^4$
ppb_v ($ppb_{CO_2(g)}$)	$= \frac{p_{CO_2}}{p_{total}} \cdot 10^7$

Table 8 Common units used for CO_2 measurement in the gas phase and associated equations ; p_{total}: system pressure; p_w: water pressure; p_{CO_2}: partial pressure of CO_2.

2.3.2 Liquid phase measurement units

Liquid phase measurements can be expressed in absolute concentration units such as mg/L or mmol/L or in relative units such as % saturation. Only if the Henry constant for a solution is known can the CO_2 content can be expressed in absolute concentration units.

For dynamic processes, such as bioprocesses, the Henry constant changes throughout the process and therefore cannot be accurately determined. In such processes it is most common to use % saturation or pressure units to display dissolved CO_2. The calibration of these processes is typically performed at starting conditions because this is where the liquid phase is the most repeatable. These units provide a measure that is relative to the conditions defined at the time of calibration. Table 9 shows common units and associated equations that are used for dissolved CO_2 measurement. For the InPro 5000i, the available units of measurement on a transmitter are partial pressure, % saturation CO_2(aq) (expressed as % CO_2) and concentration (mg/L).

Equations	
Partial pressure CO_2 (p_{CO_2}) [Pa, Bar, ATM, Torr, psi]	$= x_{CO_2} \cdot (p_{total} - p_w)$; see also Equation 2
%Saturation CO_2 (aq) (% CO_2 (aq)) [%]	$= \%\,CO_2(g) \cdot \frac{p_{total}}{(p_{total} - p_w\,(@100\,\%\ \text{saturation}))}$
CO_2 (aq) Concentration [mg/L]	$= p_{CO_2} \cdot a$
CO_2 (aq) Concentration [mmol/L]	$= \frac{p_{CO_2} \cdot a}{44.01}$
ppm_v ($ppm_{CO_2(aq)}$) [mg/L]	$= CO_2$ (aq)
ppb_v ($ppb_{CO_2(aq)}$) [mg/L]	$= CO_2$ (aq) $\cdot\ 10^3$

Table 9 Common units used for CO_2 measurement in the liquid phase and associated equations with p_{total}: system pressure; pw: water pressure; p_{CO_2}: partial pressure of CO_2; a: Henry's constant.

2.4 Influence of temperature

Temperature has a wide impact on the physical behavior of CO_2 and the InPro 5000i sensor's measurement technology. The impact on Dalton's law is primarily in regards to vapor pressure which increases with temperature (the relationship between partial pressures is not changed because temperature impacts all gases proportionally). For Henry's law, the impact is well defined: as temperature increases, normally the solubility decreases. This is why Henry's constant is temperature dependent. In Chapter 3 (InPro 5000i CO_2 measurement technology) the temperature impact on the InPro 5000i's measurement technology is discussed.

3 InPro 5000i CO_2 measurement technology

The InPro 5000i sensor's measurement technology is based on the Severinghaus principle which utilizes the correlation between dissolved CO_2 and pH of a liquid. Technology based on this principle is commonly relied upon in the biopharmaceutical industry as seen in off-line analyzers such as blood gas analyzers. The following sections provide details on the Severinghaus principle, the design of the InPro 5000i, and its measurement accuracy.

3.1 The Severinghaus principle

The potentiometric Severinghaus electrode utilizes a bicarbonate buffer system in contact with the measured media through a selective CO_2-permeable membrane. The pH value in the buffer system is related to the partial pressure of dissolved CO_2 present in the process media as described in the following equation:

$$CO_2(aq) + H_2O \overset{k_1}{\longleftrightarrow} \underset{\text{Carbonic acid}}{H_2CO_3} \overset{k_2}{\longleftrightarrow} H^+ + \underset{\text{Bicarbonate}}{HCO_3^-}$$

Equation 5 CO_2 chemical reactions in water.

By combining the equilibrium constants from Equation 5 with Henry's law from Equation 4, pH can be made a function of p_{CO_2} as shown in Equation 6.

$$pH = -\log\left[\frac{K_{CO_2}a(p_{CO_2})}{(HCO_3^-)}\right]$$

Equation 6 Relationship between pH and CO_2 partial pressure.

The two constants K_{CO_2} and "a" are both temperature dependant. Their values are specific for CO_2 and the buffer solution (i.e., CO_2 electrolyte) used in the InPro 5000i.

3.2 Design of the InPro 5000i

The InPro 5000i is hygienically designed and suitable to withstand sterilization and cleaning in place. The sensor employs a patented CO_2 selectively permeable silicone membrane and a specially engineered flat membrane pH sensor. The CO_2 from the medium diffuses across the membrane until it equilibrates within the buffer solution. A change in the CO_2 partial pressure results in a pH change in the electrolyte, which is detected by the inner body pH electrode. A built-in temperature sensor is used to provide accurate temperature compensation.

A diagram of the key internal components of the InPro 5000i is shown in Figure 6.

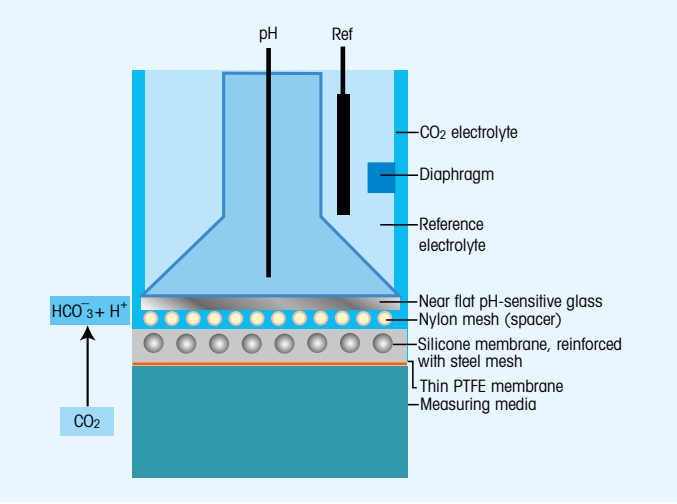


Figure 6 Key internal components of the InPro 5000i.

3.2.1 Sensor parts and assembly

The individual parts of the InPro 5000i are shown in Figure 7. The sensor design eliminates the need to dispose of durable sensor parts when exchanging the inner body.

Detailed instructions on how to assemble and disassemble these parts can be found in the InPro 5000i inner body manual.

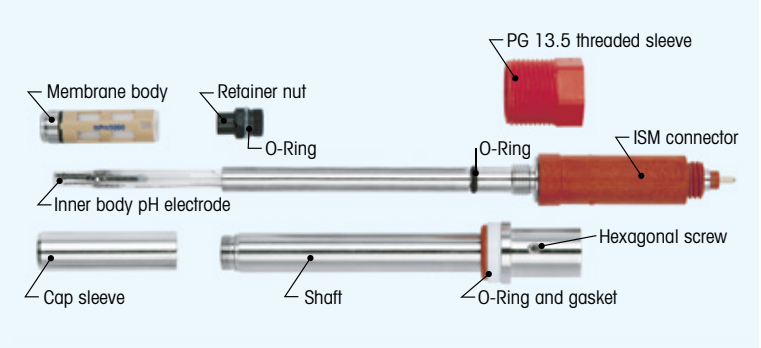


Figure 7 The changeable parts of the InPro 5000i.

From this point in the guide, we will refer to "CO_2 sensor" as the completely assembled sensor (Figure 8a), and "inner pH electrode" as the assembly without cap sleeve and membrane body (Figure 8b).

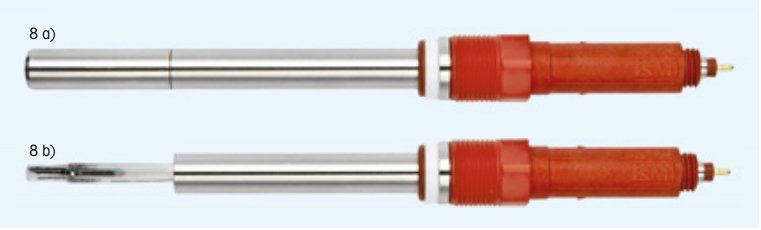


Figure 8 a) CO_2 electrode and b) inner pH electrode.

3.2.2 Inner pH electrode

The design of the internal pH sensor has a significant impact on the robustness of the CO_2 measurement. Since the CO_2 measurement is directly a function of the pH of the electrolyte, a highly sensitive and stable pH measurement is pivotal for a robust CO_2 measurement. In order to produce a pH sensor small enough to fit within the 12 mm diameter of the complete sensor, the pH inner electrode of the InPro 5000i has a flat design. As a consequence of this, the resistance of the glass is three times higher than a typical pH sensor and requires particular care during cleaning or calibration (see Section 4.1).

The glass pH electrode used in the InPro 5000i is a combined glass electrode consisting of a reference and measurement system. The reference system uses a gel electrolyte with a ceramic diaphragm. The measurement system uses the specially engineered flat glass. As with traditional pH electrodes, the inner pH electrode is also primarily characterized by a zero point (mV output when in a solution of pH 7) and a slope (the mV per pH unit). The electrode measures pH as a potential, this relationship is defined by the Nernst equation, as shown in Equation 7 and Figure 9.

$$\mathbf{U = U^0 - S(T) \cdot pH}$$

Equation 7 The Nernst equation: pH as a function of voltage potential. U is the electrode potential (mV). U^0 is the standard electrode potential (mV), S(T) is slope of the pH electrode (mV/pH), and $[H^+]$ is the proton concentration in solution (mol/L).

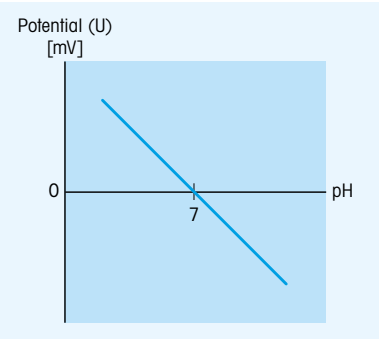


Figure 9 Graph of the Nernst equation showing the calibration curve of a potentiometric pH electrode.

A slope is established by a 2-point calibration (Figure 10 b). Performing a 1-point calibration or process calibration does not change the slope, but rather horizontally shifts the established slope as a zero point shift (also known as an offset, Figure 10 a) to align the known pH value with the intrinsic mV output of the sensor.

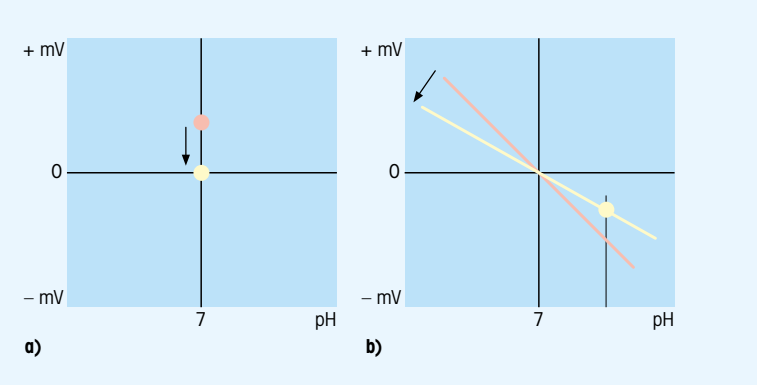


Figure 10 a) Zero point adjustment in pH buffer 7;
b) Slope correction in a 2-point calibration.

3.2.3 Membrane body and CO_2 electrolyte

The membrane body is a single use item. It contains a patented CO_2-permeable silicone membrane coated with PTFE and should be always filled with CO_2 electrolyte before operation. The selective permeability for CO_2 ensures that the electrolyte reaction (Equation 6) is influenced only by CO_2 partial pressure and not by other changes in the process medium. Known interferences are observed in the presence of hydrogen sulphide (H_2S) and ammonia (NH_3), but under typical fermentation conditions no problems should be expected. The PTFE coating of the membrane reduces biofilm build up. The "window" design of the external surface protects the glass pH electrode from high pressure due to CO_2 electrolyte expansion during sterilization; therefore, it is important to ensure that the windows are clean and dry after each sensor maintenance.

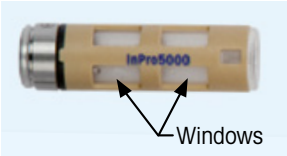


Figure 11 The membrane body is designed to have "windows" that protect the glass electrode from high vapor pressure during sterilization.

The CO_2 electrolyte should be replaced after each run and new CO_2 electrolyte should not be used if outside of expiry. The validity period of the electrolyte is two years.

3.3 Measurement accuracy

The InPro 5000i measurement range is from 10 to 1000 mbar P_{CO_2}. Using partial pressure units, the accuracy is ± 10 % of the CO_2 value when measuring from 10 to 900 mbar, and ± 15 % of the measurement value when measuring greater than 900 mbar. Converting to % saturation units means that the accuracy increases with decreasing % CO_2 saturation, as shown in Figure 12. In the low-mid measurement range, 1 to 50 % saturation CO_2 (light green area in Figure 12), the error is lower than ± 5 %.

The ideal physiological concentration for cell cultures, 5 – 20 % saturation CO_2 (dark green area in Figure 12), is well within the high accuracy range of the InPro 5000i.

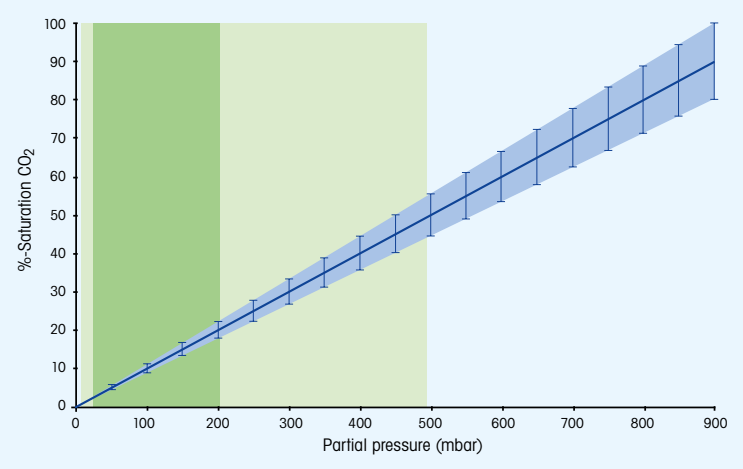


Figure 12 Measurement accuracy as a function of measurement value; in light green the high accuracy measurement range is 1 to 50 % saturation CO_2, with measurement error below ±5 %; in dark green is the typical measurement range for cell cultures, 5–20 % saturation CO_2.

This can best be explained by plotting pH vs p_{CO_2} from Equation 11, as shown in Figure 13. We see from the graph that the resolution is the greatest when the slope of the line is the most negative, this is when an incremental change in CO_2 causes the largest change in pH. The CO_2 electrolyte operative range is 6 to 10 pH; calibration of the inner pH body should also be performed in this pH range for best accuracy.

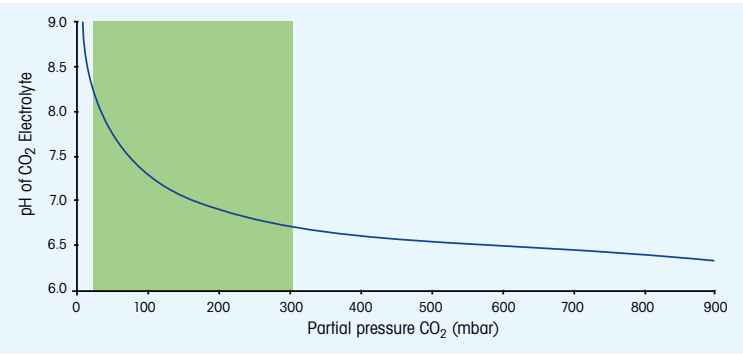


Figure 13 Relationship between pH and P_{CO_2} according to Equation 11.

3.3.1 Impact of calibration frequency on measurement accuracy

The specified measurement accuracy as described in the previous paragraph relies on correct calibration of the pH electrode. Measurement (line above) drift at typical process temperatures (37 °C) is very low allowing for long calibration intervals (1 to 3 months depending on the specific application).

After sterilization, a zero point shift is normally observed and can be adjusted by performing a CO_2 process calibration. The slope value is instead very stable for several sterilization cycles, therefore eliminating the need for 2-point calibration. Figure 14 shows how the slope of the electrode changes with sterilization cycles.

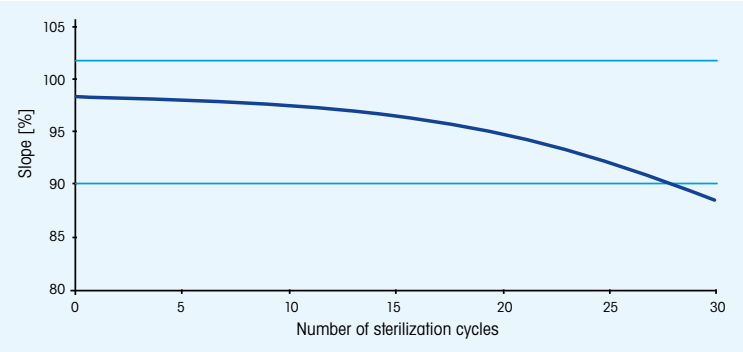


Figure 14 Stable value of slope after several sterilization cycles eliminates the need to perform 2-point calibrations of the inner pH electrode after sterilization.

For the highest accuracy, we recommend performing a CO_2 process calibration at the average concentration and temperature range expected during operations.

By performing a CO_2 process calibration at the average conditions expected during operations, you are able to minimize the impact that slope change or slope error can have on measurement accuracy.

In order to operate the InPro 5000i within the specified measurement accuracy, performing a 2-point calibration is never required.

4 Sensor calibration and maintenance

As described in Section 3.2.2, the inner pH electrode of the InPro 5000i is a pH sensor and has to be calibrated in the same way as a traditional pH sensor. Sensors with Intelligent Sensor Management (ISM™) such as the InPro 5000i, are pre-calibrated in the factory. In some situations the user will still prefer to perform calibrations of the inner pH electrode prior to use. This can be due to internal requirements or as a means to check functionality. One and two-point calibrations are possible for the inner body, as described in Section 4.1

In a typical process, the assembled CO_2 electrode (see Section 3.2) is mounted in the bioreactor and sterilized in situ before operation. For optimal operation, we recommend performing a process calibration after each sterilization cycle. A process calibration can be performed in both gas and liquid phases; recommendations for process calibrations are discussed in Section 4.2.

Calibration and maintenance frequency are discussed in Section 5.3.

4.1 Calibration of the inner pH electrode

4.1.1 Inner pH electrode: preparing for calibration or function check

Depending on the state of the sensor, the inner pH electrode can be accessed by removing the cap sleeve and the membrane body or by removing the wetting cap. Check the inside of the electrode for the presence of any bubbles. Remove bubbles by gently swinging the sensor in a vertical plane.

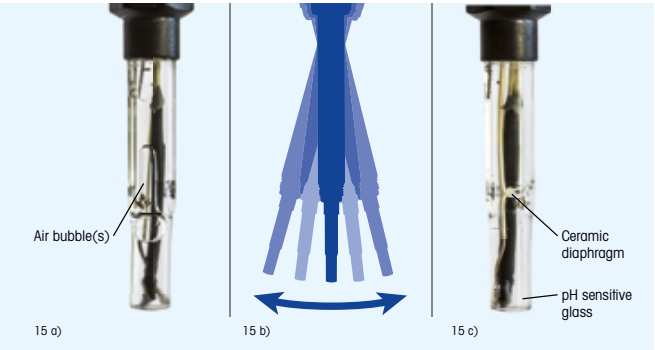


Figure 15 a) Air bubbles in the pH electrode will cause an incorrect measurement
b) Swing the sensor on the vertical plane until the bubbles are removed
c) pH electrode ready to measure.

The image below shows the minimum immersion depth in buffer solutions for calibration and function check.

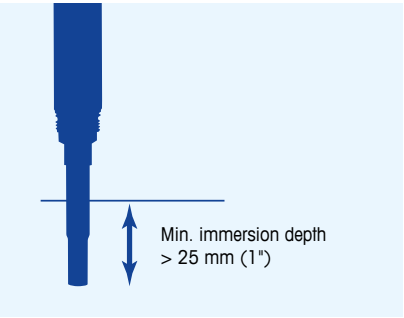


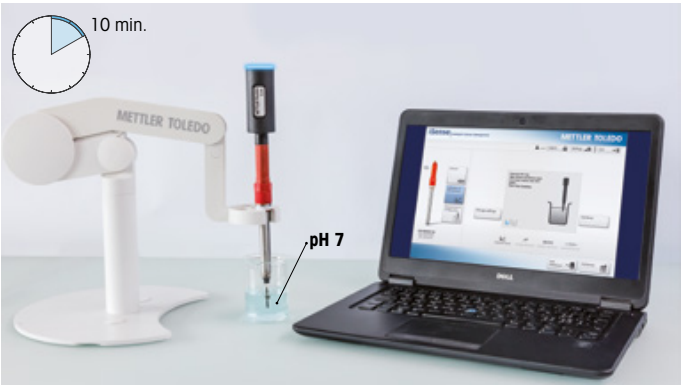
Figure 16 Minimum immersion depth for measurement in buffer solutions.

As a best practice, it is recommended to condition the inner pH electrode for 10 minutes in pH 7 buffer before calibration and function check. To avoid electrostatic charges, it is best never to rinse the sensor with water nor wipe dry the electrode. Calibration should be performed using two clean beakers for each buffer: the first beaker to rinse the electrode, the second for the calibration. This way it is possible to change from one buffer to the other without any drying step, therefore eliminating the risk of electrostatic charge building up on the electrode.

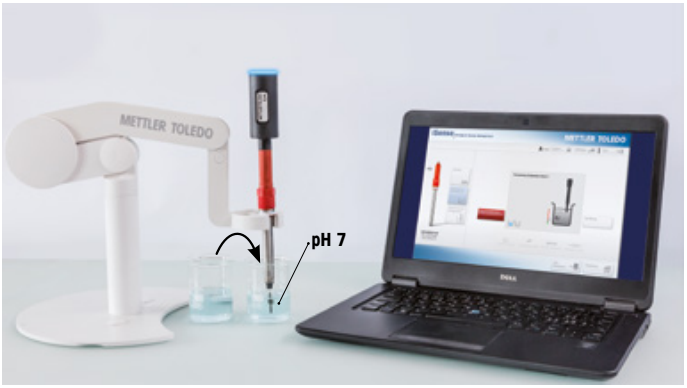
4.1.2 Inner pH electrode: 2-point calibration

For a 2-point calibration, it is recommended to use the buffer solutions pH 7 and 9.21. Using these buffers provides the highest accuracy for the measurement range of the pH electrode in this application (see Section 3.3). The first calibration point should be in buffer 7 for determining the zero point, then the second calibration point in buffer 9.21 for determining the slope.

Because of its special design, the inner pH electrode of an InPro 5000i electrode is very sensitive to handling: it should never be rinsed with anything other than buffer solution and it should never be wiped dry. The recommended steps for an accurate two-point calibration are described in Figure 17.

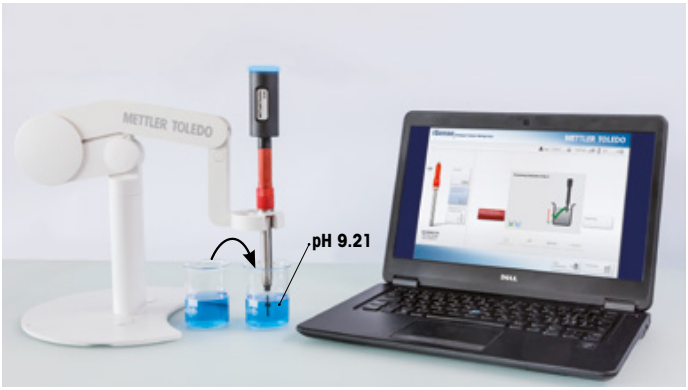


1 – Immerse the glass membrane in buffer pH 7 for about 10 mins.



2 – Use a second beaker with fresh buffer pH 7 for the first calibration point.

3 – Rinse the sensor in a beaker of buffer pH 9.21 solution.



4 – Use a second beaker with fresh buffer pH 9.21 for the second calibration point.

Figure 17 The pH inner body should be rinsed only with buffer solutions and never dried. Follow this four step procedure for an accurate calibration.

A 2-point calibration can be performed using a METTLER TOLEDO transmitter or iSense™ software (see section 5.3).

The calibrated electrode can be stored in the watering cap or membrane body with CO_2 electrolyte solution.

4.1.3 Inner pH electrode: 1-point calibration

For a 1-point calibration, it is recommended to use pH 7 buffer solution. Follow the recommendations made in Section 4.1.2 and steps 1 and 2 in Figure 17.

4.1.4 Inner pH electrode: sensor check function

The pH inner body is a consumable part and has to be replaced after 10 to 15 sterilization cycles. In order to check the functionality of the inner body, a function check can be performed using METTLER TOLEDO's iSense software. Measurement values in pH buffers pH 7 and 9.21 are used by the software to evaluate the sensor health and recommend appropriate maintenance actions or inner body replacement. The sensor check function can be used in place of a 2-point calibration to verify sensor function.

4.1.5 Acceptable calibration and function check values

As previously discussed, calibration of the CO_2 electrode is an offset of the pH-mV calibration curve. Acceptable offset values after calibration are shown in Table 10.

Criteria	Unit	Alarm	Warning	Good	Warning	Alarm
Offset	mV	< –60	–60 … –30	–30 … +30	+30 … +60	> +60
Slope	%	< 80	80 … 90	90 … 102	102 … 103	> 103

Table 10 Recommended ranges for calibration results.

4.2 Calibration of the CO_2 electrode

The CO_2 electrode is the assembled InPro 5000i inlcuding CO_2 electrolyte, the membrane body, and the cap sleeve. A process calibration to adjust the offset is required after each sterilization cycle. A process calibration is a 1-point calibration typically performed while the sensor is mounted in a vessel or a pipe.

For a process calibration, it is necessary to place the sensor in a media of known CO_2 partial pressure. This can be achieved either by using a gas mixture with a known CO_2 content (i.e., calibration gas), or by using an off-line analysis device. In the following section the different methods that can be used to process calibrate the CO_2 electrode are discussed.

4.2.1 CO_2 electrode: preparing for calibration, function check or use

Proper calibration of the inner pH electrode and fresh CO_2 electrolyte is a prerequisite for calibration and use of the CO_2 sensor. The steps below outline preparation of the CO_2 electrode.

1. Ensure that the pH electrode is calibrated and within the acceptable range. New electrodes do not need to be tested because they are factory pre-calibrated and individually tested.
2. Rinse the pH electrode with CO_2 electrolyte, do not wipe dry.
3. Ensure there are no air bubbles in the membrane body (refer to Section 4.1.1, Figure 16).
4. Fill half of a new membrane body with fresh CO_2 electrolyte, as shown in the image below. Ensure the electrolyte is within expiry.

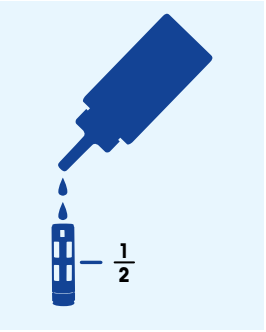


Figure 18 Electrolyte filling level

5. Slip the pH electrode into the membrane body and wipe dry the displaced CO_2 electrolyte from the outer membrane body.
6. Slip the cap sleeve over the membrane body and hand tighten.

4.2.2 In situ process calibration in liquid phase with calibration gas

This method is commonly applied using a benchtop bioreactor as in the example in Figure 19: the sensor is sterilized in the vessel and a calibration gas is used to saturate a liquid. The sensor will be used to measure in liquid; therefore, when possible, a calibration in liquid should always be preferred to a calibration in the gas phase (Section 4.2.3). In some cases, especially with large vessels, this procedure could become time consuming and expensive. Section 4.2.4 describes alternative setups for bigger vessels.

Detailed steps for the calibration are as follows:

1 After sterilization, allow the sensor to cool and equilibrate at process temperature for at least 30 minutes.
2 Fill the vessel with process media. At process temperature, with good mixing, saturate the liquid with the calibration gas, see Section 4.2.6 for guidance on the calibration gas.
3 Select the unit that will be used for the calibration value input. If you choose to use % CO_2 make sure to input the total pressure in the parameter settings (navigate to CONFIG/Measurement/Parameter Setting/TotPres transmitter) or in the calibration settings (iSense and iSense mobile).
4. Start the calibration.
5 Allow the sensor signal to stabilize then press "enter" to capture the measurement value that will be used for calibration.
6 Return to the calibration screen.
7 Input the partial pressure or % CO_2 of the calibration. Use Equation 8 below to convert the mole fraction of CO_2 in the calibration gas into partial pressure. If you are using % CO_2 you can refer to Equation 9.

$$p_{CO_2} = p_{total} \cdot x_{CO_2} = p_{total} \cdot \frac{\%C_{CO_2}(cal\ gas)}{100}$$

p_{total} = calibration pressure
x_{CO_2} = known mole fraction of CO_2 in the calibration gas
$\%CO_2$ (cal gas) = known % CO_2 in the calibration gas

Equation 8 Calculate partial pressure from the mole fraction of CO_2 in the calibration gas

$$\%\,CO_2\,(aq) = x_{co_2} \cdot 100 = \%\,CO_2\,(cal\ gas)$$

x_{CO_2} = known mole fraction of CO_2 in the calibration gas
$\%\,CO_2$ (cal gas) = known %-CO_2 in the calibration gas

Equation 9 Calculate %-CO_2 from the mole fraction of CO_2 in the calibration gas

8. Adjust or save calibration.
9. If the process pressure setting in the transmitter was changed to reflect a calibration pressure, this pressure needs to be updated to reflect the process pressure. (CONFIG/Measurement/Parameter Setting/TotPres).

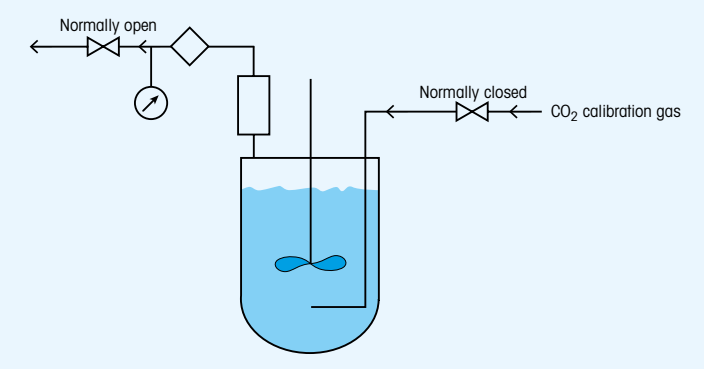


Figure 19 Typical calibration setup for a benchtop bioreactor. Inlet valve for calibration gas is normally closed; the off gas valve is normally open and preceded by a filter and a manometer.

Independently from the method of choice, a correct calibration can only be performed if the total pressure is known; when calibrating in a closed vessel a manometer should be available to measure the total pressure at the moment of calibration (see example in Figure 19).

4.2.3 In situ process calibration in gas phase with calibration gas

A process calibration in liquid phase at the process temperature should always be the first choice. When not possible, a gas phase calibration with calibration gas is a good alternative. After sterilization, let the sensor equilibrate at process temperature for at least 30 min. Purge and

then pressurize the vessel with the calibration gas, and then proceed as described in the previous paragraph (steps 3 to 8). For more information about calibration gas refer to Section 4.2.5.

4.2.4 At line process calibration using METTLER TOLEDO retractable housing gas calibration chamber

Figure 20 InTrac 797

This method is recommended for use in large bioreactors where the use of calibration gas could be impractical, or too expensive. For this method the sensor is calibrated in the gas phase at line outside of the vessel using the InTrac™ 797 retractable housing (Figure 20). The double flushing chamber design of the InTrac 797 (see also Figure 21) ensures that after sterilization, the lower flushing chamber remains sterile when the electrode/sensor is reinserted into the process. This is because the electrode section which enters the lower flushing chamber during reinsertion into the process has been sterilized in the upper flushing chamber.

This retractable housing is commonly used in bioprocessing especially for longer batches as extended fed-batch or perfusion. The sterility test report of the housing can be found in Appendix II. The report confirms that the retractable electrode housing InTrac 796/797 is sterilizable. No infections could be detected in the reactor or on the electrode housing (both positions were tested: electrode in measuring and maintenance positions) during the test period.

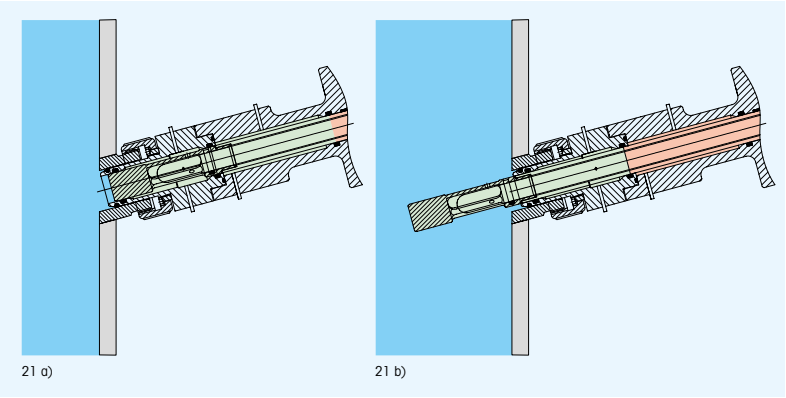


Figure 21 The double flushing chamber design of the InTrac 797; In green the two sterile chambers. a) Retracted position, b) Measuring position

Details of the recommended steaming procedure can be found in the InTrac 797 instruction manual. The general calibration procedure is described below:

1 Install the InPro 5000i into the InTrac 797 housing connected to the vessel
2 Sterilize the sensor with the InTrac 797 housing in the retracted position as shown in Figure 22 a. Sterilize the bioreactor separately while the InTrac 797 housing is in this same position. The sequence of sterilization can occur simultaneously or in any order.

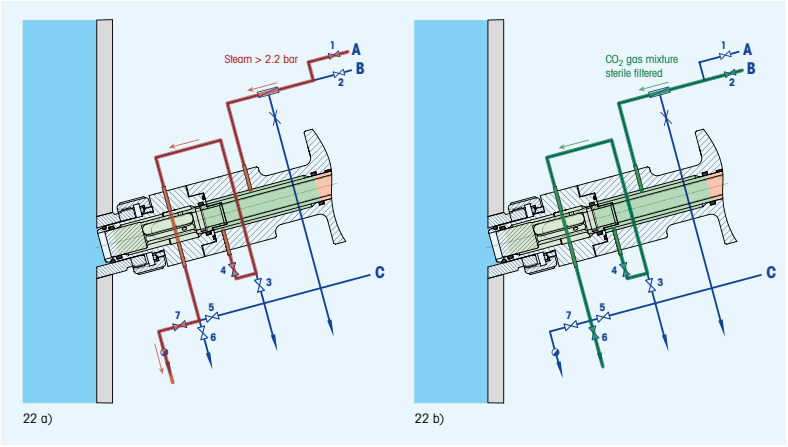


Figure 22 InTrac 797 setup during sterilization and calibration; a) Sensor in the retracted position being sterilized at line of the vessel; b) Sensor in the retracted position being calibrated at line of the vessel.

3 After sterilization allow the sensor to equilibrate at process temperature for at least 30 mins.

4 Continuously flush the housing with sterile filtered calibration gas (Section 4.2.5) as shown in Figure 22 b. Regulate the pressure using a pressure gauge at the exit line.

5 Select the unit that will be used for the calibration value input. If you choose to use % CO_2 you must input the total pressure in the parameter settings (navigate to CONFIG / Measurement / Parameter Setting / TotPres transmitter) or in the calibration settings (iSense and iSense mobile).

6 Start the calibration.

7 Allow the sensor signal to stabilize then press "enter" to capture the measurement value that will be used for calibration.

8 Return to the calibration screen

9 Input the partial pressure or % CO_2 of the calibration. Use Equation 8 to convert the mole fraction of CO_2 in the calibration gas into partial pressure. If you are using % CO_2 you can refer to Equation 9 (Section 4.2.2).

10 Adjust or save calibration.

11 If the process pressure setting in the transmitter was changed to reflect a calibration pressure, this pressure needs to be updated to reflect the process pressure. (CONFIG/Measurement/Parameter Setting/TotPres).

4.2.5 In situ process calibration without calibration gas in the liquid phase

For this method the sensor is sterilized in the vessel and an off-line or at-line analyzer is used to calibrate the CO_2 sensor. It is a valid alternative for a large bioreactor when an InTrac® 797 housing is not available. This method can also be used to verify/calibrate the CO_2 sensor without interrupting the process. When using an analyzer to perform the CO_2 calibration, it is important to minimize measurement error associated with the analyzer (some of these errors are discussed in Table 2). The calibration procedure is as follows:

1 After sterilization allow the sensor to equilibrate at process temperature for at least 30 minutes.
2 Transfer the media or liquid containing the CO_2 into the vessel. This step and the following ones can also be done during the process.
3 Bring the vessel to steady state conditions, as you would for a pH process calibration.
4 Select the unit that will be used for the calibration value input. If you choose to use % CO_2 you must input the total pressure in the parameter settings (navigate to CONFIG/Measurement/Parameter Setting/TotPres transmitter) or in the calibration settings (iSense and iSense mobile).
5 Start the calibration.
6 Allow the sensor signal to stabilize then press "enter" to capture the measurement value that will be used for calibration. At the same time grab a sample for analysis.
7 Return to the calibration screen.
8 Input the measurement from the at-line or off-line analyzer. If the analyzer provides partial pressure units these can be directly entered for calibration. If the analyzer measures in units of % volume CO_2 (g), the equation below can be used to convert into p_{CO_2}.

$$p_{CO_2} = \frac{\%\,\text{Volume}\ C_{CO_2}(g) \cdot p_{total}}{100}$$

p_{total} = calibration pressure
% Volume $CO_2(g)$ = measurement from the analyzer

Equation 10 Calculate partial pressure from the %-Volume CO_2 (g).

The above equation assumes the analyzer compensates for humidity and the calibration pressure.

3 Adjust or save calibration.
4 If the process pressure setting in the transmitter was changed to reflect a calibration pressure, this pressure needs to be updated to reflect the process pressure. (CONFIG / Measurement / Parameter Setting / TotPres).

4.2.6 CO_2 calibration gas guidance

Most calibration methods require a gas mixture with a known mole fraction of CO_2. We recommend using a gas mixture with a CO_2 partial pressure as close as possible to the measurement range, typically within 5 to 200 mbar. The highest measurement accuracy of the InPro 5000i is also achieved within this range.

In general, the CO_2 partial pressure in the gas mixture has to be within 10 mbar to 1000 mbar. This gas mixture can be purchased or created by the user.

Method 1: Purchase calibration gas

Medical grade CO_2/N_2 gas mixtures can be purchased under request from your local gas vendor.

Method 2: Create calibration gas via thermal mass flow controllers (TMFC)

The CO_2 calibration gas can be made by dilution or concentration of gases with known CO_2 mole fractions by using mass flow controllers. The volumetric flow rates of the two gases must be adjusted to reach the desired CO_2 partial pressure.

Method 3: Create calibration gas via vessel pressure indicator (bench scale bioreactors)

The CO_2 calibration gas can be made by dilution or concentration of gases with known CO_2 mole fractions using vessel pressure indicators. An example of this would be purging a vessel with N_2 then pressurizing a vessel to 0.8 ATM with N_2 then pressurizing the vessel to 1 ATM with CO_2. This would create a gas mixture in the vessel that is 20 % CO_2 by volume with a CO_2 partial pressure of 0.2 ATM.

4.3 Calibration and maintenance frequency recommendations

The recommended workflow for optimal performance of the InPro 5000i is described in Figure 21. The inner bodies of new sensors are factory calibrated, therefore calibration is not required. After being assembled with a fresh membrane and electrolyte, the CO_2 sensor can be installed on the bioreactor or retractable housing.

Following sterilization, a process calibration should be performed with a method of choice (see previous section for more details). The sensor is now ready for the process.

At the end of the batch, the inner pH body has to be disassembled again. A calibration is not necessary because the slope characteristic is very stable for 10 – 15 sterilization cycles (see Section 3.3.1). An inner body sensor check using iSense software detects if the performance of the inner body is declining and needs to be replaced. The membrane body and CO_2 electrolyte should be replaced after each batch.

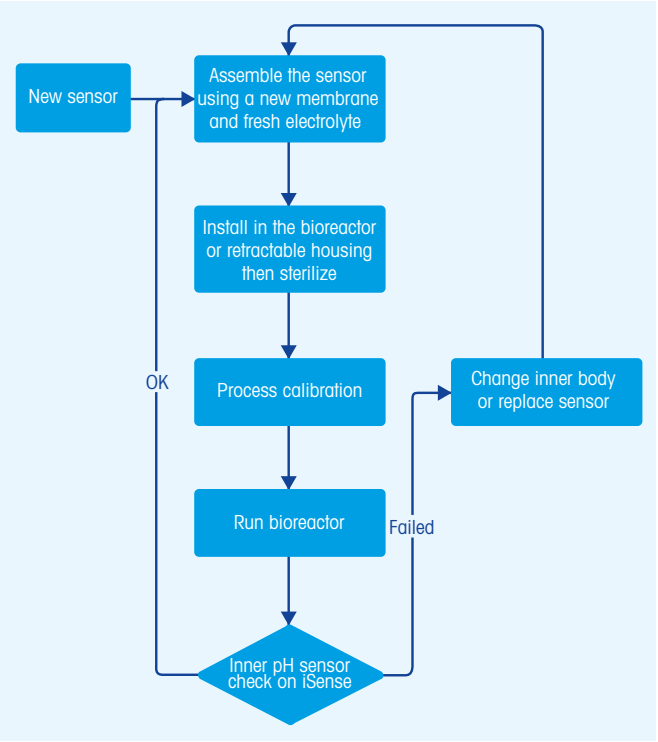


Figure 23 This workflow shows an optimal approach for using the InPro 5000i. Some users will choose to add steps to this workflow for additional function checks and calibrations.

5 Intelligent Sensor Management (ISM) ISM

The InPro 5000i probe features ISM, METTLER TOLEDO's digital platform for process analytics systems. ISM sensors provide a robust digital signal, allow for simplified sensor handling workflows, and significantly reduced cost of ownership. This chapter outlines how to best leverage the benefits of ISM with the InPro 5000i CO_2 probe.

5.1 Robust digital signal

All ISM sensors provide signal conversion from analog to digital. Unlike analog systems, the InPro 5000i features a microprocessor in the sensor head that converts the analog mV signal into CO_2 partial pressure in a digital form. Electromagnetic fields, a frequent problem in analog systems, have no influence on the digital ISM signal. This is particularly important for the InPro 5000i because the high resistance glass electrode is more susceptible to noise. ISM therefore results in greater accuracy of the measurement received at the transmitter.

5.2 Plug and Measure

The InPro 5000i CO_2 internally stores calibration data as well as other relevant information such as sensor type and serial number. When the sensor's connected to an ISM transmitter, the transmitter automatically reads the sensor information and the stored calibration data without the need for any operation intervention. Plug and Measure allows the operator to not only reduce the time required for sensor calibration and installation, but removes the possibility of human error during installation.

5.3 Sensor calibration and verification in the lab

Calibrating at the measurement point is not only unsafe for the operator, it is also inconvenient and prone to errors. As an ISM sensor's calibration data is stored in the sensor, probes can be calibrated in any convenient location, for example a lab or workshop.

iSense is Windows-based software that guides the user through calibration, maintenance and sensor configuration. Using iSense does not require highly qualified personnel because calibration and maintenance procedures are shown step-by-step with simple visual animations.

Calibration reports are generated in electronic form. Up to three calibrations are saved in the sensor memory; the process calibrations performed on the transmitters can be read and documented on a report using the read-out function. The full sensor history is saved in the iSense database.

As mentioned previously, a sensor verification step for the InPro 5000i is recommended after each sterilization. The sensor check function in iSense guides the operator through the procedure and provides recommendations on how to restore the sensor's operating condition if required.

iSense CFR is a version of iSense specifically designed for use in GMP environments. Full traceability is provided thanks to an automated Audit Trail, while paperless documentation can be achieved using electronic signatures.

6 Appendix I

Technical specifications of the InPro 5000 i sensor

Measuring range	0 … 1000 mbar p_{CO_2} (1 mbar = 1 hPa)
Lower detection limit	10 mbar p_{CO_2}
Accuracy	± 10 % (p_{CO_2} 10 – 900 mbar) ≥ ± 10 % (p_{CO_2} > 900 mbar)
Response time	90 % of final value < 120 sec (at 25 °C [77 °F] from air to CO_2)
Temperature range	0 … 60 °C (32 … 140 °F) for measurement
Sterilization temperature	≤ 130 °C (266 °F) in situ or autoclavable
Pressure range	0.2 … 2 bar absolute for measurement (3 … 30 psi)
Mechanical pressure resistance max.	3 bar at 25 °C (77 °F) (max. 42 psi)
Known interferences	SO_2, NH_3, H_2S
Measuring principle	potentiometric (Severinghaus)
Wetted metal parts	stainless steel DIN 1.4435 (similar AISI 316L) surface roughness N5 Ra < 0.4 µm (16 µ inch)
Membrane material	silicone reinforced / PTFE
O-ring materials	Viton®, silicone (FDA compliant)
Plug head	Pg 13.5 thread, washer PTFE, O-ring Viton®
Certificate	3.1B for stainless steel parts EHEDG

7 Appendix II

Sterility test of the METTLER TOLEDO retractable housing InTrac 796/797

[H S W]
HOCHSCHULE WÄDENSWIL

Testing the sterilisation reliability of the Mettler-Toledo retractable electrode housings InTrac 796/797

Final report
August 2001

Project execution: Ulrike Hahnemann, Daniel Hans
Hochschule Wädenswil
Switzerland

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Contents

1. Abstract

For testing the sterilisation reliability of the retractable electrode housings InTrac 796/797, different sterility tests were performed. In each case the test procedure comprised well defined contamination of reactor and electrode housing with the electrode in the maintenance position, as well as sterility tests on the reactor and electrode housing with the electrode in the maintenance and measurement positions. Aerobic test germs were used as bioindicators.
The retractable electrode housings proved to be sterilisable if the manufacturer's instructions were complied with. In the sterility tests with the electrode in the maintenance and measurement positions, infections could be detected neither in the reactor nor in the electrode housing.

2. Materials and methods

2.1 Bioindicators used

2.1.1 Organisms

- *Bacillus subtilis* DSM 347
- *Bacillus stearothermophilus* ATCC 7953
- *Brevundimonas diminuta* DSM 1635

2.1.1 Media

- CS broth (casein peptone soy peptone)

Fluka Tryptic Soy Broth, 500g, order no. 22092

- CS agar (casein peptone soy peptone agar)

Fluka Tryptic Soy Agar, 500g, order no. 22091

- CS broth was used as the rinsing solution.

2.1.3 Preparation of the bacterial suspensions

100 ml of CS broth were innoculated with a single colony of *B. subtilis* on CS agar and incubated overnight at 30 °C and 170 rpm.
100 ml of CS broth were innoculated with a single colony of *Br. diminuta* on CS agar and incubated overnight at 30 °C and 170 rpm.
100 ml of CS broth were innoculated with a single colony of *B. stearothermophilus* on CS agar and incubated for 24h at 55 °C and 170 rpm.

2.2 Preparation of the retractable electrode housing

Injection sockets with creeping steam valves (new: valves 8 and 9) were welded to the branches in the lines between valves 3 and 4 and between the inner chamber and valve 6 (see Fig. 1). The adaptation was carried out by the Bio T company, Bühlstrasse 1, CH-

8855 Wangen. The solutions used in the sterility test (CS broth) were introduced into the apparatus at valves 8 and 9.

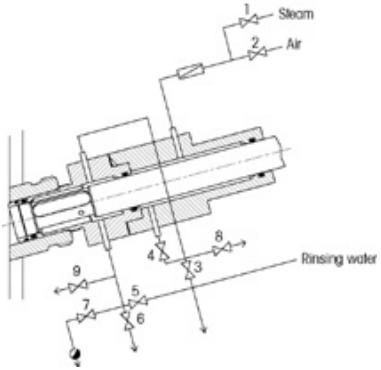

Fig. 1 Drawing of the retractable electrode housing InTrac 796/797 with the additional injection sockets and creeping steam valves 8 and 9 for sterilisation of the injection membranes in the sockets.

3. Procedures and results

3.1 Sterility tests with the pH electrode in the maintenance position

3.1.1 Execution of the test procedures

The sterility tests on the housings were performed according to the following procedure:

1. Sterilisation of the housing with steam (2.4 bar) for 30 min.
2. Ventilation (30 min.)
3. Sterility test on the inner chamber: circulation by pumping sterile CS broth for 15 min.
4. Infection of the inner chamber: circulation by pumping the bacterial suspension for 30 min. and allow to act for 2.5 h.
5. Blowout of the bacterial suspension into sterilisable containers.
6. Rinsing of the inner chamber with water: collection of the wastewater for sterilisation.
7. Sterilisation of the entire housing: collection of all condensates for sterilisation.
8. Ventilation (30 min.)
9. Sterility test on the inner chamber: circulation by pumping sterile CS broth for 15 min.

For monitoring the sterilisations, the reached external temperatures of all valve inlets and outlets were measured with contact thermometers. Within the sterile area, which is limited

by valves 3, 6, 7, 8 and 9, the temperature, required for sterilisation with saturated steam of at least 121 °C, was maintained for 30 min. Outside of these barriers there is no overpressure, so that there only lower temperatures can be attained (see Table 1).

Table 1: Temperatures during sterilisation of the retractable electrode housing

Position	Temperature on 1st sterilisation	Temperature on 2nd sterilisation
Valve 4 - above	123	124
Valve 4 - below	122	122
Valve 5 - below	123	122
Valve 8 - below	122	122
Valve 8 - injection socket, beaker and blind plug, condensate line*	120 / 81* 87** / 90**	117 / 83* 89** / 93**
Valve 3 - rear side	103*	108*
Valve 3 - below	92**	93**
Valve 7 - below	121	122
Condensate drain	100*	104*
Valve 6 - rear side	105*	104*
Valve 6 - below	91**	98**
Valve 9 - injection socket, beaker and blind plug, condensate line*	117 / 83* 84** / 96**	114 / 84* 89** / 91**
Valve 9 - below	123	124
Valve 9 - above	123	121
Condensate and wastewater line*	89**	90**

The measurement points on the sterile barriers are marked with * and those outside the sterile limits with **. In each case the external temperature was determined.

3.1.2 Results

Five days after performing the tests with the electrode in the maintenance position, no infections could be detected in the CS broth used for rinsing of the housing chambers. The growth test on the organisms in the bacterial suspensions used was positive (see Tables 2-5).

3.1.2.1 Tests with the retractable electrode housing InTrac 796

Table 2: Results of the sterility test on the housing InTrac 796 with *B. stearothermophilus*

Designation	Result
CS broth before infection of the housing	no contamination
CS broth after infection of the housing	no contamination
Growth capability of the bacteria before use	positive
Growth capability of the bacteria after use	positive

Table 3: Results of the sterility test on the housing InTrac 796 with *Br. diminuta*

Designation	Result
CS broth before infection of the housing	no contamination
CS broth after infection of the housing	no contamination
Growth capability of the bacteria before use	positive
Growth capability of the bacteria after use	positive

3.1.2.2 Tests with the retractable electrode housing InTrac 797

Table 4: Results of the sterility test on the housing InTrac 797 with *B. subtilis*

Designation	Result
CS broth before infection of the housing	no contamination
CS broth after infection of the housing	no contamination
Growth capability of the bacteria before use	positive
Growth capability of the bacteria after use	positive

Table 5: Results of the sterility test on the housing InTrac 797 with *B. stearothermophilus*

Designation	Result
CS broth before infection of the housing	no contamination
CS broth after infection of the housing	no contamination
Growth capability of the bacteria before use	positive
Growth capability of the bacteria after use	positive

3.2 Sterility tests with the pH electrode in the measuring position

3.2.1 Execution of the test procedures

The sterility tests on the housing were performed according to the following procedure:

1. Sterilisation of the housing with the electrode in the measuring position with steam (2.4 bar) during the reactor sterilisation for 30 min.
2. Sterility test on the reactor for 5 days.
3. Bring electrode into the maintenance position, infect the inner chamber: circulation by pumping the bacterial suspension for 30 min. and allow to act for 2.5 h.
4. Blowout of the bacterial suspension into sterilisable containers.
5. Rinsing of the inner chamber with water: collection of the wastewater for sterilisation.
6. Sterilisation of the entire housing: collection of all condensates for sterilisation.
7. Ventilation (30 min.)
8. Sterility test on the inner chamber: circulation by pumping sterile CS broth for 15 min.
9. Bring electrode into the measuring position, sterility test on reactor.

For monitoring the sterilisations, the reached external temperatures of all valve inlets and outlets were measured with contact thermometers. Within the sterile area, which is limited by valves 3, 6, 7, 8 and 9, the temperature required for sterilisation with saturated steam of at least 121 °C was maintained for 30 min. Outside of these barriers there is no

overpressure, so that there only lower temperatures can be attained (see sterility test series in the maintenance position, Table 1).

3.2.2 Results

Five days after performing the tests, no infections could be detected in the CS broth of the housing chambers. The growth test on the organisms in the bacterial suspensions used was positive (see Tables 6-9). In the case of the retractable electrode housing InTrac 796 in the test with *B. stearothermophilus,* no contamination occurred after 5 days in the reactor (see Tables 6-9).

3.2.2.1 Tests with the retractable electrode housing InTrac 796

Table 6: Results of the sterility test on the housing InTrac 796 with *Br. diminuta*

Designation	Result
Reactor	no contamination
CS broth before infection of the housing	no contamination
CS broth after infection of the housing	no contamination
Growth capability of the bacteria before use	positive
Growth capability of the bacteria after use	positive

Table 7: Results of the sterility test on the housing InTrac 796 with *B. stearothermophilus*

Designation	Result
Reactor	no contamination
CS broth before infection of the housing	no contamination
CS broth after infection of the housing	no contamination
Growth capability of the bacteria before use	positive
Growth capability of the bacteria after use	positive

3.2.2.2 Tests with the retractable electrode housing InTrac 797

Table 8 Results of the sterility test on the housing InTrac 797 with *Br. diminuta*

Designation	Result
Reactor	no contamination
CS broth before infection of the housing	no contamination
CS broth after infection of the housing	no contamination
Growth capability of the bacteria before use	positive
Growth capability of the bacteria after use	positive

Table 9: Results of the sterility test on the housing InTrac 797 with *B. stearothermophilus*

Designation	Result
Reactor	no contamination
CS broth before infection of the housing	no contamination
CS broth after infection of the housing	no contamination
Growth capability of the bacteria before use	positive
Growth capability of the bacteria after use	positive

4. Discussion and conclusions

On use of the housings InTrac 796/797, no infections were observed in the reactor with the electrode either in the maintenance or the measuring position.
The retractable electrode housings InTrac 796/797 may be reliably sterilised if the installation is properly executed for sterilisation and its operation is appropriate. Proper installation comprises the feeding and draining of steam, rinse water and compressed air.

To avoid contamination of the running bioprocess on exchange of the electrode, foreign infection of the inner housing chamber on the reactor side should be prevented by a clean work process. The O-ring between the reactor and the interior of the housing, which acts as a sterile barrier, must be checked after each process and replaced if necessary. Since microorganisms can become deposited in the O-ring groove, this region must be carefully cleaned to prevent carry-over of possible sources of infection.

Ulrike Hahnemann

Daniel Hans

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