

Vero Cell-based Vaccine Production: Cell lines, Media and Bioreactor Options

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Executive Summary

The recent Covid-19 pandemic introduced new challenges for the vaccine industry, it has also brought in new innovations in vaccine development including DNA/RNA based vaccines. The pandemic also increased demand for well-established cell-based vaccine production technologies.

We hereby review the strategies for optimizing Vero cell based vaccine production using rabies and influenza as examples. The Vero cell line is one of the most satisfactory vaccine production hosts based on its infectability, stability and well-documented performance in quality and quantity of viral yield. It is one of the first cell lines that received FDA approval for vaccine use and is used throughout the world. Cell culture media technology has advanced drastically in recent years, and a number of serum free and protein free options are available through commercial suppliers. Because serum tends to bind toxins and contaminants, its elimination calls for adoption of the highest quality reagents and careful monitoring of culture conditions in order to achieve optimal performance.

Vero cell-based vaccine production often utilizes micro carriers or similar types of cell attachment matrix. The advancements of 3D cell culture matrix such as Fibracel® have been important additions to the range of possible choices for optimizing in vitro production systems. With a wide array of bioreactor options available, high-density

attachment cell culture will continue to be one of the most productive methods for vaccine production.

In this era of daunting challenges from the Covid-19 pandemic, Eppendorf is constantly upgrading and expanding its cell cultivation technologies to meet the demands of this unprecedented public health emergency.

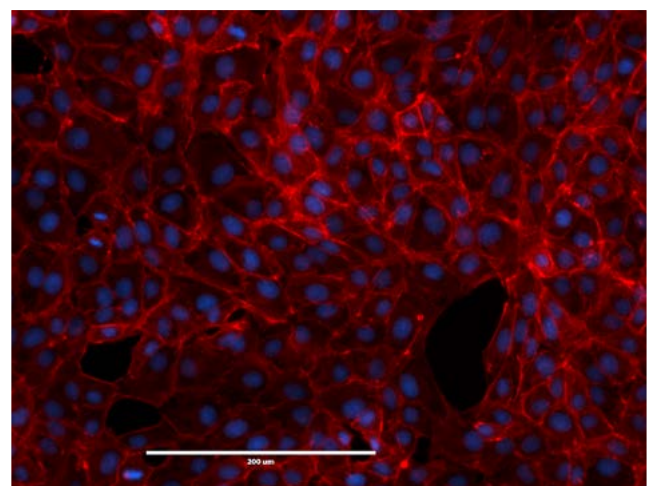


Fig. 1. Fluorescent image of confluent Vero cells. DAPI-stained nuclei appear blue, and actin filaments stained with rhodamine-conjugated phalloidin appear red (Eppendorf Inc.).

Introduction

Long before Covid-19, various viral diseases, including rabies and influenza, have been worldwide challenges to the international biomedical community. WHO notes that in 1998 over 32,000 deaths due to rabies were reported, while influenza has been responsible for millions of deaths worldwide over the course of the last century [1].

Rabies is often transmitted to humans from infected domestic animals. Dogs infected with rabies can become extremely aggressive and attacks on humans are widespread, especially in certain Asian countries where using unleashed dogs for home security is common, and the dogs were often not vaccinated. The virus is spread through their saliva and bites by infected animals can be fatal. In China, the disease is referred to as “Kuang Quan Bing”, i.e. “Mad Dog Disease”. The annual number of deaths caused by rabies had grown to about 59,000 worldwide according to the World Health Organization (WHO) [2].

Since the 18th century, vaccination has proven to be the most successful (and perhaps the only) route to the elimination of viral diseases. The history of smallpox is well known, as is the introduction of the use of cowpox virus from lesions in infected animals by Jenner in 1796 [3]. Despite his work and that of others, smallpox epidemics continued throughout the 19th century, due to improperly applied or non-existent vaccination regimes. The work of Louis Pasteur and others toward the end of the 19th century put vaccination on a sound scientific footing [4].

Influenza has been a permanent worldwide scourge long before Covid-19 virus. The CDC Influenza Division reported an estimated range of deaths between 151,700 and 575,400 individuals resulting from the 2009 H1N1 virus infection during the first year that the virus circulated [5]. Annual deaths in the United States reached 61,000 in the 2018 season with over 800,000 hospitalizations according to CDC statistics [6]. Anti-viral drugs are employed for acute treatment, but vaccination remains far and away the most effective approach for combating viral illnesses.

There has been for years a constant, underlying concern regarding the possibility of the emergence of a truly deadly virus strain, on a level with the 1918 influenza outbreak, the “Spanish Flu” that caused ~50 million deaths throughout the world. This catastrophic possibility was realized this year (2020) with the appearance and pandemic spread of the Covid-19 virus, now responsible for over 50 million cases

and over 1.3 million deaths worldwide in its first year alone.

Currently numerous Covid-19 vaccine development programs are underway throughout the world, many of them utilizing the tried and true cell culture methods, including Vero, to produce Covid-19 virus fragments and/or spike protein for vaccine development purpose. The Vero cell line has been used for years in various virus vaccine development, and is recognized as a safe and efficient production tool. With the current demands of pandemic vaccines development, we believe the need for bioreactor-based Vero cell culture will continue to grow.

Biological Systems for Viral Cultivation

Today the expanding demand for vaccine products has necessitated the development of a range of techniques for growing large quantities of antigenic proteins. Traditionally, viruses have been grown in embryonated hen’s eggs, but numerous shortcomings compromise their utility. These include a bottleneck in the availability of high quality, pathogen-free eggs, as well as low titers of emerging viruses [7]. Another major concern is that when viruses are cultivated through extended passages in hens’ eggs, there is an evolutionary process in the amnion and allantoic cavity of the egg resulting in the selection of certain viral subpopulation, antigenically and biochemically distinct from the original inoculum. Because of these and other factors, well characterized permanent cell lines are coming to dominate the field.

As an alternative to egg-based vaccine production, the advantages of mammalian cell culture systems have been widely recognized. Cultured cells provide much shorter lead times, a more controlled production process that takes advantage of closed-system bioreactors, a reduced risk of microbial contamination, and the opportunity to cultivate viral stocks without significant egg passage-dependent antigenic changes [8].

A WHO conference some years ago expressed concern regarding the rapid emergence of pandemic viral strains. It was concluded that insufficient time would be available to generate the large quantity of high quality, fertile hens’ eggs that would be required to the demands of a worldwide pandemic. In recent years, situation has only exacerbated. Thus, the cell culture alternative provides a flexible and scalable platform that can make use of the well-established biopharmaceutical bioreactor cell culture infrastructure for vaccine production.

Cell Line Options

Over the years, a number of stable cell lines have been approved by regulatory authorities for influenza virus production, such as the *Spodoptera frugiperda* insect cell line (Protein Sciences/Sanofi [9]), the Madin-Darby canine kidney (MDCK) and the Vero cell line from African green monkey, one of the most widely used. Another example is the PER.C6® cell line, which was recently announced for use in a Covid-19 vaccine development program from J&J [10].

It is noteworthy that certain cell lines may provide an environment favoring selection of viral subpopulations, and these types may be inappropriate for vaccine production. Anez et al attempted production of Dengue virus vaccine candidates using FRhL-2 diploid fetal rhesus monkey lung cells [11]. However, passage in this cell line resulted in the accumulation of a mutational variant which was responsible for reduced infectivity and immunity in Rhesus monkeys. This phenomenon was not observed in viruses passaged in the Vero cell line. Other lines of investigation support the Vero cell line as the candidate of choice for viral vaccine production, including: efficiency of primary virus isolation and replication to high infectivity titers; genetic stability of the hemagglutinin molecule, while maintaining the antigenic properties of human-derived viruses; and similarities in the

pattern of protein synthesis and morphological changes between virus-infected Vero and MDCK cells [12,13].

Given the regulatory acceptance as well as the abundance of vaccines already successfully developed on Vero platform (Table 1), The attachment culture of Vero cells remains to be one of the most attractive options for cell based viral vaccine production. The continued interest has also driven the scientific community towards further development of Vero cells into suspension cell lines, further expands the capability of the Vero cells in the vaccine development and production market [14].

Media Alternatives

There are a variety of different Vero isolates available from commercial suppliers (Vero, Vero 76, Vero E6, Vero B4), but all are quite similar, and their nutritional needs are comparable [17]. The search for the ideal mammalian cell culture medium began in the 1950s, with the holy grail being an economical, protein-free, serum-free medium that would provide strong growth support and have the property of scalability to large volumes, up to thousands of liters, while coming in at an affordable price.

Table 1. Anti-viral vaccines using Vero cell culture production technologies. Modified from Barrett et al [15], and Kiesslich and Kamen [16].

Anti-viral Vero Cell-Based Vaccines

Study (year)	Disease	Vaccine Type	Genus
Wang et al (2008)	Chikungunya Fever	Live attenuated	Alphavirus
Howard et al (2008)	Chikungunya Fever	Inactivated	Alphavirus
Blaney et al (2008)	Dengue Fever	Live attenuated or live chimeric	Flavivirus
Tauber et al (2008)	Japanese encephalitis	Inactivated	Flavivirus
Valneva Austria GmbH (Ixiaro, 2019)	Japanese encephalitis	Inactivated	Flavivirus
Ruis-Palacios et al & Vesikari et al (2006)	Rota gastroenteritis	Live attenuated	Rotavirus
GSK (RotaRix, 2008)	Rota gastroenteritis	Live attenuated	Rotavirus
Montagnon (1989)	Polio	Live attenuated Inactivated	Picornovirus
Aycardi E (2002)	Rabies	Inactivated	Lyssavirus
Spruth et al (2006)	Severe acute respiratory syndrome	Inactivated	Cornovirus
Qu et al (2005)			
Qin et al (2006)			
Monath et al (2004)	Smallpox	Live attenuated	Orthopoxvirus
Lim et al (2008)	West Nile Encephalitis	Inactivated	Flavivirus
Monath et al (2006)	West Nile Encephalitis	Live attenuated	Flavivirus
Baxter International Inc. (PREFLUCEL)	Influenza	Inactivated	Orthomyxovirus
Chan and Tambyah (2012)	Influenza	Inactivated	Orthomyxovirus
Merck & Co. (Ervebo 2019)	Ebola	Live-attenuated	Zaire Ebolavirus
Wu et al., (2015)	Hand-foot-and-mouth disease	Inactivated	Non-polio enterovirus
Pereira et al., (2015)	Yellow fever	Inactivated	yellow fever virus

Serum provides a protective function to cultured cells and binds toxins and other contaminating materials. Thus serum-free media must be extremely carefully formulated. Albumin can be substituted for serum, but it may impede the downstream steps of purification [18].

Chen et al. have tested five different serum free media, combined with Cytodex 1 microcarriers [19]. The following were evaluated: OptiPro SFM (Invitrogen®), VPSFM (Invitrogen), EX-CELL® Vero SFM (SAFC® Biosciences), Provero-1 (Lonza®) and HyQ SFM4MegaVir (HyClone®). The EX-CELL Vero SFM gave one of the highest cell densities, demonstrating that the use of serum free media has become routine for Vero cell cultivation. Comparable results were observed with a commercial serum-free medium MDSS2N (manufactured under the name AXCEVIR-Vero™ by Axcell Biotechnologies). In this case, Vero cells were compared with MDCK cells grown in T-flasks and microcarrier cultures.

Rabies Virus Cultivation Strategies

The Brazilian group led by Frazatti-Gallina has been active in the field of Rabies vaccine production [20]. Using Vero cells adhered to microcarriers and cultivated in a bioreactor with serum-free medium, they generated an effective rabies vaccine. With the aid of tangential filtration, they purified the Rabies virus by chromatography and inactivated it using beta-propiolactone.

Their protocol states that 350 cm² T-flasks were harvested and inoculated into a 3.7 liter New Brunswick™ CelliGen® bioreactor, at a proportion of 16 cells per microcarrier (Cytodex® 3-GE), yielding an initial seeding of 2.5 × 10⁵ cell/ml. The cells were grown in serum-free MDSS2 medium (Axcell Biotechnologies).

The serum-free VP-SFM medium, according to the manufacturer, was developed for Vero, BHK-21 and CHO cell growth. This medium drives the adherence of the Vero cells to the microcarriers. After 4 days of cultivation in VP-SFM medium, the cells were infected with PV rabies virus (multiplicity of infection = 0.08). The harvests of the culture supernatant were carried out 3 days after the virus inoculation and four times thereafter at intervals of 24 h. During this period, culture conditions were maintained at 60 rpm at a pH of 7.15 and 5% dissolved oxygen. Only the temperature varied from 36.5 °C in the cellular growth phase of the culture to 34°C after virus inoculation. In the course of the program, seven batches of virus suspensions were produced in the bioreactor (16L per cycle) at a mean viral titer of 10⁴. FFD50/0.05 mL.

The effectiveness of the preparation was demonstrated by immunizing mice with three doses of the new vaccine (seven batches), comparing it with the commercial Verorab and HDCV (Rabies vaccine). Mean titers of neutralizing antibodies of 10.3-34.6, 6.54 and 9.36 IU/mL were found, respectively.

The choice of the serum-free medium was fortunate. In this case the amount of contaminating DNA was very low, and tolerable, less than 22.8 pg per dose of vaccine. The authors argue that this protocol is especially applicable in the developing world, where rabies is a constant hazard and a major public health problem.

Yu et al. sought methods to increase yield in Vero cell culture systems over that obtained using roller bottles [21]. In a recent review, they summarized the production technology developed over the course of the last seven years. They have adopted the 30 L New Brunswick BioFlo® 4500 fermenter/bioreactor.

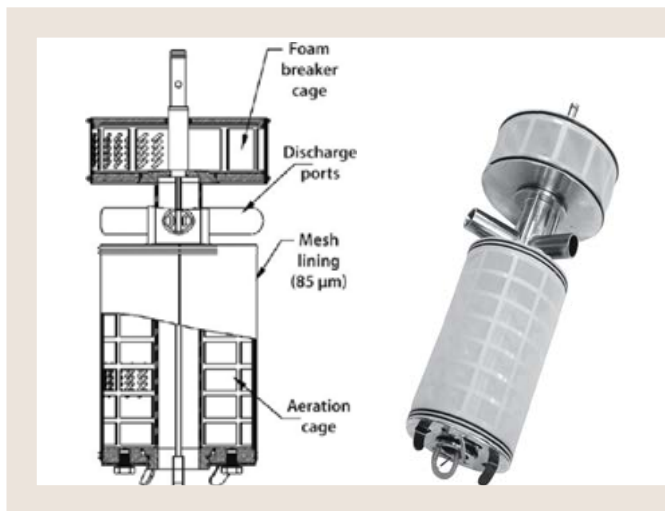


Fig. 2. New Brunswick Cell Lift Impeller (Eppendorf Inc). Patented design consists of three discharge ports located on the impeller shaft to provide uniform circulation without traditional spinning blades for conducting microcarrier cultures under ultralow-shear conditions. The flow is driven by centrifugal force, the rotation of the three ports creates a low-differential pressure at the base of the impeller shaft, lifting microcarriers up through the hollow shaft and expelling them out through its ports (The discharge ports must be submerged during operation). Bubble-shear is eliminated by the Cell Lift impeller, which utilizes a ring sparger generating bubbles only within the aeration cage, so that the oxygenation works without any bubbles coming into contact with the cells.

The cells were cultivated in media containing 10% serum, first grown as a monolayer, and when the cell density reached $1.0\text{--}1.2 \times 10^6$ cells/mL, they were transferred to the bioreactor containing 25 g/L of Cytodex-1 for perfusion culture. The virus preparations, also cultured in roller bottles, were infected with the PV2061 virus strain, harvested and transferred to the bioreactors.

Wang et al have described a purified Vero cell rabies vaccine that has been widely produced in China, which is responsible for almost two-thirds of the total rabies vaccines used in Asia [22]. This successful offering is a purified Vero cell vaccine, referred to as ChengDa Vaccine (Liaoning ChengDa Biological Co., Ltd., Shengyang, China [23]). It is grown on a Vero cell line utilizing the L. Pasteur 2061 strain of rabies virus, inactivated with β -propiolactone, lyophilized, and reconstituted in 0.5 mL of physiological saline. It fulfills the WHO recommendations for potency.

The process used at ChengDa was developed by Aycardi [24]. A single Eppendorf bioreactor was capable of producing one million dose of rabies vaccine per year. The method uses ultra-high density microcarrier cell cultures adapted to a 30 L New Brunswick CelliGen bioreactor equipped with a patented Cell Lift Impeller (Figure 2), specifically configured for a perfusion system to feed the growth media into the bioreactor. A specially designed decanting column (New Brunswick Scientific) was used to prevent perfusion loss of microcarrier and keep the cells in high concentration. The system delivers high oxygen transfer, high nutrient level and low shear stress, thus allowing cell growth up to 1.2×10^7 million cells/mL under continuous perfusion for up to 20 days.

ChengDa Vaccine was licensed by the Health Ministry of China and the State Food and Drug Administration of China (SFDA) in 2002 and has been marketed throughout the country since that time.

Influenza Virus Cultivation Strategies

The application of Vero cells for the propagation of influenza virus in animal-derived component free (ADCF) media was extensively described by Wallace et al in their US patent (no. 7,534,596 B2) [25]. The patent application includes the steps of attaching ADCF-adapted cells to a microcarrier (SoloHill® Engineering Inc.) and infecting the cells with vaccine media, producing virus within the cells and harvesting of the virus. The influenza viruses produced by this method achieved higher titer than that of the egg produced vaccine (Table 2).

Table 2. Comparing egg-based influenza production with Vero-cell-based production using Hillex II microcarriers (SoloHill Engineering).

Production System	Panama H1N1 Titers (log ₁₀ TCID ₅₀ /mL)
Egg	7.8
Vero: Serum-containing	7.9
Vero: Serum-free ADCF	8.0

A method for microcarrier-based expansion of cells from a 0.2 L spinner culture to a 2 L and 10 L bioreactor culture was developed (Figure 3). A New Brunswick CelliGen 310 bioreactor with a 5 L vessel was used for the 2 L culture stage. The vessel was equipped with a ring sparger, spin filter, 3-segmented pitched blade impeller (up-pumping), and 4-gas control at 100 mL/min (Air, N₂, CO₂, and O₂).

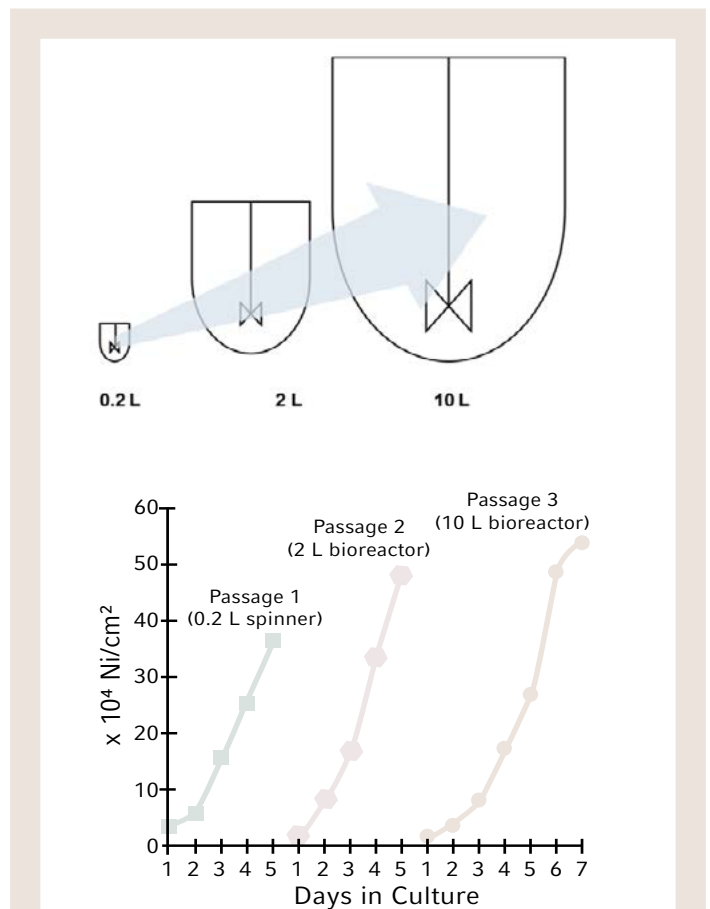


Fig. 3. Vero-based expansion on microcarriers; seed train of Vero cells cultured on Hillex II microcarrier beads (SoloHill Engineering).

Top: Diagram detailing bioreactor based expansion scheme; Bottom: Scale-up from Spinner flask to industrial bioreactors.

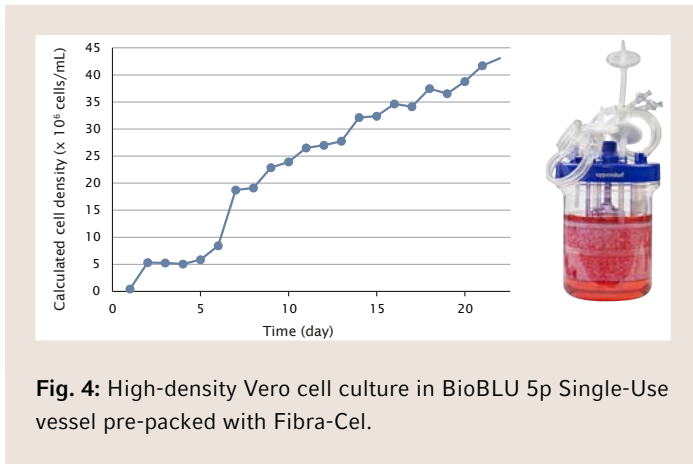


Fig. 4: High-density Vero cell culture in BioBLU 5p Single-Use vessel pre-packed with Fibra-Cel.

This expansion strategy couple with the demonstration of viral productivity represents an ideal closed system platform for vaccine production.

A similar method using Vero cell line for influenza vaccine production was demonstrated by chen et al [26]. Using Cytodex 1 microcarrier beads, these investigators were able to achieve cell densities of 2.6×10^6 /mL in serum free, protein free medium. These findings were obtained using a 250 ml Bellco microcarrier spinner flask equipped with a paddle impeller, inoculated with 2.5×10^5 /mL Vero cells in 5% CO₂ atmosphere. In a subsequent expansion phase, starting from an initial number of 5×10^5 /mL, the cells were expanded in a 3L bioreactor. After 24 hours the cells had adhered to the microcarriers and the virus was added together with fresh medium. Using these procedures, the authors were able to obtain high virus titers up to 10 Log₁₀ TCID₅₀/mL. They conclude that their approach could serve as a basis for large scale commercial production of influenza virus.

In 2011, Baxter International Inc. announced the approval for PREFLUCEL, the first Vero Cell based seasonal influenza vaccine, available for 13 participating European Union countries, including Germany, Spain, UK and the Scandinavian countries. Preflucel is comprised of purified, inactivated split influenza virions, manufactured using Baxter’s adaptation of the Vero cell platform. Although not approved for sale in the United States, data from a U.S. Phase III study with over 7,200 healthy individuals has shown that Preflucel provided 78.5% protective efficacy against subsequent culture-confirmed influenza infection, and robust immune responses against the three viral strains contained in the vaccine.

Recent Examples of Vero Cell Cultivation in Bioreactors

Although bioreactor based Vero cell culture has been widely used in vaccine production, the cultivation methods were typically guarded by vaccine manufactures. With increasing demand from our customer base, Eppendorf bioprocess applications lab developed a number of bioreactor application notes for attachment Vero cell culture.

In stirred-tank bioreactors, including both in R&D and in actual vaccine production, Vero cells are often grown on microcarriers or 3D support matrix such as Fibra-Cel. We have evaluated our Fibra-Cel disks as an attachment matrix because of their auspicious surface to-volume ratio. We find that they provide an optimal three-dimensional environment, protecting cells from damaging shear forces, allowing the realization of much higher cell densities by enabling perfusion culture. In perfusion bioprocessing, 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. We cultivated the cells in perfusion mode, which ensures a consistent supply of nutrients and the removal of toxic byproducts. We cultivated Vero cells in BioBLU® 5p Single-Use vessels pre-packed with Fibra-Cel, regulating the process with a BioFlo 320 bioprocess control station. We achieved high cell densities, up to 4.3×10^7 cells per mL (Figure 4) [27]. We believe that this provides strong support for Vero-cell-based vaccine production using Fibra-Cel packed-bed vessels (Table 3).

Table 3. 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	7200
T-175	172	1028
Roller bottle	850	212
10-layer stacked plate	6300	29

In another program to provide a method to inoculate larger bioreactors packed with Fibracel, we have evaluated spin filter as the means to increase inoculation yields. The spin filter allows easy perfusion of microcarrier based cell culture without the need for acquiring external perfusion devices [28]. We cultivated Vero cells on Cytodex 3 microcarriers at a density of 10 g/L in an Eppendorf 3 L glass vessel using a microcarrier spin filter coupled with a pitched-blade impeller. This device 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 (Figure 5). The process was controlled with a BioFlo 320 bioprocess control station without needing any additional perfusion devices. The design of the spin filter permits cultivation of anchorage-dependent Vero cells in perfusion mode on microcarriers while ensuring a consistent supply of nutrients and the removal of toxic byproducts.

At a modest microcarrier loading density of 10 g/L, we achieved attachment Vero cell culture density of 8.0×10^6 cells/mL in 9 days [28], sufficient for inoculation of a 40 L CelliGen 510 Fibracel packed-bed bioreactor designed for vaccine production. The CelliGen 510 has become the leading Rabies vaccine production platform in China, and the method, including CelliGen 510 itself, has been written into Chinese FDA guidelines as part of their production method recommendations. However, due to the very high surface capacity, it typically requires several stacked-plate culture devices to produce enough cells for inoculation. We believe this simple spin-filter perfusion platform has great potential to replace stacked plates for inoculation of larger bioreactors in vaccine production.

Conclusions

The combination of advances reviewed here provides strong support for the use of cell culture systems for virus vaccine production. The fact that Vero cells have been approved for vaccine products represents an important step on the road to technologies that do not rely upon hens' eggs for generation of adequate quantities of viruses. Advances in culture media enable the elimination of serum, thus driving the rapid and efficient purification of proteins. Whereas serum-containing media may continue to occupy a default position, it is now generally recognized that serum-free media are the optimal choice. The use of microcarrier or Fibracel add to the efficiency of the culture technology, allowing greatly increased cell densities to be reached. Finally, improvements in bioreactor design combined with these various technological advances result in a greatly improved and more functional production train.

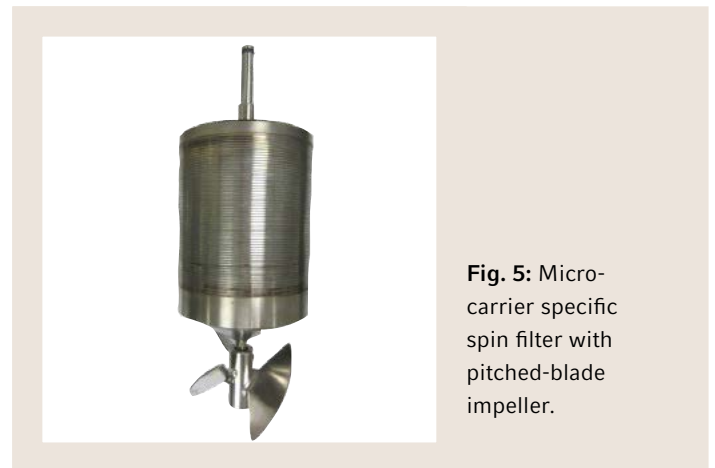


Fig. 5: Micro-carrier specific spin filter with pitched-blade impeller.

The tumultuous events of the past year, dominated by the Covid-19 pandemic, have brought us the overarching need for a Covid-19 vaccine. The range of Eppendorf bioreactors offers important opportunities for vaccine research, development as well as production. Our vaccine-enabling cell culture technologies are not limited to Vero cells, but can be used for other attachment mammalian cell culture and vaccine production as well.

According to our existing vaccine customer base, the Eppendorf packed-bed platform is the most effective attachment cell-based production platform on the market, achieving well over 100 million cells/mL in mammalian cell culture. With our expertise and specialized equipment, ranging from microcarrier spin filter, cell-lift impeller, packed-bed bioreactors, and single-use solutions, we offer a wide range of technologies well suited for vaccine research and manufacturing.

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