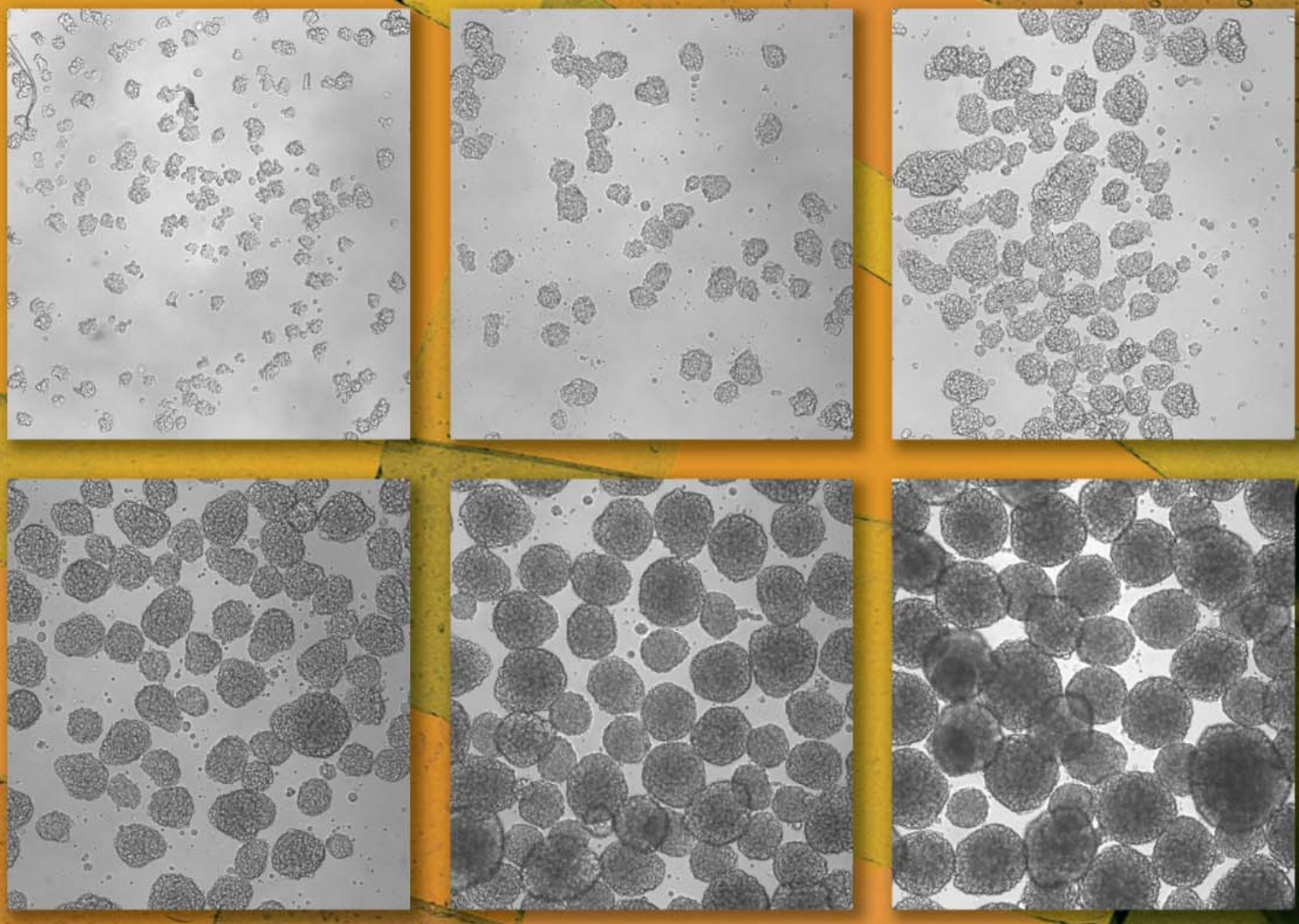
A Publication of the
WilBio Institute for BioProcess Technology

Fall 2007
ISSN 1538-8786

BioProcessing JOURNAL

Trends and Developments in BioProcess Technology



Vol. 6/No. 3

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Vero Cells in Vaccine Production

By JONATHAN H. MORGAN

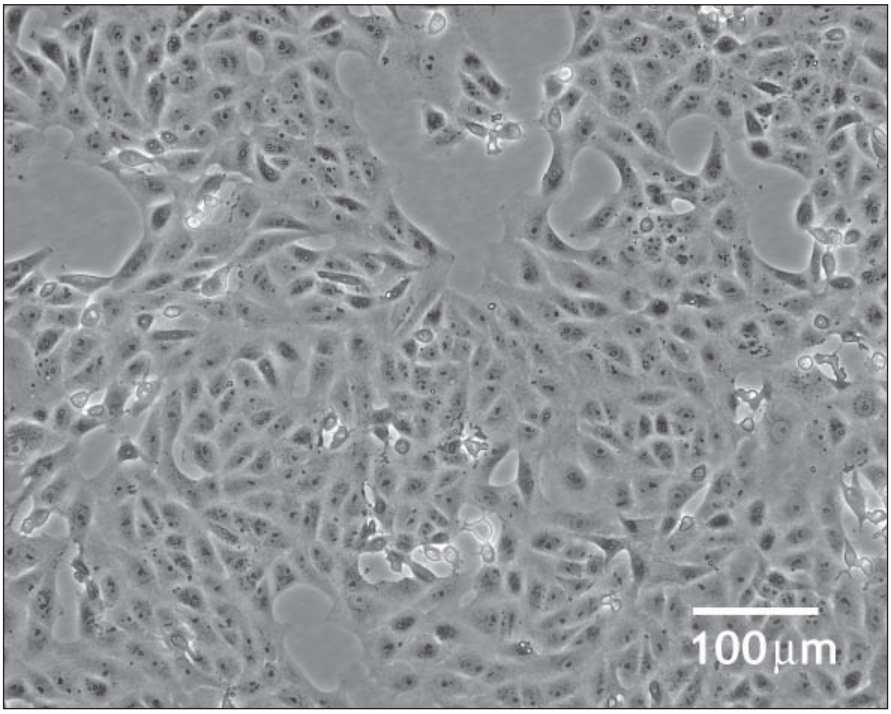

Figure 1. A monolayer of Vero cells near confluence.

The Vero cell line is one of the most widely used continuous cell lines in the world, cited in over 10,000 publications. Though originally developed as a host for viral replication, uses for this highly adaptable cell line have expanded far beyond the research laboratory to include diagnostic practices in hospitals, epidemiological surveys, *in vitro* fertilization clinics, bacterial toxin assays, and vaccine production. ATCC has played a pivotal role in this expansion by distributing the Vero cell line, ATCC® CCL-81™, to the worldwide scientific research community. Recently, ATCC developed a fully-characterized master cell bank of Vero cells prepared under current good manufacturing practice (cGMP) conditions (ATCC® CCL-81.4™). This report traces the history of the Vero cell line from its origins in the laboratory of Dr. Yosihiro Yasumura to its use as a continuous cell substrate for vaccine manufacturing.

Vero cells were initiated from the kidney of an apparently normal adult African green monkey (AGM, *Cerocopithecus aethiops*) on March 27, 1962 by Dr. Yasumura at the Chiba University in Chiba, Japan.[1] Dr. Yasumura's primary goal in establishing the Vero cell line was to find a suitable host cell to study simian vacuolating virus 40 (SV40). From his experience with poliovirus vaccine manufacturing, he believed a continuous monkey kidney cell line would support the growth of a wide range of viruses and be quite useful to virologists. Unfortunately, the establishment of such a line was fraught with problems owing to simian viral contaminants and other adventitious agents. While establishing the Vero line, Dr. Yasumura went to great lengths to minimize the potential for contamination, taking such steps as autoclaving his cell culture medium and reducing (~2%) or eliminating bovine calf serum from the medium. After several years of effort, including more than 18 attempts using tissue from five different monkey species, he achieved success with the establishment of the Vero line.

Dr. Yasumura derived the name of these cells by combining the Esperanto words "verda" (for green) and "reno" (for monkey's kidney). In Esperanto grammar, "VE + RE" changes to "VE + RO" when used as a noun. The term VERO also means "truth" in Esperanto; hence Vero has two meanings in its name.[2]

Dr. Bunsiti Simizu brought the Vero cell line from Chiba University to the United States in 1964 to the Laboratory of Tropical Virology (LTV), National Institute of Allergy and Infectious Diseases (NIAID), and the National Institutes of Health (NIH) at the 93rd passage. At that time, Dr. Simizu was a visiting fellow in the laboratory of Dr. Ned Wiegenga. The cells were subsequently deposited with ATCC in March 1967 by Drs. J. S. Rhim and W. D. Hann (also at the LTV) at the 113th passage. During this transition, the cells were adapted to grow in Eagle's minimal essential medium (EMEM) supplemented with non-essential amino acids and between 5% and 10% fetal bovine serum (FBS). More recently, Vero cells have been adapted to grow in serum-free and animal component-free medium.[3]

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True to Dr. Yasumura's vision, Vero cells support the growth of a wide variety of viruses and are extensively used for viral isolation, replication, and plaque assays. Viruses that readily replicate in Vero cells (or in any one of the several Vero clones such as Vero C1008,[4] ATCC® CRL-1586™, Vero 76,[4] ATCC® CRL-1587™, and B-Vero[5]) include members of the *Adenoviridae*, *Arenaviridae*, *Bunyaviridae*, *Flaviviridae*, *Flivoviridae*, *Herpesviridae*, *Orthomyxoviridae*, *Paramyxoviridae*, *Picornaviridae*, *Polyomaviridae*, *Poxviridae*, *Reoviridae*, *Rhabdoviridae*, *and Togaviridae* families.[6,7] Following viral infection, Vero cells do not produce interferon, though they remain responsive to it.[8] Apparently, the cell line lacks the structural genes for interferon α and β_1 due to a chromosomal deletion.[9] Further, the expression of the interferon-induced double-stranded RNA-dependent protein kinase (PKR), a key antiviral enzyme, is significantly attenuated in Vero C1008 cells.[10] These facts may account for the line's exceptional viral susceptibility.

Vero cells also support the growth of several families of parasitic and communalistic bacteria such as *Mycoplasmataceae*,[11] *Rickettsiae*,[12] and *Chlamydiaceae*.[13] They are also used to screen for various bacteria toxins including Shiga toxin and Shiga-like toxin (initially called Verotoxin).[14]

Depending upon the culture conditions and culture density, Vero cells can demonstrate either a fibroblast-like or epithelial-like morphology (Figure 1). Unless reasonable care is taken to ensure appropriate and consistent culturing conditions,[15] the cells will permanently change their phenotype, particularly upon prolonged passage (see the discussion below on *in vivo* tumor formation by Vero cells at high passage numbers). Indeed, this phenotypic plasticity was exploited to establish the several unique Vero subclones mentioned above.

When examined at passage 115,[16] 117,[15] or 123 (Table 1), greater than 90% of the cells were found to contain between 55 and 62 chromosomes with a modal number of 58. This is less than the 60 diploid chromosomes found in normal AGM cells.[17] Additionally, there were significant variations in the morphology among chromosome constituents. Over 50% of the chromosomes in each cell complement belonged to structurally altered marker chromosomes: A3, A4, B4, and B5 were absent; B2, B3, and B7 were occasionally paired; and B9, C1, and C5 were mostly paired.[15] Direct comparison between normal AGM and Vero cell chromosomes was difficult, if not impossible.[16] Presumably, the structural changes of the chromosomes found in Vero cells today were induced during the 100-plus passages after the line was first developed by Dr. Yasumura.

Vero Cells and Poliovirus Vaccines

Beginning in the mid-1970s, scientists at Institut Mérieux (IM, now known as Sanofi Pasteur), led by Bernard Montagnon, began the development of

Table 1. Characterization of the Vero MCB at passage 123. All tests were performed in compliance with applicable requirements of the US FDA GMP regulations as found in Title 21 *CFR* Parts 58, 210, and 211. All tests for adventitious agents and *in vivo* tumorigenicity were negative. Identity of the cell line was confirmed by both isoenzyme and cytogenetic analysis. Upon thaw, the cells had a viability of 98%.

Adventitious Agent Testing	
STUDY	**TEST**
Sterility	USP (United States Pharmacopeia) 27
Bacteriostasis and Fungistasis	Direct inoculation method into FTM (3 bacteria panel) and SCDM (3 fungi panel)
Mycoplasma	*Points to Consider in the Characterization of Cell Lines Used to Produce Biologicals* (1993), US FDA
General Viral Testing	28-day *in vitro* assay using MRC-5, Vero, and primary AGMK as indicator cell lines monitoring for both cytopathic effect (CPE) and hemadsorption
	Inoculation into guinea pigs, mice (weanling and suckling), and embryonated chicken eggs (*in vivo*)
	Ultrastructural evaluation of cell cultures for viral particles, with characterization and tabulation of retrovirus-like particles by transmission electron microscopy
General Retroviral Testing	PCR-based reverse transcriptase assay (PBRT) using fluorescence detection of amplification
Bovine Viral Testing	9-virus panel monitoring for CPE, hemadsorption, and immunofluorescence in indicator cell lines
Porcine Viral Testing	8-virus panel monitoring for CPE, hemadsorption, and immunofluorescence in indicator cell lines
Human Viral Testing	Cytomegalovirus (CMV) by PCR
	Human immunodeficiency virus (HIV) by PCR
	Human T-cell lymphotropic virus (HTLV), type 1 and 2, by PCR
	Epstein-Barr virus (EBV), type 1 and 2, by PCR
	Hepatitis A virus (HAV) by RT-PCR
	Hepatitis B virus (HBV) by PCR
	Hepatitis C virus (HCV) by RT-PCR
Simian Viral Testing	Simian T-lymphotropic virus (STLV) by PCR
	Simian immunodeficiency virus (SIV) by PCR
	Simian foamy virus (SFV) by PCR
	Simian virus 40 (SV40) by PCR
	Simian retrovirus (SRV-1/2/3) by PCR
Identity Confirmation	Isoenzyme and cytogenetic analysis
***In Vivo* Tumor Formation**	Tumorigenicity in athymic nude mice at 24 and 87 days post-injection, *Points to Consider in the Characterization of Cell Lines Used to Produce Biologicals* (1993), US FDA

a new enhanced potency inactivated polio vaccine (IPV). At that time there were two effective vaccines against polio virus infections: the inactivated Salk vaccine and the live-attenuated Sabin oral polio vaccine (OPV). Both were prepared in either primary monkey cells or diploid human cells. The Sabin OPV was preferred over the Salk IPV because it provided greater immunity, had lower manufacturing costs, and was easier to administer.[18] Nonetheless, there was a pressing need for a more immunogenic IPV, particularly for immunocompromised patients.

Since high potency of the new IPV was critical for success, the IM team elected to use the industrial-scale microcarrier bioreactor production system developed by van Wezel.[19] Initial attempts to manufacture the vaccine with primary monkey kidney cells were unsuccessful because the vaccine potency was too low. For that reason, the IM team turned to Vero cells (obtained from ATCC) at the suggestion of Dr. Jonas Salk.[20] Vero cells were an obvious choice since they were derived from the same species and tissue as the primary cells used for polio vaccine manufacturing, and they were presumed to be non-tumorigenic.

On the one hand, by using a continuous cell line, the IM team was able to mitigate the numerous drawbacks inherent in the use of primary cells including: a) uncertainty of supply; b) lot-to-lot variations in the final vaccine; c) complications with using bioreactor and serum-free culturing conditions; d) economic and ethical issues associated with catching wild monkeys or maintaining monkey colonies; and e) the risk of potentially fatal contamination with adventitious agents such as Marburg virus[21] and SV40.[22]

On the other hand, by selecting a continuous cell line in place of primary monkey kidney cells or a diploid human cell line like WI-38 or MRC-5, the IM team faced a unique challenge—up to that time, no continuous cell line had been used as a substrate for a licensed vaccine. Because vaccines are administered to otherwise healthy individuals with the intention of preventing disease, they and their cell substrates are held to a higher level of safety by regulatory agencies than other licensed products. Generally, whole virus vaccines, either live-attenuated or inactivated, cannot be as thoroughly purified as vaccines or biologicals derived from recombinant-expressed proteins, complicating matters even further. This is due to either: a) the complexity of the virus itself; b) the need to enhance the vaccine's immune response; or c) a desire to achieve a specific cost-of-goods target. Consequently, there is a greater chance for components of the cell substrate, particularly cellular DNA, to pass into the final licensed product and ultimately, the patient.

During the course of their work, the IM team developed and employed several procedures for working with continuous cell substrates that assured the safety of the final vaccine. They incorporated a cell banking system (seed – master – working) where they verified the identity and source of the cell line, tested to ensure the absence of adventitious agents, determined the amount of residual cellular DNA in the final vaccine, and then tested the cell line for *in vivo* tumorigenicity. All of these steps are now key criteria for the manufacture of continuous cell substrates used in vaccine production in guidance documents from the World Health Organization (WHO),[23] the European Agency for the Evaluation of Medicinal Products (EMEA),[24] the Japanese Ministry of Health, Labor and Welfare,[24] and the US Food and Drug Administration (FDA).[11]

While Vero cells were initially thought to be non-tumorigenic, and the IM team found no evidence for *in vivo* tumor formation,[20] later studies demonstrated that cells at high passage numbers were tumorigenic.[25-29] However, the passage number required to form tumors *in vivo* was higher than those attained by the IM team in their vaccine manufacturing runs.

The initial enhanced IPV developed by the IM team was licensed in France in 1985,[30] in the United States in 1990,[31] and is now licensed in over 60 countries worldwide. Subsequently, the IM team produced a live-attenuated OPV and a rabies vaccine using Vero cells.[32] Vero cells have been used to produce a vaccine against Japanese encephalitis.[33] Recently, two rotavirus vaccines manufactured in Vero cells (RotaTeq® from Merck & Co., and Rotarix® from GlaxoSmithKline) have been licensed.

Following their success with the enhanced IPV, the IM team donated a bank of Vero cells to the World Health Organization (WHO 10-87, passage number 134).[34] This bank was designated as a master cell bank (MCB) by the organization in 1998[23] and is distributed to national control authorities and producers of biologicals by ATCC and the European Collection of Animal Cell Cultures (ECACC). As recommended by a WHO study group, the establishment of such a bank ensured a constant supply of cells with stable properties for vaccine manufacturing.[35]

Bovine Spongiform Encephalopathy and Cell Vaccine Substrates

Beginning in 1991, the FDA's Center for Biologics Evaluation and Research (CBER) issued a series of letters and guidance documents to vaccine manufacturers over concerns about possible transference of agents that may cause spongiform encephalopathies, particularly those of bovine origin.[36] This action was taken following the emergence of bovine spongiform encephalopathy (BSE, "mad cow disease") in Europe in the 1980s. Cell lines in use since 1980 may have been exposed to media containing serum or other protein-containing components derived from bovine sources. Consequently, these bovine sources might have been contaminated with the causative agent of BSE. Similar guidance documents were issued by EMEA.[37]

To minimize the possibility of contamination during the manufacturing processes, CBER recommends that manufacturers not use materials derived from cows that were born, raised, or slaughtered in countries on the BSE list. This list, maintained by the US National Center for Import and Export Veterinary Services (NCIE),[38] includes all countries where BSE is known to exist, as well as countries in which import requirements or surveillance are inadequately restrictive for import into

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the United States—even when there is no record of BSE in the region. A similar list is maintained by the European Food Safety Authority (EFSA).[39]

In July 2000, CBER convened a joint meeting of the Transmissible Spongiform Encephalopathy Advisory Committee and the Vaccines and Related Biological Products Advisory Committee to evaluate the risk for BSE transmission from vaccines manufactured with bovine-derived materials. They concluded that the risk was low. However, the joint committee recommended that in addition to using fully traceable reagents for all future vaccine manufacturing runs, current lots of vaccines and working cell banks established after 1980 should be re-derived using fully traceable reagents.[40] Any bovine-derived reagents used for this effort needed to be obtained from countries not on the BSE list. EMEA issued similar regulations for European licensed vaccines.[41] Since then, all manufacturers of licensed vaccines have complied.

In 2003, following this recommendation, the WHO Monitoring Group on Cell Banks found possible deficiencies in the production records of the 10-87 bank and these might have regulatory implications for the MCB status of that bank.[42] The WHO re-designated their 10-87 Vero master cell bank from "MCB status" to "well-characterized cell seed" status. While these cells should no longer be used for direct vaccine manufacturing, they could be used to derive subsequent MCBs.[43]

The WHO further noted that the re-designation of the bank might lead vaccine investigators to use the cells at passage levels higher than previously recommended should they re-derive an MCB.[43] By starting at passage number 134, cells from the 10-87 bank could easily exceed the WHO's recommended 150 end-of-production passage number[23] when cultured through the master bank, working bank, and manufacturing stages of vaccine production. Vero cells above passage number 150 have a high risk of tumor formation *in vivo*.

ATCC's Vero MCB

When the WHO's Vero MCB was re-designated, ATCC saw a need to develop a replacement cell substrate that specifically met the requirements for vaccine development and manufacturing. Making such a well-qualified and highly-characterized bank available would save vaccine investigators time and money by eliminating the need for them to derive their own MCB. Additionally, in concordance with the original WHO goal, it would ensure a constant supply of cells with stable properties.

The ATCC bank (ATCC® CCL-81.4™) was started from one of the initial vials of Vero cells deposited with ATCC in 1967, thereby establishing a known pedigree while allowing for full traceability. This deposit date was well before the FDA-established 1980 cut-off date for risk concerns of BSE in materials used in vaccines. The cells were grown under cGMP conditions using fully traceable and documented culture reagents (EMEM 90%, and FBS 10%). A full passage history of this bank is shown in Figure 2.

The cells were characterized under cGMP conditions using fully validated tests for identity, adventitious agents

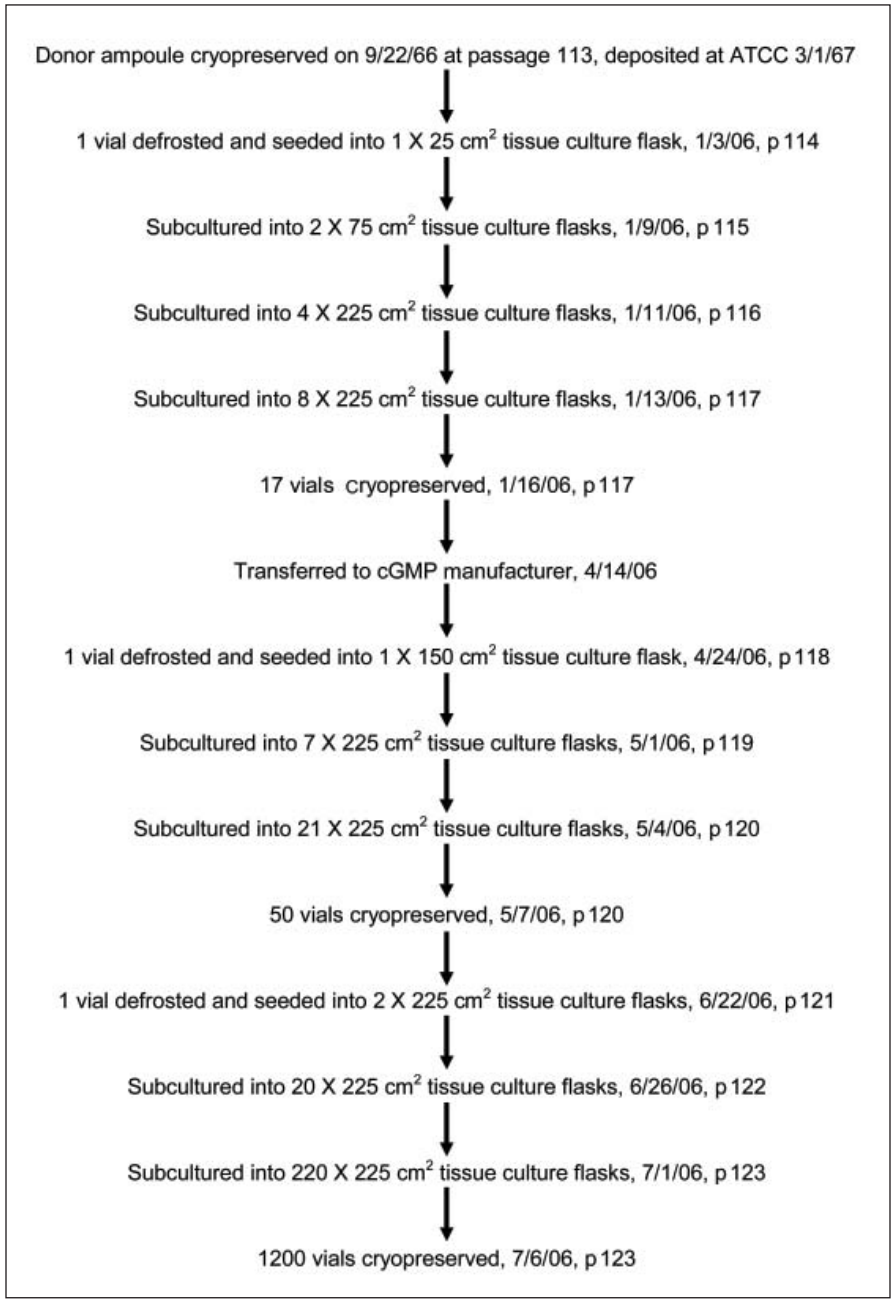

Figure 2. Passage history of ATCC® CCL-81.4™ Vero MCB.

and tumor formation *in vivo*. No adventitious agents were found, and the cells did not form tumors *in vivo* after 87 days. A list of these characterization tests is provided in Table 1. At cell passage number 123, the bank ensures the manufacturing of effective, high-titer vaccines with minimal likelihood of *in vivo* tumor formation by the end-of-production passage.

This ATCC Vero MCB is available for use by vaccine investigators. It is currently being used by the nonprofit organization PATH for developing and manufacturing a new type of vaccine against rotavirus.[44]

To date, Vero cells are the only continuous cell substrate used in the production of FDA-licensed vaccines.[45] This cell line's ability to support the high-titer growth of a wide range of viruses, their capacity to grow to high densities in bioreactors under animal component-free culture conditions, and the excellent safety record of the vaccines generated from them[46] all combine to make Vero cells the cell substrate of choice for viral-vaccine development—an enduring testament to Dr. Yasumura's pioneering efforts.

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