Changes to be made by the Author(s):  
  
1. Please take this opportunity to thoroughly proofread the manuscript to ensure that there are no spelling or grammar issues. The JoVE editor will not copy-edit your manuscript and any errors in the submitted revision may be present in the published version.  
  
2. The abstract in your editorial manager account and the Long Abstract in your manuscript do not match. Please ensure that after revising the manuscript, the abstract in your editorial manager file is updated to match the Long Abstract in the manuscript as well.

**Correction made**  
3. Please highlight 2.75 pages or less of the Protocol (including headings and spacing) that identifies the essential steps of the protocol for the video, i.e. the steps that should be visualized to tell the most cohesive story of the Protocol. The highlighted steps should form a cohesive narrative with a logical flow from one highlighted step to the next. Remember that non-highlighted Protocol steps will remain in the manuscript, and therefore will still be available to the reader. Section 2 (the calibration of the pH sensor) could be excluded from highlighting and filming to meet this length restriction.

**2.75 pages of protocol text highlighted**   
  
4. Please include scale bars for applicable images and define them in the Figure Legends.

**Scale bar added to figure 4 to indicate the size of micro-carriers, and text edited in figure legend to indicate such..**   
  
5. Please include the “doi” in the citations according to the following format: [Lastname, F.I., LastName, F.I., LastName, F.I. Article Title. *Source*. **Volume** (Issue), FirstPage – LastPage, doi:DOI, (YEAR).]

**Correction made**

6. What instrument is being powered up in step 1.1?

**Changed step 1.1 text to read: “Power up Bioreactor”**  
Reviewers' comments:  
  
Reviewer #1:   
This manuscript describes a simple bioreactor system that allows real time assessment of temperature, pH and DO in culture and can be potentially useful in industrial setting. This bioreactor system may be useful for laboratory research as well as commercial application for development of biotherapeutics. This paper contains sufficient background and clear rationale for developing this bioreactor system.  
  
Because this bioreactor system can monitor pH, DO and temperature overtime during culture, it will be very informative showing figures with pH, DO and temperature changes during culture.   
  
In figure 1, please define PBS.

**Text was added to Figure 1 to define PBS.**  
  
  
Reviewer #2:   
*Manuscript Summary:*   
The manuscript "Cultivation of Mammalian Cells using a Single-Use Pneumatic Bioreactor System" by Cummings et al. describes a simple yet useful application for growing anchorage-dependent cells. The rationale and the instruction on how to use this system, including setting up, calibrating, inoculating, running, and sampling are very clear and detailed.  
  
*Major Concerns:*  
\* Please provide a more detailed comparison to other currently available bioreactor types for growing anchorage-dependent cells on microcarriers.

**The following sentence was added to text lines 79-81.**

**“Currently, the types of bioreactors where adherent cells are grown on micro-carriers include spinner flasks and stirred tank systems, which use axial impellers to maintain suspension of the cell coated micro-carriers.”**

Also, please comment on different types of microcarriers that can be used in this bioreactor system.

**The following sentences were added to text lines 74-76.**

**“Depending on the attachment characteristics of the cell, several different types of micro-carriers are commercially available, such as, dextran, peptide, or collagen coated.”**

\* Please comment on the maximum cell density that the current pneumatic air wheel setup can support without oxygen transfer limitation.

**The following was added to line 320: “with cell densities reaching over 30 million cells/mL.”**

\* Please provide figures at a better resolution since the morphology of these cells on microcarriers is a critical indicator. All Excel graphs and tables are also at very low resolution and quality.

**Files were submitted as per JoVE manuscript instructions, thus figures and graphs with higher resolution will not be provided.**

\* Please comment on any difficulty using this bioreactor system for long-term culturing of the cells as compared to harvesting.

**Not part of protocol**  
*Minor Concerns:*  
Typos and formatting issues are found throughout the manuscript. Several examples are listed below:

**All corrections made.**  
  
\* Line 36: "system, for" ⎝system for  
  
\* Line 80: "sheer stress" ⎝ shear stress  
  
\* Line 80: "nutrient & metabolite" ⎝ nutrient and metabolite  
  
\* Line 207: "full polarized" ⎝ fully polarized  
  
\* Line 227: "source,by" ⎝ source by  
  
\* Line 245: "infect th" ⎝ infect the  
  
\* Line 313: "It extremely well suited" ⎝ It is extremely well suited  
  
\* Line 346: there should not be a large space between "1." and "Freshney" as compared to other references

Reviewer #3:   
This work presents an experimental procedure that may be useful and interesting. However, many points are not clear, and need to be explained more carefully.   
  
The bioreactor system itself is not described at all: how is it constructed? How does it work?

**The assembly and operation of the system will be demonstrated in the video, so no additional text will be added to address this comment.**

Which gas is used (line 93)?

**Depending on the cell’s growth characteristics and medium buffering parameters, different gas mixtures would be required, therefore, no additional text will be added to address this comment.**

Figure 2 has no interest in its present form: it brings no information.

**Additional text added to line 101 to indicate the comparison to stirred tank bioreactor systems:**

**“In contrast to stirred tank bioreactors, the vertical impeller of this single use reactor is turned by a stream of gas bubbles within the vessel, which allows for gentle and uniform medium mixing (Figure 2).”**

Where does the fluid go in? out? Where are the cells? Is something moving? Where is the "air-wheel"? why is it "single-use"?

**The assembly and operation of the system will be demonstrated in the video, so no additional text will be added to address this comment.**

Fig.1 seems to indicate that the bioreactor working volume may be up to 2000 or 2500 liters: is it an error?

**Additional text was added (line 100-101) to clarify that larger Air-Wheel bioreactor systems can accommodate 2000 or 2500 liter volumes.**

**“As compared to the stirred tank reactor, the pneumatic reactor has low wall shear stress even with high volume Air-wheel bioreactor systems (Figure 1).”**  
Line 96 and 145: what does "vessel" mean in this context?

**Not found**   
  
The procedure: why infect the cells with the addition of a virus inoculum? What conclusions do the authors expect from this part of the procedure? This is not clearly explained.

**No additional changes were made based on this comment because we stated in lines 94-96 the purpose of using a virus inoculum.**

**“In this video, using the single use pneumatic (Air-Wheel) bioreactor system, we demonstrate the culture of human lung carcinoma cells (A549) cells on micro-carriers for production of an oncolytic adenovirus.”**   
  
In several parts of the paper, the authors give some affirmations without any demonstration:  
  
- in the abstract: "this bioreactor system eliminates many of the challenges associated with currently available systems by minimizing hydrodynamic shear and nutrient gradient formation, and allowing for uniform culture medium aeration" …" This bioreactor system also has tremendous potential for scale-up of adherent and suspension mammalian cells for production of a variety therapeutic proteins, monoclonal antibodies, stem cells, biosimilars, and vaccines".

**The video portion of the submission will address these comments.**   
  
- Lines 65-67 in the Introduction: "The pneumatic bioreactor demonstrated in this video has proven suitable for the production of such mesenchymal stem cells for this application"

**The statement in lines 65-67 was referenced with: “(Serra et al., personal communication, 2013)”, so no additional clarification will be added.**

- Lines 278-279: "In previous experiments, we have found that monitoring and adjusting gas flow is critical to maximizing cell growth".   
  
**The following was added to the end of the sentence: “(unpublished data).”**