Propagation of influenza virus in Vero cells using Cytodex microcarriers in WAVE Bioreactor systems

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Propagation of influenza virus in Vero cells using Cytodex™ microcarriers in WAVE Bioreactor™ systems

The aim of this work was to establish a process to produce influenza virus in Vero cell culture using single-use equipment. The end result would be a fast and simple process enabling scale-up and adaptation to industrial production. The following parameters were evaluated: medium composition, cell detachment, medium supplements, agitation conditions, and infection conditions. Different combinations of media and proteases were evaluated for cell growth in static culture. As part of optimizing the conditions for cell growth in WAVE Bioreactor system, the effects of a number of supplements and different rocking conditions were evaluated. Next, in order to find the optimal infection parameters, both trypsin concentration and different virus stock dilutions were evaluated. Finally, the robustness and reproducibility of the entire workflow for virus propagation in Vero cells using microcarriers and WAVE Bioreactor system was investigated. The data show that a robust process using WAVE Bioreactor system for propagating influenza virus in Vero cells attached to Cytodex 1 microcarriers has been developed. WAVE Bioreactor 20/50 system using a single-use Cellbag™ bioreactor (Fig 1) offers easy set-up with no cleaning required, making it a fast and convenient alternative to stainless steel bioreactors.

Fig 1. WAVE Bioreactor 20/50 system with Cellbag bioreactor and WAVEPOD™ II Integrated Controller.

Introduction

The traditional method for propagating influenza virus for vaccine production uses fertilized hen eggs. This method is labor-intensive and requires large facilities with limited scalability.

As the threat of an influenza pandemic rises, so too does the need for methods to rapidly produce mass quantities of influenza vaccine. Production of influenza vaccine in eggs is dependent on the limited supply of fertilized eggs. A shift from egg-based to cell culture-based vaccine production is one way to secure a more rapid response. A switch to cell culture methods and single-use products gives flexibility that would significantly reduce the time to vaccine clinical trials and approval, effectively decreasing the time-to-market.

Several cell lines are available for producing vaccines in a cell culture-based system. Vero cells were used in this study because of their well-known reputation as a suitable platform for the production of numerous human vaccines. Because it is desirable in human vaccine production to avoid animal-derived components, our cell culture process is animal-component-free.

Materials and methods

Cell line, maintenance, and expansion

Vero cells, derived from African green monkey kidney cells, were obtained from ATCC (Nr. CCL81). The cells were cultivated in different formulations of animal-component-free media with three supplements: Pluronic®F-68, soy peptone (Fluka), and soybean-derived trypsin inhibitor (Sigma-Aldrich; 1 mg/mL in phosphate-buffered saline [PBS]). Vero cells on Cytodex 1 microcarriers were propagated and subsequently infected in Cellbag bioreactors using WAVE Bioreactor 20/50 systems.

For maintenance cultivation in T-flasks or cell expansion in Cell Factories (Nunc), the Vero cells were washed with PBS-EDTA (Sigma-Aldrich) prior to addition of recombinant protease for detachment using either TrypLE™ Select or Accutase®.

Microcarrier preparation

Cytodex 1 was used at a final concentration of 3 g/L (1). The microcarriers were hydrated in siliconized (Sigmacote®) glass vessels, and washed three times with PBS, followed by autoclaving for 15 min at 121°C (2). They were then washed with cultivation medium before transfer to a Cellbag bioreactor.

WAVE Bioreactor system

A WAVEPOD II controller was used with WAVE Bioreactor 20/50 system to control the pH, temperature, oxygen, CO_2, and mixing parameters. Vero cells were cultivated in a Cellbag-10L, with a working volume of 2 L. The culture conditions were 37°C, pH 7.2, 5% CO_2. Dissolved oxygen was monitored with an optical probe.

Cell sampling and counting

For routine culture Vero cells were passaged in T-flasks twice a week. Cell factories were used for cell expansion. A sample of the detached Vero cells was counted in a Cedex® HiRes cell analyzer using trypan blue exclusion.

Cells grown in the WAVE Bioreactor were sampled daily to determine concentration and morphology. A sample of the cell suspension was removed via the sample port while the base unit was in continuous rocking motion. Sample (1 mL) was transferred to a tube, after which 800 µL of 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 then vortexed for 45 sec, and nuclei were counted in a Bürker chamber.

Cell morphology and attachment

Cell morphology and attachment to microcarriers were evaluated using an inverted microscope with attached camera (Eclipse TS100, Nikon).

Vero cell attachment to Cytodex 1

Different shaking parameters and media compositions were evaluated to determine optimal conditions for attachment of Vero cells to Cytodex 1 microcarriers. Cell cultivation was performed in a Cellbag-10L at a working volume of 2 L. All cultures were inoculated with 4×10^5 cells/mL. The rocking parameters described in (reference 3) were slightly modified as listed below:

- Intermittent rocking for 6 h (16 rpm/4.5° for 2 min, then 0 rpm/0° for 18 min). To improve cell spreading, the culture was then left unagitated for 2 h before switching to continuous rocking (11 rpm/4.5°).
- Intermittent rocking for 6 h (16 rpm/4.5° for 2 min, then 0 rpm/0° for 8 min). To improve cell spreading, the culture was then left unagitated for 2 h before switching to continuous rocking (11 rpm/4.5°).
- Continuous rocking (11 rpm/4.5°).
- Continuous rocking (12 rpm/5°).
- Continuous rocking (12 rpm/5°) followed by an increase in speed (14 rpm/5°) after 24 h.

Virus stock preparation

Influenza A/Solomon Islands/3/2006 (H1N1) IVR145 WHO (egg-derived) was adapted through a number of passages in Vero cells. A working virus stock was created and tested for infectivity using tissue culture infectivity $dose_{50}$ $(TCID)_{50}$ calculations (4). The mean value of five $TCID_{50}$ analyses was used to calculate the titer of the virus stock as $10^{6.9}$.

Process development

The process development was divided into two parts: cell attachment and growth; and infection with virus.

Optimization of cell attachment and growth

The following parameters were evaluated:

Medium composition

- VP-SFM (Invitrogen)
- OptiPRO™-SFM

Recombinant proteases (for cell detachment prior to subculture)

- TrypLE Select
- Accutase

Media supplements

- *Pluronic F-68*, a nonionic, copolymer of ethylene and propylene oxide, is used as an anti-foaming agent and cell membrane stablilizer against shearing during culture. It is often used in WAVE Bioreactors.
- *Soy peptone* is used as a substitute for animal serum. It is a papaic/pancreatic digest of defatted soybean meal and is an excellent source of vitamins and carbohydrates.
- *Soybean trypsin inhibitor* is used in cell culture applications to inhibit tryptic activity during cell dissociation, which minimizes cell damage/death.

Rocking conditions

- Rocking conditions were optimized as described above, in *Vero cell attachment to Cytodex 1*.

Vero cell growth in T- flasks

Vero cells were expanded in T-flasks using VP-SFM or OptiPRO-SFM. Cell subcultivation was performed by washing the T-flask once with PBS-EDTA then adding either TrypLE Select or Accutase and incubating for approximately 2 min at 37°C. Cell number and viability were determined in a Cedex HiRes cell analyzer. Cells were seeded into a fresh T-flask at a cell density of 4×10^4 cells/cm^2 and cultivated at 37°C with 5% CO_2.

Vero cell growth in WAVE Bioreactor system

Cell culture medium was supplemented with soy peptone, trypsin inhibitor, and Pluronic F-68 in order to support cell growth in the absence of serum and to protect cells from shear forces in the WAVE Bioreactor system. The rocking conditions listed above (see page 2) were evaluated with respect to cell growth using different media supplements.

Optimization of infection parameters

Infection in T-flasks and WAVE Bioreactor system

Infections in T-flasks and WAVE Bioreactor system were performed using virus dilutions in the range of 1:1000 to 1:5000. Trypsin (porcine pancreas, Sigma-Aldrich) was used at a concentration of 5 to 25 µg/mL. Virus titers were measured using the $TCID_{50}$ assay. Briefly, Vero cells were grown in 96-well microplates, infected with 10-fold serial dilutions of virus suspension, and incubated for 5 d. Wells showing cytopathic effects were counted, and the $TCID_{50}$ titer was calculated according to the method of Reed and Müench (4).

Hemagglutinin (HA) was measured using a Biacore™ assay (5).

Results

Optimization of cell attachment and growth

Vero cell growth in T-flasks

Vero cell growth and morphology were evaluated in T-flasks over seven passages in either VP-SFM or OptiPRO-SFM. The average doubling time with VP-SFM was 30 h; with OptiPRO-SFM, a slightly shorter doubling time of 25 h was observed. Either TrypLE Select or Accutase recombinant protease was used to detach cells for each passage.

No significant difference in doubling time was observed between these two proteases. However, the cultures treated with TrypLE had a slightly better morphology based on microscopic observation (Fig 2A vs. 2B).

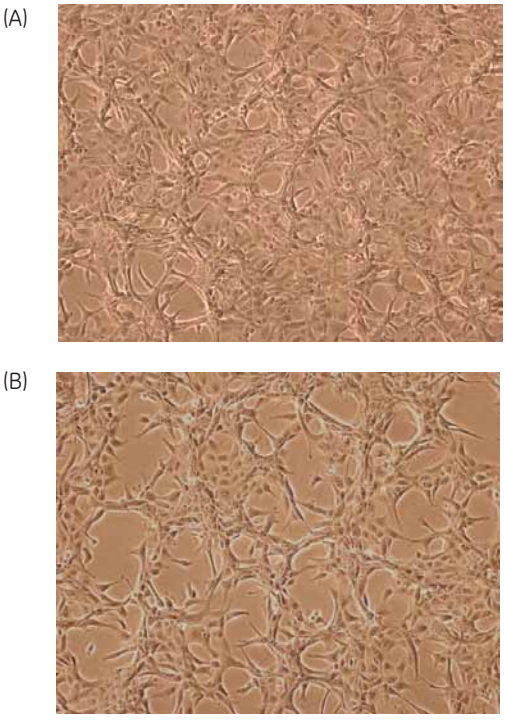

Fig 2. Vero cells grown for 48 h in OptiPRO-SFM using TrypLE or Accutase for cell detachment. (A) OptiPRO-SFM with TrypLE; (B) OptiPRO-SFM with Accutase.

Vero cell attachment and growth on microcarriers

To investigate cell attachment and growth, Vero cells were grown in 24-well plates on Cytodex 1 in OptiPRO-SFM. Prior to being seeded on Cytodex 1, the cells were dislodged using TrypLE.

After 1 h of incubation, cells began to attach to microcarriers as seen in Figure 3A. Cells were spreading and starting to proliferate after 22 h (Fig 3B). After 48 h, the cells were evenly distributed on the microcarriers and approached confluence (Fig 3C).

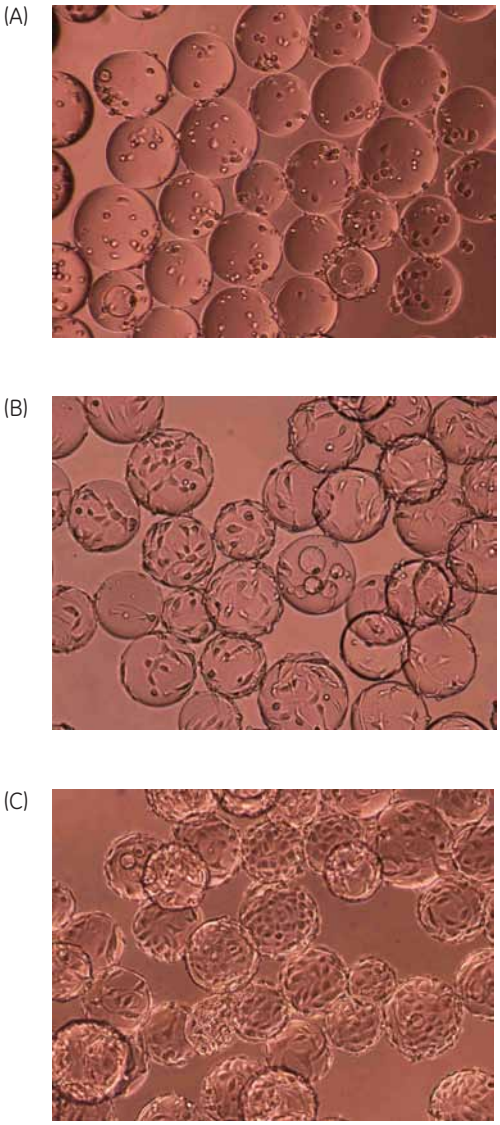

Fig 3. Morphology of Vero cells on Cytodex 1 microcarriers after incubation in OptiPRO-SFM. Cell morphology after incubation for (A) 1 h; (B) 22 h; and (C) 48 h.

Vero cell growth in WAVE Bioreactor system

Moving from static culture to an agitating system means that cells are subject to shear forces. To compensate for this, the shear protectant Pluronic F-68 can be added to the medium. To obtain conditions free of animal components, soy peptone can be used instead of serum, and trypsin inhibitor can be used to inactivate proteases.

When comparing intermittent and continuous rocking conditions (see page 2), a clear difference in distribution of cells on microcarriers was observed. The evenness was improved when using continuous rocking conditions. To obtain a robust inoculation during continuous conditions, the effect of soy peptone was evaluated by testing three different concentrations (0.1%, 0.2%, and 0.3%) while the concentrations of trypsin inhibitor and Pluronic F-68 were kept constant at 20 mg and 0.2%, respectively. The optimal concentration of soy peptone was found to be 0.3% based on microscopic evaluation of attachment and growth (data not shown). The amount of required trypsin inhibitor depends on the amount of TrypLE used for cell detachment.

For example, 20 mg of trypsin inhibitor was used when 100 mL of TrypLE was added to a 2-L culture. Addition of three supplements (Pluronic F-68, 0.2%; soy peptone, 0.3% and trypsin inhibitor, 20 mg in a 2-L culture) is required for optimal cell growth as shown in Figures 4 and 5.

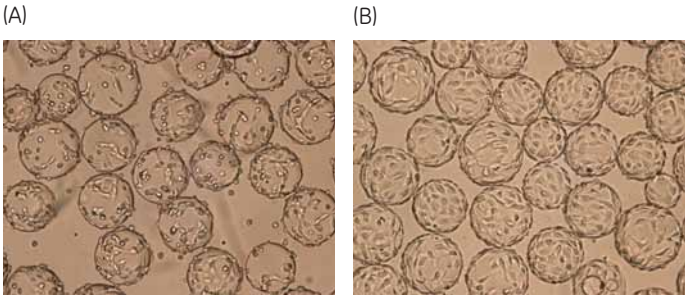

Fig 4. Cell morphology after 48 h of growth. (A) without supplements; (B) with supplements.

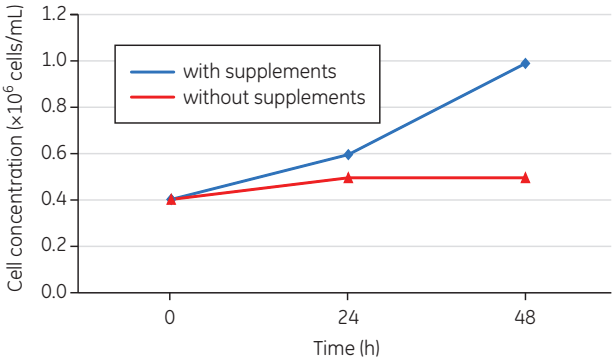

Fig 5. The effect of supplements on cell growth.

Optimization of infection parameters

Infection parameters were optimized in T-flasks. Cell density at time of infection (TOI) was approximately 1.1×10^6 cells/mL and the time of harvest (TOH) was 3 d post-infection. The cells were visually inspected daily to observe the cytopathic effects (CPE). The harvested material was analyzed using $TCID_{50}$ (data not shown). With respect to both CPE and virus titer, the most favorable conditions for infection were a trypsin concentration of 12.5 µg/mL and a virus dilution of 1:1000. Multiplicity of infection (MOI) was calculated based on the virus stock titer of $10^{6.9}$, virus dilution of 1:1000, and cell density of 1.1×10^6 cells/mL. MOI was calculated to be 0.004.

Propagation of influenza virus in WAVE Bioreactor system

To investigate robustness and reproducibility, a number of 2-L batches in WAVE Bioreactor system were prepared using Cytodex 1 at a concentration of 3 g/L and OptiPRO-SFM supplemented with 0.2% Pluronic F-68, 0.3% soy peptone, and 20 mg trypsin inhibitor. To obtain homogenous and robust cell growth, the following rocking conditions were selected: 12 rpm/5° for 24 h, then increased to 14 rpm/5° until harvest. The starting Vero cell density was 0.4×10^6 cells/mL.

The microcarriers were nearly confluent after 48 h (Fig 6). The infection parameters were: MOI, 0.004; TOI, 48 h; TOH, 72 h; and temperature, 37°C. All batches were visually inspected with respect to CPE (Fig 7) and analyzed using $TCID_{50}$ assay (Fig 8). Early cytopathic effects were seen 24 h post-infection (Fig 7A), and cells were detached from the microcarriers and lysed by the virus 72 h post-infection (Fig 7B).

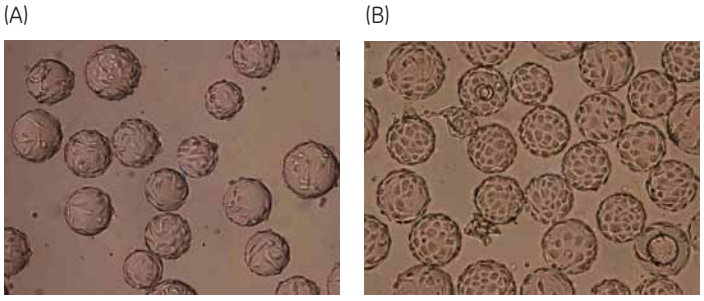

Fig 6. Vero cell morphology after inoculation of microcarriers in a WAVE Bioreactor system. A) 5 h and B) 48 h post-inoculation.

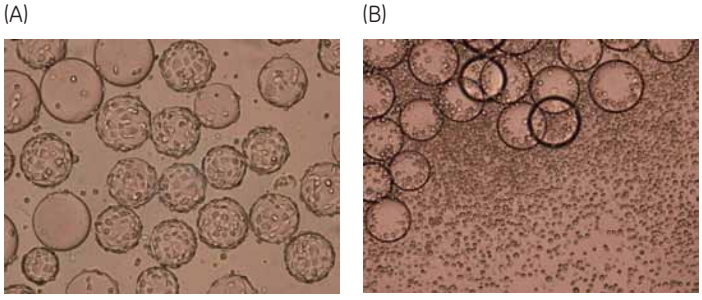

Fig 7. CPE after virus infection of cultures in a WAVE Bioreactor system. (A) 24 h post-infection; (B) 72 h post-infection.

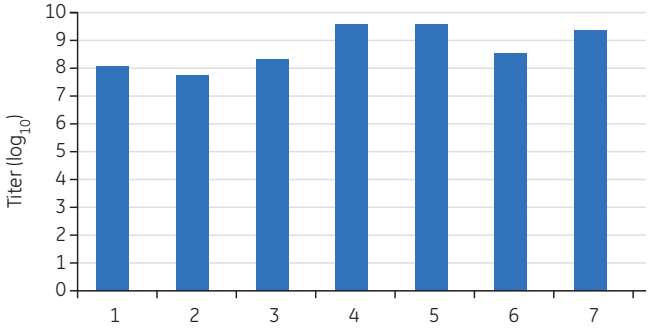

Fig 8. $TCID_{50}$ values from seven different 2-L influenza batches propagated in a WAVE Bioreactor system (TOH 72 h).

Kinetics of hemagglutinin concentration and virus titer during culture

Samples were taken at different time points post-infection. The concentration of hemagglutinin (HA) was measured using a Biacore assay, and the infectious titer was analyzed using $TCID_{50}$. The results are shown in Figure 9. Infectivity peaked relatively early (72 h post-infection), whereas HA concentration increased slowly for several additional days.

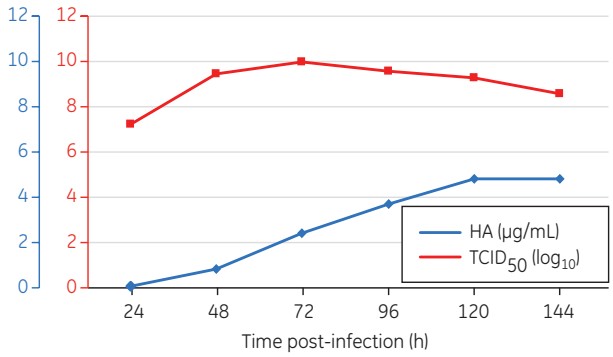

Fig 9. Kinetics of HA and $TCID_{50}$ during culture.

Discussion and conclusions

The aim of this work was to establish a process for producing influenza virus in Vero cell culture using single-use materials. The end result would be a fast and simple process enabling scale-up and adaptation to industrial production. For example, the production of influenza virus in WAVE Bioreactor 20/50 system using a Cellbag bioreactor is a fast and convenient alternative to stainless steel bioreactors, because it is easy to set up and no cleaning is required.

We have been able to grow the cell cultures in animal-component- free conditions. It is important from a regulatory perspective to avoid animal components in human vaccine production, and it is critical to live virus vaccines because there are limited options for inactivating adventitious virus.

Appropriate rocking conditions for the WAVE Bioreactor system were identified. A number of parameters were tested in order to determine the optimal conditions with regard to minimizing shear forces, as assessed by morphological examination of the Vero cell-populated microcarriers. It is important to find the conditions that give even distribution of cells on the carriers.

The optimal TOH based on infectivity peaked relatively early post-infection. For a split- or subunit-based vaccine, infectious activity is not relevant, and thus it is possible to harvest later in order to obtain the maximum amount of viral antigen (in this case, HA).

In this study we did not investigate the effects of scale-up. However, all the methods and equipment used here are currently used for commercial large-scale vaccine production.

Calculations based on $TCID_{50}$ values indicate that approximately 50 000 doses of monovalent live influenza virus (LIV) vaccine could be produced from a 2-L culture assuming a total process yield of 20% and a dose of 10^7 infectious virus particles. Thus, a 100L-L reactor should hypothetically be able to produce 2 500 000 doses of monovalent live vaccine.

A simple workflow of the optimized vaccine production process is shown in Figure 10.

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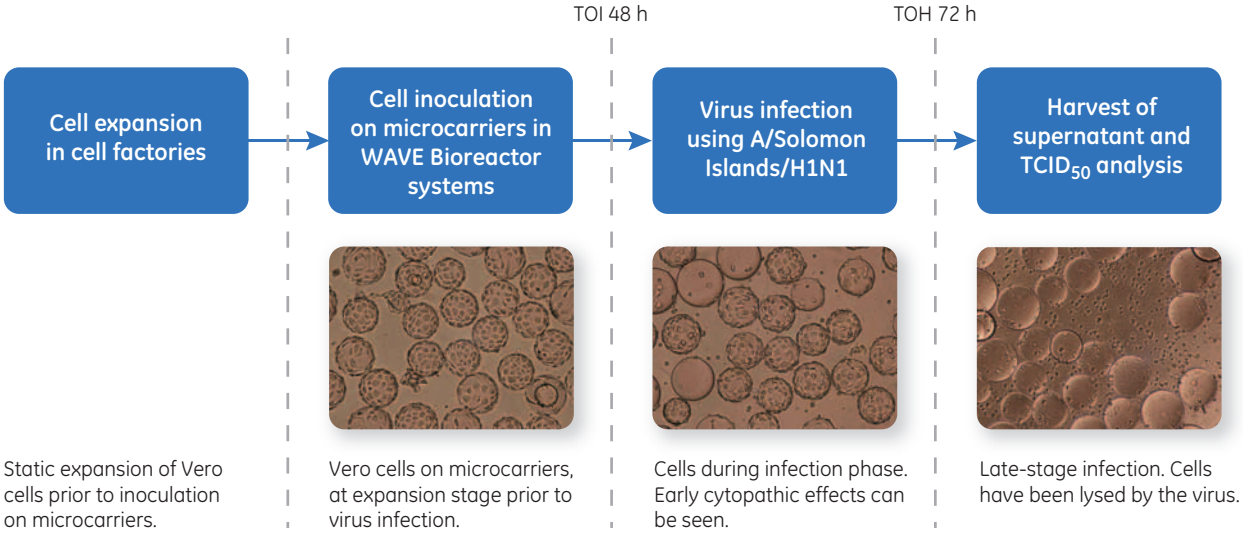

Fig 10. Workflow of influenza virus propagation in Vero cells using Cytodex microcarriers in WAVE Bioreactor systems.

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