



# Scale-up of adherent Vero cells grown on Cytodex microcarriers using single-use bioprocessing equipment

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# Scale-up of adherent Vero cells grown on Cytodex™ microcarriers using single-use bioprocessing equipment

In cell culture-based vaccine production, scale-up of adherent cells is challenging. This study shows a process for scaling up adherent Vero cells from static cell factories to influenza production at 50 L scale using WAVE Bioreactor™ systems and ReadyToProcess™ single-use bioprocessing equipment. Vero cells were grown to high cell density on Cytodex microcarriers in 10 L working volume. The cells were detached with trypsin and used to seed a 50 L production culture with the same microcarrier concentration. The cells were allowed to reattach and grow on the new microcarriers in a larger Cellbag™ bioreactor chamber. Cells were subsequently infected with influenza virus. The results show a repeatable scale-up procedure. The cell growth was similar in the 10 L seed cultures as in the 50 L production cultures in three consecutive experiments, with an obtained hemagglutinin (HA) concentration of approximately 12 µg/mL and a virus titer of 10<sup>9</sup> virus particles/mL.

## Introduction

Compared to an egg-based vaccine production, cell culture-based vaccine production is seen as an alternative way for a more rapid response to pandemic challenges. Traditionally, cell-based vaccine production is performed in stainless steel bioreactors requiring extensive cleaning procedures. With disposable bioreactor systems without the need for cleaning and validation, the start-up time for GMP manufacturing can be significantly reduced (1).

Adherent cells, such as Vero cells, are almost exclusively used for human cell-based vaccine production. Vero cells are susceptible to a broad range of viruses and are widely used for the production of viral vaccines like polio, rabies, or



Fig 1. Overview of the production process of whole, live influenza virus using ReadyToProcess equipment.

enterovirus (2, 3). Recently, influenza vaccine was successfully produced at industrial scale using Vero cells (4). Vero cells can only proliferate when provided with a suitable surface. The scale-up of such cell cultures is challenging because of the required detachment and reattachment of cells during passaging. The aim of this study is to present a process for scaling up adherent Vero cells for influenza propagation. The cultures were run at a working volume of 50 L using WAVE Bioreactor system and ReadyToProcess single-use equipment (Fig 1). When scaling up to 50 L from a 10 L bioreactor culture, the cells were detached by trypsinization and subsequently used to seed new Cytodex microcarriers of the same concentration in the larger Cellbag bioreactor. The cells reattached and grew on the new microcarriers and were subsequently infected with influenza virus. Our results show that the scale-up procedure using single-use WAVE Bioreactor systems is a fast and convenient option for virus production.

## Materials and methods

### Cell line and cultivation media

Vero cells were obtained from ATCC (No. CCL81, ATCC-LGC Standards, Teddington, Middlesex TW11 0LY, UK). The cultivation medium used was Dulbecco's modified Eagle medium (DMEM)/Ham's F12 (GE Healthcare Life Sciences) supplemented with glucose (1 g/L), glutamine (0.9 g/L),  $\beta$ -cyclodextrin (0.1 g/L) (Sigma-Aldrich Co., St. Louis, MO, USA), and soy peptone (2 g/L) (Kerry, Norwich, NY, USA). For bioreactor cultivations, Pluronic™ F-68 was added (2 g/L) (Sigma-Aldrich Co.). For cell propagation, the cultivation medium was supplemented with fetal bovine serum (50 g/L) (GE Healthcare Life Sciences).

Inoculum was grown in Nunc™ T-flasks and Cell Factory™ systems (Thermo Fisher Scientific Inc., Waltham, MA, USA). For detachment, cells were washed with PBS-EDTA and incubated with Accutase™ (GE Healthcare Life Sciences).

### Microcarrier preparation

In bioreactor cultivation of the Vero cells, the microcarrier concentration was 3 g/L. Cytodex 1 microcarriers (GE Healthcare Life Sciences) were hydrated in PBS in a siliconized glass bottle (Sigmacote™, Sigma-Aldrich Co.) and washed three times with PBS prior to autoclaving for 15 min at 121°C. Before use, the microcarriers were washed with cultivation medium.

### Bioreactor cultivation

Cultures at 10 L scale were grown in a WAVE Bioreactor 20/50 system (GE Healthcare Life Sciences). WAVEPOD II controller (GE Healthcare Life Sciences) was used to control temperature, pH, DO, and agitation. The culture parameters were maintained at 37°C, pH 7.1, DO 30%, and the agitation settings were 10 rpm/5°. Cultures at 50 L scale were run in a WAVE Bioreactor 200 system (GE Healthcare Life Sciences). Cultivation conditions were the same as for the 10 L cultures except for the agitation setting, which were changed to 6 rpm/5°.

### Scale-up procedure and bead to bead transfer

Inoculum grown in cell factories was used to seed a 10 L microcarrier culture. The 10 L culture was subsequently used to inoculate a 50 L culture by bead to bead transfer. For cell transfer to the 50 L microcarrier culture, the heating and agitation were switched off and the microcarriers were allowed to settle. The cells were washed twice in PBS-EDTA inside the Cellbag 50L bioreactor (GE Healthcare Life Sciences). For wash, the supernatant (90% of the volume) was removed and partly replaced with PBS-EDTA. After a second wash, the microcarrier suspension was transferred to a Sigmacote siliconized glass bottle. In a laminar flow hood, the residual buffer was removed and trypsin-containing buffer solution (0.2% trypsin and 0.02% EDTA in PBS w/o Ca<sup>2+</sup>, Mg<sup>2+</sup>), corresponding to 250% of the settled carrier volume, was added. The bottle was incubated at 37°C in a water bath and the suspension was mixed every 5 min. After 20 min, cell detachment was verified microscopically and the supernatant transferred to a glass bottle. The microcarriers were washed twice with cultivation medium and the supernatants were pooled. The cell suspension was used to inoculate a 50 L culture in a Cellbag 200L bioreactor (GE Healthcare Life Sciences).

## Cell sampling and counting

During bioreactor cultivation, samples were taken daily to determine cell concentration and morphology as well as concentrations of selected metabolites. Microcarrier suspension was withdrawn via the sample port while the base unit was rocking continuously. A 1 mL sample was transferred to a tube. After settling of the microcarriers, 800  $\mu$ L supernatant was removed and replaced with an equal volume of 0.1% crystal violet in 0.1 M citric acid and 1% Triton™ X-100. The suspension was vigorously mixed for 45 s and the released nuclei were counted using a hemocytometer. Cell morphology and attachment to microcarriers were determined using an inverted microscope with attached camera (Eclipse TS100, Nikon Instruments Inc., Melville, NY, USA).

The concentrations of glucose, lactate, glutamine, glutamate, and ammonium were measured in a Bioprofile Flex™ analyzer (Nova Biomedical Corporation, Waltham, MA, USA).

## Virus stock preparation and infection

Influenza A/Solomon Islands/3/2006 (H1N1) IVR145 WHO (egg-derived) was adapted through a number of passages in Vero cells. A working virus bank was established and tested for infectivity by using 50% tissue culture infective dose (TCID<sub>50</sub>). Cells were washed in serum-free medium prior to infection. For washing, the heating and agitation were switched off, the microcarriers were allowed to settle inside the Cellbag bioreactor, and 80% of the supernatant was removed and replaced with serum-free medium. Two washings were performed for efficient removal of serum. The cells were infected during the exponential growth phase at a multiplicity of infection of  $4 \times 10^{-3}$  (the ratio infectious virus particles to number of cells) and a trypsin concentration of 2 mg/L.

## Harvest

During virus growth, samples were taken daily to visually inspect the cells for cytopathic effect (CPE) and to determine HA concentrations and virus titers. The cultures were harvested 3 to 4 d after infection.

The supernatant was clarified by depth filtration and influenza virus was purified as described in application note 29-0435-49 AA.

## Analytical methods

The amount of infectious virus particles was measured by TCID<sub>50</sub> assay. This assay measures the dilution of the sample that generates CPE in 50% of the cells. Vero cells were seeded in 96-well microplates and incubated in 5% CO<sub>2</sub> at 37°C overnight. The cells were infected with 10-fold serial dilutions of virus sample, followed by incubation for 5 d in 5% CO<sub>2</sub> at 33°C. The cells were visually inspected for CPE and the TCID<sub>50</sub> titer was calculated according to the method of Reed and Muench (5). The outcome is presented as a log<sub>10</sub> titer (10<sup>xx</sup>TCID<sub>50</sub> units/mL).

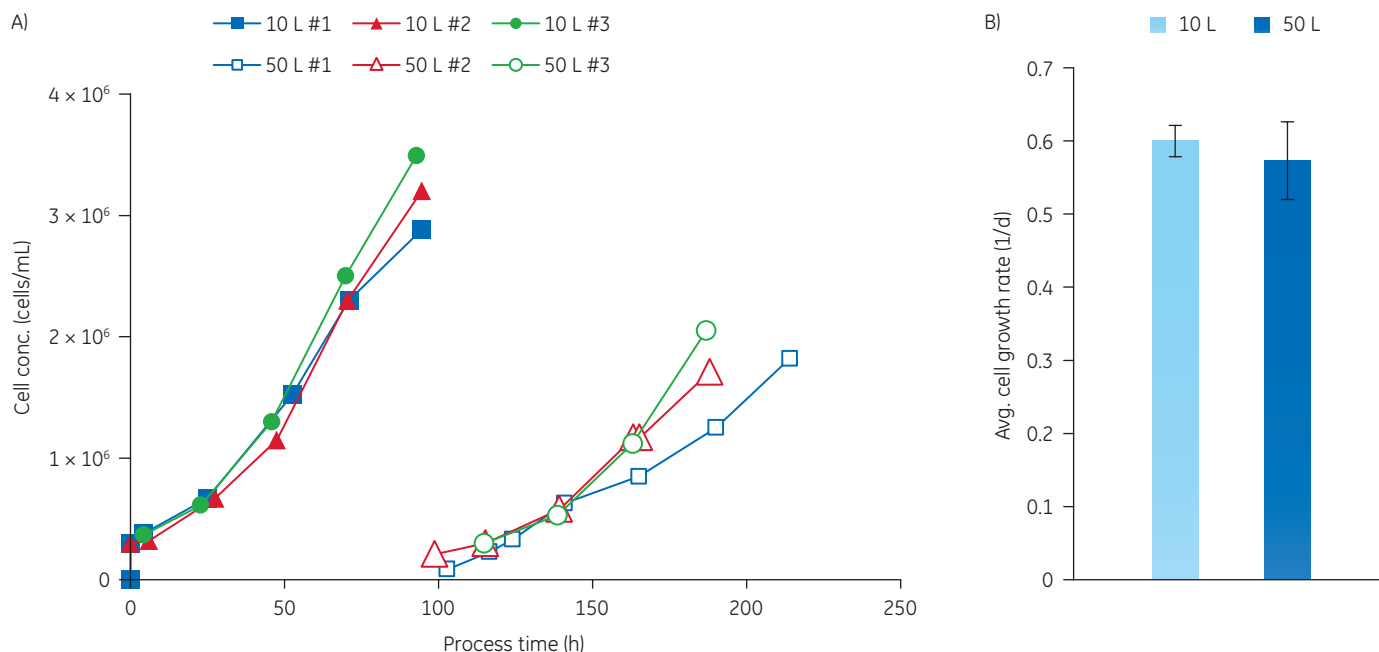
Total amount of virus particles was measured in Virus Counter 2100 (ViroCyt, Denver, CO, USA). The measurement is based on flow cytometry. Proteins and nucleic acids in the sample were labelled with two different fluorescent dyes. A simultaneous signal of protein and nucleic acid was counted as an integral virus particle. Virus Counter measures all virus particles present, both infective and non-infective.

HA was quantified in Biacore T200 with sensor Chip CM5 (GE Healthcare Life Sciences). Recombinant HA protein was immobilized on the dextran matrix. Virus-containing samples were mixed with a fixed concentration of antiserum and injected over the chip surface. Free antibodies (not bound to virus at equilibrium) bound to the surface HA. Low concentration of virus in the sample resulted in a high antibody binding to the surface, while a high virus concentration resulted in low antibody binding. A HA calibration curve, based on reference material from the National Institute for Biological Standards and Control (NIBSC), was included three times in each run: at start, in the middle, and at the end. These calibration curves were used for software normalization to obtain accurate concentrations of the samples.

## Results and discussion

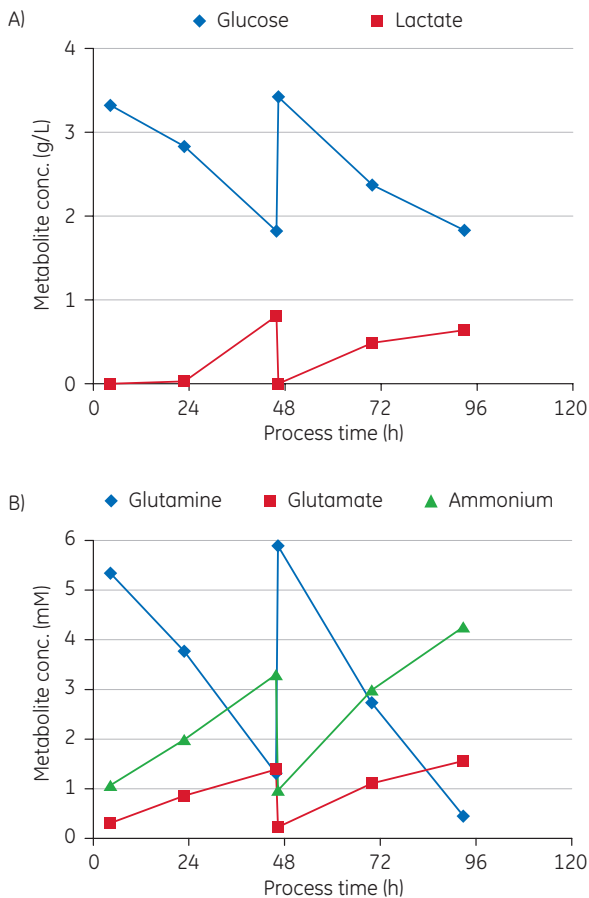
The cell growth in the 10 and 50 L cultures is shown in Figure 2. Similar growth was observed in three consecutive experiments. In the seed cultures, final cell concentrations of about  $3 \times 10^6$  cells/mL were achieved. Cells from the seed cultures were used to inoculate the 50 L production culture. A split ratio of 1:5 was used throughout this study. In both cultivation volumes, Vero cells attached to the microcarriers and started to grow without a lag phase. In previous experiments, delayed cell growth was observed after the bead to bead transfer (data not shown). Reducing the incubation period with trypsin from 30 to 20 min seemed

to overcome this problem. Most probably, this is caused by a reduced effect of the protease on the cell surface receptors, especially integrins, which are required for reattachment and growth of adherent cells (6). As shown in Figure 2, the growth rates in the 10 and 50 L culture were similar. The 50 L cultures were infected with influenza virus at a cell concentration of about  $2 \times 10^6$  cells/mL that was obtained about 4 d after the bead to bead transfer. The first 50 L culture was extended by one day to reach the desired cell concentration. The reason was probably the lower inoculum concentration compared to the following two runs.



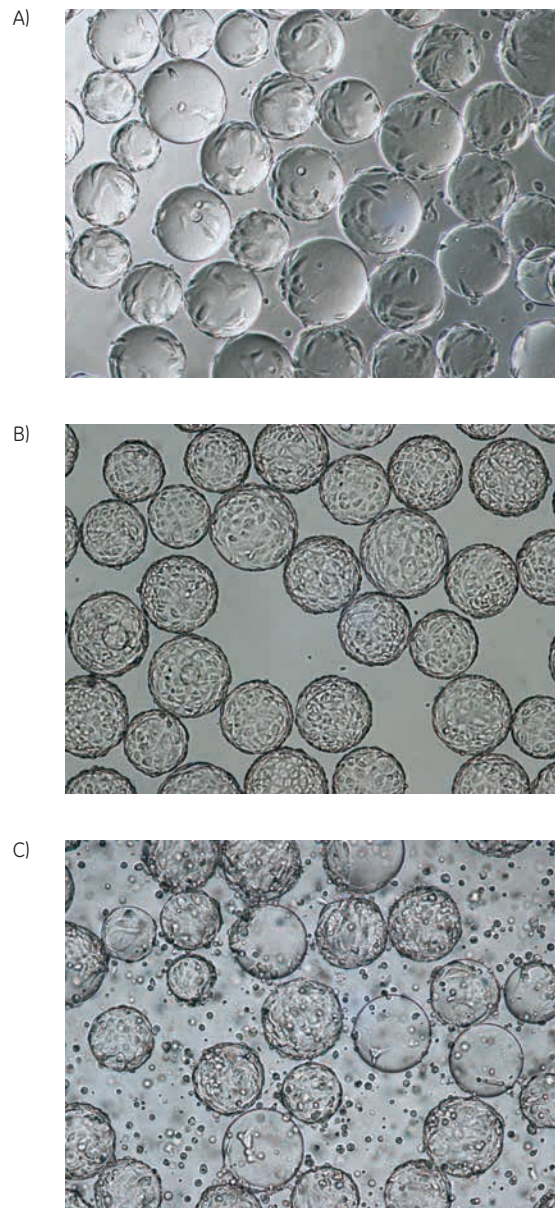
**Fig 2.** (A) Cell growth and (B) average growth rate in the 10 L seed cultures and 50 L scaled-up cultures. Error bars show one standard deviation.

Representative metabolite concentrations during the four-day culture are shown in Figure 3. In both cultivation volumes, an 80% to 90% medium exchange was performed after 2 days of processing to maintain sufficient nutrient concentrations. As shown in the graph, the residual glucose concentration was kept above 1.5 g/L and the lactate concentration was maintained below 1 g/L. As a key energy source, glutamine is a critical nutrient for cells and heavily consumed during growth. Hence, a medium exchange was required after two days of cultivation in order to prevent limiting concentrations. It is crucial to prevent depletion, as this can trigger apoptosis (7). Medium exchange may be more favorable than plain feeding of glutamine, as the accumulated ammonium concentration already exceeded 4 mM. The washing with serum-free medium before virus infection supplied the cultures with fresh nutrients and removed catabolites. A low ammonium concentration seems to be desirable during virus propagation, as it has been shown that elevated concentrations of this catabolite can prevent infection of the host cell (8).



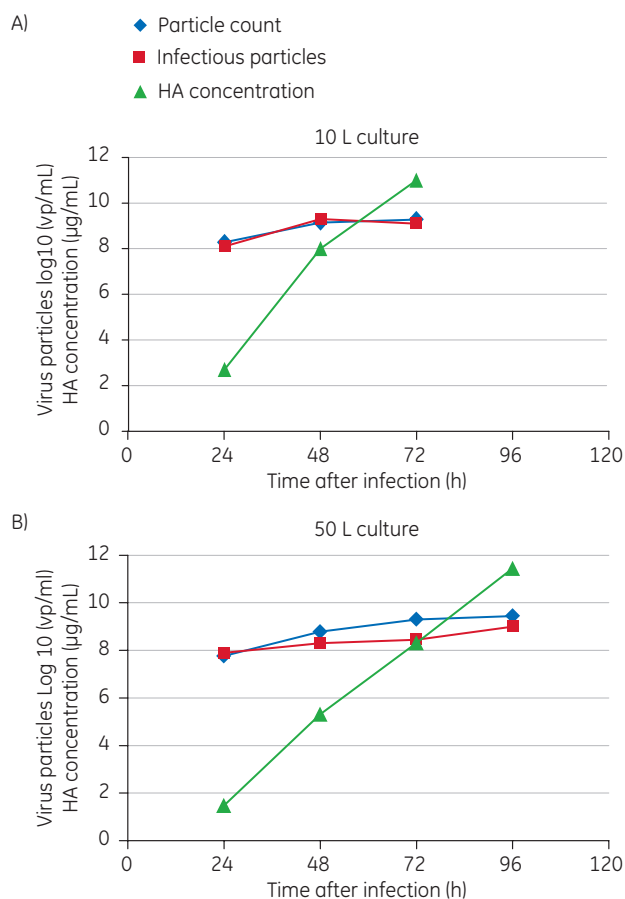
**Fig 3.** Metabolite concentrations during the 10 L culture. Medium exchanged was performed after 2 d of processing.

The scale-up strategy from static cell factories to 10 L seed cultures, and then further expansion to 50 L cultures by bead to bead transfer, was shown to be reproducible. The cells attached and grew on the new microcarriers also after bead to bead transfer. However, it is important to optimize the cell concentration at bead to bead transfer. The optimal cell concentration is dependent on the cell line and cultivation conditions. The morphology of Vero cells at different stages of the process is shown in Figure 4. The cells spread out and reached confluence on the microcarriers. CPE was observed 2 to 3 days after virus infection.



**Fig 4.** Morphology of Vero cells in the 50 L culture (A) 24 h after bead to bead transfer, (B) when reaching cell confluence, and (C) after 72 h cultivation showing CPE. Magnification was 100-fold.

In Figure 5, a comparison between virus growth in a 10 L and a 50 L culture is shown. The smaller volume was inoculated directly from Cell Factory, the larger volume via bead to bead transfer as described above. Both cultures were infected at similar cell concentrations of approximately  $2 \times 10^6$  cells/mL. The amount of infectious virus particles, as determined by a TCID<sub>50</sub> assay, as well as the total virus amount, according to virus count measurements, increased with cultivation time. The majority of virus particles were formed during the first two days after infection. The HA concentration increased continuously throughout the infection phase and peaked at the time of harvest. While a slightly slower virus growth was observed in the 50 L culture, the same final concentrations were obtained. At harvest, the infectious virus concentration in both cultures was  $10^9$  TCID<sub>50</sub> units/mL. The total amount of virus particles showed a good correlation to the TCID<sub>50</sub> measurements. Interestingly there was no significant difference in virus count between the ViroCyt system and the TCID<sub>50</sub> assay. The HA concentration at harvest was about 12 µg/mL in both cultivation volumes.



**Fig 5.** The amount of virus particles measured with ViroCyt Virus Counter and infectious virus particles measured with TCID<sub>50</sub>. The HA concentration was measured with Biacore T200. (A) 10 L culture, (B) 50 L culture. Samples were taken daily during virus growth.

## Conclusions

This case study shows a robust and reproducible scale-up procedure demonstrated by three consecutive cultivation experiments, in which TCID<sub>50</sub> values of  $10^9$  infectious virus particles/mL, was achieved. Assuming a recovery of 25% for the overall process and a dose requirement of  $10^7$  TCID<sub>50</sub>, more than 1.5 million doses of monovalent live attenuated influenza vaccine could be produced from a 50 L cell culture. The processes were run in single-use WAVE Bioreactor 20/50 and WAVE Bioreactor 200 systems. Compared to virus production in stainless steel bioreactors, the setup and turnaround time can be substantially reduced by the use of disposable systems that make time-consuming cleaning and sanitization procedures redundant. The described process is a fast and convenient alternative to more traditional methods.

The case study described in this application note is not a fully optimized process. Further optimization of the process is necessary prior to use in vaccine manufacturing.

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## Ordering information

Product	Quantity	Code number
WAVE Base 20/50	1	28-9413-42
WAVE kit	1	28-9416-46
WAVE Bioreactor 200	1	29-0016-41
WAVEPOD II	1	29-0016-32
Cellbag 50L	1	CB0050L10-31
Cellbag 200L	1	CB0200L10-31
Cytodex	1	17-0448-03
DMEM/Ham's F12	1	G0014,3050
FBS	1	A15-101

### Related literature

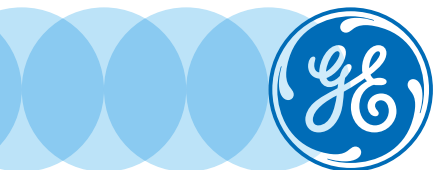
Downstream scale-up purification of influenza virus using single-use bioprocessing equipment, application note	29-0435-49
Overview of a scale-up of a cell-based influenza virus production process using single-use bioprocessing equipment, white paper	29-0435-51
Microcarrier cell culture principles and methods, handbook	18-1140-62



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First published Apr. 2013

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