

# Inexpensive low-oxygen incubators

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**Although the evidence is overwhelming that ambient oxygen is at least somewhat damaging to most normal cells in culture, the expense and effort involved has resulted in few laboratories growing their cells under physiological oxygen conditions. We here describe how to produce, from commercially available plastic wide-mouth jars, very simple gas-tight containers that can be flushed with prepared gas mixtures to produce low-oxygen environments for standard cell culture. This permits any laboratory to easily try the effects of physiological oxygen on their system without the need for dedicated incubators and substantial monetary investments.**

## INTRODUCTION

Oxygen tension in most mammalian tissues range from 1% to 6%<sup>1</sup>, much lower than the approximately 21% oxygen present at sea level. As a consequence, cells being cultured under normal laboratory conditions are actually being exposed to a physiologically hyperoxic environment. It was shown almost 30 years ago that growing normal human diploid cells in 2% oxygen significantly extended their lifespan<sup>2</sup>, and since then a multitude of papers has confirmed the deleterious effects of ambient oxygen. Mouse cells are extremely sensitive to ambient oxygen, and the DNA damage that occurs under routine culture conditions is sufficient to induce growth arrest after 10–15 doublings (or many fewer if knockout mice deficient in certain DNA repair factors are used). Although mouse cells can ‘spontaneously’ inactivate the ARF/p53 pathway, resume cell division and become ‘immortal’, normal mouse cells appear to be intrinsically immortal<sup>3,4</sup> and can divide reasonably indefinitely without undergoing ‘senescence’ if grown under physiologic oxygen conditions<sup>5</sup>. Similarly, even after expressing telomerase, some human cells fail to immortalize unless grown in low-oxygen environments<sup>6</sup>, demonstrating that the growth arrest of these cells in atmospheric oxygen was not due to telomere-based replicative senescence.

Given the increasing efforts being devoted to culturing a variety of normal cell types and stem cells, it seems very ill-advised to grow them under conditions known to produce DNA damage. A number of different companies now manufacture tri-gas incubators that allow investigators to adjust the levels of carbon dioxide, oxygen and nitrogen in order to obtain the desired gas mixture, or oxygen controllers that can be used to convert a normal incubator plus a nitrogen source to a controlled oxygen. However, there are a variety of disincentives to the use of these devices. Not only is the cost of the actual incubator expensive, but one also needs to provide a continuous source of nitrogen gas, usually from the evaporation phase of a liquid nitrogen tank. This then requires not only space to put liquid nitrogen tank, but also the ongoing expense of having to replace it every few weeks. Most laboratories using these dedicated incubators also run them at 5% oxygen in order to reduce the large nitrogen consumption required to reduce the oxygen to lower levels. Although 5% oxygen provides a very significant improve-

ment over 21% oxygen, it is at the upper range of tissue tensions. The initial and ongoing expenses produce such a high threshold for the use of such incubators that most laboratories, even those aware of the potential importance of low oxygen, choose to simply ignore it and culture cells in ambient oxygen as they have done in the past.

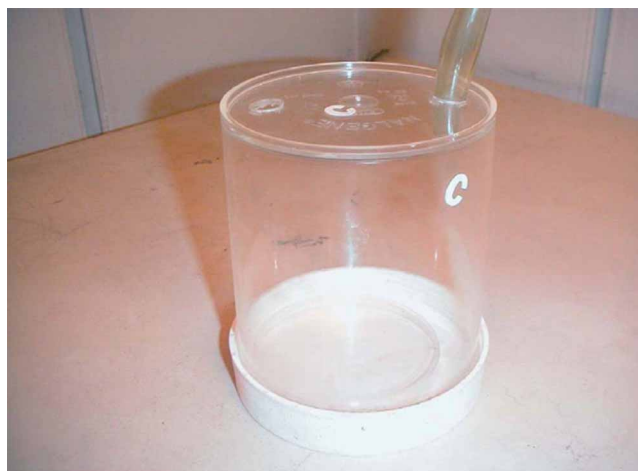
Modular incubators that can be flushed with different gas mixtures are also available (for example, from Billups-Rothenberg). Our experience with these is that after a relatively short period of time they develop leaks and need to be re-gassed several times per week. In addition, they are sufficiently large that, functionally, one puts a variety of cell types in a single modular incubator. In order to look at any particular cell type, one then needs to open the entire unit, thus requiring gas re-equilibration for all of the different cell types. Although the equilibration of the gas phase within a 10-cm dish in such an incubator is 90% complete within 15 min, it takes more than 3 h for the liquid medium to reach 90% equilibration<sup>7</sup>. As a consequence, there is a conflict between examining or manipulating one cell type and maintaining other cell types undisturbed in the same modular incubator.

We have devised a simple, convenient and inexpensive alternative to dedicated low-oxygen incubators that permits a flexible approach and can easily be tried by the average laboratory.



**Figure 1** | Low-oxygen chambers in use. Low-oxygen chambers containing flasks and dishes in a normal laboratory incubator can be mixed with dishes cultured in atmospheric oxygen.

**Figure 2** | Chamber construction. Chamber illustrating the two half-inch holes drilled in the top, the 10-cm dish lid placed in the bottom to give a flat surface, and the bubble tubing cut to fit into one of the holes that connects to the gas mixture. After gassing, the holes are plugged with silicon rubber stoppers.



Individual modular incubators are constructed by inverting 1-liter wide-mouth plastic jars and drilling holes in what used to be the jar bottom but now becomes the top. The holes are plugged with silicone stoppers. Flushing these incubators for 2 min with a pre-made gas mixture of 2% oxygen, 5% CO<sub>2</sub> and 93% nitrogen suffices to produce the desired low-oxygen environment. Each modular incubator is sufficiently inexpensive so that dozens can be made, and laboratory CO<sub>2</sub> incubators can be filled with whatever mixture is desired of cells growing outside of containers in ambient oxygen or within containers in controlled oxygen environments, thus avoiding the need for dedicated incubators. The modular incubators are sufficiently small that each is used for a maximum of four 10-cm dishes, so that individual experiments or cell types can be placed in different incubators, avoiding the problems of commercially available modular incubators in which

gas re-equilibration of all of the experiments within the much larger incubator is required each time it is opened and can easily be tried by the average laboratory (**Figs. 1 and 2**).

**MATERIALS**

**REAGENTS**

• Commercially prepared gas tank containing desired gas mixture (for example, 2% oxygen, 5% CO<sub>2</sub> and 93% nitrogen), fitted with appropriate regulator and a gauge sensitive enough to lower the delivery pressure to 2 p.s.i. (**Fig. 3**; for example, VWR cat no. 55850-388). ▲ **CRITICAL** Most laboratory regulators have gauges that are inaccurate at low pressures, so it is convenient to either purchase a medical oxygen regulator that has a low-pressure component or cobble together something from a local hardware store that provides the ability to deliver 2 p.s.i. of pressure. This is a somewhat arbitrary value that we chose for reproducibility, where

2 min of continuous flow was about 1 min in excess of that needed to get the oxygen tension inside the chambers down to ~2%.

**EQUIPMENT**

- Silicone vacuum grease
- Nalgene 2117 Straight-Side Wide-Mouth Jars, polymethylpentene with white polypropylene screw-top lids, autoclavable, 1,000 ml (cat. no. 2117-1000)
- Size 15D silicone rubber stoppers
- Bubble tubing: for example, MG Scientific T618-1: ID 1/8 inch, OD 1/4 inch, bubble ID 1/2 inch

**PROCEDURE**

- 1| **Figure 1** illustrates the modular incubator setup. First drill two half-inch holes into the clear bottoms of Nalgene 1,000-ml Straight-Side Wide-Mouth Jars (**Fig. 2**). Although this can be done by a bioengineering department, adequate holes are produced using a home drill press and a flat 1/2-inch wood drill bit.
- 2| Invert the jar so that the white plastic lid becomes the bottom of the incubator and the holes are at the top. Plug the holes with size 15D silicone rubber stoppers.
- 3| The lid has a small bump in its center that prevents dishes from lying flat on its surface. Form a flat surface by placing the lid from a 10-cm plastic petri dish on the white lid.

- 4| Coat the threads of the jar with silicone vacuum grease so that it closes smoothly and forms a gas-tight seal.

- 5| Bubble tubing provides a very convenient means of connecting the tank to the chambers. Cut one of the expanded sections before it tapers to the small diameter, providing the tubing with a good, snug fit into one of the 1/2-inch holes in order to flush the chambers.



**Figure 3** | Low-pressure delivery system. Although a regulator with a second stage capable of delivering 2 p.s.i. can be purchased, one can cobble together a low-pressure gauge with materials available from a local hardware store. We interposed a shutoff valve (with the red handle) so that, once everything is set up, all one needs to do is turn the shutoff valve to on for 2 min to flush the chambers.

6| Connect to a tank containing a special three-gas mix consisting of 2% oxygen, 5% CO<sub>2</sub> and 93% nitrogen.

7| Chambers must be re-gassed each time they are opened to observe or feed the cells. There is no need to re-gas unopened chambers (for example, if cloning cells, they can be left for several weeks without re-gassing).

**▲ CRITICAL STEP** An important consideration in the use of these chambers is to avoid adding too many dishes. T25 flasks contain 75 ml of gas space and a 10-cm dish contains 100 ml of gas space. The 1-liter jar actually contains 1.2 liters of gas space. A stack of four 10-cm dishes thus contains one-third the total gas space, so that even if all of the exterior volume initially contains 2% oxygen, after it equilibrates the oxygen will be  $[(0.4 \text{ liter} \times 21\%) + (0.8 \text{ liter} \times 2\%)]/1.2 \text{ liter} = 8\%$  oxygen final. Thus you should not put in too many dishes, or else you should re-gas the chamber after 1 h to drive the oxygen lower if you really need it to be very low (tissue O<sub>2</sub> is 1–6%).

## ? TROUBLESHOOTING

We have not found these chambers to leak. Chamber integrity and lack of leaks is easily tested by placing a dish with tissue culture medium that contains phenol red in the low-oxygen chamber; gas the chamber, and then simply place it on a bench for a few weeks. If the chamber does not leak, the 5% CO<sub>2</sub> will be retained and the medium will not become alkaline and will maintain its orange-red color for many weeks. Polystyrene is equally impermeable to both CO and O<sub>2</sub>, so this test confirms the ability of the chambers to maintain gas concentrations.

The effect of low oxygen can easily be verified by comparing clone sizes at 20% versus 2% oxygen. Typically the number of clones will be approximately the same, but after 2–3 weeks of growth the low-oxygen colonies will be 2–3 times larger than those in atmospheric oxygen. This is particularly true for normal diploid cells, whereas the effect on tumor cells is often less.

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