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Easy Perfusion for Anchorage-dependent Cell Culture using an Eppendorf Vessel equipped with Microcarrier Spin Filter

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Abstract

Anchorage-dependent cells, such as Vero cells, are widely used as a platform for viral vaccine production. In perfusion bioprocesses it is possible to constantly add nutrients and to remove byproducts, while retaining the cells in the bioreactor. Therefore higher cell densities can be reached than in conventional batch or fed-batch processes. In this study we tested the suitability of a spin filter as cell retention device. We cultivated Vero cells on Cytodex® 3 microcarriers (10 g/L) in an Eppendorf 3 L glass vessel using a microcarrier spin filter coupled with a pitched-blade impeller. The microcarrier spin filter is a cylinder-shaped cage that spins with the impeller shaft and is covered with a large 75-micron screen designed to prevent microcarriers from being collected with the waste media during perfusion. The process was controlled with a BioFlo® 320 bioprocess control station and there were no additional devices needed for the perfusion. With the unique design of this spin filter, we easily cultivated anchorage-dependent Vero cells in perfusion mode on microcarriers and ensured a consistent supply of nutrients and the removal of toxic byproducts.

At a modest microcarrier loading density of 10 g/L, we achieved Vero cell density of 8.0×10^6 cells/mL in 9 days, sufficient for inoculation of a 40 L CelliGen® 510 packed-bed bioreactor designed for vaccine production, assuming > 50% cell recovery. We demonstrated great potential of using microcarrier spin filter for attachment cell based vaccine production scale-up.

Introduction

Fig. 1: Spin filter with marine impeller (left) and pitched-blade impeller (right).

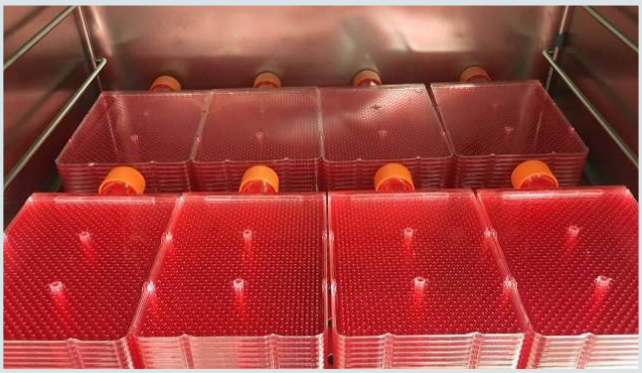

Fig. 2: Vero cell culture in HYPERFlask vessels.

Viral diseases, including but not limited to rabies, rotavirus, and influenza, are worldwide challenges to the international biomedical community. WHO noted that in 1998, over 32,000 deaths due to rabies were reported [1]. The annual deaths from rabies virus grew to 55,000 by 2006 [2], while influenza virus was responsible for millions of deaths worldwide over the course of the last century.

As a result, the demand for vaccine products for viral diseases has necessitated the development of more sophisticated production techniques based on bioreactor cell culture systems. Vero cell lines have become one of the most widely used cell lines for viral vaccine production [3].

Bioreactor based Vero cell culture using Eppendorf's low-shear Cell-Lift Impeller has been widely adopted for rabies vaccine production [4]. In this study, we tested the suitability of a spin filter as cell retention device. Spin filters are used to keep cells inside the vessel during continuous or perfusion culture. Spin filter with a low-shear marine impeller are offered for suspension and microcarrier applications, and it is ideal for production of secreted proteins or vaccines. We cultivated Vero cells on Cytodex 3 microcarriers (10 g/L) in an Eppendorf 3 L glass vessel using a microcarrier spin filter coupled with a pitched-blade impeller. The microcarrier spin filter is a cylinder-shaped cage that spins with the impeller shaft and is covered with a large 75-micron screen designed to prevent microcarriers from being collected with the waste media during perfusion. The process was controlled with a BioFlo 320 bioprocess control station and there were no additional devices needed for the perfusion. With the unique design of this spin filter, we easily cultivated anchorage-dependent Vero cells in perfusion mode on microcarriers and ensured a consistent supply of nutrients and the removal of toxic byproducts.

Materials and Methods

Equipment:
> BioFlo 320 bioprocess control station
> ViCELL® XR Cell Viability Analyzer (Beckman Coulter®)
> Cedex® bioanalyzer (Roche Custom Biotech)

Vessels:
T-175 cell culture flasks
> HYPERFlask® M cell culture flask (Corning 10030)
> Eppendorf 3L glass vessel with 75 micron spin filter impeller

Cell line:
> adherent Vero cell (ATCC®, CCL-81™)

Cell culture medium:
Gibco® DMEM (ThermoFisher®, 11965084), Antibiotic-antimycotic (ThermoFisher, 15240-062), and HI FBS (ThermoFisher, 10438018), 0.25% Trypsin (ThermoFisher, 25200114), Antifoam C Emulsion (Sigma Aldrich®, A-8011).

Microcarrier:
Cytodex® 3 (GE Healthcare®, 17-0485-02)

Cell lysis reagents:
Reagent A100, Reagent B100 (ChemoMetec®, Denmark)

Procedure

Bioreactor inoculum preparation

The adherent Vero cells were cultivated in DMEM medium supplemented with 1 x Antibioticantimycotic and 1% (V/V) HI (heat inactivated) FBS. One 1 mL vial of Vero cells (5×10^6 cells) was thawed by following the ATCC Vero cell protocol [5] and used to inoculate a T-175 flask containing 20 mL of pre-warmed medium. The cells were initially cultured in a Galaxy® 170R CO_2 incubator at 37°C, 5% CO_2 with passive humidification. On day four, when the Vero cell culture reached full confluency, the cells were trypsinized and passaged into two T-175 flasks each containing 25 mL of freshly made, pre-warmed medium at a density of 5×10^6 cells/flask. After three days, when the cells reached 100% confluence again, they were further expanded to five T-175 flasks with a seeding density of 5×10^6 cells/flask. When cells reached full confluence, all cells were harvested, combined and inoculated into five HYPERFlask vessels, at a seeding density of ~1.9×10^7 cells/flask, each flask was filled full of cell culture medium according to manufacturer instructions [6]. The HYPERFlask vessels were then incubated for another six days (Figure 2) to full confluence, the cells were then harvested according to manufacturer's protocol and were combined into a 1-liter sterile addition bottle for the inoculation of the bioreactor vessel. The total inoculum volume was 280 mL with a cell density of 5.0×10^6 cells/mL and cell viability of 99%. A schematic diagram of the Vero cell expansion process for bioreactor inocultaion is displayed in Figure 3.

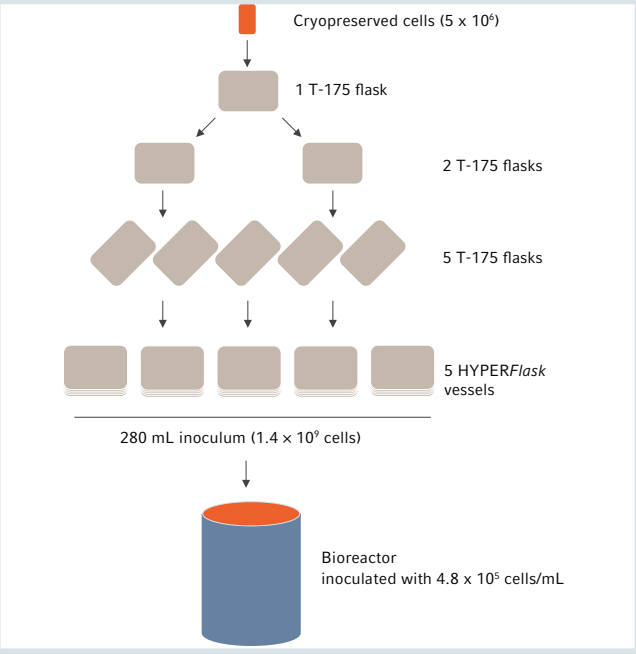

Fig. 3: Schematic of Vero cell expansion process

Table 1: Overview of process parameters and setpoints.

Parameter	Device/setpoint
Bioprocess controller	BioFlo 320
Vessel	3 L glass vessel, water jacket, with spin filter for microcarrier (75 µm)
Inoculation density	4.8×10^5 cells/mL
Working volume (L)	3 L
Sparger	Ring sparger
Gassing control	Sparging: 3-Gas Auto gas mixing, combined flow 0.002 – 0.5 SLPM Air flow 0.002 – 0.3 SLPM; O_2 flow 0 – 0.5 SLPM
Dissolved oxygen (DO)	50 %
Agitation	Spin filter with pitched-blade impeller; 40 rpm
pH	7.1 ± 0.1 Cascade to CO_2 (acid) and Cascade to 0.45 M sodium bicarbonate (base)
Temperature	37°C

Bioreactor control and process parameters

An Eppendorf 3 L glass vessel with 75 micron spin filter impeller and a BioFlo 320 bioprocess control system were used for this experiment (Figure 4). Before inoculation, 30 g of Cytodex 3 microcarriers were sterilized by following the manufacturer's instruction, and added in the vessel with 2 L serum free DMEM media. Dissolved oxygen (DO) was measured using a polarographic ISM® sensor. DO was controlled at 50% by automatic gassing at a flow of 0.002 – 0.5 SLPM in the 3-Gas Auto mode. In order to reduce the foaming issue caused by high gas flow in late culture stage, air flow was limited from 0.002 – 0.3 SLPM and oxygen flow was limited from 0 – 0.5 SLPM. The pH was measured using an Mettler-Toledo® pH sensor and controlled at 7.1 (deadband = 0.1) via a cascade to CO_2 (acid) and 0.45 M sodium bicarbonate (base) (Table 1).

After the microcarriers and 2 L media were equilibrated to the setpoints, 280 mL of the Vero cell inoculum was added into the vessel pre-set with an agitation speed of 40 rpm. Two minutes later, the agitation was stopped to increase the chance for cell attachment. The culture was stirred intermittently (40 rpm agitation for two minutes followed by 28 min-

utes of incubation without agitation) for the next three hours. After three hours, another 720 mL of DMEM media contained 150 mL FBS were added into the vessel with continuous 40 rpm agitation, and the final working volume was 3 L with medium containing 5% FBS, and 10 g/L microcarriers.

Feeding and perfusion control

A glucose concentration between 3 and 5 g/L was targeted for the perfusion experiment. The perfusion rate was started at 0.2 Vessel Volumes per Day (VVD) and was gradually increased to 1.2 VVD at the end of the run (Table 2). The perfusion rate was determined by monitoring the level of ammonium. The goal was to keep ammonium level below 4 mM. In addition to perfusion, based on the glucose level in the bioreactor at the end of every day, extra glucose bolus feeding (200 g/L glucose stock solution) was performed 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 next day. Anti-Foam C was added when it was needed.

Analytical methods and cell counting method

Cell metabolites were measured daily with a Cedex Bio Analyzer, including glucose, glutamine, lactate and ammonium levels. Cell density was measured daily using Vi-CELL cell

Fig 4. BioFlo 320 bioprocess control station equipped with a 3L glass vessel with microcarrier spin filter.

Table 2: Perfusion rates in the Vero cell microcarriers perfusion process.

Day	Perfusion Rate (VVD)
0-2	0
3-6	0.25 - 0.6
7-10	0.9 - 1.2
Working volume (L)	3 L

analyzer for counting of cell nuclei. 1 mL sample of homogeneous microcarrier culture was lysed by using 1 mL of NucleoCounter Reagent A100, then neutralized with 1 mL of NecleoCounter Reagent B100. The lysis sample was filter with through a 40-micron cell strainer and the measurement was conducted using Vi-CELL. The cell type used for nuclei counting by Vi-CELL was "default" within the selection menu.

Results and Discussion

Results of cell expanding in flasks

Vero cells were initially cultured in a T-75 flask, then expanded into five T-175 flask vessels, and eventually cultured in five HYPERFlasks with a seeding density of 1.9×10^7 cells/flask. After a 6-day expansion period in HYPERFlask vessels, total viable harvested Vero cells were 1.4×10^9 cells (Figure 5).

Results of perfusion culture of Vero cells on Cytodex 3 microcarriers in spin-filter impeller vessel.

The perfusion run of Vero cells on Cytodex 3 microcarriers was performed for 10 days. 280 mL inoculum was prepared with cell density of 5.0×10^6 cells/mL, and 99% cell viability. The working volume of the bioreactor was 3 L, and the inoculation density was 4.8×10^5 cells/mL. In the course of the culture, Vero cells growth on Cytodex 3 microcarriers was observed using a microscope (Figure 6), and cells reached full confluency on day ten.

The perfusion was started on day three because the glucose level became low and the toxic waste of lactate and ammonium became high. Our perfusion strategy was to maintain glucose concentration between 3-5 g/L, and ammonium level below 4 mM. Perfusion rate was increased accordingly to maintain these target levels.

The metabolic profiles of Vero cells in the bioreactor were shown in Figure 7. For the glucose line, the spikes indicated

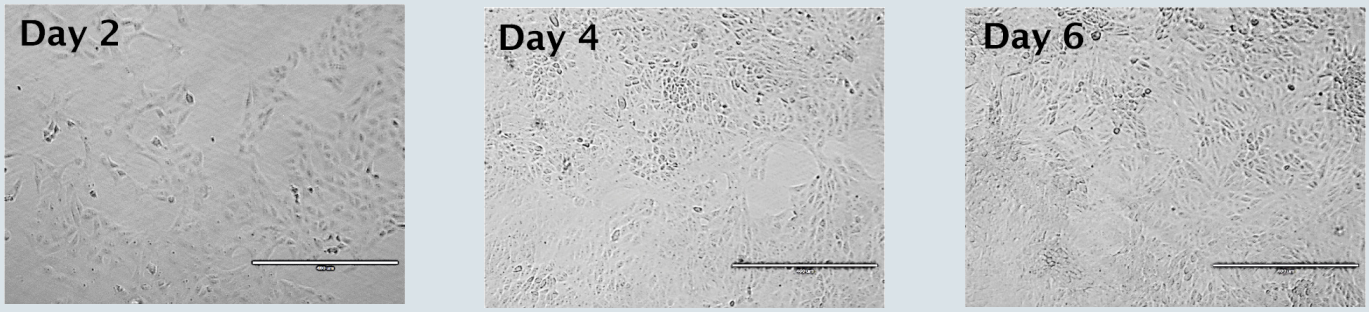

Fig. 5. Vero cells cultured in HYPERFlask vessel on day 2, day 4, and day 6.

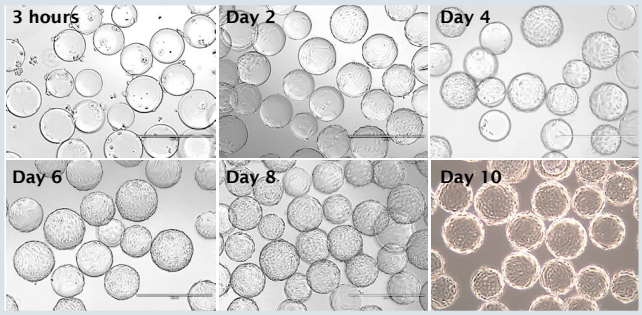

Fig. 6. Vero cells cultured on Cytodex 3 microcarriers 3hours after inoculation and on day 2, 4, 6, 8, 10

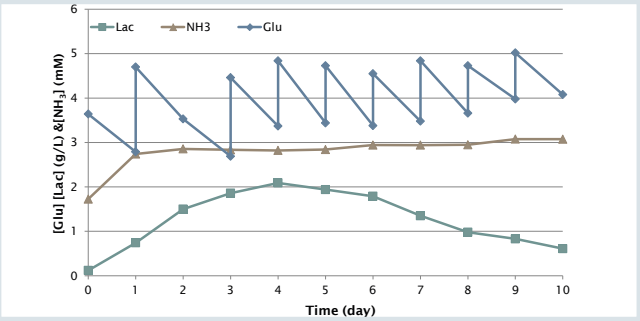

Fig. 7. Metabolic files of Vero cells perfusion culture.

that the glucose bolus feeding was performed daily from day one.

The culture performed for 10 days. Cell density was measured daily by using Vi-CELL to count nuclei as described before. Figure 8 showed the Vero cell growth curve. With 10 days culture of 3 L working volume with 10 g/L Cytodex 3 microcarriers, the final Vero cell density of the culture reached 8 × 10^6 cells/mL.

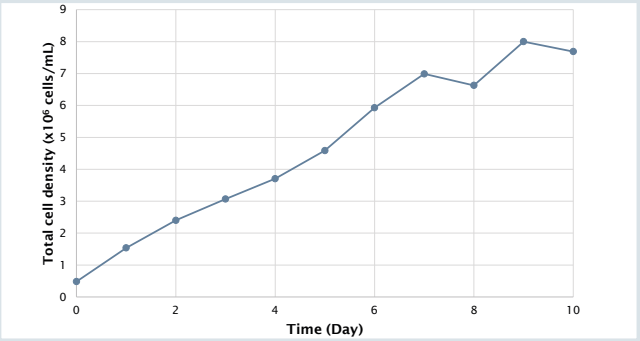

Fig. 8. Vero cell growth curve on Cytodex 3 microcarriers.

Conclusion

We have demonstrated that the BioFlo 320 vessel equipped with spin filter for microcarrier is an excellent platform for high density Vero cell culture. We achieved Vero cell density of 8 million cells per ml with a modest microcarrier loading density of 10 g/L. At this level of Vero cell density, a 3 L spin filter culture can provide sufficient cells for inoculation of a 40 L CelliGen® 510 packed-bed bioreactor suitable for vaccine production.

Literature

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Ordering information

Description	Order no. international	Order no. North America
BioFlo® 320 Control Station	1379963011	1379963011
75 Micron spin filter impeller	M1379-1136	M1379-1136
Galaxy® 170R		
CO_2 Incubator	CO17301001	CO17301001
Cell culture flasks T-75	0030711122	0030711122
Cell culture flasks T-175	0030712129	0030712129
Addition bottle kit, 1L	M1362-9901	M1362-9901
Polarographic DO sensor, ISM	P0720-6653	P0720-6653

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