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High-Density Vero Cell Perfusion Culture in BioBLU® 5p Single-Use Vessels

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Abstract

Vero cells are anchorage-dependent cells that are widely used as a platform for viral vaccine production. In stirred-tank bioreactors, they are ordinarily grown on microcarriers. Fibra-Cel® disks are a promising alternative attachment matrix with a high surface-to-volume ratio. They provide a three-dimensional environment that protects cells from damaging shear forces, helping to achieve high cell densities.

In this study, we cultivated Vero cells in Eppendorf BioBLU 5p Single-Use Vessels pre-packed with Fibra-Cel. The process was controlled with a BioFlo® 320 bioprocess control station. We cultivated the cells in perfusion mode, which ensures a consistent supply of nutrients and the removal of toxic byproducts.

We achieved the very high Vero cell density of approximately 43 million cells per mL, demonstrating great potential for Vero-cell-based vaccine production using Fibra-Cel packed-bed vessels.

Introduction

Viral diseases, including but not limited to rabies, rotavirus, and influenza, are worldwide challenges faced by the international biomedical community. The WHO notes that rabies causes tens of thousands of deaths every year [1]. And the influenza virus was responsible for millions of deaths worldwide over the course of the last century.

Strong demand for vaccines for viral diseases required the development of more productive manufacturing techniques, including those based on scalable bioreactor cell culture systems. The Vero cell line has become one of the most widely used cell lines for viral vaccine production [2].

Stirred-tank bioreactors filled with microcarriers and equipped with the Eppendorf low-shear cell-lift impeller have been used for rabies vaccine production in Vero cells [3]. Packed-bed basket impellers combined with Fibra-Cel disks as growth support are a promising alternative culture system

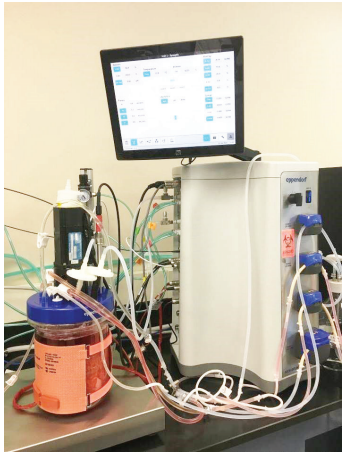

Fig. 1:
Vero cell perfusion culture setup. A BioBLU 5p Single-Use Vessel was controlled with a BioFlo 320 bioprocess control station.

that provides a three-dimensional, low-shear environment. This method is used by vaccine manufacturers, although the results have not yet been published.

In this study, we cultivated Vero cells in Eppendorf BioBLU 5p Single-Use Vessels pre-packed with Fibra-Cel disks. We analyzed the glucose consumption of the culture as a measure of cell growth. In addition, we introduced a method for directly measuring cell numbers at the end of the cell culture run by counting nuclei, and established a correlation between glucose consumption rate and Vero cell densities.

Material and Methods

Cell line and medium

We used adherent Vero cells (ATCC®, CCL-81™) for this study. We cultivated the cells in Dulbecco's modified eagle medium (DMEM, Thermo Fisher Scientific®, USA), supplemented with 1x Antibiotic-Antimycotic (Thermo Fisher Scientific, USA) and 1 % (v/v) heat-inactivated fetal bovine serum.

Bioreactor and cell culture growth surface

We used a BioBLU 5p Single-Use Vessel with a built-in basket impeller pre-packed with Fibra-Cel disks (Figure 1). Each BioBLU 5p vessel contains 150 g of Fibra-Cel disks. The growth surface of one gram of Fibra-Cel disks is 1,200 cm^2. One BioBLU 5p thus provides a growth surface of 180,000 cm^2, which is equivalent to about 212 roller bottles, or 29 10-layer, stacked-plate, cell culture vessels (Table 1).

Table 1: Comparison of growth surfaces of different cell culture vessels

Vessel	Total growth surface (cm^2)	Growth surface equivalent to (number of BioBLU 5p vessels)
BioBLU 5p Single-Use Vessel	180,000	1
T-25 flask	25	7,200
T-175 flask	175	1,028
Roller bottle	850	212
10-layer stacked plate	~6,300	29

Bioreactor inoculum preparation and inoculation

For the preparation of the bioreactor inoculum, we thawed one vial of Vero cells (5 x 10^6 cells) following the Vero cell protocol provided by ATCC [4]. We used the cells to inoculate a T-175 flask containing 20 mL of pre-warmed medium. We cultured the cells in a Galaxy® 170R CO_2 incubator (Eppendorf, Germany) at 37°C and 5 % CO_2, with passive humidification. On day 4, when the Vero cell culture reached full confluence, we trypsinized the cells (0.25 % Trypsin, Thermo Fisher Scientific, USA) and passaged them into three T-175 flasks each containing 25 mL of freshly made, pre-warmed medium at a density of 5 x 10^6 cells/flask. After 3 days, when the cells reached 100 % confluence again, they were further expanded to eight T-175 flasks with a seeding density of 5 x 10^6 cells/flask. When the cells reached full confluence, all were harvested, combined, and inoculated into eight HYPER*Flask*® M cell culture flasks (Corning®, USA) at a seeding density of ~1.9 x 10^7 cells/flask. We filled each flask with cell culture medium according to the manufacturer's instructions [5]. The cells were cultivated in the HYPER*Flasks* for another 5 days to full confluence and then harvested according to the manufacturer's protocol. We combined all the cells into a 1-L sterile addition bottle (Eppendorf, Germany) and used them for the inoculation of one BioBLU 5p Single-Use Vessel. The total volume of the inoculum was 530 mL, with a cell density of 3.0 x 10^6 cells/mL and a cell viability of 99 %.

Figure 2 illustrates the Vero cell expansion process.

Bioreactor control and process parameters

We used a BioBLU 5p Single-Use Vessel controlled with a BioFlo 320 bioprocess control system (Figure 1).

We cultivated the Vero cells at 37°C.

Dissolved oxygen (DO) was measured using a polarographic ISM® sensor (Mettler Toledo®, Switzerland). DO was controlled at 50 % by automatic gassing at a flow of 0.002 – 0.5 SLPM in the 3-Gas Auto mode. To reduce foaming caused by high gas flow in the late culture stage, we limited air flow to 0.002 – 0.2 SLPM and oxygen flow to 0 – 0.5 SLPM.

The BioBLU 5p Single-Use Vessel was equipped with an optical pH sensor and the pH was controlled at 7.1

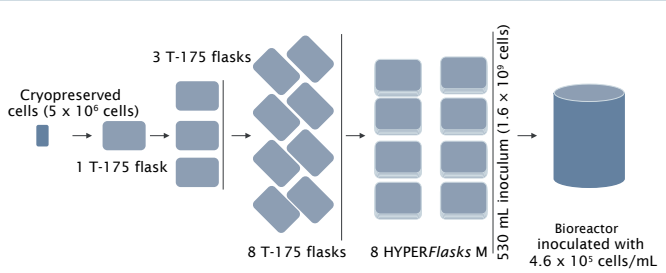

Fig. 2: Schematic of the Vero cell expansion process

(deadband = 0.1) via a cascade of CO_2 (acid) and 0.45 M sodium bicarbonate (base).

We added Antifoam C Emulsion (Sigma-Aldrich®, USA) when it was needed.

The culture parameters are summarized in Table 2

Table 2: Overview of process parameters and setpoints

Parameter	Device/setpoint
Inoculation density	4.6 x 10^5 cells/mL
Working volume	3.5 L
Sparger	Macrosparger
Gassing control	Sparging: 3-Gas Auto Gas mixing, combined flow 0.002–0.5 SLPM Air flow 0.002–0.2 SLPM; O_2 flow 0–0.5 SLPM Overlay: 3-Gas Auto gas mixing, 0.05–1 SLPM
Dissolved oxygen (DO)	50 %
Agitation	Basket impeller; 100 rpm
pH	7.1 ± 0.1 Cascade to CO_2 (acid) and 0.45 M sodium bicarbonate (base)
Temperature	Heat blanket; 37 °C

Feeding and perfusion control

We started with a perfusion rate of 0.2 vessel volumes per day (VVD), and gradually increased it to 1.5 VVD at the end of the run. We determined the perfusion rate by monitoring the level of ammonium. It was measured daily with a Cedex® Bio Analyzer (Roche Diagnostics®, Germany). The goal was to keep the ammonium concentration below 4 mM. The perfusion rates are summarized in Table 3.

We targeted a glucose concentration >3 g/L. The glucose concentration was measured with a Cedex Bio Analyzer. In addition to perfusion, based on the glucose level in the bioreactor at the end of every day, we performed extra glucose bolus feeding (200 g/L glucose stock solution) to bring the glucose level in the bioreactor close to the glucose concentration in the perfusion medium (4.7 g/L) at the beginning of the next day.

Table 3: Perfusion rates

Day	Perfusion rate (VVD)
0-2	0
3-7	0.2-0.5
8-11	0.7-1.0
12-21	1.1-1.5

The concentrations of glutamine and lactate were measured as well, using a Cedex Bio Analyzer.

The rate of glucose consumption (R) in grams per day can be calculated based on the total glucose added to the bioreactor minus the residual glucose. In addition to the glucose provided in the culture medium and perfusion medium, we also supplemented the culture with additional glucose through a bolus feed. The daily total amount of glucose added to the bioreactor is therefore equal to the amount of glucose in the vessel at the start of the day ($G_{vessel\text{-}start}$), combined with the glucose supplied through perfusion ($G_{perfusion}$), and the extra glucose added via bolus feed (G_{bolus}). By subtracting the amount of glucose remaining in the vessel at the end of the day ($G_{vessel\text{-}end}$) as well as the amount of glucose remaining in the harvested perfusate ($G_{harvest}$), we arrive at the amount of glucose consumed (g) per day (24 h). The daily glucose consumption rate (R) is represented by the following equation:

$$R = (G_{vessel\text{-}start} + G_{perfusion} + G_{bolus} - G_{vessel\text{-}end} - G_{harvest}) \text{ /day}$$

> R = Rate of glucose consumption per day (g/day)
> $G_{vessel\text{-}start}$ = Amount of glucose in the vessel at the start of the day (g)
> G_{bolus} = Amount of glucose added through bolus feed for the day (g)
> $G_{perfusion}$ = Amount of glucose added through perfusion for the day (g)
> $G_{vessel\text{-}end}$ = Amount of glucose in the vessel at the end of the day (g)
> $G_{harvest}$ = Amount of glucose in the harvest perfusate at end of the day (g)

The glucose amount in the vessel at the start and the end of the day can be calculated by multiplying the glucose concentration in the medium by the working volume. The amount of glucose added by bolus feed can be calculated based on the concentration of the glucose stock solution and the volume of the bolus feed. The amount of glucose supplied through perfusion can be calculated based on the glucose concentration in the perfusion medium and the perfusion volume. The amount of glucose remaining in the harvested perfusate can be calculated based on its volume

and the concentration of glucose in the perfusate for each day.

Crystal violet nucleus counting assay

We determined the number of cells in the BioBLU 5p bioreactor by a crystal violet nuclei counting assay (Chemglass Life Science, USA, CLS-1332-01). After the completion of the cell culture process, we cut the vessel open below the head plate and took the Fibra-Cel basket out of the vessel. The Fibra-Cel disks are accessible after removing the two soft silicone plugs located on the side-wall of the basket. We collected three samples of Fibra-Cel disks from two different locations in the basket, and transferred them to 50 mL conical tubes. Each sample contained 15 Fibra-Cel disks. We extracted the cell nuclei from the disks and stained them according to instructions supplied with the Crystal violet dye nucleus count kit, as follows: The disks with cells from each sample were soaked in 4 mL of crystal violet dye and incubated at 37°C for one hour. During the incubation, we vortexed the samples for one minute every 30 minutes. After one hour, we transferred the 4 mL crystal violet dye from each sample tube into a new tube as the first extraction of the samples. We added 3 mL of fresh crystal violet dye to each Fibra-Cel-containing tube and incubated at 37°C for the second extraction. Again, the sample tubes were vortexed for one minute every 30 minutes at the beginning, middle, and end of the one-hour incubation. The cell nuclei from the first and second sample extractions were then counted using a Vi-CELL® XR Cell Viability Analyzer (Beckman Coulter®, USA) after 10x dilution in deionized water. We combined the results. The default cell-type setting was used for cell counting on the Vi-CELL.

Results

Cell expansion in flasks

We cultivated Vero cells initially in a T-75 flask, expanded them into eight T-175 flasks, and then cultured them in eight HYPER*Flasks* with a seeding density of 1.9 x 10^7 cells/flask. After a 5-day expansion period in the HYPER*Flasks*, the Vero cells reached a density ~3.17 x 10^8 cells/flask. Microscopic images of Vero cells grown in the HYPER*Flasks* are shown in Figure 3. When the cells reach full confluence, a 1 cm^2 culture area contains approximately 1.84 x 10^5 Vero cells.

Perfusion culture of Vero cells in the BioBLU 5p Single-Use Vessel

We cultivated Vero cells in the packed-bed BioBLU 5p for 21 days.

We inoculated the culture with a 530 mL inoculum with a cell density of 3.0 x 10^6 cells/mL, and 99 % cell viability. The working volume of the bioreactor was 3.5 L, and the inoculation density was 4.6 x 10^5 cells/mL. To assess Vero cell attachment on the Fibra-Cel disks, we took a culture sample 30 minutes after inoculation. No cells were observed in suspension, indicating that the Vero cells had attached to the Fibra-Cel disks rapidly. We started the perfusion on day 3, because the glucose level became low and the concentration of the toxic byproducts lactate and ammonium increased. Our perfusion strategy was to maintain the glucose concentration between 2-4 g/L, and the ammonium level below 4 mM, and we increased the perfusion rate accordingly to maintain these target levels. The metabolic profiles of Vero cells in the bioreactor are shown in Figure 4. For the glucose line, the spikes indicated that a bolus feeding

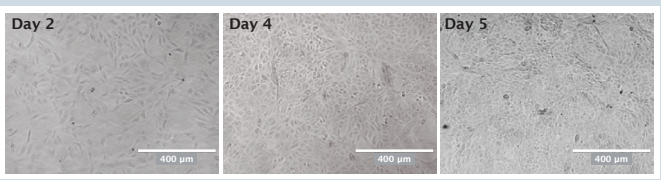

Fig. 3: Vero cells cultured in HYPERFlasks on day 2, day 4, and day 5

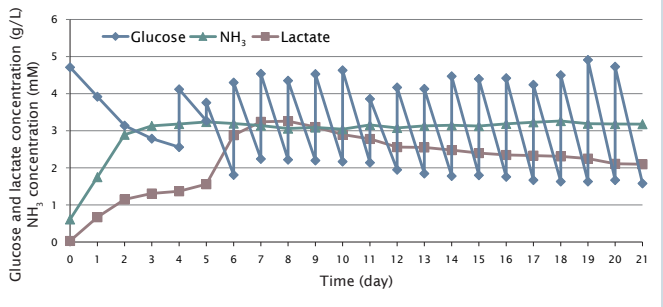

Fig. 4: Metabolic profile of Vero cell perfusion culture

with glucose was performed daily from day 4.

Because the cells adhered to the Fibra-Cel disks, we could not directly measure the cell density during the culture. As an indirect measure of cell growth we tracked the glucose consumption rate of the culture (Figure 5). We calculated the total glucose consumption of the culture and the daily glucose consumption rate. For the first four days, the daily glucose consumption rates were around 2.7 g/day, indicating that cells were in lag phase. From day 5, the glucose consumption rate started to increase to 4.4 g/day, and even doubled to 9.7 g/day on day 6, indicating that cell growth had entered the log phase. Cells were growing continuously, and the glucose consumption rate doubled again to 19.4 g/day on day 17. On the last day of the run, the glucose consumption rate was still increasing, reaching 22.4 g/day. In total, the cells consumed 273 g glucose during the 21 days.

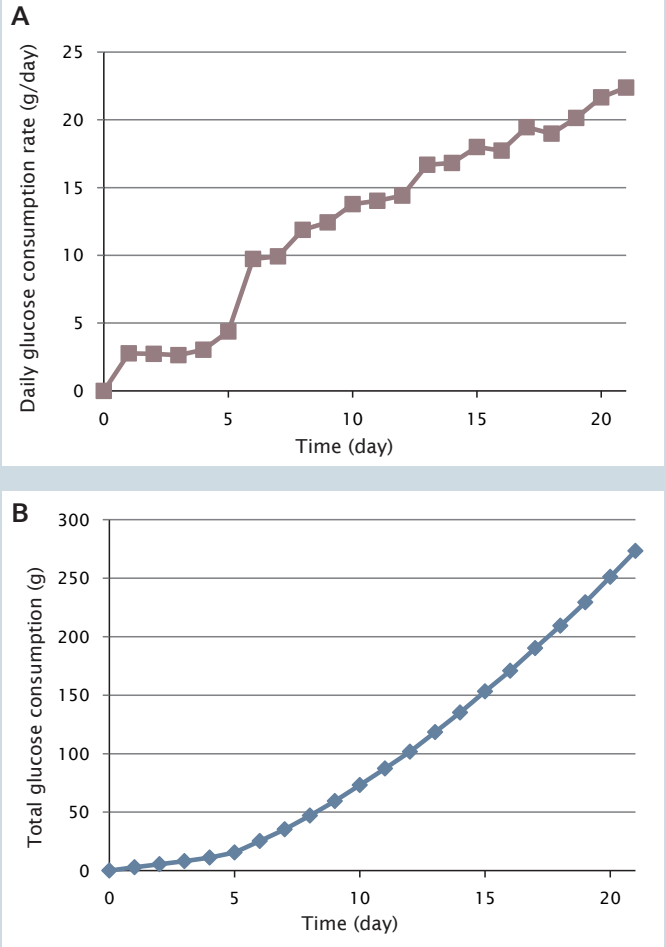

Fig. 5: A: Daily glucose consumption rate of the culture **B:** Total glucose consumption of the culture

pH control using optical pH sensing technology

The BioBLU 5p vessel features optical pH-sensing technology. We achieved precise pH control within the deadband of 7.0 to 7.2 (Figure 6). We compared the data derived from the pH sensing dot with offline data from an external pH meter and found that it was not necessary to recalibrate the optical sensor. This indicates that the pH sensing dot is compatible with the Vero culture medium.

Results of the crystal violet nucleus counting assay

After double extraction of the Vero cell nuclei from the Fibra-Cel disks, we counted the nuclei in the samples with a Vi-CELL analyzer, using the default cell type. The results are shown in Table 4. For one Fibra-Cel disk sample, the average cell count was 4.43 x 10^6 cells. From this number, we extrapolated the total number of cells in the culture.

To calculate how many pieces of Fibra-Cel disks are in each BioBLU 5p basket, we weighed 50 unused, dry Fibra-Cel disks as 0.22 g. Each BioBLU 5p basket vessel contains 150 g of Fibra-Cel disks, which means that each BioBLU 5p contains approximately 34,091 Fibra-Cel disks. One Fibra-Cel disk retrieved at the end of the culture contained 4.43 x 10^6 cells. As a result, the total number of Vero cells in the BioBLU 5p vessel at the end of the perfusion culture was calculated as 1.51 x 10^{11} cells. The final cell density at the end of the culture, based on the 3.5 L working volume, was 4.31 x10^7 cells/mL.

Based on the cell density at the end of the culture (4.31 x10^7 cells/mL) and the glucose consumption rate on

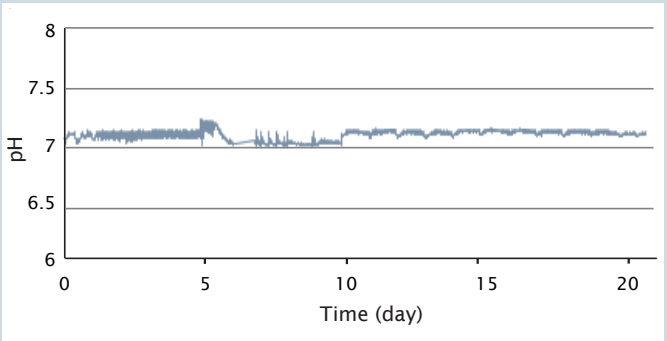

Fig. 6: Optical pH control of the Vero cell culture in BioBLU 5p Single-Use Vessels within the deadband of 7.0 to 7.2

Table 4: Nuclei counting results of samples containing 15 Fibra-Cel disks

	Sample 1	Sample 2	Sample 3
First extraction (4 mL)	$14.55 \times 10^6 \times 4 = 58.2 \times 10^6$	$13.24 \times 10^6 \times 4 = 52.96 \times 10^6$	$16.33 \times 10^6 \times 4 = 65.32 \times 10^6$
Second extraction (3 mL)	$2.04 \times 10^6 \times 3 = 6.12 \times 10^6$	$2.53 \times 10^6 \times 3 = 7.59 \times 10^6$	$3.10 \times 10^6 \times 3 = 9.30 \times 10^6$
Total cells in 15 Fibra-Cel disks	64.32×10^6	60.55×10^6	74.62×10^6
Total cells in one Fibra-Cel disk	4.29×10^6	4.04×10^6	4.97×10^6

the last day (22.37 g), we obtained the glucose-consumption-to-cell-density-conversion ratio at the end of the culture. Assuming that this conversion ratio remained relatively unchanged over the duration of the culture, we converted the daily glucose consumption rate into daily cell density. The calculated Vero cell growth curve is shown in Figure 7.

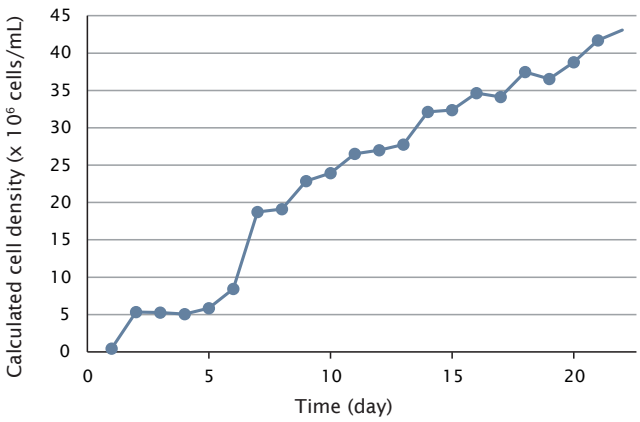

Fig. 7: Calculated Vero cell growth curve

Conclusion

We have demonstrated that the Eppendorf BioBLU 5p Single-Use Vessel pre-packed with Fibra-Cel disks is an excellent platform for high-density Vero cell culture. In the 3.5 L vessel, we achieved a high Vero cell density of approximately 43 million cells per mL, demonstrating great potential for Vero cell-based vaccine production. Since viral particles are much smaller than cells, they can be directly eluted from packed-bed cell culture vessels without removal of cells. In addition, as high Vero cell densities can be translated into high virus production yield, i.e. high titer, the Fibra-Cel packed-bed presents an ideal platform for high-titer viral vaccine production.

Acknowledgement

The nuclei counting method was suggested by Mr. Zhenggang Xie from Eppendorf China.

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Ordering information

Description	Order no.
BioFlo® 320, All configured units include the same base control station	
Base Control Station	1379963011
BioBLU® 5p Single-Use Vessel, Macrosparger	M1363-0133
Optical pH Sensor, incl. cable, for BioBLU® 5p	P0300-2372
Polarographic DO sensor, ISM®, 12/220 mm	P0720-6653
Addition/Harvest bottle kit for aerobic processes, includes a clear Pyrex® glass bottle with aseptic reservoir cap including a full lenght stainless-steel dip tube and 0.2 µm vent filter, 1 L	M1362-9901
Galaxy® 170 R, CO_2 Incubator, 170 L, Standard	CO17301001
Eppendorf Cell Culture Flasks T-75, Sterile, pyrogen-, DNase-, RNase-, human and bacterial DNA-free. Non-cytotoxic, TC treated, with filter cap, 20.0 mL, 80 flasks (16 bags × 5 flasks)	0030711122
Eppendorf Cell Culture Flasks T-175, Sterile, pyrogen-, DNase-, RNase-, human and bacterial DNA-free. Non-cytotoxic, TC treated, with filter cap, 30.0 mL, 48 flasks (12 bags × 4 flasks)	0030712129

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