BIOSTAT® Cplus stirred tank bioreactor for microcarriers-based Vero cells culture

Production of Peste des Petits ruminants virus vaccine

1. Introduction

Scalable and robust bioreactor systems that can be readily applied in a large-scale, industrial setting are of paramount importance in biotechnology. Stirred tank bioreactor (STB) is the most frequently used bioreactor system for such purposes. It encompasses several advantages such as feasibility, scalability and reusability, making them an ideal platform for cell-based production of complex biopharmaceuticals, such as virus-based vaccines [1].

The BIOSTAT® Cplus (Figure 1) from Sartorius Stedim Biotech (Sartorius) fulfills industry requirements for bioreactor with state-of-the-art technology and design.

Figure 1. BIOSTAT® Cplus 20L STB.

The cylindrical shape vessel is designed based on proven engineering principles, with a height to diameter ratio of 2:1 and a bottom clearance of 10 cm (equivalent to 4 L). The convex bottom encloses a harvesting port at the lowest position. Stirring is ensured by two 3-blade segment impellers, 30° angled, appropriate for homogeneous mixing at low shear rates, mounted on a rigid impeller shaft. Aeration is promoted via headspace or submerged ring-sparger with typical pore diameter of 0.8 mm. The BIOSTAT® Cplus is equipped with a feedback control system that modifies gas stream composition (Air, O_2, N_2 and CO_2 pressures in gas inlet) to guarantee cells oxygen requirements and automatically adds acid and base or CO_2 and base to control pH throughout the culture. Pre-defined ready to use packages include all components needed for powerful and convenient operation in cell culture applications (e.g. mesh tube for medium exchange or spinfilter of 75 μm pore size for perfusion). Further enhancement of system performance can be achieved using BioPAT® MFCS/DA, a powerful software package for supervisory process control, extended visualization, data acquisition and trend display.

In this application note we present data demonstrating feasibility of the BIOSTAT® Cplus 20L STB for microcarriers (MC)-based Vero cells growth and production of a vaccine against Peste des Petites Ruminants Virus (PPR), a highly contagious disease affecting small ruminants in Africa and Asian with a relevant negative economic impact [2]. This process will be used by African manufacturers to eradicate PPR targeted by the Food and Agriculture Organization (FAAO) for 2030. A novel/scalable PPRV vaccine production process is herein proposed using Vero cells growing on MC, serum-free medium (SFM) and STB (Figure 2). This includes a new method for *in situ* cells detachment from MC and perfusion culture in 2L STB, used as seed bioreactor.

2. Material and Methods

a. Cell culture under static conditions

The Vero African Green monkey kidney cell line obtained from ECACC (Cat.# 84113001) was adapted to grow in ProVero™-1 SFM (Lonza) supplemented with 5 μg/L of Epithelial Growth Factor (EGF) (data not shown). Adapted

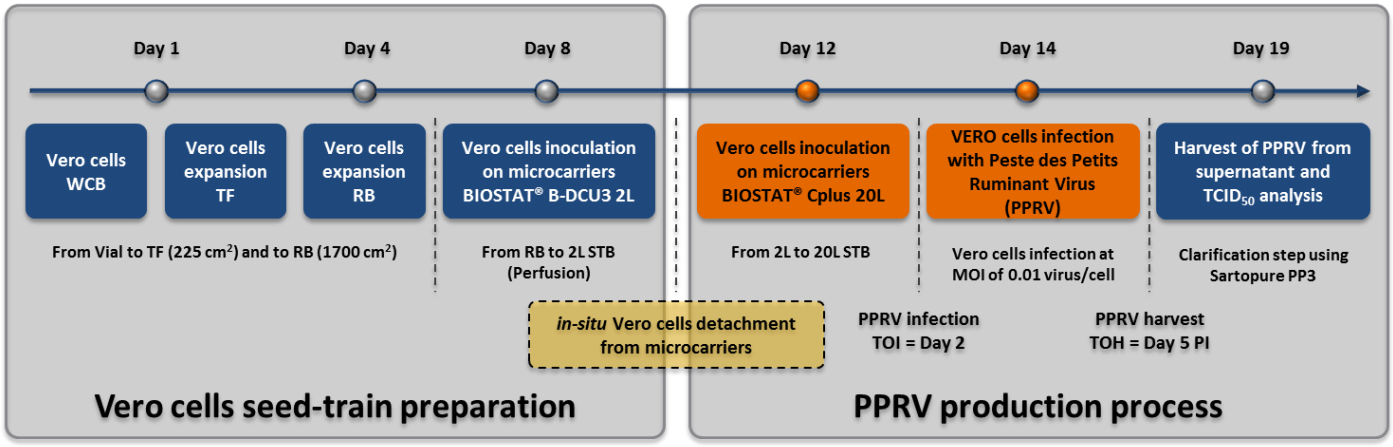

Figure 2. Vero cells seed-train preparation and PPRV production process in the BIOSTAT® Cplus 20L STB. Abbreviations: MOI – Multiplicity of infection; RB – Roller bottle; TF – Tissue culture flask; TOH – Time of harvest; TOI – Time of infection; WCB – Working cell bank

cells were routinely sub-cultured every 3-4 days using initial cell density of $2x10^4$ cell/cm^2. TrypLE Select 1x was used for Vero cells detachment from MC and trypsin inhibitor was used to inactivate TrypLE Select. Cultures were kept at 37ºC in humidified atmosphere of 5% CO_2.

b. Cell culture in bioreactors

Bioreactor system and configuration: Vero cells were cultured in the 2L BIOSTAT® B-DCU3 and the 20L BIOSTAT® Cplus, both from Sartorius. The 2L BIOSTAT® B-DCU3 was equipped with an internal stainless steel spin-filter of pore size 75 µm (Sartorius) to allow perfusion cultures (for Vero cells seed-train preparation). All bioreactors were equipped with two 3-blade segment impellers, 30° angled, appropriate for homogeneous mixing at low shear rates. Aeration was promoted via headspace or submerged ring-sparger with pore diameter of 0.8 mm.

Culture medium: Cells were cultured on Cytodex®-1 MC (prepared according to manufacturing instructions) at 3 g/L using ProVero™-1 supplemented with 5 µg/L of EGF and 0.1 % (v/v) of Pluronic™ F-68.

Cell inoculation and MCs colonization: Cells were seeded at a concentration of $0.1x10^6$ cell/mL and MC colonization promoted using intermittent stirring for 5 h: ON for 2 min (at corresponding Njs - Table 1) and OFF for 18 min.

Aeration mode: STB were operated using dual aeration: (i) day 0-2 post-inoculation, aeration was performed via headspace at a rate of 0.1 vvm; (ii) day 2 onwards, aeration was performed via gas sparging at a rate of 0.005 vvm. Anti-foam C at a final concentration of 0.01 % (v/v) was used.

Operational conditions and control strategies: pO_2 was set to 40 % (in air saturation) and controlled by varying N_2 and O_2 pressures in gas inlet. pH was maintained at 7.2 using CO_2 and $NaHCO_3$ addition. Temperature was set to 37ºC. Stirring rates (Table 1) and scale-up criteria were defined based on engineering correlations (specific power input, shear stress rate and Kolmogorov length scale) [3, 4].

Table 1. Stirring rates used in the 2L and 20L STBs for Vero cell seed-train preparation and PPRV production processes.

Scale (L)	Nc (RPM)	Njs (RPM)
2	70-90	120
20	30-50	65-70

Nc – Stirring rate for cell culture; Njs – Stirring rate for off-bottom suspension of MC.

c. Vero cells seed-train preparation

Perfusion culture: Perfusion was initiated when cells reached $0.4x10^6$ cell/mL (onset of exponential cell growth phase) and dilution rate was set to 0.5-1 day^{-1}.

***In situ* Vero cells detachment from MC:** Based on the work of Nienow *et al.* (2014) [5] for mesenchymal stem cells, an enzymatic and mechanical method for *in situ* Vero cells detachment from MC was designed and successfully implemented in the BIOSTAT® B-DCU3 2L STB, with detachment yields above 80%; no impact on cells re-attachment to MC or on virus productivity was observed (data not shown). Briefly, when cells reached $2.0x10^6$ cell/mL, stirring was turned off and MC allowed to settle down. Spent medium was removed (90% of 2L working volume) and TrypLE Select added at a ratio of 1.5 volumes of TrypLE Select to 1 volume of settled MC. The suspension of cells and MC was then agitated continuously during 6

cycles of 7 min at 155 rpm, with a 5 seconds pulse at 250 rpm in between cycles. Once maximum cell detachment efficiency was reached, trypsin inhibitor was added to the suspension culture at a ratio of 1 volume of trypsin inhibitor to 1 volume of TrypLE Select. Combined with perfusion, this enabled the scale-up to the BIOSTAT® Cplus 20L STB directly from the 2L BIOSTAT® B-DCU3, surpassing the need for a mid-scale platform and thus reducing seed-train preparation time.

d. Production of PPRV

Vero cells at 0.2x10^6 cell/mL were infected with PPRV strain Nig 75/1 (kindly provided by Dr. Geneviève Libeau, CIRAD-EMVT, France) at a multiplicity of infection (MOI) of 0.01 virus/cell. Prior to infection, complete medium exchange was performed. Cultures were harvested at day 5 post-infection and PPRV in the supernatant clarified using a Sartopure PP3 depth filter. Prior to virus clarification, MC were allowed to settle-down.

e. MFCS for supervisory control and data acquisition

BioPAT® MFCS/DA (Multi Fermenter Control System) was used for supervisory bioprocess control and data acquisition of Vero cell culture and PPRV production in STB.

f. Analytics

Cell counting and viability: Viable cells were counted in a Fuchs–Rosenthal hemocytometer chamber using trypan blue dye exclusion method. Cell viability and MC colonization were assessed by staining cells with fluorescein diacetate (green, viable cells) and propidium iodide (red, non-viable cells) as described in Serra *et al.* (2010) [6], followed by visual inspection under a fluorescence microscope. Total cell concentration was determined using crystal violet staining.

PPRV titration: PPRV titration was performed using the $TCID_{50}$ method as described in Silva *et al.* (2008) [7]. All virus samples were titrated in triplicate.

Metabolite analysis: Glucose, glutamine and lactate concentrations were assessed using YSI 7100MBS. Ammonia concentration was quantified enzymatically using the Ammonia, UV method. All samples were analyzed in triplicate.

3. Results and discussion

a. Microcarriers colonization

The kinetics of MC colonization during cell attachment phase (up to 24h) for the 2L and 20L STB are presented in Figure 3. Results show that more than 90% of MC are colonized after 24 hours in both STB.

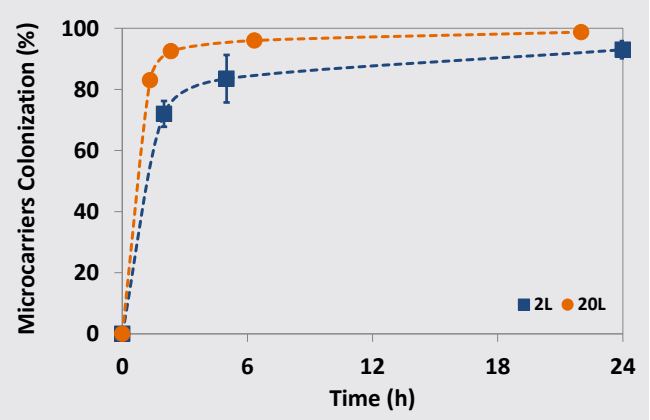

Figure 3. Kinetics of MC colonization for the 2L (blue) and 20L (orange) STB. Error bars represent standard deviation of 3 independent measurements.

b. Vero cells growth and infection

The kinetics of Vero cells growth before and after infection by PPRV for the 2L and 20L STB are depicted in Figure 4. Cells were able to grow until day 4 post-infection in both STB, reaching similar maximum cell concentrations.

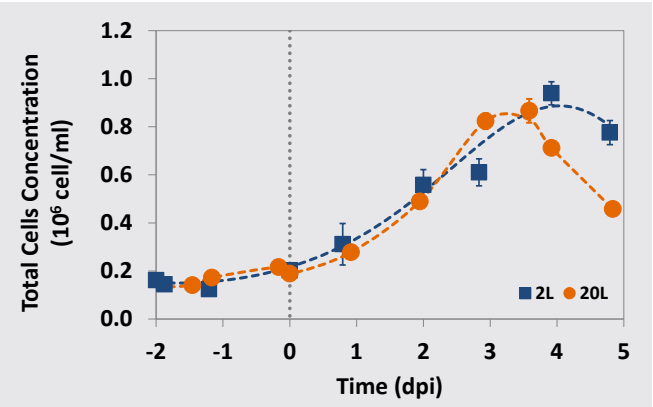

Figure 4. Vero cells growth before and after infection by PPRV for the 2L (blue) and 20L (orange) STB. Dashed gray line: time of infection. DPI: days post-infection. Error bars represent: (i) for the 2L STB - standard deviation of two independent cultures, (ii) for the 20L STB - standard deviation of 4 independent cell counts.

The morphology of Vero cells during PPRV infection process is shown in Figure 5. In both STB (2L and 20L), cells are attached and spread on MC at the time of infection (Day 0

PI), become swollen as infection progresses (Day 3 PI), and start lysing and/or detaching from MC at day 5 PI.

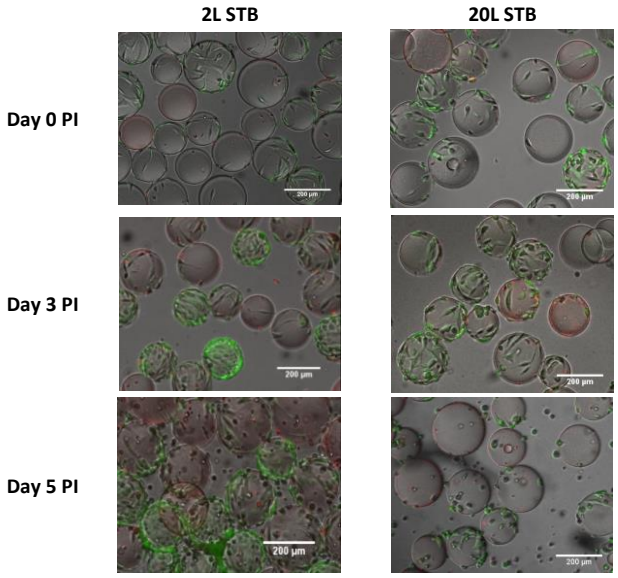

Figure 5. Morphology of Vero cells during PPRV infection process for the 2L and 20L STB. PI denotes post-infection. Cells were stained with fluorescein diacetate (green, viable cells) and propidium iodide (red, non-viable cells).

Metabolic profiles of Vero cells before and after infection by PPRV for the 2L and 20L STB were assessed, and cell specific consumption and production rates estimated (Table 2). No significant differences were observed between both systems, and values recorded are within the normally obtained for batch animal cell cultures [8].

Table 2. Cell specific metabolic rates of glucose, glutamine, lactate and ammonia during Vero cell growth and PPRV production for the 2L and 20L STB.

Scale (L)	Cell specific metabolic rates (pmol/cell.h)			
	Glucose	Glutamine	Lactate	Ammonia
2	0.1-0.3	0.05	0.2-0.5	0.04-0.05
20	0.1-0.4	0.04-0.08	0.2-0.4	0.05-0.06

c. PPRV production and clarification

The kinetics of PPRV production for the 2L and 20L STB is shown in Figure 6. Infectious PPRV titers increase over culture time in both 2L and 20L STB, reaching a maximum of 4.5-4.9x10^6 $TCID_{50}$/mL (approx. $TCID_{50}$/cell = 5) at day 4-5 post-infection. The titers (*per* mL and *per* cell) achieved are within those reported for PPRV production in STB and serum-free medium [7]. PPRV recovery yields after clarification using PP3 depth filters were comparable (85 ± 9% in the 2L STB and 90 ± 17% in the 20L STB).

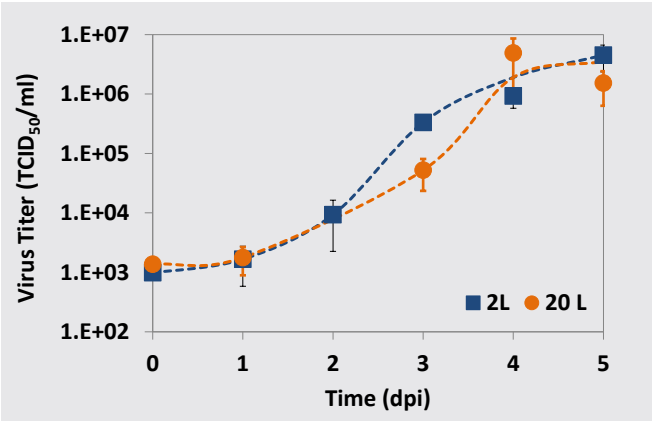

Figure 6. PPRV production kinetics for 2L (blue) and 20L (orange) STB. DPI denotes days post-infection. Error bars represent standard deviation of 3 independent measurements.

4. Conclusion

The BIOSTAT® Cplus 20L STB is comparable to conventional STBs and fully scalable. The results herein presented demonstrate the feasibility and scalability of the BIOSTAT® Cplus for MC-based Vero cells growth and production of a PPRV vaccine. The kinetics of MC colonization, Vero cells growth before and after infection, and PPRV production obtained in the BIOSTAT® Cplus and the BIOSTAT® B-DCU3 2L STB are very similar. In addition, virus clarification efficiencies obtained in both scales are comparable, thus confirming the potential of Sartopure PP3 filter for PPRV clarification.

5. Literature

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[3] Croughan M *et al*. 1988. DOI: 10.1002/bit.260320805

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[7] Silva A *et al*. 2008. DOI: 10.1016/j.vaccine.2008.03.077

[8] Zeng A *et al.* 1998. DOI: 10.1021/bp9800337