FDA Briefing Document
Vaccines and Related Biological Products Advisory Committee Meeting
September 19, 2012

Cell Lines Derived from Human Tumors for Vaccine Manufacture

Table of Contents

Cell Lines Derived from Human Tumors for Vaccine Manufacture

Attachments:

1. Guidance for Industry (February 2010): "Characterization and Qualification of Cell Substrates and Other Biological Materials Used in the Production of Viral Vaccines for Infectious Disease Indications"

2. Review Article (Anal. Chem. 83, 4327-4341; May 25, 2011): "Landscape of Next-Generation Sequencing Technologies" by Thomas P. Niedringhaus, Denitsa Milanova, Matthew B. Kerby, Michael P. Snyder, and Annelise E. Barron

1. Introduction

This meeting of the Vaccines and Related Biological Products Advisory Committee (VRBPAC) is being held to discuss the use of cell lines derived from human tumors as substrates for the production of preventive viral vaccines.

Over the last decade, it has become clear that the current repertoire of cell substrates is inadequate to manufacture the next generation of viral vaccines (i.e., certain viruses cannot be propagated or grow poorly in the available cell lines). Therefore, manufacturers have submitted additional cell lines to the FDA for consideration for use in the production of viral vaccines. All of the new mammalian cell lines being considered are immortal, having been transformed by various oncogenes or are spontaneously immortalized, and some are derived from human tumors. Over the last 15 years, when new types of cell substrates have been proposed, starting in 1998, CBER has presented its review approach to the Advisory Committee to address the issues raised first by the use of immortalized mammalian cell lines and then tumorigenic cell lines. The purpose of those discussions with the Advisory Committee was to obtain their input and to make public a discussion of the issues. This current VRBPAC meeting is a continuation of this process.

To date, only a limited number of preventive vaccines made in tumorigenic cell lines have entered clinical trials. Some live-vector vaccines made in laboratory-immortalized human cells have entered clinical trials (VRBPAC 2001), as have some inactivated vaccines manufactured in non-human tumorigenic cell lines (VRBPAC 2005). However, tumor-derived cell lines have not been used for production of prophylactic vaccines (see Section 2 for definitions). The purpose of the present VRBPAC meeting is to discuss the scientific issues associated with the use of cell lines derived from human tumors for the production of both live and inactivated prophylactic viral vaccines and to identify safety concerns and determine how they can be addressed. As background, this briefing package includes a brief description of the history of cell substrates used for viral vaccine manufacture in the U.S., information on the potential concerns associated with the use of tumor-derived cells, and finally, information on the Agency's current practices on reviewing new cell substrates with respect to the presence of adventitious agents and residual cell-substrate DNA.

2. Background

In this document, tumor-derived cell lines refer to lines established from cells isolated from tumors. Tumorigenic cell lines refer to cells capable of forming tumors in an immune-compromised rodent. Continuous cell lines are immortal and may be established from cells isolated from tumors or from cells originally isolated from healthy tissues and transformed *in vitro* spontaneously or by infection with an oncogenic virus or by DNA transfection with oncogenes or known immortalizing genes such as TERT. On the other hand, diploid cells are mortal (*i.e.*, they have a limited lifespan in culture) and diploid cell lines are established from cells isolated from healthy tissues. While diploid cell lines are

not expected to be tumorigenic, continuous cell lines may or may not exhibit a tumorigenic phenotype.

2.1 History of Cell Substrates for Viral Vaccines Manufactured in the U.S.: Primary, Diploid and Tumorigenic Cells

The following are selected highlights of the use of cell substrates for growth of virus for production of preventive vaccines in the U.S. (see Appendix 1 for details on discussions with the VRBPAC and other meetings on cell substrates):

1950s and 1960s: Primary cell cultures derived from rhesus macaque kidneys were used to grow virus to manufacture inactivated poliomyelitis vaccine (IPV) and live-attenuated oral poliovirus vaccine (OPV).

Embryonated hens' eggs or primary chick embryo fibroblast cultures were used to grow virus to manufacture vaccines against yellow fever, influenza, measles, and mumps. These substrates were considered acceptable both for live-virus vaccines (yellow fever, measles, and mumps) and inactivated-virus vaccines (influenza), since it was believed that adventitious agents that could infect chickens would not infect and be pathogenic for humans.

1960s to 1990s: Human diploid cell lines derived from fetal lung tissue were used to grow virus for the production of a rubella vaccine (WI-38 cells) and for a polio vaccine, a hepatitis A vaccine, and a rabies vaccine, and the varicella vaccine (MRC-5 cells). A diploid cell line derived from rhesus monkey lung cells (FRhL-2) was used for a rotavirus and a rabies vaccine.

1990s to 2000: A continuous, non-tumorigenic cell line derived from the kidney of an African green monkey (Vero) was first used for the manufacture of an inactivated poliomyelitis vaccine (IPV) and then a live-attenuated oral poliomyelitis vaccine (OPV). The use of Vero cells to grow virus for live viral vaccines was the topic of discussion at a VRBPAC meeting in 2000. The VRBPAC supported the use of Vero cells for production of inactivated vaccines. However, some members expressed the concern that Vero cells had the capacity to become tumorigenic with prolonged passage in culture and recommended that the cells not be used beyond a specific number of passages. In addition, several advisory committee members expressed reservations regarding the use of Vero cells to manufacture live virus vaccines; however, following additional discussion and refinement of manufacturing controls to mitigate potential risks, three live viral vaccines that were made using low-passage non-tumorigenic Vero cells have been licensed in the US (Smallpox vaccine in 2007; and two rotavirus vaccines in 2006 and 2008).

1990s: The use of tumorigenic cell lines to grow virus to produce vaccines against emerging pathogens was discussed by the VRBPAC in 1998. The topic for discussion was the appropriate approach/method to assess the risks associated with the use of such cells in the production of these vaccines. The Defined Risks Approach, which

experimentally estimates the likelihood of a risk event occurring, was presented to the committee. This approach requires identification of specific risk events and experimental measurement of their frequency using quantitative assays. The committee generally concurred with this approach.

2000s: The first tumorigenic cell line to be considered for use in the production of a live viral vaccine was PER.C6 for the production of a replication-defective adenovirus vectored HIV-1 vaccine (VRBPAC 2001). Since the adenovirus type 5 (Ad5) vector was deleted in the early region 1 (E1 region), human cells expressing the E1 genes in *trans* were required to complement the defect in the viral vector. The PER.C6 cell line was generated in a laboratory by immortalizing human fetal retinal cells with the E1 region of Ad5. This was the first example of a "designer-cell substrate", since it was developed specifically to eliminate the production of replication-competent adenovirus during manufacture. In addition, because the mechanism of immortalization was known, the level of concern with these cells was reduced. Following evaluation of the comprehensive testing plan for these cells and consideration of the purification steps during manufacture, the VRBPAC supported use of PER.C6 cells for manufacture of replication-defective Ad5-vectored vaccines against HIV-1. The testing plan included quantification of the tumorigenic phenotype of PER.C6 cells and evaluation of the oncogenicity of PER.C6 DNA and cell lysates in newborn nude mice, newborn hamsters, and newborn rats. Although this extensive oncogenicity testing for the characterization of a vaccine cell substrate was done for the first time and the assays were not standardized, the VRBPAC considered that, in the absence of other assays, they were acceptable for this purpose. In addition, testing for adventitious agents was more extensive than the usual *in vivo* and *in vitro* tests recommended at the time and included a comprehensive list of specific PCR tests for potential virus contaminants.

The next tumorigenic cell line under consideration was the Madin-Darby canine kidney (MDCK) cell line, which was proposed for the production of inactivated influenza virus vaccines (VRBPAC 2005). The MDCK cell line is a spontaneously immortalized cell line established from the kidney of an apparently normal dog. Some variants of MDCK cells are highly tumorigenic, while others are not [reviewed in Omeir *et al*. (64)]. The Committee accepted the use of tumorigenic MDCK cells for inactivated influenza vaccines and their evaluation in clinical trials.

Additionally, a non-tumorigenic MDCK cell line was discussed for the manufacture of a live, attenuated influenza virus vaccine (VRBPAC 2008). Since this clone of MDCK cells was not tumorigenic, the level of residual cell-substrate DNA in the final product was low and reduced in size, and the route of vaccine administration was intranasal, the majority of the Committee supported the evaluation of this vaccine in clinical trials.

2.2 Cell Substrates Derived from Human Tumors Proposed for the Manufacture of Viral Vaccines

Three cell lines derived from human tumors will be discussed during the September 2012 VRBPAC meeting: the CEM leukemia T cell line, the A549 lung adenocarcinoma cell

line, and the HeLa cervical carcinoma cell line. These cell lines serve as the basis for this discussion because sponsors have proposed their use in the production of vaccines for evaluation in clinical trials. CBER requests that these cells be viewed as representative of this type of cell substrate so that the recommendations of the advisory committee will be applicable to other tumor-derived cell lines (human and non-human) proposed for vaccine manufacture in the future.

Over the last 20 years, it has been recognized that cell lines derived from tumors may be the optimal and in some cases the only cell substrate that can be used to propagate certain vaccine viruses. For example, to produce HIV-1 in sufficient quantities for inactivated HIV-1 vaccines, cell lines derived from human CD4-positive T cells are preferred over primary T cells. The first such vaccine submitted to the FDA was an inactivated HIV-1 produced in the T-cell line HuT78, which was derived from a human lymphoma (24). The vaccine was an inactivated HIV-1 intended as a therapeutic vaccine for the treatment of HIV-1-infected individuals and was evaluated in clinical trials in the 1990s (59, 60). The appropriateness of using HuT78 cells to produce an HIV-1 vaccine was discussed by the VRBPAC in 1998, and while there were questions as to the need to use such cells, the Committee accepted that alternative cell substrates were not feasible.

The cell lines under consideration for this meeting are:

- The CEM T-cell line established from cells from an individual with leukemia (22) and is highly permissive for HIV-1 strains that use the co-receptor CXCR4.
- The A549 cell line established from cells excised from the lung of an individual with adenocarcinoma of the lung (51). The A549 cell line is highly susceptible to human adenovirus infection and is used both to detect adenoviruses and to produce replication-competent adenoviruses for certain investigational vaccines (4).
- The HeLa cell line, which was established from cells of a cervical carcinoma in 1952 (25, 26). These cells contain approximately 50 copies of the HPV-18 genome and constitutively express the viral oncoproteins E6 and E7 (38, 45, 79). The HeLa cell line is permissive to many human viruses. HeLa cells have been engineered to be producer lines for adeno-associated virus (AAV) (87). AAV has been used to produce investigational gene-therapy products (3, 21, 96) and investigational vaccines (90).

Summary:

Historically, each new type of cell substrate used for viral vaccine production has presented novel issues that have been addressed by CBER after scientific consultations with the VRBPAC, first in 1998 and then in 2000, 2001, 2005, and 2008. The present VRBPAC is a continuation of these discussions. The current repertoire of cell substrates is inadequate for manufacture of certain types of new vaccines. To address this limitation, sponsors have proposed the use of several novel cell substrates that are tumorigenic or derived from human tumors. Because these cell lines that have not hitherto been accepted by regulatory authorities for the manufacture of prophylactic vaccines, CBER has entered into discussions with the VRBPAC so that issues associated with these types of cells will be discussed in an open scientific forum.

3. Considerations Regarding the Use of Tumor-derived Cell Lines

When tumorigenic cell lines were first considered for vaccine manufacture in 1998 and then proposed for vaccine manufacture in 2001, three major safety concerns were identified that needed to be addressed: 1) the presence of residual live cells in the vaccine that might have the potential of being tumorigenic in humans; 2) the presence of residual DNA from the cell substrate; and 3) the potential presence of adventitious agents, including adventitious viruses that might have contributed to the tumorigenic phenotype. These same concerns also apply to tumor–derived cell lines from human tumors. In addition, with cells derived from human tissues there is also a heightened concern that unknown oncogenic factors may be present and contribute to the development of an oncogenic phenotype if they are co-purified with the vaccine virus.

To assist in the discussion of the risks associated with use of human tumor-derived cell lines for the manufacture of a viral vaccine and the development of strategies to mitigate these risks, this section of the briefing document summarizes the available information regarding how normal cells become tumor cells.

3.1 Mechanism of Tumorigenesis and Considerations for Characterization of Cell Substrates

Experimental data on the mechanism of cancer development has demonstrated that the transformation of a normal human cell to one with a tumorigenic phenotype is a multi-stage process. The tumorigenic transformation of normal cells can be caused by infectious agents (i.e., oncogenic viruses), somatic mutations, and epigenetic changes. The fact that multiple oncogenic events are required to establish a tumor has been considered to provide an additional safety margin for vaccines manufactured using a tumor-derived cell substrate for virus growth. However, while it has been reported that several oncogenic events are required to convert a normal human cell into a cell that can establish a tumor in an experimentally inoculated mouse (31), the concern remains that a single oncogenic “hit” can predispose a cell to subsequent oncogenic events that will, perhaps over many years, lead to a malignant cell. At this time, if such initiating events occur it is not possible to detect them. Nevertheless, because this is a theoretical possibility, regulatory authorities have required that multiple methods be used to reduce potential oncogenic factors in vaccines produced in such cell substrates.

The transformation of a normal cell to one with a tumorigenic phenotype is a multi-stage process. Below is a brief description of each of the mechanisms of tumorigenesis and how they can affect the safety profile of human-derived tumor cells as substrates for vaccine production.

3.1.1 Oncogenic Viruses

The first cancer virus described was the Rous sarcoma virus, a retrovirus that causes sarcomas in chickens (72, 73). The determination that the gene responsible for the neoplastic transformation was the *src* gene, a gene acquired from the chicken genome and modified during multiple virus infection cycles (41, 56, 57), was instrumental in establishing the field of oncogenes (85, 91). The subsequent identification of cellular analogues of many other viral oncogenes followed rapidly (7-9, 15, 92, 93, 100), as did the discovery of viruses that can cause human tumors (12, 18, 78).

Viruses can be oncogenic in several ways. They can carry dominant oncogenes that directly induce a transformed phenotype in the infected cell or they can integrate into the host genome and cause the activation of cellular oncogenes or the inactivation of tumor-suppressor genes. For example, viruses such as papillomaviruses, adenoviruses, polyomaviruses, gamma herpesviruses (such as EBV), and replication-defective and replication-competent retroviruses, such as MC29 and RSV, respectively, have been shown to carry oncogenes. However, in most cases, expression of the viral oncoproteins alone is insufficient to result in cancer, and subsequent genetic and/or epigenetic changes are necessary to convert the initially transformed cell into a malignant cell.

The presence of oncogenic adventitious viruses would represent the most likely source of oncogenic activity in a tumorigenic cell substrate, and these viruses would have the greatest potential for transmitting cancer. Thus, it is important to address whether there are any infectious adventitious viruses present in the cell substrate. To accomplish this, various virus-detection assays and clearance strategies are recommended for evaluating the safety of a tumorigenic cell substrate for use for vaccine manufacture (see Section 4.1).

3.1.2 Somatic Mutations

The acquisition and accumulation of somatic mutations contributes to the development of many cancers. For example, point mutations can result in conversion of proto-oncogenes to oncogenes, and chromosomal rearrangements and insertional mutagenesis, either through infection with an exogenous retrovirus or the mobilization of endogenous retroviruses or retrotransposons, can result in activation of proto-oncogenes by elevating their expression, as can gene amplification. Insertional mutations can also inactivate tumor-suppressor genes. However, because the two copies of tumor-suppressor genes are generally expressed, the oncogenic consequences of such an insertional mutation would require inactivation of the other allele, which would be a rare event. Tumor-suppressor genes can acquire gain-of-function mutations to become dominant-negative proteins, which can interfere with the function of the normal protein; only in this case would the oncogenic risk of DNA be increased.

The main safety issue related to transformation by somatic mutations is the presence of residual cell-substrate DNA in a vaccine and whether this DNA represents an oncogenic or an infectious risk. As discussed in Section 4.2, the oncogenic and infectious risk of

DNA can be reduced by lowering the amount of DNA, and/or decreasing the size of the DNA (by nuclease digestion), and/or by reducing the activity of the DNA (by chemical treatment or gamma irradiation).

3.1.3 Epigenetic Changes

Epigenetic mechanisms of gene regulation include: chromatin remodeling, histone modifications, DNA methylation, and altered microRNA (miRNA) expression. Although such mechanisms have been described to alter gene expression, it was not thought that the consequences of epigenetic events could be heritable, *i.e.*, passed on from one somatic cell generation to the next and thus permanently modify the cell's phenotype. However, recently it has been recognized that treatment of cells with certain agents or proteins can induce phenotypic changes through epigenetic processes, and that these phenotypes can be passed on to subsequent cell generations.

If a vaccine cell substrate becomes tumorigenic through epigenetic mechanisms, the oncogenic risk associated with using these cells for vaccine production is reduced for the following reasons:

- If the stimulus that induced the phenotypic change(s) in the cells was endogenous, either it is no longer present in that cell or, if still present, then its concentration in the vaccine produced from the cell would likely be too low for it to exert a biological effect in the vaccine.
- If the stimulus that had induced the phenotypic change in the cells was exogenous, it would not be present in the cell substrate and thus would not be present in the vaccine.
- If the outcome of the stimulus on the cells was to change the phenotype through alterations in gene expression *via* DNA methylation, the risk would be reduced to one associated with the DNA or chromatin. However, because changes in gene expression often involve multiple normal cellular genes, transmission of the tumorigenic phenotype to cells in the vaccine recipient *via* DNA or chromatin would be highly improbable, since it would require the transfer (uptake) and co-expression of multiple genes. Because these genes would not be expected to be linked, the co-transfer of two or more genes would be improbable. And because these genes are normal cellular genes, their expression would likely not be transforming. In addition, since oncogenesis through DNA methylation frequently involves the silencing of tumor-suppressor genes (6, 14, 19, 30, 39, 40, 43, 70), the uptake of such a silenced gene by cells would not be expected to alter the cell's phenotype due to lack of expression.
- If the outcome of the stimulus on the cells was to change the phenotype through alterations in gene expression *via* histone modifications and chromatin remodeling, the risk would again be reduced to one associated with the chromatin and transmission of these traits to other cells would be improbable as the factors affecting these modification requires the transfer and expression of multiple genes.

In summary, if the tumorigenic phenotype arose through epigenetic mechanisms, the capacity to transfer this phenotype to vaccine recipients *via* residual cellular components would be improbable or involve hitherto unrecognized mechanisms.

3.2 Metastatic Potential

Several types of cells can form tumors when inoculated into immune-compromised rodents; however, not all of these tumors can metastasize. The steps involved in metastasis are: the invasion of the basement membrane (invasion), entry of the blood or lymphatic systems (intravasation), survival in these tissues and migration to distant sites through the blood vessels or, more commonly, the lymphatics, traversal of the vessel walls (extravasation), and colonization of distant tissues (89). The mechanisms whereby cells become metastatic are unknown, and it is not clear how the fact that a cell forms metastases in an immune-compromised rodent influences the degree of risk for a vaccine manufactured in such a cell.

Several proposed mechanisms for the acquisition of a metastatic phenotype are briefly presented below (17). If a mechanism involving the activation of additional proto-oncogenes were operating, then compared with a non-metastatic cell, the DNA from a cell capable of metastasis might represent more of an oncogenic risk. However, if the generation of a metastatic cell results from the increased expression of proteins involved in invasion, intravasation, *etc*., or the reduced expression of proteins that maintain cell/cell junctions, then any residual DNA from metastatic cells in a vaccine would not represent more of a risk than DNA from non-metastatic cells.

If the generation of a metastatic phenotype involves single or multiple activated oncogenes, demonstration of DNA clearance in the vaccine can address this concern. Furthermore, for mechanisms involving multiple genes, the conversion of a normal cell to a metastatic cell would require the simultaneous uptake of multiple DNA fragments by a single cell, which would be an improbable event. Thus, with appropriate manufacturing controls, the use of tumorigenic cells that can metastasize in immunocompromised rodents may not represent an additional safety concern for the use of these cells for vaccine production.

Summary: Although the possibility of unknown oncogenic factors remains as a theoretical concern with cells derived from human tissues, current knowledge related to the mechanisms of oncogenesis supports the notion that it is highly improbable that any whole cell or complex of cellular factors from a tumor-derived cell will be present in sufficient quantities and lead to the development of tumors in the vaccine recipient. Thus, cellular DNA and adventitious agents are considered the major safety concerns for vaccines manufactured in tumorigenic cell lines or cell lines derived from human tumors. Therefore, extensive characterization for adventitious agents in the cell substrate and validation of DNA removal through the manufacturing process are required. The current recommendations for cell-substrate and vaccine testing with respect to adventitious agents and residual cellular DNA are discussed in Section 4.

4. Recommendations for Testing of Cell Substrate and Vaccines

Current recommendations for testing of mammalian cell substrates and vaccines are generally based on three main safety concerns: the presence of residual, potentially tumorigenic, live cells in the vaccine; the presence of residual DNA from the cell substrate; and the potential presence of adventitious agents.

The characterization of cell substrates includes tumorigenicity testing. This testing is described in the 2010 guidance document [*Guidance for Industry for Characterization and Qualification of Cell Substrates and Other Biological Starting Materials Used in the Production of Viral Vaccines for the Prevention and Treatment of Infectious Diseases]* (included as Attachment 1). This testing is performed to determine if the cell substrate is capable of forming tumors after inoculation into animals to characterize the tumorigenic phenotype of a cell line. In the case of a tumorigenic cell substrate, an extended observation period of 4 months or more is recommended with additional characterization of the tumorigenic phenotype by dose-response studies.

Because infectious or oncogenic viruses could be transmitted through whole cells present in a vaccine, the demonstration of removal of cells is required for vaccines manufactured in the U.S. in any cell substrate.

4.1 Adventitious Agents

4.1.1 Background

The presence of adventitious agents is a safety concern in the use of any cell substrate and throughout the manufacture of any biological product. Adventitious agents are defined as microorganisms that are not intended to be present in a biological product and include bacteria, fungi, mycoplasma/spiroplasma, mycobacteria, rickettsia, protozoan parasites, transmissible spongiform encephalopathy agents, and viruses. In addition to specific regulatory requirements for adventitious agent testing, FDA has issued regulatory guidance for industry and points to consider documents to help manufacturers formulate a comprehensive testing regimen for viral contaminants based upon the cell substrate, raw materials, and manufacturing steps for each vaccine. These documents are further supplemented by information in relevant International Conference on Harmonization (ICH) documents and World Health Organization (WHO) documents.

The use of human tumor-derived cell lines poses added safety concerns regarding the potential presence of unexpected and unknown viruses. These include viruses that may be present in the cell line due to their existence in the patient tissue such as oncogenic, latent DNA viruses (e.g., adenoviruses, hepadnaviruses, herpesviruses, papillomaviruses, polyomaviruses) and, 2) endogenous retroviruses (ERVs), which exist normally in a quiescent state in the host cell DNA of all species, but can become activated and produce virus particles in tumor cell lines (36, 74).

Furthermore, many tumor-derived cell lines support infection and replication of a wide variety of viruses and therefore may be more susceptible to infectious viruses present in the host (e.g., some RNA viruses such as LCMV) or through contaminated human or animal-derived reagents used during cell-line derivation and passage history. There may be additional concerns in the case of virus-like particles (VLPs) such as co-packaging of "unwanted" oncogenic RNAs or DNAs that may be transferred to the recipient by vaccination.

All cell substrates need to be assessed for the potential presence of adventitious viruses during cell-line qualification and demonstrated to be free of infectious virus.

4.1.2 Current Recommendations for Characterization and Testing of Cell Substrates

CBER's current recommendations for adventitious virus testing for preventive vaccines and approaches for development of mitigation strategies to reduce the risk of virus contamination are describe in the *Guidance for Industry for Characterization and Qualification of Cell Substrates and Other Biological Starting Materials Used in the Production of Viral Vaccines for the Prevention and Treatment of Infectious Diseases* (2010). The recommended adventitious agent testing listed in this guidance is shown in Appendix 2.

Adventitious agent testing of mammalian cells includes non-viral and viral agents. Non-viral agent testing includes tests for mycoplasma, bacteria and fungal sterility, and mycobacteria. Adventitious-virus testing includes: general tests for virus detection, i.e., *in vivo* assays generally in adult mice, suckling mice, and embryonated hens' eggs; *in vitro* cell-culture tests for cytopathic and hemadsorbing/hemagglutinating viruses using three cell lines (same species and tissue type as used in production, human diploid cells, and monkey kidney cells); and assays for retroviruses, such as transmission electron microscopy (TEM), PCR-based reverse transcriptase assays (e.g., PERT assay), and infectivity assays. In certain cases, retrovirus-detection assays may include drug treatment to induce latent viruses (discussed below).

Assays for species-specific adventitious viruses are also recommended. These are based not only on the animal species from which the cells were derived, but also on the passage history and the raw materials of animal origin used to culture the cells from original isolation to the time of use. Recommended tests include: 1) antibody-production assays such as MAP for mouse viruses, HAP for hamster viruses, and RAP for rat viruses and challenge assay for lymphocytic choriomeningitis virus (LCMV), in the case of rodent cell lines or potential exposure to rodent materials; and 2) tests for bovine and porcine viruses in the case of cells lines derived from these species or exposure to bovine serum and porcine trypsin. Virus-specific assays should also be included based upon donor species and passage history (e.g., PCR and infectivity assays for human pathogens in case of human cell lines).

While safety concerns related to cell tumorigenicity associated with residual intact cells or residual cell DNA may be addressed by removal of whole-cells and reduction of DNA size and amount (as discussed above), demonstrating the absence of latent and occult viruses in the cell substrate is more challenging. Therefore, additional testing recommendations include investigating for the presence of latent viruses such as DNA viruses and endogenous retroviruses using chemical induction assays, and to detect oncogenic viruses by testing cell lysates *in vivo* in three newborn rodent species. For detection of latent viruses, a step-wise strategy was developed at CBER for treating cells with four chemical inducers that have different mechanisms of action to activate endogenous retroviruses and DNA viruses, followed by detection of induced viruses using various general and specific assays (42, 52, 53). Detection of induced viruses may be performed through the use of transmission electron microscopy (TEM) or PCR, which is highly sensitive and compared with TEM is more specific, as detection is based on primer design from known sequences.

The detection of known and novel viruses may be further facilitated by the use of broad virus detection technologies such as massively parallel sequencing, long range PCR with mass spectrometry, and virus microarrays, as discussed below.

4.1.3 Emerging Technologies for Viral Detection

Technologies such as massively parallel sequencing (MPS) or deep sequencing, virus microarrays, and broad-range PCR with electrospray ionization mass spectrometry (PCR/ESI-MS; commercial name PLEX-ID) have enabled rapid identification of novel virus strains and novel-virus discovery in research and clinical samples (These technologies are further detailed in Appendix 3). These emerging methods have also been used by some researchers and testing labs to investigate cells and source materials used in manufacture of vaccines as well as to test commercially available vaccines to demonstrate the potential applications of the technologies. Such studies resulted in the discovery of porcine circovirus-1 (PCV-1) in a rotavirus vaccine (Rotarix) by virus microarrays and MPS (95), bluetongue virus in Vero cells by PLEX-ID (76), as well as a xenotropic murine leukemia virus-related virus designated as XMRV that was initially discovered in a human clinical sample using virus microarrays (88) and later found to be a novel recombinant murine retrovirus (66). The use of MPS to screen raw materials such as bovine serum has resulted in identification of several novel bovine viruses and the detection of a large number of noninfectious viral genome fragments (65). These results highlight the need for development of a follow-up strategy for evaluation of a positive signal with any of these nucleic acid-based technologies to assess the biological significance and relevance of such findings to product safety. A logical approach is to determine if any detected sequence is a part of a complete virus genome, if the genome is particle associated, and whether the particle is infectious. Further evaluation of potential risk would be to determine if the particle is able to replicate in human cells.

Emerging technologies that can detect both known and novel viruses have generated great interest and extensive discussions regarding the application of these methods for the evaluation of biological products. Although the advantages of these methods are

recognized, currently they have not been recommended for use due to technical challenges that are being addressed. These challenges include standardization and validation, data analysis, interpretation, and storage, and development of follow-up strategies for investigation of a positive result. Additionally, more data are needed to determine when these technologies should be applied, for which product types, and at what stage of manufacturing.

4.1.4 Viral Clearance by the Manufacturing Process

In addition to the testing of the cell substrates and other raw materials, vaccine safety may be further assured through assessment of the capacity of the manufacturing steps to clear potential contaminating viruses. Clearance includes both virus removal during the vaccine manufacturing process, as well as specific inactivation steps incorporated into the production process (2). Clearance of potential adventitious agents can be assessed quantitatively by spiking with model viruses of various types to determine the reduction in titer afforded by each step in the manufacturing process to derive a log-clearance value. Inactivation steps (e.g., chemical treatment, gamma irradiation) cannot typically be used with live-virus vaccines, and consequently, the extent of adventitious agent clearance is usually higher for an inactivated vaccine than with a live-virus vaccine. The extent of viral clearance by the manufacturing process is product dependent, and there are limitations inherent in the studies to assess viral clearance (e.g., number of model viruses evaluated). Thus, viral clearance studies are a useful contribution to the evaluation of vaccine safety, but they cannot by themselves ensure the absence of adventitious agents.

Summary: A comprehensive evaluation of vaccine cell substrates for the presence of adventitious agents has been described in the current FDA guidance document. This evaluation currently includes recommendations for conventional *in vitro* and *in vivo* tests for unknown adventitious agents, as well as specific tests (e.g., PCR) for agents relevant to the origin, passage, and use of the cell in vaccine production. Evaluation of adventitious virus removal and/or inactivation also plays an important role in establishing the safety of vaccine products. As recent examples illustrate, emerging technologies (e.g., massively parallel sequencing) may have the potential to be powerful tools for the detection of known and unknown adventitious agents and may be especially useful for evaluation of novel cell substrates. Nevertheless, at the present time, these emerging technologies are not standardized, nor is there a defined path for follow-up of potential signals generated during their application. Further discussions, including input from the current September 2012 VRBPAC, is needed to determine the role of such technologies in evaluating novel cell substrates, including cell lines derived from human tumors.

4.2 Residual Cell-substrate DNA

4.2.1 Background: DNA Oncogenicity, DNA Infectivity, and DNA Integration

Small amounts of residual cell substrate DNA unavoidably occur in all viral vaccines as well as other biologics produced using cell substrates. There are several potential ways

DNA could be a risk factor. DNA can be oncogenic or infectious; in addition, it can cause insertion mutagenesis through integration into the host genome.

DNA Oncogenicity

A major concern about residual cell-substrate DNA has been the potential for the induction of cancer, particularly if the DNA was from a tumorigenic cell or from a cell line established from a human cancer.

Cellular oncogenes were originally identified based on their homology with viral oncogenes. For example, the viral *src* gene (v-*src*), the transforming gene of the Rous sarcoma virus (RSV), the first tumor virus identified (73), was found to have counterparts in cellular DNA, first in the chicken genome (85, 94) and then in all mammalian genomes (27, 67). Additional cellular versions of viral oncogenes, termed proto-oncogenes, were identified (34, 93). However, proto-oncogenes are normal genes and are involved in normal cellular processes. It is only when "activated" (most frequently through mutation) that they become dominant oncogenes. It is the potential presence of such activated dominant oncogenes in the genomes of certain cell substrates, such as continuous cell lines and tumorigenic cells, that has raised concern over residual DNA in vaccines prepared using such cell substrates, since complete removal of DNA from vaccines is not possible. Therefore, the issue has been whether the low levels of residual cell-substrate DNA in vaccines could be a risk factor in recipients of these vaccines. This issue has been debated over many years with the conclusion that residual DNA amount and size should be controlled. A summary of these discussions is presented in Appendix 4.

DNA infectivity

If the DNA from the cell substrate contains the genome of an infectious virus, either as an extrachromosomal element or integrated into the host genome of the cell, uptake of that DNA could result in expression of the corresponding virus, and if that virus could establish a productive infection in the human host, the consequences could potentially be pathogenic.

Although there are publications on the infectivity of genomes of DNA and RNA viruses, few studies have determined the specific infectivity of virus DNA. Studies at CBER have shown that cloned HIV-1 DNA was infectious *in vitro* at 1 pg, and DNA from HIV-1-infected cells was infectious at a level of 2 µg (83). Apart from determining the specific infectivity of an HIV-1 genome, this study demonstrated that an integrated copy of a viral genome has similar infectivity to a non-integrated copy of the same genome. In addition, this *in vitro* infectivity assay has been used to quantify the reduction in DNA activity afforded by various treatments commonly used during vaccine manufacture. Such treatments as β-propiolactone (BPL) for inactivated vaccines and benzonase digestion for live vaccines can reduce the infectivity of DNA by more than 100,000 fold (83). Combining the reduction in the biological activity of DNA afforded by manufacturing steps with lowering the permitted amount of residual DNA to 10 ng per dose or below provides safety factors in excess of 10^7 (82, 83).

DNA integration

While it is theoretically possible that integration of DNA could induce oncogenicity, either through the activation of a proto-oncogene or the inactivation of a tumor-suppressor gene, this risk depends on the efficiency with which naked DNA integrates. Integration was a concern when DNA vaccines were developed in the 1990s, and a VRBPAC meeting was convened in 1997 to discuss the issue. It was decided that it was unlikely that DNA integration occurred at a high enough frequency to be a concern, although no data were available at the time. Subsequently, this decision was confirmed experimentally, when Ledwith and colleagues demonstrated in a rodent model that integration of a plasmid DNA vaccine occurred at a very low efficiency (46, 47, 97), with 4 integrants being identified following injection of 50 µg plasmid DNA. At this low level of integration (at the most, 1 integration event per 150 cells), the oncogenic consequences of DNA integration would not be a significant concern. In addition, studies at CBER have shown that up to 25 µg of a plasmid containing a strong promoter failed to induce tumors in sensitive mouse strains, whereas tumors were induced by the same vector expressing oncogenes (81).

As stated above, the major risk from DNA is considered to be through the introduction of a dominant activated oncogene or an infectious genome, particularly one from a virus that can establish a productive infection in the human host.

4.2.2 Current Recommendations for Control of Whole Cells and Host-cell DNA Content in Vaccines

Potential infectious or oncogenic agents could be transmitted through whole cells present in the vaccine; therefore, demonstration of removal of cells from vaccines has been required in the U.S. for several decades.

The current testing recommendations in viral vaccines for the testing of host-cell DNA and cell lysates from tumorigenic cells was established when the first tumorigenic cell line for vaccine production was reviewed and presented to the VRBPAC in 2001. Although there were no standardized assays to test for oncogenic activity of DNA, the Committee agreed that cell DNA and lysates should be evaluated in three newborn rodent species. The species were newborn nude mice, newborn hamsters, and newborn rats. These species were selected based on their broad sensitivity to oncogenic viruses. The recommendations were to administer by the subcutaneous route in the three newborn species 100 µg or more of DNA or a lysate prepared from 10^7 cells. Animals should be monitored for more than four months, and any tumors would need to be assessed for their species of origin to determine whether they arose spontaneously or were induced by the test article. Such testing of cell lysates in animal species could also contribute to the detection of oncogenic viruses capable of withstanding the cell lysis preparation.

The oncogenic and infectious risk of residual DNA in vaccines can be reduced by the implementation of manufacturing steps designed to lower the amount of DNA, decrease the size of the DNA, and/or to reduce the activity of residual DNA by chemical treatment or gamma irradiation. Studies with BPL and benzonase demonstrated that DNA

infectivity can be reduced by several orders of magnitude (83). Current recommendations are that the level of residual cell-substrate DNA should be ≤10 ng per dose and a median DNA size of 200 bp or lower.

Summary: Although current testing recommendations include evaluation of the oncogenicity of host cell DNA and cell lysates *in vivo*, the oncogenic and infectious risk of DNA is primarily addressed by lowering the amount of DNA, decreasing the size of the DNA (by nuclease digestion), and/or by reducing the activity of the DNA (by chemical treatment or gamma irradiation).

5. Summary

The current repertoire of cell substrates is inadequate for manufacture of certain types of new vaccines. To address this issue, sponsors have proposed the use of several novel cell substrates that are tumorigenic or derived from human tumors. In fact, it has been recognized that such cell lines derived from tumors may be the optimal and in some cases the only cell substrate that can be used to propagate certain vaccine viruses. However, at the present time, only a limited number of vaccines made in tumorigenic cell lines have entered clinical trials. In each case, the use of a specific tumorigenic cell substrate has been discussed with the vaccines advisory committee before clinical trial initiation. The purpose of the present VRBPAC meeting is to discuss the scientific issues associated with the use of cell lines derived from human tumors for the production of both live and inactivated prophylactic viral vaccines and to identify safety concerns and determine how they can be addressed. The use of such cell lines for the manufacture of prophylactic vaccines has not hitherto been discussed nor have such products entered clinical trials.

In previous discussions regarding the possible use of tumorigenic cell lines for vaccine manufacture, three major safety concerns were identified that needed to be addressed: 1) the presence of residual live cells in the vaccine that might have the potential of being tumorigenic in humans; 2) the presence of residual DNA from the cell substrate; and 3) the potential presence of adventitious agents, including adventitious viruses that might have contributed to the tumorigenic phenotype. The current data suggest that these are also the major issues associated with tumor-derived cells, including cell lines derived from human tumors. In the current meeting of the VRBPAC, the committee will review and discuss these issues and the acceptability of the use of human tumor-derived cell substrates for production of preventive vaccines. In addition, the committee will discuss the overall approach used to characterize such cell substrates, including the role of new technologies in evaluating novel cell substrates.

APPENDIX 1
Prior VRBPAC Discussions and other Meetings on Cell Substrates

This appendix expands on information presented in the body of this briefing document. It includes some additional details on discussions and meetings related to critical decisions made regarding the use of new cell lines for vaccine manufacturing. Also included are links to the transcripts of earlier VRBPAC meetings as described in the text.

In the 1970s, the first continuous cell line was used for the production of a vaccine intended for the general public. Pasteur Mérieux Sérums & Vaccins (now known as *sanofi pasteur*) used Vero cells, a continuous cell line derived from the kidneys of the African green monkey by serial passage *in vitro* (101), for the manufacture of an inactivated poliomyelitis vaccine (IPV) and then for the production of a live-attenuated poliomyelitis oral vaccine (OPV). While both vaccines are licensed in Europe, only the Vero-cell produced IPV is licensed in the US.

By the 1990s, additional types of cells were being considered for the production of new vaccines such as those against agents of bioterrorism, novel infections viruses, and pandemic influenza. The Office of Vaccines Research and Review (OVRR) in CBER entered into internal discussions to identify what are the issues associated with the use of cells that are tumorigenic or were derived from human tumors. The discussions resulted in a Defined Risks Approach (50), which consists of identifying the risk events, determining the frequency of these events by developing quantitative assays to measure them experimentally, and then from these results estimating the likelihood of the risk event. A component of this approach was to enter into a dialogue with the VRBPAC. OVRR considered that this dialogue would be an ongoing enterprise both to inform the committee as to OVRR's evolving thinking on cell-substrate safety issues and to gain their advice. The Defined Risks Approach was presented to the VRBPAC, in 1998, and the transcripts of that meeting are available (www.fda.gov/ohrms/dockets/ac/98/transcpt/3476t1.pdf). A component of the OVRR approach was that an international meeting was to be organized at which the issues identified were to be discussed, gaps in knowledge identified, and future studies to address these gaps considered. The meeting was held in 1999; the proceedings were published in the Developments in Biologicals in 2001 (11).

In 2000, a VRBPAC meeting was convened to discuss whether the use of the continuous non-tumorigenic Vero cell line represented a safety concern for the production of live, attenuated viral vaccines and if so how these concerns could be addressed. The transcripts from this meeting are available (www.fda.gov/ohrms/dockets/ac/00/transcripts/3616t2a.pdf www.fda.gov/ohrms/dockets/ac/00/transcripts/3616t2b.pdf). Members of the VRBPAC supported the use of the non-tumorigenic Vero cells for production of inactivated vaccines; however, some members expressed some concern that, because Vero cells have the capacity to become tumorigenic, they might not be suitable for the production of live, attenuated vaccines until the mechanism whereby these cells become tumorigenic is understood. The Committee recommended that OVRR investigate the mechanism whereby the cells become tumorigenic to ascertain whether this capacity itself

represented a safety concern. Nevertheless, with additional testing and discussions, several live viral vaccines produced in Vero cells have been licensed in the United States over the last decade: smallpox vaccine (2007) and two rotavirus vaccines (2006 and 2008). In addition, Vero cells are the substrate for a number of investigational vaccines.

In 2001, OVRR was asked to consider the first tumorigenic cell substrate for the manufacture of a replication-defective adenovirus-vectored vaccine against HIV-1 infection. Propagation of replication-defective adenovirus type 5 (Ad5) vectors requires a human cell line that expresses *in trans* the genes deleted in Ad5 vectors. The Ad5 vector under discussion expressed the transgene in place of the early region 1 (E1 region), and thus a human cell line that expresses E1A and E1B is necessary for vector production. The first such line constructed for this purpose was the 293 line (29), which contains a single copy of the left 12% of the Ad5 genome (84) and expresses E1A and E1B. However, a problem with 293 cells for the production of Ad5-vectored products was that a measurable level of replication-competent adenovirus (RCA) was generated by recombination with the integrated Ad5 E1 region (33). This was considered undesirable for prophylactic vaccines administered parenterally, and new lines were developed that produced no measurable RCA, such as the PER.C6 line (20) for E1-deleted Ad5 vectors. PER.C6 cells are tumorigenic, although dose-response tumorigenicity studies (35, 49, 86) in the nude mouse have shown that they are "weakly" tumorigenic, since 10^7 cells are required to form a tumor (28, 80).

The PER.C6 line and the Ad5 vectored HIV-1 vaccine were presented to the VRBPAC in 2001. This was the PER.C6 cell line generated by immortalizing human fetal retinal cells by the early-region 1 (E1) of adenovirus type 5 (Ad5). For this discussion, the Committee included several experts in tumorigenicity and oncogenicity. Issues associated with the potential risks from DNA were discussed at the meeting. Based on a comprehensive testing regimen for these cells, it was decided that these cells could be used for the safe manufacture of replication-defective Ad5-vectored vaccines against HIV-1. In addition to quantifying the tumorigenic phenotype of PER.C6 cells, the testing involved evaluating the oncogenicity of PER.C6 DNA and cell lysates in newborn nude mice, newborn hamsters, and newborn rats for oncogenic activity. This extensive oncogenicity testing was done for the first time and, while the assays were not standardized, the VRBPAC considered they were appropriate at the time. In addition, testing for adventitious agents was more extensive than the usual *in vivo* and *in vitro* tests recommended at the time and included a comprehensive list of specific PCR tests for potential virus contaminants. Transcripts from that meeting are available (www.fda.gov/ohrms/dockets/ac/01/transcripts/3750t1_01.pdf www.fda.gov/ohrms/dockets/ac/01/transcripts/3750t1_02.pdf www.fda.gov/ohrms/dockets/ac/01/transcripts/3750t1_03.pdf www.fda.gov/ohrms/dockets/ac/01/transcripts/3750t1_04.pdf).

The next meeting to discuss safety issues associated with novel cell substrates occurred in 2004 in Rockville, MD, and was organized by the National Institutes of Health (NIH) and the International Alliance for Biological Standardization (IABS). The proceedings were published in 2006 in the Developments in Biologicals series (68). At that meeting the

first data on the oncogenicity of DNA obtained from studies conducted in the OVRR were presented.

In 2005, tumorigenic Madin-Darby canine kidney (MDCK) cells were proposed for the production of two inactivated influenza virus vaccines, and OVRR requested that the VRBPAC evaluate their suitability for this purpose. The MDCK cell line is a spontaneously immortalized cell line established from dog kidney in 1958 by Madin and Darby, although there is no publication reporting its derivation. [However, kidney cell lines were established by the same group from sheep and cattle, and these were described (54, 55); thus, it is likely that similar methods and reagents were used to establish the MDCK cell line.]

Over the years, conflicting data have been published as to whether this spontaneously immortalized cell line is tumorigenic [reviewed in Omeir *et al*. (64)]. This confusion is most likely due to the inherent behavior of the MDCK cells themselves, since it is known that MDCK cells have an unstable phenotype with respect to several characteristics. For example, their predominantly epithelial morphology can be changed to a fusiform cell type by treatment with various cytokines such as hepatocyte growth factor (37), and they can be induced to undergo epithelial to mesenchymal transition (EMT), a process that occurs during normal development and one that also occurs during metastasis, in response to growth factors (5, 44, 58, 71), transcription factors (63), or activated oncogenes (16, 61, 62, 71). Nevertheless, what seems to be clear is that MDCK cells currently available from the American Type Culture Collection are tumorigenic in both adult and newborn athymic nude mice (64).

At a VRBPAC meeting in 2005, OVRR presented data on the use of both the infectivity and oncogenicity of DNA to estimate risks and manufacturers presented data on the vaccine purification process. Because of the demonstrated low level of residual small size DNA in the final product and the fact that the vaccines were inactivated, the majority of the Committee recommended that the use of tumorigenic cells did not represent a significant risk. The transcripts from that VRBPAC meeting are available. (www.fda.gov/ohrms/dockets/ac/05/transcripts/2005-4188t1.pdf)

In 2008, the VRBPAC were asked to consider the use of non-tumorigenic MDCK cells for the manufacture of a live, attenuated influenza virus vaccine. Because this line of MDCK cells was not tumorigenic, the level of DNA was low and reduced in size, and the route of vaccine administration was intra nasal, the majority of the Committee supported the evaluation of the vaccine in clinical trials. Nevertheless, it should be noted that because some MDCK cell lines were tumorigenic, some members remained concerned that residual MDCK DNA might escape digestion to a small size. The transcripts from that VRBPAC meeting are available.
(www.fda.gov/ohrms/dockets/ac/08/transcripts/2008-4384T1_1.htm
www.fda.gov/ohrms/dockets/ac/08/transcripts/2008-4384T1_2.htm)

APPENDIX 2
Cell Substrate Recommended Testing from "Guidance from Industry: Characterization and Qualification of Cell Substrates and Other Biological Materials Used in the Production of Viral Vaccines for Infectious Disease Indications"

Multi-stage testing with a variety of different assays is used to assure product safety and ensure product quality. This includes testing all the biological materials in vaccine manufacture, especially the initial components, which are the virus seed and the cell substrate. Different stages for testing are selected based on the maximum likelihood of adventitious agent detection and effect on product quality. This table shows a typical testing scheme. Selected tests may be recommended on a case-by-case basis depending upon the cell substrate and other biological materials used for virus propagation as well as production conditions for vaccine manufacture.

Table 1: Example of Biosafety Testing Scheme for Manufacture of a Viral Vaccine

	Cell Testing				Testing of Intermediates and Final Product				
	MCB	WCB	Control Cells	EOP	Virus Seed Harvest	Virus Seed Final	Vaccine Harvest	Vaccine Bulk	Final Filled Product
IDENTITY & PURITY[1]	+	(+)		(+)	(+)	+		(+)	+
STABILITY	(+)			(+)		(+)			
STERILITY									
Bacteria and fungal	+	+	+	+	+	+	+	+	+
Mycoplasma or Spiroplasma	+	+	+	+	+	+	+		
Mycobacteria	(+)	(+)		(+)	(+)[2]		(+)		
AA TESTING									
Cytopathic and hemadsorbing viruses			+						
In vitro cell culture	+	+	(+)	+	+		(+)		
In vivo assays	+		(+)	+	+		(+)		
TEM[4]	+			+					
Antibody production assay	(+)				(+)				
Bovine viruses	(+)	(+)	(+)	(+)	(+)		(+)		
Porcine viruses	(+)	(+)	(+)	(+)	(+)		(+)		
Retroviruses[4]	+		(+)	+	+		(+)		
Specific viruses[4]	(+)	(+)	(+)[3]		(+)		(+)		
TUMORIGENICITY				+					
ONCOGENICITY				(+)					
OTHER AGENTS									
Residual cell DNA								+	
Residual cell protein								+	
Residual serum, antibiotics, benzonase, ancillary reagents[2]								+	
GST									+
ENDOTOXIN									+

[1] tests may be different for cells versus intermediate and final products
[2] if applicable
[3] including ALV test in case of chicken cells
[4] may be combined with virus induction assays, as applicable
+ required stage for testing
(+) alternative stage for testing
MCB: master cell bank; WCB: working cell bank; EOP: end of production cells; AA: adventitious agent; TEM: transmission electron microscopy; GST: general safety test

APPENDIX 3
Summary of Selected Emerging Technologies for Virus Detection

Massively parallel sequencing (MPS), are high throughput sequencing technologies that result in massive quantities of data that present significant challenges for data storage, analysis, and management (48). The details of the different MPS platforms are reviewed in Attachment 2 and briefly summarized here. The currently established sequencing platforms are the Roche/454 Genome Sequencer FLX System based upon pyrosequencing with optical detection, the Illumina/Solexa Genome Analyzer System based upon reversible terminator sequencing by synthesis with fluorescence/optical detection, and the Applied Biosystems (ABI) SOLiD Analyzer System based upon sequencing by ligation with fluorescence/optical detection. Other emerging and platforms are PacBio RS (Pacific Biosciences) based upon real-time, single molecule DNA sequencing with fluorescence/optimca detection, Personal Genome Machine (PGM) sequencer (Ion Torrent /Life Technologies) based upon sequencing by synthesis with change in pH detected by ion-sensitive field effect transistors (ISFETs) for detection and gridION (Oxford Nanopore) based upon nanopore exonuclease sequencing using current detection. Selecting the appropriate platform requires consideration of the nature of the sample and targeted application (for example, whole genome or transcriptome sequencing) and the desired outcome (for example, virus detection or characterization). It is possible that a hybrid approach using more than one technology may be needed to obtain complete information. A major challenge of any of the MPS platforms is data analysis, transfer, and storage, which virology and bioinformatics expertise, the availability of specialized analysis programs and high-capacity data transfer and storage systems. Periodic re-analysis of data is necessary as new sequences become available in the databases.

Virus microarrays contain short single-stranded DNA oligonucleotides attached to a solid surface that use hybridization to detect the presence of nucleic acids from viruses (13, 23). The oligonucleotides are selected based upon known sequences and represent multiple regions of the viral genome for accurate identification. The DNA and cDNA samples are labeled with fluorescent dyes before hybridization on a microarray to allow visualization of bound probes. Virus microarrays can generate results quickly but the results are sequence-specific and also depend upon the availability of virus sequences for primer design. The arrays need to be updated based upon the addition of new sequences to the databases.

PLEX-ID, the commercial name for the Ibis bios sensor platform, couples PCR amplification with electrospray spectrometry (75, 77). Multiple pairs of primers are used that have been selected and designed based upon their broad conservation in virus families. Following PCR amplification, the mass spectrometer determines the base composition of each amplicon present, which is compared with a data-base of calculated base compositions derived from known sequences or in-house reference standards. The PLEX-ID provides results quickly and can detect virus families using broad PCR primers; however, these are dependent upon available sequences.

APPENDIX 4
WHO Deliberations: Issues Associated with the Presence of Residual Cell-substrate DNA in Vaccines

Deliberations of a WHO Study Group in 1986
In 1986, a WHO Study Group was convened in Geneva to discuss the safety concerns with the use of continuous cell lines for the production of biologicals. The background papers from these discussions were published (69) and the full deliberations of the Study Group were published by the WHO (1). The conclusions from the discussions with respect to DNA was that for biologic products manufactured in continuous cell lines, the amount of DNA per parenteral dose should be 100 pg or less, a value that was considered to represent an insignificant risk. This was a conservative decision and was based predominantly on the results of studies on the oncogenic activity of polyoma virus DNA. Briefly, this viral DNA induces tumors in newborn hamsters, and the amount of viral DNA that induced tumors in 50% of the hamsters was 2 µg, which defines the tumor-inducing dose at a 50% endpoint for polyoma virus DNA. (We also refer to this as the OD_{50}, or the oncogenic dose at which tumors are induced in 50% of the inoculated animals.) To estimate the risk of a tumor-inducing dose from a dominant activated oncogene in 100 pg of cellular DNA, several factors need to be considered: the tumor-inducing dose of polyoma virus DNA (2 µg), the size of the polyoma virus genome ($5x10^3$ base pairs), and the size of the haploid mammalian genome ($3x10^9$ base pairs). The frequency of an oncogenic event from 100 pg of polyoma virus DNA calculates to be: $100 \div (2 \times 10^6) = 0.5 \times 10^{-4}$.
However, because the size of the mammalian genome is much larger than the size of the virus genome, the frequency of a tumor-inducing dose of 100 pg of cellular DNA calculates to be: $0.5 \times 10^{-4} \div (2 \times 10^6) = 0.25 \times 10^{-10}$.
This calculated frequency was considered by the WHO Study Group to provide a sufficient margin of safety with respect to residual cell-substrate DNA.

Deliberations of a WHO Study Group in 1997
The value of 100 pg of host cell DNA per vaccine dose remained the recommended standard for a decade. However, the issue was revisited in 1997 for several reasons. First, vaccine manufacturers could not always meet this level of residual cell-substrate DNA for some viral vaccines, such as with certain enveloped viruses. Second, more information was available as to the oncogenic events in human cancers, where it has been established that multiple events, both genetic and epigenetic, are required (31, 32, 89, 98, 99). And third, for continuous non-tumorigenic cell lines such as Vero, the major cell substrate that was being considered at the time, the presence of activated dominant oncogenes in these cells was unlikely. The outcome of the 1997 WHO meeting was that the amount of residual cell-substrate DNA allowed per dose in a vaccine produced in a continuous cell line and one administered by the parenteral route was raised from 100 pg to 10 ng (10).

REFERENCES

1. 1987. Acceptability of cell substrates for production of biologicals. World Health Organization, Geneva.
2. 1998. Q5A Viral Safety Evaluation of Biotechnology Products Derived from Cell Lines of Human or Animal Origin, p. 30. International Conference on Harmonisation.
3. Aitken, M. L., R. B. Moss, D. A. Waltz, M. E. Dovey, M. R. Tonelli, S. C. McNamara, R. L. Gibson, B. W. Ramsey, B. J. Carter, and T. C. Reynolds. 2001. A phase I study of aerosolized administration of tgAAVCF to cystic fibrosis subjects with mild lung disease. Hum Gene Ther 12:1907-1916.
4. Alexander, J., S. Ward, J. Mendy, D. J. Manayani, P. Farness, J. B. Avanzini, B. Guenther, F. Garduno, L. Jow, V. Snarsky, G. Ishioka, X. Dong, L. Vang, M. J. Newman, and T. Mayall. 2012. Pre-clinical evaluation of a replication-competent recombinant adenovirus serotype 4 vaccine expressing influenza H5 hemagglutinin. PLoS ONE 7:e31177.
5. Balkovetz, D. F. 1998. Hepatocyte growth factor and Madin-Darby canine kidney cells: in vitro models of epithelial cell movement and morphogenesis. Microsc Res Tech 43:456-463.
6. Baylin, S. B. 2005. DNA methylation and gene silencing in cancer. Nat Clin Pract Oncol 2 Suppl 1:S4-11.
7. Bishop, J. M. 1983. Cellular oncogenes and retroviruses. Annu Rev Biochem 52:301-354.
8. Bishop, J. M. 1990. Nobel Lecture. Retroviruses and oncogenes II. Biosci Rep 10:473-491.
9. Bishop, J. M. 1986. Oncogenes and proto-oncogenes. J Cell Physiol Suppl 4:1-5.
10. Brown, F., E. Griffiths, F. Horaud, and J. C. Petricciani (ed.). 1998. Safety of Biological Products Prepared from Mammalian Cell Culture, vol. 93. Karger, Basel.
11. Brown, F., A. M. J. Lewis, K. Peden, and P. Krause (ed.). 2001. Evolving Scientific and Regulatory Perspectives on Cell Substrates for Vaccine Development, vol. 106. Karger, Basel.
12. Butel, J. S. 2000. Viral carcinogenesis: revelation of molecular mechanisms and etiology of human disease. Carcinogenesis 21:405-426.
13. Chen, E. C., S. A. Miller, J. L. DeRisi, and C. Y. Chiu. 2011. Using a pan-viral microarray assay (Virochip) to screen clinical samples for viral pathogens. J Vis Exp:e2536.
14. Cheung, H. H., T. L. Lee, O. M. Rennert, and W. Y. Chan. 2009. DNA methylation of cancer genome. Birth Defects Res C Embryo Today 87:335-350.
15. Cooper, G. M., and M. A. Lane. 1984. Cellular transforming genes and oncogenesis. Biochim Biophys Acta 738:9-20.
16. Davies, M., M. Robinson, E. Smith, S. Huntley, S. Prime, and I. Paterson. 2005. Induction of an epithelial to mesenchymal transition in human immortal and malignant keratinocytes by TGF-beta1 involves MAPK, Smad and AP-1 signalling pathways. J Cell Biochem 95:918-931.
17. Duffy, M. J., P. M. McGowan, and W. M. Gallagher. 2008. Cancer invasion and metastasis: changing views. J Pathol 214:283-293.
18. Eckhart, W. 1998. Viruses and human cancer. Sci Prog 81 (Pt 4):315-328.
19. Ehrlich, M. 2009. DNA hypomethylation in cancer cells. Epigenomics 1:239-259.
20. Fallaux, F. J., A. Bout, I. van der Velde, D. J. van den Wollenberg, K. M. Hehir, J. Keegan, C. Auger, S. J. Cramer, H. van Ormondt, A. J. van der Eb, D. Valerio, and R. C. Hoeben. 1998. New helper cells and matched early region 1-deleted adenovirus vectors prevent generation of replication-competent adenoviruses. Hum Gene Ther 9:1909-1917.
21. Flotte, T. R., P. L. Zeitlin, T. C. Reynolds, A. E. Heald, P. Pedersen, S. Beck, C. K. Conrad, L. Brass-Ernst, M. Humphries, K. Sullivan, R. Wetzel, G. Taylor, B. J. Carter, and W. B. Guggino. 2003. Phase I trial of intranasal and endobronchial administration of a recombinant adeno-associated virus serotype 2 (rAAV2)-CFTR vector in adult cystic fibrosis patients: a two-part clinical study. Hum Gene Ther 14:1079-1088.
22. Foley, G. E., H. Lazarus, S. Farber, B. G. Uzman, B. A. Boone, and R. E. McCarthy. 1965. Continuous culture of human lymphoblasts from peripheral blood of a child with acute leukemia. Cancer 18:522-529.
23. Gardner, S. N., C. J. Jaing, K. S. McLoughlin, and T. R. Slezak. 2010. A microbial detection array (MDA) for viral and bacterial detection. BMC Genomics 11:668.
24. Gazdar, A. F., D. N. Carney, P. A. Bunn, E. K. Russell, E. S. Jaffe, G. P. Schechter, and J. G. Guccion. 1980. Mitogen requirements for the in vitro propagation of cutaneous T-cell lymphomas. Blood 55:409-417.
25. Gey, G. O. 1954. Some aspects of the constitution and behavior of normal and malignant cells maintained in continuous culture. Harvey Lect 50:154-229.
26. Gey, G. O., W. D. Coffman, and M. T. Kubicek. 1952. Tissue culture studies of the proliferative capacity of cervical carcinoma and normal epithelium. Cancer Research 12:264-265.

27. Gibbs, C. P., A. Tanaka, S. K. Anderson, J. Radul, J. Baar, A. Ridgway, H. J. Kung, and D. J. Fujita. 1985. Isolation and structural mapping of a human c-src gene homologous to the transforming gene (v-src) of Rous sarcoma virus. J Virol 53:19-24.
28. Graham, F. L. 1987. Growth of 293 cells in suspension culture. J Gen Virol 68:937-940.
29. Graham, F. L., J. Smiley, W. C. Russell, and R. Nairn. 1977. Characteristics of a human cell line transformed by DNA from human adenovirus type 5. J Gen Virol 36:59-74.
30. Greger, V., E. Passarge, W. Hopping, E. Messmer, and B. Horsthemke. 1989. Epigenetic changes may contribute to the formation and spontaneous regression of retinoblastoma. Hum Genet 83:155-158.
31. Hahn, W. C., C. M. Counter, A. S. Lundberg, R. L. Beijersbergen, M. W. Brooks, and R. A. Weinberg. 1999. Creation of human tumour cells with defined genetic elements. Nature 400:464-468.
32. Hahn, W. C., and R. A. Weinberg. 2002. Rules for making human tumor cells. N Engl J Med 347:1593-1603.
33. Hehir, K. M., D. Armentano, L. M. Cardoza, T. L. Choquette, P. B. Berthelette, G. A. White, L. A. Couture, M. B. Everton, J. Keegan, J. M. Martin, D. A. Pratt, M. P. Smith, A. E. Smith, and S. C. Wadsworth. 1996. Molecular characterization of replication-competent variants of adenovirus vectors and genome modifications to prevent their occurrence. J Virol 70:8459-8467.
34. Hesketh, R. 1997. The Oncogene and Tumour Suppressor Gene FactsBook, Second Edition ed. Academic Press, London.
35. Hill, R. P. 1980. An appraisal of in vivo assays of excised tumours. Br J Cancer Suppl 4:230-239.
36. Hirschl, S., O. Schanab, H. Seppele, A. Waltenberger, J. Humer, K. Wolff, H. Pehamberger, and T. Muster. 2007. Sequence variability of retroviral particles derived from human melanoma cells melanoma-associated retrovirus. Virus Res 123:211-215.
37. Howard, S., T. Deroo, Y. Fujita, and N. Itasaki. 2011. A positive role of cadherin in Wnt/beta-catenin signalling during epithelial-mesenchymal transition. PLoS ONE 6:e23899.
38. Inagaki, Y., Y. Tsunokawa, N. Takebe, H. Nawa, S. Nakanishi, M. Terada, and T. Sugimura. 1988. Nucleotide sequences of cDNAs for human papillomavirus type 18 transcripts in HeLa cells. J Virol 62:1640-1646.
39. Jones, P. A., and S. B. Baylin. 2007. The epigenomics of cancer. Cell 128:683-692.
40. Jones, P. A., and S. B. Baylin. 2002. The fundamental role of epigenetic events in cancer. Nat Rev Genet 3:415-428.
41. Kawai, S., and H. Hanafusa. 1971. The effects of reciprocal changes in temperature on the transformed state of cells infected with a rous sarcoma virus mutant. Virology 46:470-479.
42. Khan, A. S., W. Ma, Y. Ma, A. Kumar, D. K. Williams, J. Muller, H. Ma, and T. A. Galvin. 2009. Proposed algorithm to investigate latent and occult viruses in vaccine cell substrates by chemical induction. Biologicals 37:196-201.
43. Kondo, Y., and J. P. Issa. 2010. DNA methylation profiling in cancer. Expert Rev Mol Med 12:e23.
44. Konishi, T., T. Takehara, T. Tsuji, K. Ohsato, K. Matsumoto, and T. Nakamura. 1991. Scatter factor from human embryonic lung fibroblasts is probably identical to hepatocyte growth factor. Biochem Biophys Res Commun 180:765-773.
45. Lazo, P. A., J. A. DiPaolo, and N. C. Popescu. 1989. Amplification of the integrated viral transforming genes of human papillomavirus 18 and its 5'-flanking cellular sequence located near the myc protooncogene in HeLa cells. Cancer Res 49:4305-4310.
46. Ledwith, B. J., S. Manam, P. J. Troilo, A. B. Barnum, C. J. Pauley, T. G. Griffiths, 2nd, L. B. Harper, H. B. Schock, H. Zhang, J. E. Faris, P. A. Way, C. M. Beare, W. J. Bagdon, and W. W. Nichols. 2000. Plasmid DNA vaccines: assay for integration into host genomic DNA. Dev Biol (Basel) 104:33-43.
47. Ledwith, B. J., S. Manam, P. J. Troilo, A. B. Barnum, C. J. Pauley, T. G. Griffiths, L. B. Harper, C. M. Beare, W. J. Bagdon, and W. W. Nichols. 2000. Plasmid DNA Vaccines: Investigation of Integration into Host Cellular DNA following Intramuscular Injection in Mice. Intervirology 43:258-272.
48. Lee, H., and H. Tang. 2012. Next-generation sequencing technologies and fragment assembly algorithms. Methods Mol Biol 855:155-174.
49. Lewis, A. M., D. W. Alling, S. M. Banks, S. Soddu, and J. L. Cook. 1999. Evaluating virus-transformed cell tumorigenicity. J Virol Methods 79:41-50.
50. Lewis, A. M., Jr., P. Krause, and K. Peden. 2001. A defined-risks approach to the regulatory assessment of the use of neoplastic cells as substrates for viral vaccine manufacture. Dev Biol 106:513-535.
51. Lieber, M., B. Smith, A. Szakal, W. Nelson-Rees, and G. Todaro. 1976. A continuous tumor-cell line from a human lung carcinoma with properties of type II alveolar epithelial cells. Int J Cancer 17:62-70.
52. Ma, H., Y. Ma, W. Ma, D. K. Williams, T. A. Galvin, and A. S. Khan. 2011. Chemical induction of endogenous retrovirus particles from the vero cell line of African green monkeys. J Virol 85:6579-6588.

53. Ma, W., T. A. Galvin, H. Ma, Y. Ma, J. Muller, and A. S. Khan. 2011. Optimization of chemical induction conditions for human herpesvirus 8 (HHV-8) reactivation with 12-O-tetradecanoyl-phorbol-13-acetate (TPA) from latently-infected BC-3 cells. Biologicals 39:158-166.
54. Madin, S. H., P. C. Andriese, and N. B. Darby. 1957. The in vitro cultivation of tissues of domestic and laboratory animals. Am J Vet Res 18:932-941.
55. Madin, S. H., and N. B. Darby, Jr. 1958. Established kidney cell lines of normal adult bovine and ovine origin. Proc Soc Exp Biol Med 98:574-576.
56. Martin, G. S. 2004. The road to Src. Oncogene 23:7910-7917.
57. Martin, G. S. 1970. Rous sarcoma virus: a function required for the maintenance of the transformed state. Nature 227:1021-1023.
58. Medici, D., E. D. Hay, and D. A. Goodenough. 2006. Cooperation between snail and LEF-1 transcription factors is essential for TGF-beta1-induced epithelial-mesenchymal transition. Mol Biol Cell 17:1871-1879.
59. Moss, R. B., F. Ferre, R. Trauger, F. Jensen, A. Daigle, S. P. Richieri, and D. J. Carlo. 1994. Inactivated HIV-1 Immunogen: impact on markers of disease progression. J Acquir Immune Defic Syndr 7 Suppl 1:S21-27.
60. Moss, R. B., W. Giermakowska, P. Lanza, J. L. Turner, M. R. Wallace, F. C. Jensen, G. Theofan, S. P. Richieri, and D. J. Carlo. 1997. Cross-clade immune responses after immunization with a whole-killed gp120-depleted human immunodeficiency virus type-1 immunogen in incomplete Freund's adjuvant (HIV-1 immunogen, REMUNE) in human immunodeficiency virus type-1 seropositive subjects. Viral Immunol 10:221-228.
61. Oft, M., R. J. Akhurst, and A. Balmain. 2002. Metastasis is driven by sequential elevation of H-ras and Smad2 levels. Nat Cell Biol 4:487-494.
62. Oft, M., J. Peli, C. Rudaz, H. Schwarz, H. Beug, and E. Reichmann. 1996. TGF-beta1 and Ha-Ras collaborate in modulating the phenotypic plasticity and invasiveness of epithelial tumor cells. Genes Dev 10:2462-2477.
63. Olmeda, D., M. Jorda, H. Peinado, A. Fabra, and A. Cano. 2007. Snail silencing effectively suppresses tumour growth and invasiveness. Oncogene 26:1862-1874.
64. Omeir, R. L., B. Teferedegne, G. S. Foseh, J. J. Beren, P. J. Snoy, L. R. Brinster, J. L. Cook, K. Peden, and A. M. Lewis, Jr. 2011. Heterogeneity of the tumorigenic phenotype expressed by Madin-Darby canine kidney cells. Comp Med 61:243-250.
65. Onions, D., and J. Kolman. 2010. Massively parallel sequencing, a new method for detecting adventitious agents. Biologicals 38:377-380.
66. Paprotka, T., K. A. Delviks-Frankenberry, O. Cingoz, A. Martinez, H. J. Kung, C. G. Tepper, W. S. Hu, M. J. Fivash, Jr., J. M. Coffin, and V. K. Pathak. 2011. Recombinant origin of the retrovirus XMRV. Science 333:97-101.
67. Parker, R. C., G. Mardon, R. V. Lebo, H. E. Varmus, and J. M. Bishop. 1985. Isolation of duplicated human c-src genes located on chromosomes 1 and 20. Mol Cell Biol 5:831-838.
68. Petricciani, J., and R. Sheets (ed.). 2006. Vaccine Cell Substrates, vol. 123. Karger, Basel.
69. Petricciani, j. C., and W. Hennessen. 1987. Cells products safety. Background papers from the WHO Study Group on Biologicals. Geneva, 18-19 Nov. 1986. Dev Biol Stand 68:1-81.
70. Poke, F. S., A. Qadi, and A. F. Holloway. 2010. Reversing aberrant methylation patterns in cancer. Curr Med Chem 17:1246-1254.
71. Ridley, A. J., P. M. Comoglio, and A. Hall. 1995. Regulation of scatter factor/hepatocyte growth factor responses by Ras, Rac, and Rho in MDCK cells. Mol Cell Biol 15:1110-1122.
72. Rous, P. 1967. The challenge to man of the neoplastic cell. Cancer Res 27:1919-1924.
73. Rous, P. 1911. A sarcoma of the fowl transmissible by an agent separable from the tumor cells. J Exp Med 13:397-411.
74. Ruprecht, K., H. Ferreira, A. Flockerzi, S. Wahl, M. Sauter, J. Mayer, and N. Mueller-Lantzsch. 2008. Human endogenous retrovirus family HERV-K(HML-2) RNA transcripts are selectively packaged into retroviral particles produced by the human germ cell tumor line Tera-1 and originate mainly from a provirus on chromosome 22q11.21. J Virol 82:10008-10016.
75. Sampath, R. 2011. Advanced Techniques for Detection and Identification of Viral Contaminants Using the Ibis PLEX-ID Universal Biosensor. PDA J Pharm Sci Technol 65:690.
76. Sampath, R., L. B. Blyn, and D. J. Ecker. 2010. Rapid molecular assays for microbial contaminant monitoring in the bioprocess industry. PDA J Pharm Sci Technol 64:458-464.
77. Sampath, R., K. L. Russell, C. Massire, M. W. Eshoo, V. Harpin, L. B. Blyn, R. Melton, C. Ivy, T. Pennella, F. Li, H. Levene, T. A. Hall, B. Libby, N. Fan, D. J. Walcott, R. Ranken, M. Pear, A. Schink, J. Gutierrez, J. Drader, D. Moore, D. Metzgar, L. Addington, R. Rothman, C. A. Gaydos, S. Yang, K. St George, M. E. Fuschino, A. B. Dean, D. E. Stallknecht, G. Goekjian, S. Yingst, M. Monteville, M. D. Saad, C. A. Whitehouse, C. Baldwin, K. H. Rudnick, S. A. Hofstadler, S. M. Lemon, and D. J. Ecker.

2007. Global surveillance of emerging Influenza virus genotypes by mass spectrometry. PLoS ONE 2:e489.
78. Sarid, R., and S. J. Gao. 2011. Viruses and human cancer: from detection to causality. Cancer Lett 305:218-227.
79. Seedorf, K., T. Oltersdorf, G. Krammer, and W. Rowekamp. 1987. Identification of early proteins of the human papilloma viruses type 16 (HPV 16) and type 18 (HPV 18) in cervical carcinoma cells. Embo J 6:139-144.
80. Shen, C., M. Gu, C. Song, L. Miao, L. Hu, D. Liang, and C. Zheng. 2008. The tumorigenicity diversification in human embryonic kidney 293 cell line cultured in vitro. Biologicals 36:263-268.
81. Sheng, L., F. Cai, Y. Zhu, A. Pal, M. Athanasiou, B. Orrison, D. G. Blair, S. H. Hughes, J. M. Coffin, A. M. Lewis, and K. Peden. 2008. Oncogenicity of DNA in vivo: Tumor induction with expression plasmids for activated H-ras and c-myc. Biologicals 36:184-197.
82. Sheng-Fowler, L., A. M. Lewis, Jr., and K. Peden. 2009. Issues associated with residual cell-substrate DNA in viral vaccines. Biologicals 37:190-195.
83. Sheng-Fowler, L., A. M. Lewis, Jr., and K. Peden. 2009. Quantitative determination of the infectivity of the proviral DNA of a retrovirus in vitro: Evaluation of methods for DNA inactivation. Biologicals 37:259-269.
84. Spector, D. J. 1983. The pattern of integration of viral DNA sequences in the adenovirus 5-transformed human cell line 293. Virology 130:533-538.
85. Stehelin, D., H. E. Varmus, J. M. Bishop, and P. K. Vogt. 1976. DNA related to the transforming gene(s) of avian sarcoma viruses is present in normal avian DNA. Nature 260:170-173.
86. Taghian, A., W. Budach, A. Zietman, J. Freeman, D. Gioioso, W. Ruka, and H. D. Suit. 1993. Quantitative comparison between the transplantability of human and murine tumors into the subcutaneous tissue of NCr/Sed-nu/nu nude and severe combined immunodeficient mice. Cancer Res 53:5012-5017.
87. Tatalick, L. M., C. J. Gerard, R. Takeya, D. N. Price, B. A. Thorne, L. M. Wyatt, and P. Anklesaria. 2005. Safety characterization of HeLa-based cell substrates used in the manufacture of a recombinant adeno-associated virus-HIV vaccine. Vaccine 23:2628-2638.
88. Urisman, A., R. J. Molinaro, N. Fischer, S. J. Plummer, G. Casey, E. A. Klein, K. Malathi, C. Magi-Galluzzi, R. R. Tubbs, D. Ganem, R. H. Silverman, and J. L. DeRisi. 2006. Identification of a novel Gammaretrovirus in prostate tumors of patients homozygous for R462Q RNASEL variant. PLoS Pathog 2:e25.
89. Valastyan, S., and R. A. Weinberg. 2011. Tumor metastasis: molecular insights and evolving paradigms. Cell 147:275-292.
90. Vardas, E., P. Kaleebu, L. G. Bekker, A. Hoosen, E. Chomba, P. R. Johnson, P. Anklesaria, J. Birungi, B. Barin, M. Boaz, J. Cox, J. Lehrman, G. Stevens, J. Gilmour, T. Tarragona, P. Hayes, S. Lowenbein, E. Kizito, P. Fast, A. E. Heald, and C. Schmidt. 2010. A phase 2 study to evaluate the safety and immunogenicity of a recombinant HIV type 1 vaccine based on adeno-associated virus. AIDS Res Hum Retroviruses 26:933-942.
91. Varmus, H., W. Pao, K. Politi, K. Podsypanina, and Y. C. Du. 2005. Oncogenes come of age. Cold Spring Harb Symp Quant Biol 70:1-9.
92. Varmus, H. E. 1990. Nobel lecture. Retroviruses and oncogenes. I. Biosci Rep 10:413-430.
93. Varmus, H. E. 1985. Viruses, genes, and cancer. I. The discovery of cellular oncogenes and their role in neoplasia. Cancer 55:2324-2328.
94. Varmus, H. E., R. A. Weiss, R. R. Friis, W. Levinson, and J. M. Bishop. 1972. Detection of avian tumor virus-specific nucleotide sequences in avian cell DNAs (reassociation kinetics-RNA tumor viruses-gas antigen-Rous sarcoma virus, chick cells). Proc Natl Acad Sci U S A 69:20-24.
95. Victoria, J. G., C. Wang, M. S. Jones, C. Jaing, K. McLoughlin, S. Gardner, and E. L. Delwart. 2010. Viral nucleic acids in live-attenuated vaccines: detection of minority variants and an adventitious virus. J Virol 84:6033-6040.
96. Wagner, J. A., I. B. Nepomuceno, A. H. Messner, M. L. Moran, E. P. Batson, S. Dimiceli, B. W. Brown, J. K. Desch, A. M. Norbash, C. K. Conrad, W. B. Guggino, T. R. Flotte, J. J. Wine, B. J. Carter, T. C. Reynolds, R. B. Moss, and P. Gardner. 2002. A phase II, double-blind, randomized, placebo-controlled clinical trial of tgAAVCF using maxillary sinus delivery in patients with cystic fibrosis with antrostomies. Hum Gene Ther 13:1349-1359.
97. Wang, Z., P. J. Troilo, X. Wang, T. G. Griffiths, S. J. Pacchione, A. B. Barnum, L. B. Harper, C. J. Pauley, Z. Niu, L. Denisova, T. T. Follmer, G. Rizzuto, G. Ciliberto, E. Fattori, N. L. Monica, S. Manam, and B. J. Ledwith. 2004. Detection of integration of plasmid DNA into host genomic DNA following intramuscular injection and electroporation. Gene Ther 11:711-721.
98. Weinberg, R. A. 1996. How cancer arises. Sci Am 275:62-70.
99. Weinberg, R. A. 1996. The molecular basis of carcinogenesis: understanding the cell cycle clock. Cytokines Mol Ther 2:105-110.

100. Willecke, K., and R. Schafer. 1984. Human oncogenes. Hum Genet 66:132-142.
101. Yasumura, Y., and Y. Kawakita. 1963. Study of SV40 in tissue culture. Nippon Rinsho 21:1201-1205.