

- The Biology of HIF Proteins Impacts Your Protocol
- Cells Respond to O<sub>2</sub> Changes in MINUTES
- Optimize O<sub>2</sub> Levels For Your Cells
- Pre-equilibrate All Culture Media
- Minimize Room Air Exposures
- Other Tips for Getting Started

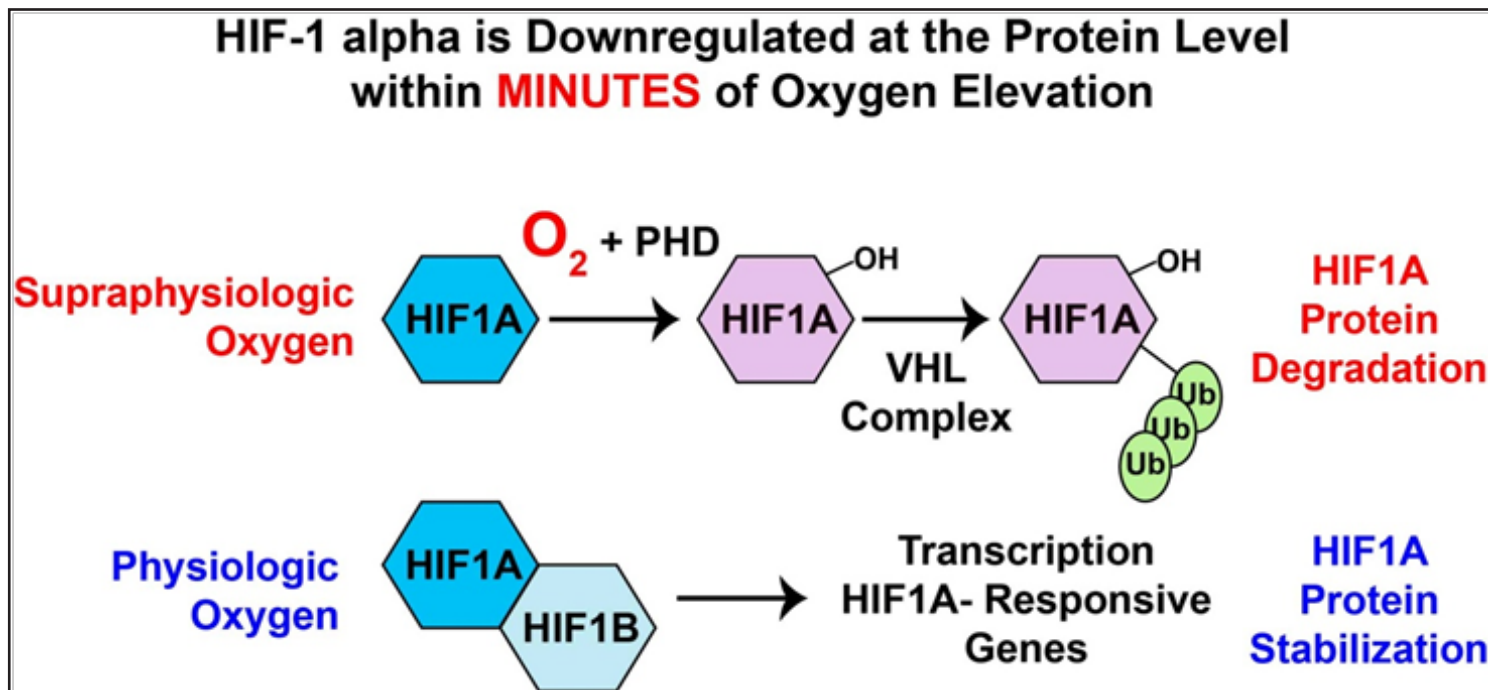


## Tips for Planning, Executing and Reporting *In Vitro* studies at Physiologically Relevant Oxygen Levels

For more information check out our  
Cytocentric Blog: [www.biospherix.com/cytocentric](http://www.biospherix.com/cytocentric)

# The Biology of HIF Proteins Impacts Your Protocol and the Outcome of Your Experiments in Physiologic Oxygen

Cellular responses to oxygen are mediated in part by hypoxia-induced factors (HIF-1 and -2 protein families). These transcription factors control a wide variety of cell functions, and are among the most rapidly regulated proteins found to date.

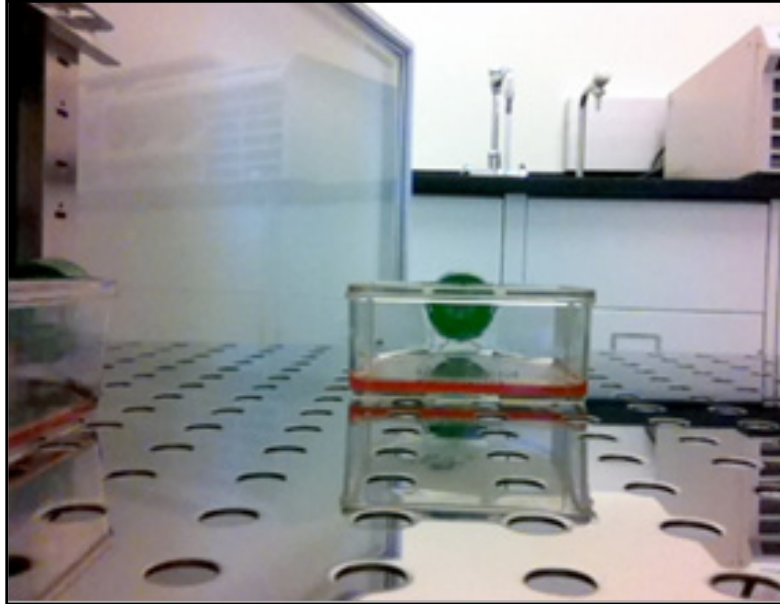


**A Simplified Model of HIF-1 $\alpha$  Regulation by Oxygen.** The regulation of HIF proteins occurs at multiple levels, but the regulation by prolyl hydroxylases (PHD) in the presence of elevated oxygen occurs on a time scale of minutes. PHD family enzymes hydroxylate proline residues on HIF1 $\alpha$  allowing interaction with Van Hippel Landau (VHL) and other proteins. HIF1 $\alpha$  is then rapidly poly-ubiquitinated and targeted for degradation.

There are numerous reviews of HIF protein regulation available. For a report specifically on timing, see Jewell, U. R., et al. **“Induction of HIF-1 $\alpha$  in response to hypoxia is instantaneous.”** The FASEB Journal 15.7 (2001): 1312-1314. Also see: Semenza, Gregg L. “HIF-1 mediates metabolic responses to intratumoral hypoxia and oncogenic mutations.” The Journal of clinical investigation 123.9 (2013): 3664.

**We strongly recommend planning your experiments to eliminate or minimize pericellular oxygen changes to avoid destabilizing HIF proteins.**

# What is the Oxygen Level in the Traditional Open Room CO<sub>2</sub> Controlled Incubators I Have Been Using All These Years?



**(Hint: It's Not 20.8%)**

Because room air is near 21% oxygen, researchers often report that incubation oxygen is as well. However, this is impossible for a traditional open room cell culture incubator set at 5% CO<sub>2</sub> or more. Room air contains only 0.03 - 0.06% CO<sub>2</sub>. Every time the inner incubator door is opened and closed, CO<sub>2</sub> is infused to replace what is lost to the room. This necessarily drives the percentage of oxygen down inside the incubator.

High humidity also drives incubator oxygen levels down as water vapor can take up 8% of the gas volume. Customers with high oxygen applications have reported to us that they could not drive oxygen levels above 87% with 5% CO<sub>2</sub> and an open water source.

We measured oxygen levels in standard open-room 5% CO<sub>2</sub>-controlled incubators after opening the inner door just long enough to place a sensor on a shelf before closing it.

We found an average of **17.2 +/- 0.3%** oxygen (n=4 incubators in 3 different institutions). This can drift up over time, if the incubator is not opened for several days to about 19%.

However, just opening the door again caused the oxygen to jump up and CO<sub>2</sub> levels to drop. Once closed, the incubator then infuses CO<sub>2</sub> to restore it to 5% and oxygen falls again. Smaller incubators had wider swings in gas levels than large ones. These variations may affect your cell cultures.

See relevant data at the Cyto-centric Blog: <http://biospherix.com/cyto-centric/375-reproducibility.html>

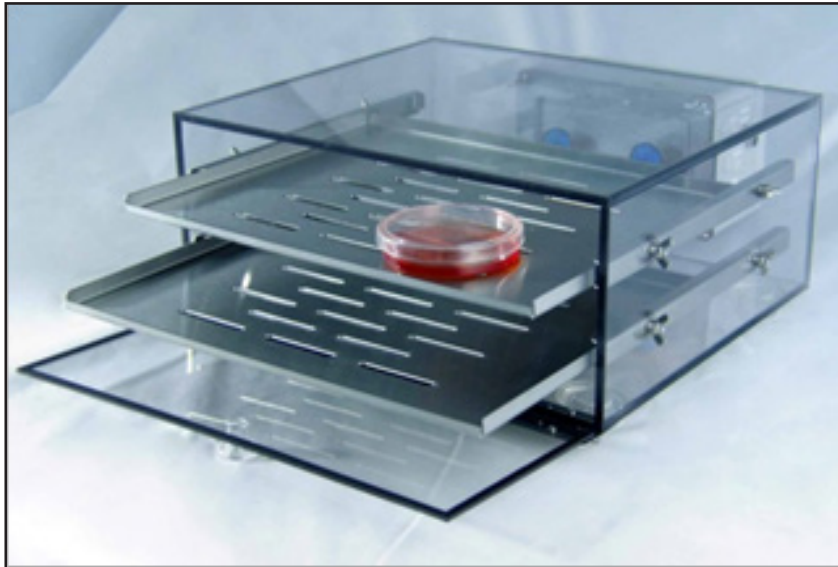
## **Summary:**

**A standard 5% CO<sub>2</sub>-controlled cell culture incubator is not at 21% oxygen.**

**The frequency and length of time the inner incubator door is open could be an overlooked source of variability in cell culture conditions.**

**Don't report 21% oxygen incubation unless you control oxygen to 21%.**

# I Have Seen Others Use 1%, 2%, 5%, 8%, or 10% Oxygen. What Oxygen Level Should I Use for My Cells?



*In vivo*, as soon as air enters the body, it mixes with CO<sub>2</sub> being expired and the percentage of oxygen decreases. While a chamber can certainly be controlled to 20.8% oxygen (room air), this is suprphysiologic.

Oxygen levels in the body vary widely depending upon the local physiology or pathophysiology. Circulating venous blood is generally accepted to contain 5% oxygen. Some tissues, like bone marrow or fetal tissues, can normally experience lower oxygen levels. Tissues with little vasculature, like cartilage or the interior of large solid tumors, can be close to anoxic (0%).

**We strongly recommend that you refer to scientific literature related to your specific cell or tissue model to determine the best oxygen range for your *in vitro* cell culture.**

These reviews may be helpful to get started:

Carreau, Aude, et al. "Why is the partial oxygen pressure of human tissues a crucial parameter? Small molecules and hypoxia." *Journal of cellular and molecular medicine* 15.6 (2011): 1239-1253.

Jež, M., Rožman, P., Ivanović, Z., Bas, T. Concise review: The Role of Oxygen in Hematopoietic Stem Cell Physiology. *Journal of cellular physiology*. 2015; 230(9): 1999-2005.

We also encourage you to do preliminary experiments, testing a range of oxygen levels, to optimize conditions for your model in your hands.

## **Summary:**

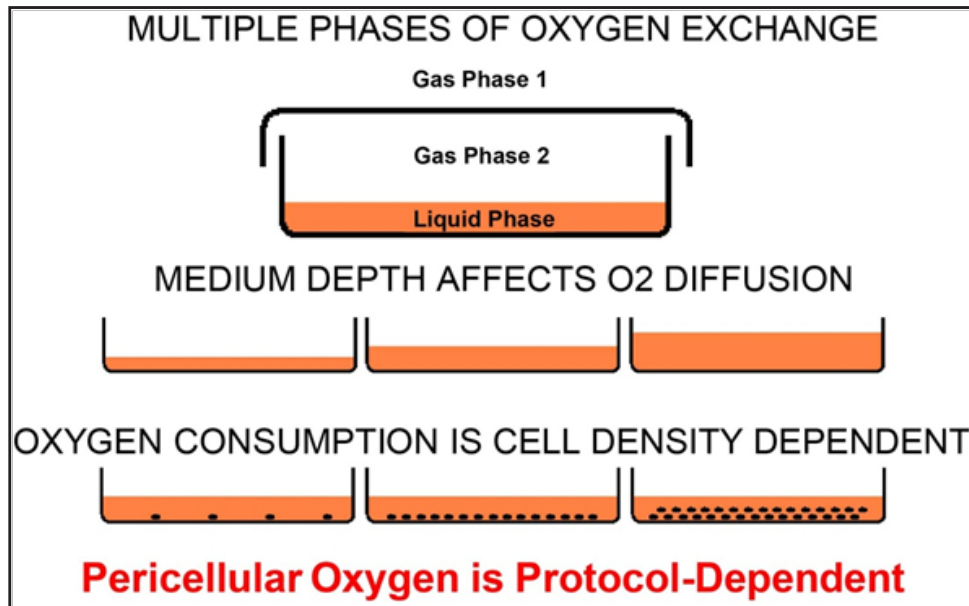
**21% oxygen is not appropriate for most *in vitro* studies.**

**Venous blood may be at 5% oxygen, but many important tissue types normally experience lower oxygen levels.**

**Find a range of oxygen levels that is appropriate for your model in the scientific literature.**

**Confirm the best oxygen level for your model in your system with preliminary experiments.**

# What Oxygen Level Does My Cell Culture Experience in the Bottom of a Cell Culture Vessel?



Cell culture oxygen levels start with the incubation chamber oxygen level, but other factors, such as cell culture vessel type, mixing, and starting medium oxygen level affect it as well. The depth of the cell culture medium can affect the equilibration time of the medium and the oxygen levels of your cells.

Oxygen-permeable cell culture vessels are available from some vendors that allow oxygen exchange through the bottom of the vessel in static culture. This reduces the diffusion distance between the chamber and your cells and reduces the time for oxygen changes to reach your cells.

Pericellular oxygen levels can change with cell density and total cell oxygen consumption. Don't let cells overgrow. Keep cell numbers within consistent ranges from experiment to experiment.

## Summary:

**Pericellular oxygen depends upon multi-stage gas exchange between the atmosphere and the cells in the culture vessel.**

**Factors in your protocol that affect oxygen diffusion to your cells include vessel type and medium depth.**

**Cell density affects oxygen consumption rates. Use consistent cell density ranges from experiment to experiment and don't let cells overgrow.**

**Some vendors sell cell culture vessels with oxygen-permeable, water-tight growth surfaces.**

# I Have to Handle My Cells in a Standard Room Air BSC. What Can I Do To Get the Best Results in Suboptimal Cell Handling Conditions?



## Pre-Equilibrate Cell Culture Media and Other Solutions

Cell culture media and other solutions for cell culture are very slow to equilibrate with incubator oxygen in static culture. Pre-equilibrate cell culture media to the desired oxygen levels before using with your cells to minimize undesirable pericellular oxygen level swings.

If you are able to do so while maintaining sterility of your media, you can sparge them with nitrogen gas to reduce the partial pressure of oxygen. In addition, if using tubing sets or other plastic-ware that will contact cells or medium in a functionally closed system, pre-equilibrate those as well.

The authors of this paper pre-equilibrated media at least six hours, but more often overnight, with occasional shaking in a low-oxygen atmosphere before applying it to cells: Mantel, Charlie R., et al. "Enhancing hematopoietic stem cell transplantation efficacy by mitigating oxygen shock." *Cell* 161.7 (2015): 1553-1565.

## While Working with Cells in a Room Air BSC, Minimize Mixing of Cell Suspensions and Media with Room Air

If cell handling must be done in room air, such as in a standard biological safety cabinet (BSC), pipette pre-equilibrated media carefully against the side of vessels.

## What is the Longest Length of Time I Can Have My Cells Out in the BSC?

The sensitivity of different cell types to oxygen changes may be different. The longer the cells are out of optimal conditions, the more likely they are to be affected by those conditions. In the incubator or in the hood, pericellular oxygen levels are completely protocol-dependent. The more media and other liquid components are mixed with HEPA-filtered room air in the BSC, the more they equilibrate with room air oxygen, which is supraphysiologic.

# I Have to Handle My Cells in a Standard Room Air BSC. What Can I Do To Get the Best Results in Suboptimal Cell Handling Conditions?

## Work Fast and Cold for HIF Analysis

To help slow HIF degradation during cell handling if you must handle your cells in room air, pre-chill tubes, centrifuges, and other equipment. Work with ice-cold PBS or other solutions for washing cells. Before you start, make sure that all of your supplies are at hand for the entire procedure. You will get the best results if you work quickly and efficiently without interruptions until your cell, protein, or RNA preparations are either used in analysis or placed in long-term storage.

### Summary:

**HIF proteins are transcription factors that are regulated at the protein level and are modulated within minutes of oxygen changes.**

**Plan and execute experiments to minimize pericellular oxygen changes.**

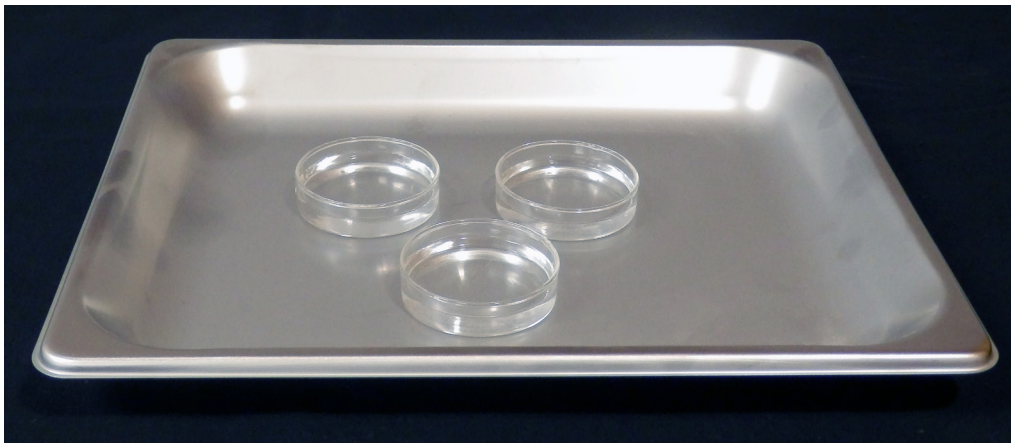
**Pre-equilibrate media and other solutions that contact cells.**

**If appropriate, pre-equilibrate tubing sets or other closed containers that contact cells.**

**Pre-chill all solutions, tubes, centrifuges, and other equipment to slow HIF degradation for protein analysis.**

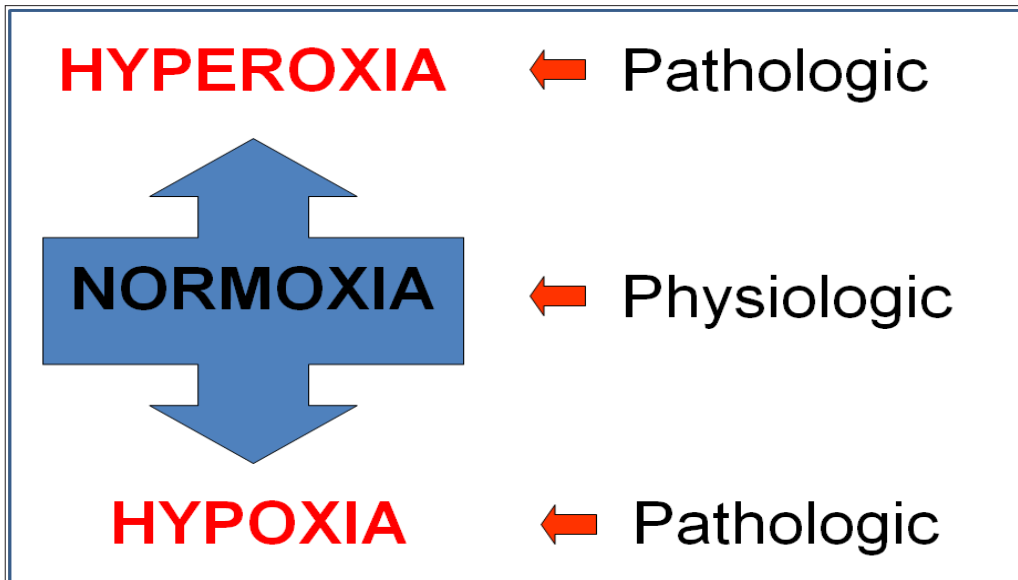
## How Much Water Should I Put in the Chamber?

We recommend starting with **3 or 4 100mm cell culture dishes** filled with sterile, distilled water placed in the water pan in the bottom of a C-chamber. Add more dishes if you observe too much evaporation from your cultures. Do not fill the pan directly with water in the bottom of the chamber as is a common practice with incubators. Too high a humidity for extended periods of time can disrupt equipment function.



Petri dishes placed in water pan. Set in bottom of C-chamber for humidity control.

## Terminology and Goldilocks: Is it “Hypoxia” or “Normoxia?”



Tissue oxygen is lower than atmospheric air, which is generally accepted to be 20.8%. Tissues oxygen levels vary widely in vivo, but tissues such as bone marrow, fetal tissues, and cartilage are profoundly low in oxygen, normally. What can you call pathologically low oxygen then? Terminology in oxygen research can be quite confusing when it is all relative and has multiple frames of reference in active use.

Room air oxygen is often called “normoxic”, but there is nothing “normal” about room air oxygen in vivo.

“Normoxic” is used to describe normal tissue oxygen levels, which vary widely. This makes room air “hyperoxic”.

“Hypoxic” is also often used to describe tissue oxygen as less than room air oxygen levels, but this is the “normal” state of most in vivo tissues.

“Hypoxic” is also used to describe tissues with less oxygen than they need. This is a pathologic state, not normal at all.

“Hyperoxic” is an oxygen level that is too high, but higher than what? Room air or “normoxic” tissue oxygen?

These relative terms don’t make any sense biologically when they are framed with room air as the reference point and they come into direct conflict when trying to compare tissue types that have different physiologic origins. For example:

Is 5% oxygen low (hypoxic), high (hyperoxic), or just right (normoxic)?

For upper airway epithelium, 5% oxygen is hypoxic.

For chondrocytes from cartilage, 5% oxygen is hyperoxic.

For venous blood, 5% oxygen is normoxic, but venous blood returned to the lungs rapidly increases in oxygen to 10% or more.

Newer terms, like physioxia and physioxic, are an improvement, in that they place a clear emphasis on the use of an appropriate physiologic frame of reference for in vivo oxygen terminology. However, these are still relative terms.

**Until a better terminology is in widespread use, avoid using relative terms and use specific oxygen levels when reporting your results for your model or cell type.**



## What About Oxygen Levels Outside the Chamber in the Incubator?



If you have cells in the incubator but outside of the C-chamber, the chamber may affect oxygen levels for those cells outside. Small amounts of nitrogen or other gases infused into the C-chamber may escape, reducing the partial pressure of oxygen and other gases outside of the chamber.

Pressurizing and decompressing cells is an old method for lysing cells. So that the equipment will not pressurize cell cultures when it infuses gas, our chambers are designed with two means of equalizing air pressure. One is the soft-seal magnetic door seal. This allows the door to “burp”, opening briefly and resealing, if chamber pressure rises above ambient air pressure.

The other mechanism is on some C-chamber models but not others; an extra hose barb on the back that releases gas pressure. Check with your sales or service representative if you are not sure if your apparatus has one. If your C-chamber has an extra hose barb on it, exhaust gas can be vented outside the incubator by placing tubing on the hose barb and running the tubing out to the room. This will minimize any gas exhaust to the incubator interior.

However, if you need to control oxygen levels outside of the C-chamber with the most accurate means, use a C-chamber and controller for each protocol or gas level that you require. With a C-chamber for each condition needed, you have the best control of the incubator atmosphere for all of your cells.



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