Introduction

This Application Report is one in a series documenting simple protocols for culturing cells in a BioFlo® 110 benchtop fermentor/bioreactor system. The modular BioFlo 110 is offered with an extensive choice of vessels, impellers and control modules to enable growth of a wide variety of cultures, including mammalian, insect, plant, yeast and bacterial cells. Complete kits are also available. This report concentrates on achieving high-density hybridoma cell cultures using NBS’ Advanced Cell Culture Kit.

The Bioreactor

Vessel

This BioFlo 110 Advanced Cell Culture Kit was equipped with a magnetic drive and water-jacketed 3.0 L vessel with a nominal working volume of 2.25 L. All BioFlo 110 Advanced Cell Culture Kits are configured with six control modules, a complete vessel assembly including a pitched blade agitation impeller, foam/level sensors, as well as dissolved oxygen and pH probes.

For this application, researchers in NBS’ cell culture laboratory used a standard 3.0L BioFlo 110 Advanced Cell Culture Kit with a magnetic drive and a water-jacketed vessel (NBS Catalog # M1273-1212). A suspension cell spin filter, with a 12-14 micron screen (NBS Catalog # M1273-3202) was used for perfusion in order to grow the hybridoma cells in a continuous mode. Additionally, BioCommand® Plus supervisory software (NBS Catalog # M1291-0000) was used to control the feeding schedule and collect data; and a NucleoCounter™ (NBS Catalog # M1293-0000) was used to count viable cells.

For this application we substituted an optional NBS spin filter impeller for the standard pitched blade impeller. The spin filter impeller is very useful for a continuous perfusion process because it is a simple device that lends itself to media exchange and can be easily scaled up. With most commonly available spin filters, screens rapidly become clogged when cells reach high cell density during perfusion. NBS, however, has designed spin filters with three different screen sizes that are suitable for most cell types. Feel free to contact NBS for screen size suggestions.
Control System
Listed below are six control modules included in an Advanced Cell Culture Kit, which were also used for this application:

<table>
<thead>
<tr>
<th>Control Module</th>
<th>Function</th>
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<tbody>
<tr>
<td>Primary Control Unit (PCU)</td>
<td>User interface for up to four vessels.</td>
</tr>
<tr>
<td>Power Controller</td>
<td>Agitation &amp; temperature control; power outlets for five peristaltic pumps.</td>
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<tr>
<td>Four-Pump Module</td>
<td>Adding and removing liquids.</td>
</tr>
<tr>
<td>pH/DO Controller</td>
<td>Maintaining dissolved oxygen and pH at setpoints.</td>
</tr>
<tr>
<td>Gas Mix Controller</td>
<td>Four valves allow automatic rationing of gasses for gentle regulation of pH and DO in cell culture, and for oxygen enhancement in fermentation. Works in concert with the pH/DO Controller.</td>
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<tr>
<td>Foam/Level Controller</td>
<td>Accepts up to 3 conduction probes for automatic control of antifoam addition and media addition/removal in a vessel. Works together with the four-pump module.</td>
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Materials and Methods
Overview
• Sterilize the vessel with phosphate buffer solution (PBS) for 60 minutes.
• Remove the PBS from the vessel.
• Add 1.5 L of media into the vessel.
• Key in control setpoints.
• Inoculate 500 ml of inoculum suspension to provide a starting cell count of $2.44 \times 10^5$ cells/ml.
• Use BioCommand software to automatically calculate and control glucose addition throughout the run.

Medium
RPM1640 medium from Invitrogen, supplemented with 5% FBS from Hyclone and 0.1% Pluronic from Gibco.

Inoculum
Hybridoma cell line 3G5 from ATCC
Inoculum was cultivated in an NBS Shaker (Innova® 2000), which was inside a CO2 Incubator (Innova CO-170 NBS Catalog # M1305-0000)

PCU: Select the Application
On the initial set-up screen, the USE... selector button will allow you to change the default Fermentation application to Cell Culture application. For further details refer to the Operation Section in the BioFlo 110 manuals.

Control Setpoints
Setpoints were keyed into the controller prior to inoculation. The vessel was allowed to equilibrate prior to inoculation. The DO loop’s Current Value remained higher than setpoint until the culture was introduced.

- Temperature: 37°C
- pH: 7.2
- DO: 50%
- Agitation: 70-130 rpm
- O2 (Gases): 4 Gas

PH Control
The pH probe was calibrated prior to the autoclave cycle (refer to BioFlo 110 Instruction Manual). CO2 gas and a liquid base were used to maintain the pH setpoint. pH control parameters were:

- Base: Sodium bicarbonate, 8% solution
- Pump: Pump 4 of the 4-Pump Module
- Transfer tubing: Narrow bore silicone tubing, as supplied (1/16" ID & 3/16" OD - NBS Catalog # P0740-2396)
- Vessel inlet: Triport adapter in the vessel headplate

DO Control
The DO probe was calibrated after the autoclave cycle (refer to BioFlo 110 Instruction Manual). DO control was set to Auto mode, which automatically defaults to 4-Gas Mode after the Gas Control (O2 loop) selection is made. We also changed the P and I value of the DO loop to meet the culture demands, as shown below.

PCU Setup: (DO loop)
- DO Control Selections: Auto (4 Gas mode)
- PID values: $P = 2.00$ and $I = 0.67$
Gas Control (O₂ loop)
The control was set to 4-Gas mode, to automatically maintain DO and pH setpoints, using 3 gases (Air, O₂, N₂) to control the DO setpoint and CO₂ to control pH setpoint.

PCU Setup: (O₂ loop)
• O₂ Control Selections: 4 Gas mode

Continuous Feed
All pumps were calibrated using the standard tubing supplied to keep track of the liquid quantities entering and exiting the vessel. Samples were taken several times a day for off-line measurement of glucose and cell density.

PCU setup: (Pump 1 & Pump 2 loop)
• Pump 1 . . . . . . . . . . plugged into “Pump A” Power Controller outlet
• Pump 2 . . . . . . . . . . plugged into “Pump B” Power Controller outlet
• Pump 1 (Nutrient) . . Vessel inlet - Triport adapter in the vessel headplate
• Pump 2 (Harvest) . . . . Vessel outlet - 12mm port using the dip tube
• Pump A Selection . . Manual mode (automatically adds liquid to the vessel with the Bio-Command control program)
• Pump B Selection . . Wet On (removes liquid from the vessel using the level probe as a trigger to turn the pump on)

Growth Conditions
Cells were inoculated into the bioreactor from T-flask cultures with a live cell concentration of 2.44 x 10⁵ cells/ml.
The rate of the perfusion was changed, based on the glucose concentration in the culture, to maintain glucose level at about 1 g/L. See figure 4 for details.

Control Program
For this study, we used NBS’ BioCommand Plus software to control nutrient addition.
For the nutrient control program, we calculated the approximate time frame profile to automatically add fresh media using pump 1.

Procedure
During the first 48 hours, the culture was run in a batch mode. Media perfusion was started on day 2. Samples were removed at regular intervals during the culture for determination of live and dead cells using the NBS NucleoCounter. To verify the accuracy of the NucleoCounter reading, we also used Trypan blue exclusion method (Sigma T8154) as well as a 12mm biomass probe, which was inserted in the vessel and connected to a biomass monitor. The biomass monitor was connected to our BioCommand software to provide an instant on-line cell concentration. See Appendix

A linear correlation of 0.993 was observed between viable cell concentration (X) and the capacitance (pf/cm) range as seen in Figure 3. See Appendix section (2) for details.

Figure 3. Relationship between viable cell densities (determined by NucleoCounter & Trypan Blue Method) and capacitance (determined by biomass probe).

Figure 4. Hybridoma cell 3G5 cells grown in a 3L BioFlo110 with a Spin Filter impeller. S1: initial batch culture stage; S2: medium perfusion culture stage; and S3: batch culture stage. T1, T2 and T3 indicate that we increased the perfusion rate, which rapidly increased cell growth.
In most perfusion processes, cell viability typically decreases over time; but in this experiment viability improved during the process and was maintained at a very high level (95%) in the late perfusion stage due to the superior control of BioFlo 110. A high cell density of $1.27 \times 10^7$ cells/ml was achieved after 240 hours of culture.

**APPENDIX**

(1) The Biomass monitor and sensor used in this experiment are not available in the marketplace as they are still undergoing test runs. When this equipment is available, we will make an announcement in the Culture Club, BioFlo 110 newsletter.

The Biomass sensor uses the principle that under the effect of an electric field between two electrodes, live cells (which have intact plasma membranes) polarize and behave as small condensers. The value of the resulting capacitance is measured by the second pair of electrodes and correlates exactly to the concentration of biomass in the culture environment.

In the first stage of culture (0-30 hrs), the pH and DO were controlled by surface aeration at a rate of 200 ml/min. After 30 hours of culture, the capacitance value began to decrease to 0.42 [pf/cm] at 45 hour, as shown in Figure 3. At this time perfusion was started at 1 liter per day, which rapidly increased the value to 0.73 [pf/cm] after 90 min. This phenomenon occurred three times (and is indicated in Figure 4 as T1, T2 and T3) during the S1 and S2 stages. We believe that the plasma membrane polarization for each cell changed, depending on the cell culture conditions.

(2) The equation below was used to calculate the linear correlation between viable cell densities (determined by NucleoCounter, Trypan Blue) and capacitance (determined by biomass probe).

$$X \ [10^6 \text{ cells/ml}] = 1.386 \times C \ [\text{pf/cm}]$$

Here, $X$ is viable cell density [$10^6 \text{ cells/ml}$] and $C$ is capacitance [pf/cm].

**Figure 5.** The viable cell densities (determined by NucleoCounter & Trypan Blue).