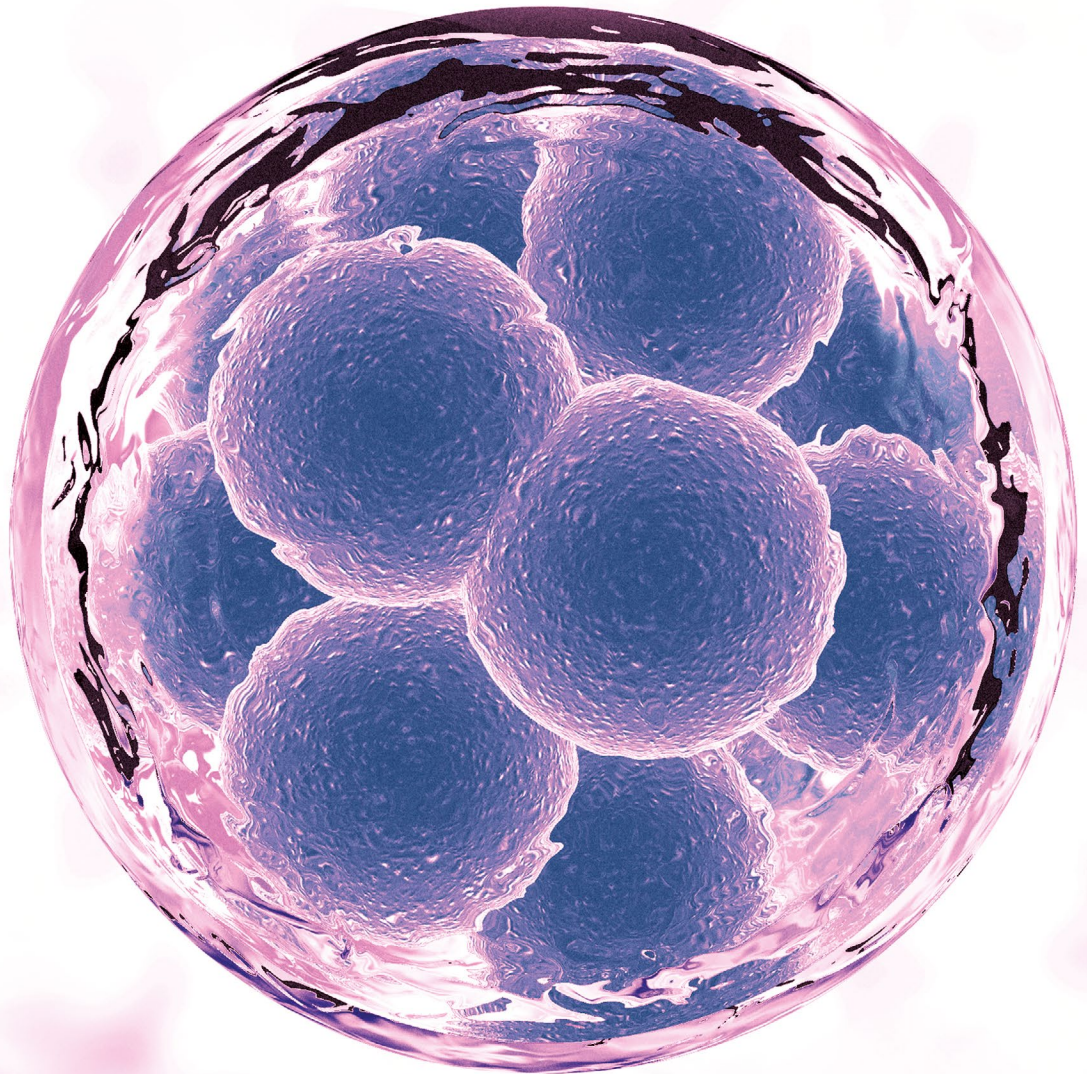




# Embryo Culture Guide

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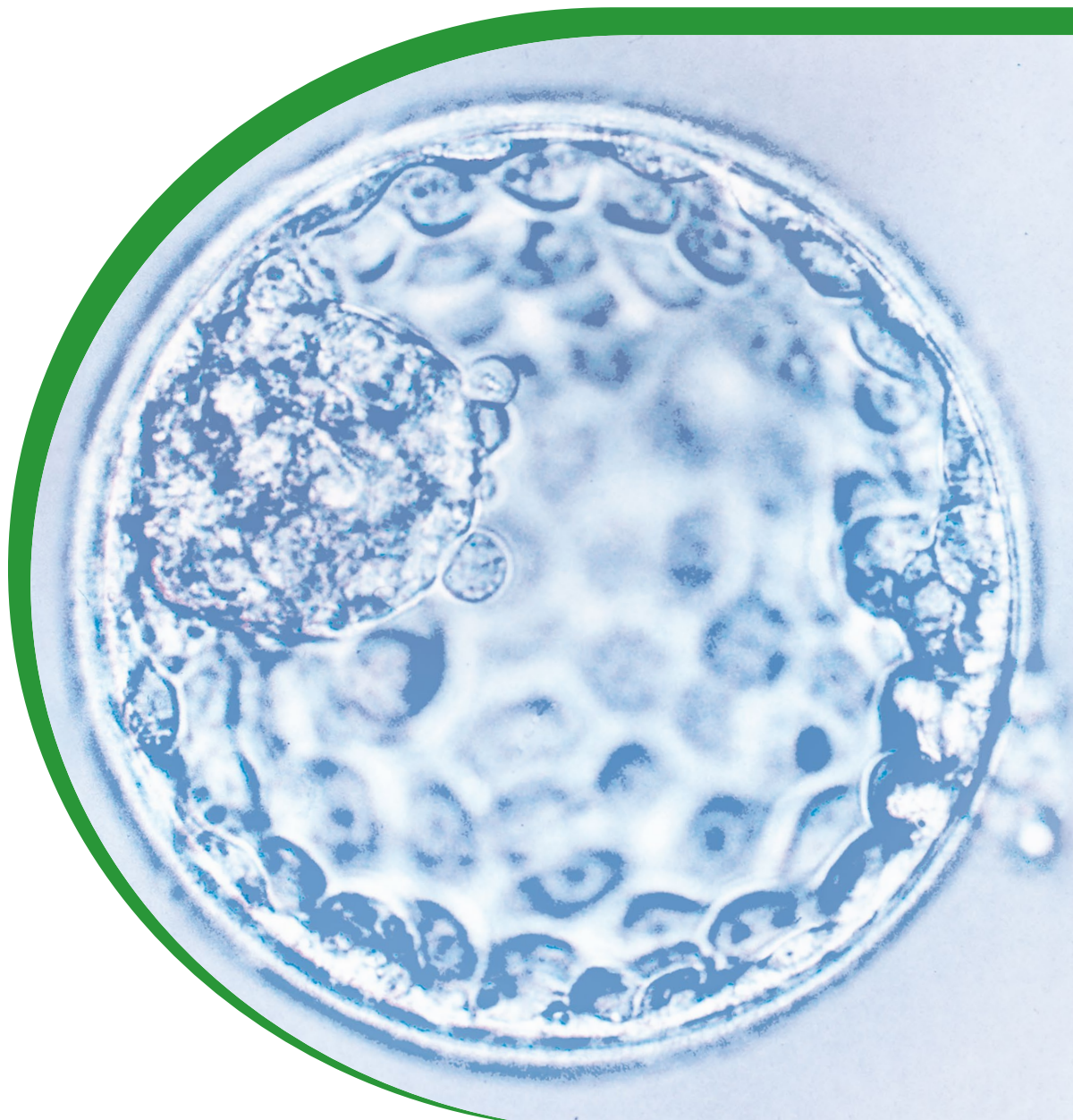


# Introduction

**CooperSurgical Fertility and Genomic Solutions' offers a comprehensive portfolio of ART equipment, products and services for the complete IVF process.**

We work alongside embryologists and medical practitioners to continuously develop next-generation solutions, including new scientific advances, services and education to help improve clinical outcomes.

When performing embryo culture there are a number of key considerations to be taken into account. These considerations, as well as variations in techniques commonly used, are explored in this guide. Where possible, the pros and cons of each technique are discussed alongside the relevant day-to-day quality control measures.



# Culture media systems

## Sequential and single step

There are two main approaches to embryo culture: the “back to nature” strategy used in sequential media and the “let the embryo choose” strategy of single step media.

Sequential media use stage-specific formulations that aim to mimic the *in vivo* environment that the embryo would encounter. Typically the components reflect the metabolic needs of the embryo. In the early cleavage stages, development is driven by the maternal genome and entails only low levels of biosynthetic activity. At this stage, there is a preference for lactate and pyruvate as an energy source. After embryonic genome activation, around the time of compaction, there is a switch to a higher level of metabolic activity and related demand for energy. For this reason, the embryo prefers glucose (Table 1).

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### Pre-compaction (1-8c)

- Low biosynthetic activity
- Lactate and pyruvate
- Non-essential amino acids
- Maternal genome
- Individual cells

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### Post-compaction (>8c)

- High biosynthetic activity
- Glucose
- Essential & non-essential amino acids
- Embryonic genome
- Differentiation
  - 2 distinct cell types

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Table 1

In the case of single phase culture, all components are provided and the embryo uses these as and when needed. This approach means the composition is not tailored to stage-specific demands, so compromises may be needed. However, careful design means a single medium can support optimal development without pushing the embryo down non-physiological metabolic pathways.

Evidence from published literature (Sfontouris et al, 2016) and clinical testing show results to be comparable in the two systems.

# Storage, handling and use of media

The way culture media are handled and stored may have a significant impact on their quality, and can affect clinical results. You should receive your media from the manufacturer in good condition. It should be stored at 2 – 8 °C out of direct light and tightly capped. It is important to monitor storage conditions for your media. Be wary of using domestic fridge-freezers as these may have a thaw cycle to limit ice formation, which results in fluctuating temperatures. It is recommended that you use a refrigerator which has data-logging.

When handling media, ensure aseptic technique is followed. Unless using a single use product, steps should be taken to limit the number of times a single bottle is opened and closed. It may be best to avoid keeping small volumes of media with a large air space in the bottle. For this reason, careful thought should be given to the bottle size you order.

During dish preparation, careful consideration should be given to time taken, airflow in the workstation, temperature and micro drop size or well culture. When using micro drop culture, it is highly recommended that a maximum of two dishes be prepared at a time to reduce the time between dispensing of drops and covering with the oil overlay. This minimizes risks associated with changes in osmolality. For an excellent description of this, see Swain et al, 2011.

If using smaller micro drops, it may be prudent to prepare dishes one at a time or maybe work in pairs to avoid any evaporation of media. In open systems, even though larger volumes are used, it is equally important to prepare dishes quickly and place them in a humidified incubator.

Once set up, medium should be fully CO<sub>2</sub> and temperature equilibrated before use, in most instances, 2-4 hours is adequate.

Bear in mind that time for equilibration will depend on drop size, thickness of the oil overlay (see Figure 1) and if the bottle has previously been opened. In some centers at high altitude, thinner air means a higher percentage of CO<sub>2</sub> must be used in the incubator to achieve the desired pH (Figure 2).

## Equilibration time in a 0.5 mL medium sample with oil overlay

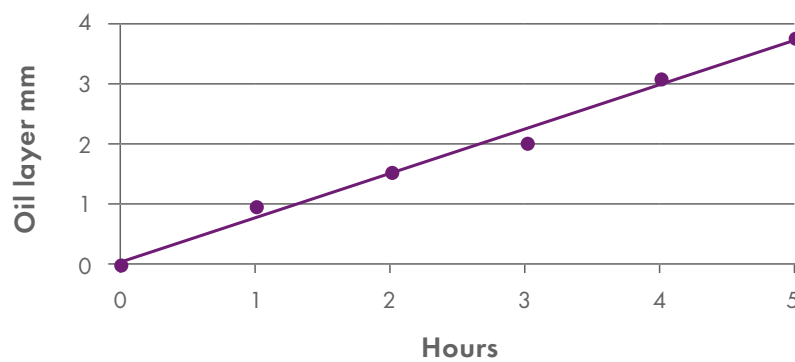


Figure 1

**Equilibration time in a 0.5 mL medium sample with oil overlay.  
Height above sea level influences the required CO<sub>2</sub> level in the incubator**

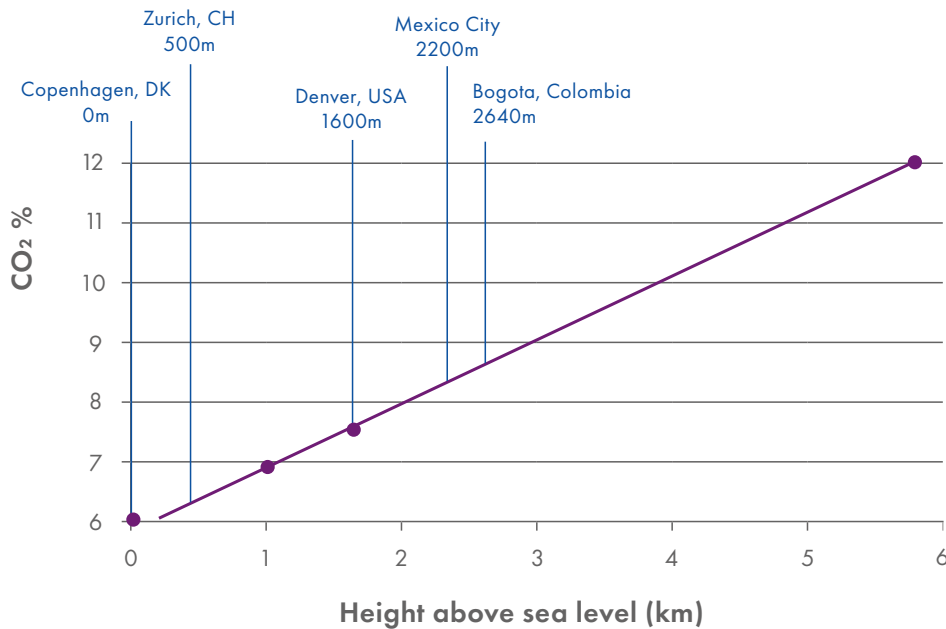


Figure 2

## Culture individually or in groups?

There is no clear consensus on the optimal volume of culture medium needed per embryo, nor the numbers of embryos that might be cultured together.

Animal data suggests that it is not a simple case of establishing an optimal volume per embryo. This is because the effects of embryo culture density vary depending on whether it is the volume that is changed and the numbers of embryos. The overall view however seems to suggest that group culture in smaller volumes may not make a difference in morphological development but seems to give higher numbers of cells in the derived blastocysts, suggesting healthier embryos (Ebner et al, 2011).

While a lot of these data are from animal models, the trend is seen in both monovular (one egg ovulated) or multiovular species so it seems reasonable to assume human embryos will show similar results. A number of studies certainly show better embryo development after group culture (eg Ebner et al, 2011).

# Gas phase and pH

## pH and oocytes/embryos: pHi vs pHe

Intracellular pH (pHi) regulates protein conformation, glycolysis and a number of other critical metabolic and transport processes in the human oocyte/embryo. Maintaining pHi is therefore imperative especially in the early embryo. Extracellular pH (pHe) of the culture medium is generally higher than pHi but is known to be a critical factor in establishing pHi.

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### What pHe to aim for?

- A precise optimum of pHe for the culture of human embryos has yet to be identified.
  - The physiological range of 7.2 - 7.4 is used, however there are numerous observations that varying pH in vitro according to developmental stage (oocyte, 2PN, cleavage embryo, blastocyst) may improve development.
  - Oocytes do not seem to pH regulate to a great extent, and pHi follows pHe. Embryos on the other hand seem to maintain a somewhat acidic pHi, even at higher pHe.
  - The pH during the culture phase should therefore be lowered to target the pHi with pHe set slightly above pHi since embryo metabolism also acidifies the cytoplasm.
- 

### How is stage-specific pH achieved in culture media?

- The level of bicarbonate determines the equilibrium pH and thus the required %CO<sub>2</sub> inflow in the incubator.
  - Optimal pH control involves CO<sub>2</sub> flow optimized for each medium/embryo stage.
  - To avoid using multiple incubators, for example a high-low-high pH regime, choose media with a buffer formulation that delivers the desired pH at a fixed CO<sub>2</sub> level.
- 

### When to test pH

- First of all, it is essential that the level of CO<sub>2</sub> in the incubator is known and is consistent. Labs should ensure that the CO<sub>2</sub> level in each incubator is independently validated, only then can pH be assessed.
- It is generally recommended that, as a minimum, pH be tested when a new batch of medium is introduced to the laboratory. Some labs may wish to perform additional tests, for example, weekly checks of each incubator.

## How to measure pH

Accurate, reproducible measurements require awareness of several issues including temperature of the medium. If the medium contains protein, then the pH meter must be cleaned after each use as protein may adhere to the pH meter.

1. Always calibrate the pH meter before each group of measurements. Ensure calibration takes place at the same temperature as the measurement (ie, 37 °C, see small table below).
2. Use fresh calibration buffers with one buffer pH close to that of the measured medium. (eg, buffers at pH7 and pH10).
3. Make sure that the calibration buffers are equilibrated to 37 °C before measuring.
4. Store the electrode in a designated storage solution.
5. Be aware of probe deterioration. Electrodes deteriorate gradually and frequently used probes must be renewed regularly, eg, every 6 months.
6. When removing CO<sub>2</sub> -equilibrated media from the incubator for testing, you must work quickly. Testing must be completed within 30 seconds or the ambient atmosphere will affect pH. Removal of media from the incubator to test pH will produce inaccurate measurements. An alternative approach is to tightly cap media prior to removal from the incubator and use a gas analyzer to measure pH.

Table 2 shows an example of measured pH error in a sample medium if the electrode is calibrated at room temperature and measured at 37 °C.

	Wrong	Correct
Calibration Temperature	22.8 °C	37.0 °C
Measured Temperature	37.0 °C	37.0 °C
CO <sub>2</sub>	5.0%	5.0%
<b>pH Results</b>	<b>7.29</b>	<b>7.38</b>

Table 2

## Low Oxygen Culture

*In vivo*, oocytes and embryos are exposed to a maximum of 5-8% oxygen (O<sub>2</sub>) in the reproductive system. Atmospheric O<sub>2</sub> may lead to elevated levels of reactive oxygen species (ROS), potentially causing oxidative stress (damage to cell organelles, lipids, membranes, DNA, gene expression), and ultimately poor embryo development.

Increasingly, research has given stronger backing to the use of lower oxygen levels during *in vitro* culture. (Meintjes et al, 2009, Kovacic et al, 2009, Waldenström et al, 2008, Kovacic & Vlaisavljevic, 2008). This is despite advanced culture media incorporating a variety of antioxidants and ROS scavengers to counter the hyperoxic effects.

The effect of low oxygen culture is reportedly greatest in extended culture to the blastocyst stage (Sobrinho et al, 2009). However, data also shows a clear benefit of low oxygen irrespective of the culture duration (Day 2-Day 6) (Kovacic et al, 2009).

Consequently ORIGIO media are also formulated for use with ambient oxygen but, wherever possible, we recommend culturing with lowered oxygen of 5%.



# Temperature control

Maintaining constant temperature at 37°C must be a top priority during all stages of culture. Deviations may harm metabolism and mitotic apparatus.

## Embryo sensitivity to temperature

Oocytes in particular are extremely sensitive to alterations in temperature. Cooling will cause spindles to depolymerize. When re-warmed, spindles will hopefully repolymerize, but there is a risk of spindle reformation failure, wrongful reattachment of the chromosomes and subsequent aneuploidy of the embryo. Likewise, early embryos are more sensitive to temperature changes, which may upset cellular metabolism, membrane stability and transport processes.

## Temperature control during incubation

Strict temperature monitoring in incubators and the use of heated biological chambers is advised. Calibrated and temperature-mapped heated stages must be employed whenever working outside the incubator or chamber.

Door opening reduces incubator temperature. Repeated openings may cause cumulative lowering of incubator (and medium) temperature, especially in larger incubators used for multiple patients (Figure 3).

Strict temperature monitoring and control is advisable. Similar precautions concern CO<sub>2</sub> level and humidity. Loss of incubator humidity will increase evaporation and medium osmolarity especially when media are without an oil overlay. Incubators with chambers for individual patients allow much greater control of the culture environment.

## Temperature control outside the incubator

Beware that most plastic dishes originally manufactured for tissue culture are designed to be stackable. Consequently the base of the dish is not in full contact with the heating stage when working under a microscope (Figure 4). Even a small air gap may cause medium cooling which could affect embryos. Proper warming and quick handling of media outside the incubator is imperative.

Time of temperature recovery after a single 5s door opening in a standard IVF incubator. Recovery time to 37°C can be >20min.

Figure 3

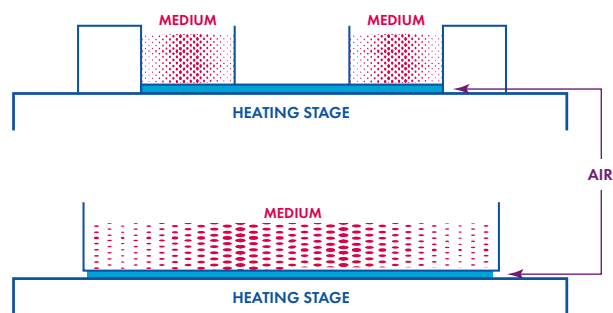
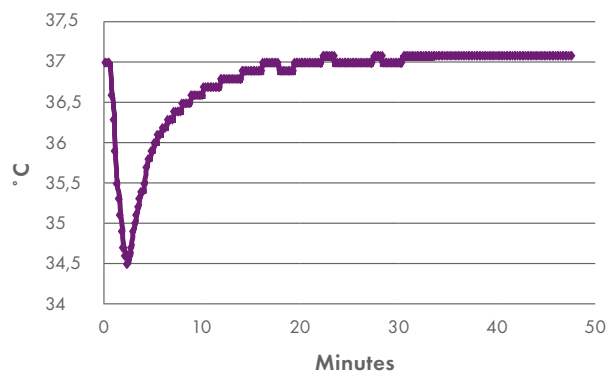


Figure 4

## Air gaps and temperature control

Air is a poor energy conductor, and gaps between the medium and heated surface will cause temperature variation.

# Overlay

When culturing under oil, the handling and use of the oil is just as important as your culture medium.

Mineral oil or liquid paraffin are commonly used in IVF to protect the culture from evaporation and contamination. Oil is a by-product of the petroleum refinement process. Mineral and paraffin oils have the same origin, however paraffin oil has been further refined, thus increasing the purity of the oil. Oils for IVF are typically supplied as either pre-washed or un-washed. However, it is generally recommended to wash the oil prior to use, as this removes potentially detrimental contaminants.

It has been reported that oil can be prone to peroxidation if allowed to age in suboptimal conditions (Otsuki et al, 2008). It is recommended that all oil be stored in dark conditions at 4-8 °C. Oils should then be equilibrated to 37 °C in an atmosphere of 5-6% CO<sub>2</sub> before use.

# Hardware

All the careful work in establishing excellent culture conditions can easily be undone if the associated plasticware and equipment is poor. It is therefore recommended that all plastics (culture vessels, pipettes, tips, etc) are MEA-tested and/or are subjected to a sensitive sperm survival assay before use. Culture dishes are available pre-tested and CE-marked, otherwise labs should perform their own tests.

In addition, labs should implement a comprehensive system of quality control that includes validation of equipment. This should ensure the optimal pH and temperature for gametes and embryos is maintained, not only in culture but during all handling steps.

# Air quality

Embryos, especially cleavage-stage embryos, are sensitive to environmental toxicants, especially volatile organic compounds (VOCs). These originate mostly from vehicles, industrial activity and solvents. For this reason, careful attention should be dedicated to your general air supply and purification measures should be taken to effectively remove VOCs. This is especially the case in metropolitan areas, where concentrations can be greatly elevated (Morbeck, 2015).

The enclosed incubator environment may have concentrated VOC levels several times higher than the ambient air. It is recommended that all incubators be fitted with an in-line gas filter.

Ensure that air entering incubators (ambient + gas) is clear of VOCs and other contaminants by using the ORIGIO Gas Line Filter.



# Quality control

Monitoring and quality control testing covers all processes from the receipt of raw materials to the final product, including product development. The final products are tested with a combination of the following QC tests according to our specifications:

- pH test: USP current edition
- Osmolality test: USP current edition
- Mouse Embryo Assay (MEA): Embryos are cultured in test medium and control medium for 72 hours (for 2-cell) or 96 hours (for 1-cell) respectively. ORIGIO requires a minimum of 80% formation rate to expanded blastocysts. All relevant final products are tested by a MEA test.
- Endotoxin LAL test: USP current edition
- Sterility test: USP current edition. All products are sterile filtered and each batch is tested for sterility.

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