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**Use of Madin Darby Canine Kidney Cells for the Manufacture of
Live, Attenuated Influenza Vaccines ^a**

Briefing Document

FDA

Vaccines and Related Biological Products Advisory Committee

Silver Spring, MD

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List of Abbreviations and Definitions

ACB	Accession Cell Bank
ADC	Animal derived components
ATCC	American Type Culture Collection
<i>att</i>	Attenuated
AVA	Adventitious agents
BSE	Bovine spongiform encephalopathy
bp	Base pairs
<i>ca</i>	Cold-adapted
CBER	Center for Biologics Evaluation and Research
CDLC	Chemically defined lipid concentrate
cDNA	Complementary DNA
cGMP	Current Good Manufacturing Practices
CTM	Clinical trial material
EOP	End of Production Cells
EU	European Union
FBS	Fetal Bovine Serum
FDA	United States Food and Drug Administration
HA	Hemagglutinin
HCD or hcDNA	Host cell DNA
HCP	Host cell protein
IM	Intramuscularly
IN	Intranasally
LAIV	Live, attenuated influenza vaccine
LOD	Limit of detection
MCB	Master cell bank
MDCK	Madin Darby Canine Kidney
mRNA	Messenger RNA

MVS	Master virus seed
NA	Neuraminidase
P	Passage
PERT	Product-enhanced reverse transcriptase
PrPSc	Prion protein scrapie
SFM	Serum-free medium
SINE	Short interspersed nuclear elements
TCID ₅₀	50% Tissue culture infectious dose
TEM	Transmission electron microscopy
TFF	Tangential flow filtration
TIV	Trivalent inactivated influenza vaccine
<i>ts</i>	Temperature sensitive
TSE	Transmissible Spongiform Encephalopathy
U.S.	United States
vRNA	Viral RNA
<i>wt</i>	Wild-type
WCB	Working cell bank

1 Executive Summary

FluMist[®] (Influenza Virus Vaccine Live, Intranasal – also known as live, attenuated influenza vaccine or LAIV) was originally licensed in the U.S. in 2003 and, similar to all U.S.-licensed influenza vaccines, is currently produced in embryonated chicken eggs. There is a compelling public health need to increase the quantity and reliability of the nation's influenza vaccine supply to prevent morbidity and mortality caused by seasonal epidemics as well as enhance pandemic preparedness. LAIV has many attributes, such as a high degree of immunogenicity in immunonaïve subjects and efficacy in children to both matched and mismatched influenza strains that can contribute to prevention of influenza. MedImmune, LLC, hereafter referred to as MedImmune, has developed a new production technology for LAIV by replacing eggs with the Madin Darby Canine Kidney (MDCK) cell line as the production substrate.

MedImmune has developed a cloned cell line of MDCK cells that has been extensively characterized. Comprehensive testing has shown that these cells contain no detectable adventitious agents, have a very low propensity to form tumors in sensitive animal models and neither the cellular DNA nor other subcellular components had any detectable oncogenic properties. In order to use this cell line in production, MedImmune has developed a robust cell culture based production and purification process that removes any intact cells and reduces the quantity of cellular proteins and DNA, thereby further enhancing the safety of the vaccine. The combination of robust production methods with a highly characterized production substrate supports the conclusion that an MDCK-produced LAIV is safe and can be produced efficiently and reliably. MedImmune is ready to initiate clinical evaluation of both seasonal and pandemic influenza vaccines produced using our MDCK cell line to support licensure of these products.

2 Background

2.1 A New Production System for Influenza Vaccines is a Public Health Imperative

MedImmune is developing cell culture based production and clinical development programs for both live, attenuated cell culture produced seasonal and pandemic LAIV. Cell culture production is expected to increase the supply of influenza vaccine compared to egg based

production as well as increase the assurance that the quality or availability of the production substrate does not limit vaccine production. The following points address the critical public health need for moving forward with cell culture produced vaccines.

- Increasing the supply of vaccine, and vaccine manufacturing capacity is essential for preparing for a pandemic emergency.
- Cell culture production has the potential to deliver larger numbers of doses in a shorter period of time than egg-based production and the scale of production can be more rapidly increased.
- All egg supplies, even the specific pathogen free eggs used to produce LAIV, are contaminated with microorganisms. Protecting the product and production facilities from contamination requires strict control of manufacturing process. Problems with these controls led to the severe shortage of inactivated influenza vaccine supply in the 2004-05 season (CDC 2004). In contrast, cell culture production is conducted with highly characterized sterile materials (media, cell banks, viral seeds) in closed, controlled, aseptic systems using modern technologies. Thus, cell culture production reduces the risk of vaccine shortages due to losses resulting from contamination of the product or the facility.
- The chicken flocks, and thus the supply of eggs, could be severely compromised by a circulating poultry pathogen, including avian influenza, during the time of a pandemic. Loss of the egg supply would result in no influenza vaccine being available for the public. Since cell culture production is initiated from well-characterized frozen cell banks, production would not be compromised from a circulating pathogen.
- Ultimately, cell culture production of influenza vaccines may alleviate problems associated with isolation and passage of human influenza viruses in eggs (Mochalova, 2003; Saito, 2004; Chen, 2008).

2.2 Evaluation of Benefits and Risks of Cell Culture Manufacture for LAIV

Cell culture production can be applied to LAIV as to most live, viral vaccines (eg, polio vaccine, varicella vaccine, and rotavirus vaccine). Cell culture production of LAIV has several potential benefits including an increased production capacity and an increase in the control and characterization of the production substrate. These attributes raise the level of assurance that influenza vaccine supplies will be available for both seasonal as well as pandemic purposes. Using a continuous cell line for production comes with a limited number of defined potential risks. Through a combination of cell line selection, cell line characterization and testing, and implementation of robust manufacturing procedures,

MedImmune has taken steps to address these risks and produce a safe and reliable vaccine. The following is a summary of the benefits and risks of cell culture production of LAIV.

- Benefits of LAIV that are critical components of both annual and pandemic vaccines
 - Studies with LAIV in children have demonstrated high levels of protection against culture-confirmed influenza illness, including significant reductions in influenza cases compared to inactivated vaccine (Belshe, 2007; FluMist Package Insert, 2008).
 - LAIV has demonstrated protection in both children and adults against influenza strains antigenically mismatched to those contained in the vaccine.
 - The immune response to LAIV appears to be particularly strong in immunonaïve individuals such as young children.
 - Intranasal delivery may provide for fast, easy administration of vaccine.
 - LAIV can be produced more efficiently than inactivated vaccine; one ml of either infected egg or cell culture fluid can contain 100 times more LAIV doses than inactivated doses.
- Benefits of cell culture production of LAIV
 - Bulk vaccine can be supplied faster using the proposed manufacturing scale (2 X 2500L bioreactors) and increasing the scale of production does not require the lead time needed to increase egg based production (approximately a 12 month lead time is needed to significantly increase the size of egg laying flocks).
 - Individuals who cannot use any egg produced influenza vaccines due to egg allergies will have the opportunity to protect themselves from influenza.
 - Cell culture increases the reliability of vaccine supply. Cell banks can be produced and extensively tested prior to use in manufacturing.
 - Cell culture may reduce the likelihood of antigenic changes in the hemagglutinin due to egg adaptation.
- Mitigation of potential risks associated with cell culture production of LAIV
 - The MDCK cell banks have undergone a comprehensive testing and characterization program demonstrating that they are safe for vaccine production.
 - The MDCK cell banks have been evaluated with over 60 tests and no adventitious agents have been detected.
 - The MDCK cell bank has a very low propensity to cause tumors in nude mice; injection of 10 million MDCK cells did not result in observable tumors even after 6 months of observation.
 - The MDCK DNA and lysates from MDCK cells did not cause tumors in any of the 3 rodent species tested.

- The modern production technologies used to produce and purify the vaccine enhance the assurance that the vaccine product is safe.
 - Multiple filtration steps in the purification process are capable of removing over 100 billion times more cells than are used during production, thereby ensuring an acellular product.
 - The purification procedures reduce the quantity and size of any residual host cellular DNA to levels below that acceptable for injectable vaccines (WHO recommendation is 10 ng for parenterally administered vaccines; Griffiths, 1999).
 - The purification procedures reduce the quantity of host cell proteins in the product.

3 MDCK Cell-Produced LAIV

3.1 Comparability of MedImmune's MDCK Cell Culture Produced Vaccine to the Commercial Egg Produced Vaccine in Preclinical Models

Attenuation of FluMist is controlled by vaccine virus genes independent of the host cell substrate; therefore, the biological properties of LAIV produced in eggs or cell culture would be expected to be very similar. Indeed, extensive preclinical characterization of MDCK cell-produced and egg-produced seasonal influenza vaccine demonstrated that the critical phenotypes, cold-adaptation (*ca*), temperature sensitivity (*ts*), and attenuated (*att*) in the ferrets, and corresponding genetic loci of the vaccine strains are preserved throughout the manufacturing processes. In addition, vaccine produced in either substrate had comparable biochemical properties (virus genome sequences and virus protein expression), physical properties (virus morphology, size, and total virus particle counts), toxicology profile, and replication properties.

Using sensitive animal models, replication of the vaccine virus was shown to be restricted to the upper respiratory tract, as expected, regardless of the production substrate (ie, MDCK cells or eggs) used to prepare the virus inoculum. Results from a GLP toxicology study in ferrets show that overall the MDCK produced vaccine was immunogenic, well tolerated and safe in this test system. Minor microscopic changes observed in respiratory tissues were consistent with changes observed in prior ferret toxicology studies using intranasally delivered egg-produced frozen or refrigerated FluMist vaccine. In a separate experiment, MDCK cell-produced and egg-produced vaccines were shown to be similarly immunogenic and protected ferrets against wild-type (*wt*) influenza virus challenges. As shown in [Table](#)

3.1-1, animals vaccinated with either cell culture or egg produced vaccine effectively limited the replication of *wt* virus following a challenge infection; similar results were obtained following measurement of the challenge virus in lung tissues (see [Appendix 1](#)).

Table 3.1-1 Viral Shedding in Nasal Washes After Challenge in Vaccinated Ferrets

Challenge Virus (FFU)	Treatment	Log ₁₀ TCID ₅₀ /ml (Mean ± SD) ^a		
		Day 49 8 hours after challenge	Day 51 Day 2 after challenge	Day 52 Day 3 after challenge
<i>wt</i> A/NewCaledonia/20/99 5.5 log ₁₀	Egg-produced Vaccine	≤ LOD	≤ LOD	≤ LOD
	Cell-produced vaccine	≤ LOD	≤ LOD	≤ LOD
	Placebo	≤ LOD	4.3 ± 0.6	3.5 ± 0.8
<i>wt</i> A/Wisconsin/67/05 7.0 log ₁₀	Egg-produced Vaccine	≤ LOD	1.8 ± 1.0	≤ LOD
	Cell-produced vaccine	≤ LOD	≤ LOD	≤ LOD
	Placebo	1.8 ± 0.6	5.6 ± 0.7	4.1 ± 0.9
<i>wt</i> B/Malaysia/2506/04 5.5 log ₁₀	Egg-produced Vaccine	≤ LOD	≤ LOD	≤ LOD
	Cell-produced vaccine	≤ LOD	≤ LOD	≤ LOD
	Placebo	≤ LOD	4.0 ± 1.1	4.4 ± 1.7

^a Limit of detection (LOD): 1.2 log₁₀ FFU/ml

Results of the preclinical testing support the conclusion that the MDCK cell manufacturing process does not change the safety or performance of the LAIV vaccine strains compared to those used for the licensed egg-produced FluMist vaccine.

3.2 Selection and Preparation of the MDCK Cells

Several criteria were used to choose a cell line for production, including the ability of the cells to support replication of adequate quantities of a wide range of influenza strains, to adapt to serum free media and to have an overall low level of tumorigenicity. Multiple cell lines, including MRC-5, CHO, 293, Vero, and WI-38, were assessed and MDCK cells were the only cells that met all requisite criteria. MDCK cells are routinely used in public health

surveillance programs (Fujii, 2002; Oh, 2008), clinical virology, and research laboratories around the world as routine substrates for influenza virus cultivation due to their sensitivity to and productivity of a wide magnitude of different influenza viruses. The MDCK cell line is a continuous cell line for which potential for tumorigenicity in animal models has been reported to range from none (Stiles, April 1976; Stiles, Sept 1976; U, 1985) to moderately low (Leighton, 1969; Leighton, 1970). At the November 2005 VRBPAC meeting, two independent vaccine manufacturers characterized the tumorigenic potential of their specific MDCK cell lines. One manufacturer had selected and produced a subclone of MDCK cells that were no longer dependent on adherence for growth, growing well in suspension cultures. These cells formed tumors after injection of as few as 10 cells into nude mice. A second manufacturer produced an MDCK subclone that grew in a contact inhibition dependent fashion; these cells also could produce tumors in nude mice; however, in contrast to the suspension cells, it took at least 10^5 anchorage-dependent, or contact inhibition dependent, MDCK cells to produce tumors in these animals. None of the investigations of the tumorigenicity studies suggested specific mechanisms of transformation related to these cells. These data demonstrate that while suspension subclones of MDCK cells can have a relatively high propensity to form tumors in animal models, the majority of data on contact inhibition dependent MDCK cells demonstrated that these cells have a relatively low ($\geq 10^5$ cells) potential to form tumors in these model systems.

Based on their potential to grow in serum free media, ability to support the growth of a range of influenza subtypes and strains, and relatively low tumorigenic potential of adherent cells, the MDCK cell line was chosen as the cell substrate for manufacture of LAIV.

3.2.1 Media Development for Growth of MedImmune's MDCK Cell Banks

In order to develop a cell culture flu vaccine with the highest possible safety margin and minimum exposure to animal-derived products, MedImmune initiated a cell culture media development program. The goal of this project was to develop a serum free cell culture medium formulation that supported growth of adherent MDCK cells and allowed high levels of LAIV virus production. A basic media formulation, MediV-100 serum free medium (SFM), was produced from a published report and served as a foundation for further development (Taub, 1979). A derivative of MediV-100, designated MediV-105 SFM, was developed that contains additional growth factors and is free of animal-derived components (ADC) with the exception of chemically defined lipid concentrate (CDLC) which contains cholesterol from Australian sheep wool. MediV-105 SFM is the media used to produce the

master and working cell banks and the data for cells grown in this media are shown in Section 3.3.

3.2.2 MDCK Biological Cell Cloning and Production of the Cell Banks

It has been reported that MDCK cells obtained from the ATCC are comprised of a heterogeneous population and subclones can be established that exhibit slightly different biochemical properties (Nakazato, 1989; Arthur, 2000). Additionally, data presented at VRBPAC in 2005 indicated that a subpopulation of suspension MDCK cells that are no longer contact dependent can be isolated. In order to ensure that MedImmune's production substrate was a uniform population of cells, MedImmune biologically cloned cells via limiting dilution cloning in medium containing serum.

MDCK cells were obtained from the ATCC (CCL-34, lot 1805449) and expanded in DMEM containing 10% FBS. Cells were cloned by limiting dilution in DMEM or DMEM/F12 containing 10% serum and the individual clones were cultured, expanded and evaluated for their ability to produce influenza virus. A small percentage of the clones produced higher titers of influenza virus than the majority of the population and one of these (Subclone 9B9-1E4) was chosen to produce a small bank of cells, designated the Accession Cell Bank (ACB) (Figure 3.2.2-1). MedImmune expanded cells from this bank with MediV-105 SFM in a cGMP environment to generate the MDCK Master Cell Bank (MCB). One vial of this MDCK MCB was expanded for four passages in serum-free MediV-105 SFM in a cGMP environment to generate the MDCK working cell bank (WCB) at Passage 101. The MDCK WCB forms the cell source for viral vaccine production. In addition, to mimic end-of-production cells, a vial of the MDCK MCB was further cultured for 16 passages, undergoing approximately 51 population doublings, to reach a passage level that was considered beyond the expected end of production passage level (EOP). This strategy of producing an MCB, WCB and EOP banks of cells are aligned with both US and EU regulatory guidance on cell banking and enables extensive testing to be conducted on the cells used for production.

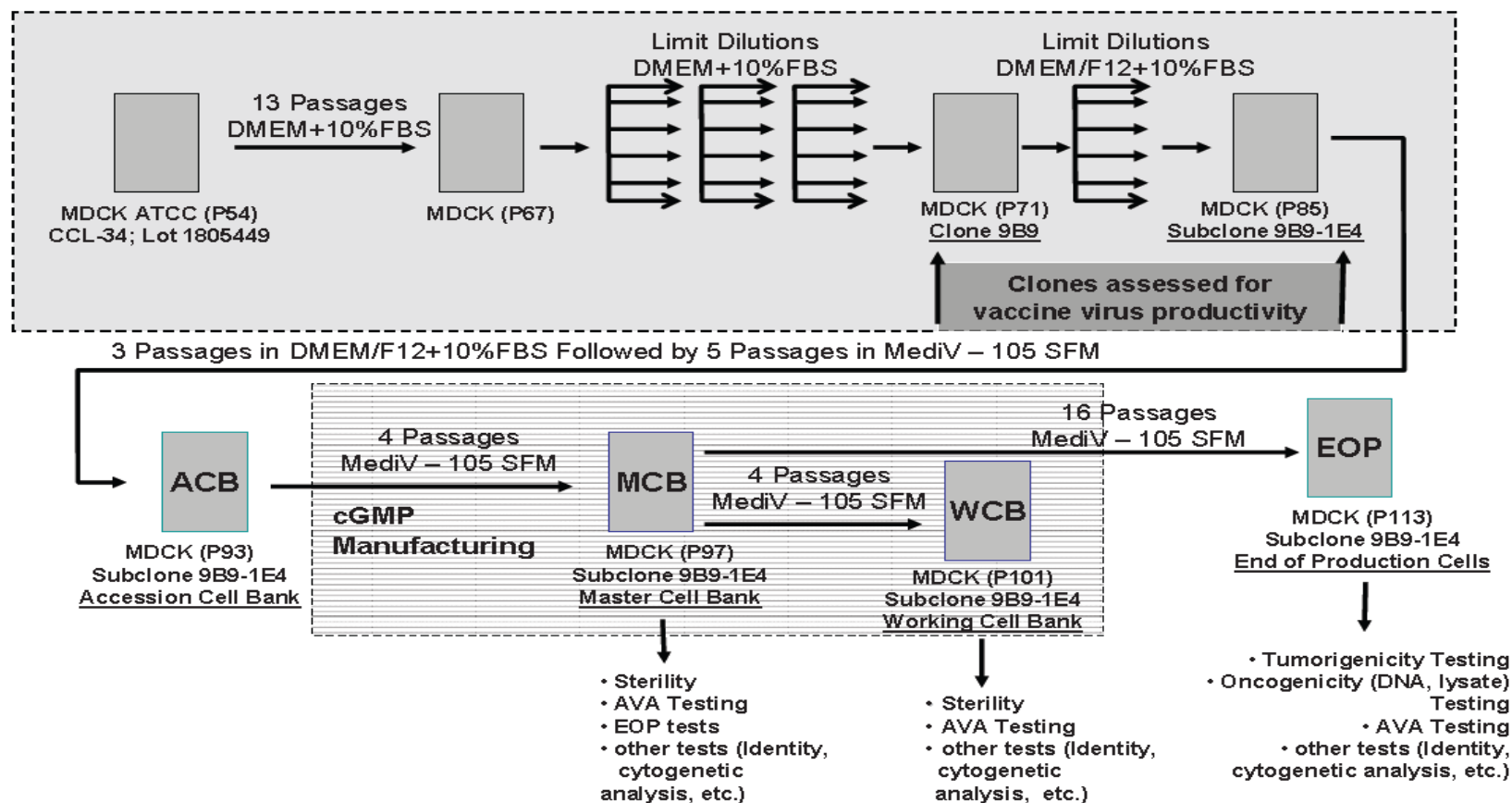


Figure 3.2.2-1 Flow Chart of MedImmune MDCK Cell Line Derivation

Genealogy of MedImmune MDCK cell banks derived from the ATCC MDCK cells (CCL-34). The cell passage level and cell culture media that were used to expand the cells and prepare the cell clones or cell banks are described for each development stage. The steps that were performed using serum-containing media are illustrated in the top gray box. All other steps used serum-free media. The manufacturing steps that were conducted under cGMP conditions are illustrated in a box with horizontal lines. Information regarding the types of cell bank tests is provided in the flow chart. The specific tests and the test results are provided in appendices as indicated in Section 3.3.2.

3.3 Cell Line Testing and Characterization to Assess the Safety of the Cell Substrate

MedImmune's MDCK cell line has been extensively tested and characterized. No adventitious agents were detected and no evidence that MedImmune's MDCK cells or subcellular components are tumorigenic or oncogenic was found in animal models. The testing and characterization studies MedImmune conducted for the MDCK cell line can be broadly grouped into three major categories:

- 1) Biological properties of the cell line, such as confirmation of canine origin, morphology, and productivity of influenza virus,
- 1) Detection of potential bacterial, mycoplasma, fungal and viral adventitious agents that might be present in canine cells, and
- 2) Assessment of the intact cells, MDCK DNA and MDCK cellular lysates to induce tumors in animal models.

MedImmune's cell bank testing regimen complies with the regulatory guidelines for the characterization and qualification of cell substrates and other biological materials used in the production of viral vaccines, as well as follows CBER's recommendations obtained during the Type B pre-IND meeting held on 20Sep2006 and during subsequent communications regarding MDCK cell bank characterization protocols.

3.3.1 Characterization of Biological Properties

The genetic identity of MDCK MCB, WCB and EOP cells were evaluated using isoenzyme and cytogenetic analyses. Based on isoenzyme migration patterns, ploidy distribution and karyology, the MCB, WCB and EOP cells were determined and confirmed to be of canine origin. Chromosomal analyses did not detect aberrations and none of the chromatids or chromosomes had been interchanged or deleted. In addition, the modal chromosome number remained consistent at approximately 78 for all the cell banks.

The MDCK cell banks maintained consistent growth kinetics, constant cellular morphology and a steady rate of population doublings throughout passaging from the MCB passage level through the EOP passage level (See [Figure 3.3.1-1](#)).

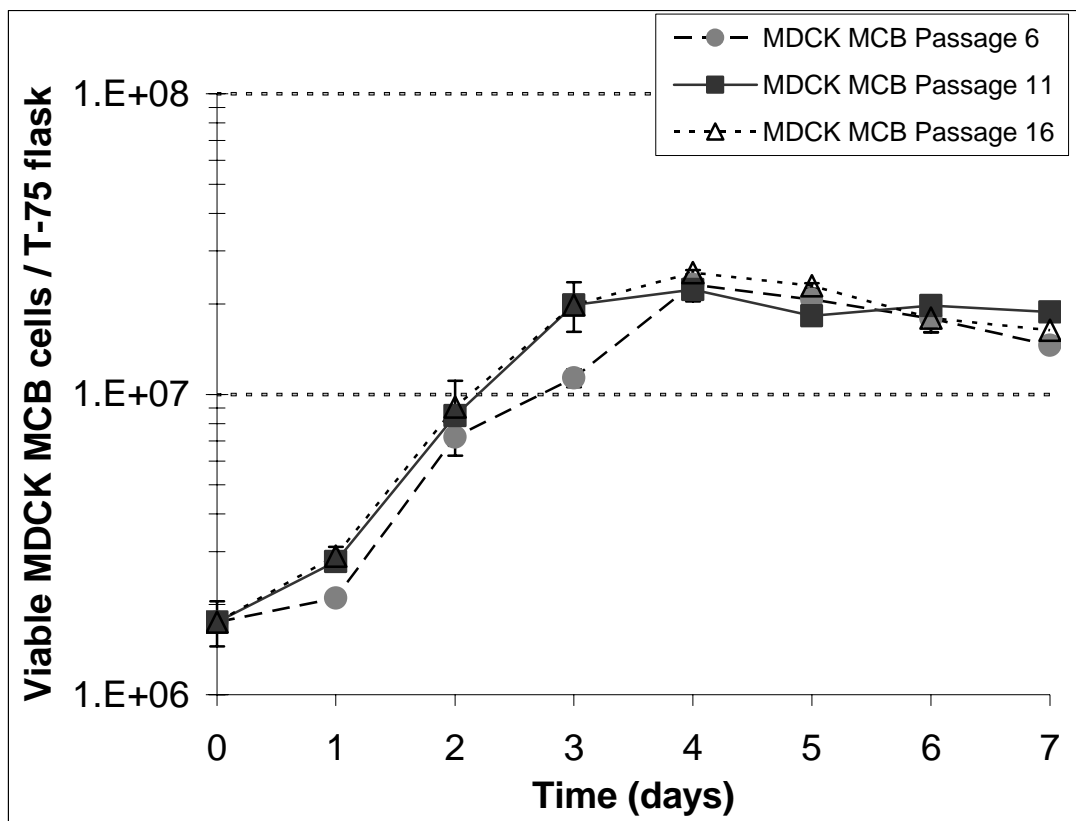


Figure 3.3.1-1 MDCK MCB Growth Curve

Graphical representation of the growth rate and increase in viable cell number observed in three distinct MDCK passages (6, 11, and 16) in MediV-105 media over 7 days.

3.3.2 Testing for Adventitious Agents

The production of a cell bank enables the characterization of many different attributes of the substrate well in advance of its use in production. In contrast, the chickens as well as some of the eggs of the egg laying flock undergo a limited amount of testing to screen for pathogens that may be problematic for egg production or quality. The eggs used for production; however, undergo virtually no testing and the levels of microbial contamination are unknown until after the vaccine is harvested.

In order to verify that no adventitious agents were detected in the MDCK cell banks, tests were performed at different stages of cell banking (MCB, WCB, and EOP). These adventitious agent tests are divided into assessment that can detect a wide array of adventitious agents and more specific ones that use sensitive methodologies such as PCR. The following are grouped into broad spectrum and specialized tests:

Broad Spectrum Tests

Broad spectrum tests are able to detect a broad spectrum of adventitious agents and include:

- 1) Microbial tests, which are generalized sterility tests that can detect bacterial and fungal agents, mycoplasma, and mycobacteria;
- 3) In vitro and in vivo adventitious agent tests, which use a variety of indicator cell lines and animal models to look for cytopathic effect of adventitious agents such as canine, bovine, equine, and porcine pathogens;
- 4) Antibody production tests, in which non-cultivable viruses that can provoke an immune response by previously naïve animals following inoculation with the test article;
- 5) Tests for occult viruses and retroviruses, which are designed to detect latent viruses in cells by using chemical inducers;
- 6) Transmission electron microscopy (TEM), which uses electron microscopy to detect virus particles.

Specialized Tests

A series of specialized molecular-based tests were performed on MedImmune's MDCK cells to detect potential pathogens from a variety of animal species. The molecular-based tests employ genetic target sequences to detect specific human, canine, or simian viruses, and viruses that could be associated with raw materials of bovine or porcine origin.

The majority of these tests were performed on the MCB; however, a subset of tests was performed on EOP cells including PCR for canine adventitious agents and tests for inducible endogenous retroviruses and latent DNA viruses. In addition, each lot of the WCB was tested to confirm that adventitious agents (canine, bovine, equine, and porcine) were not introduced during manufacture of the WCB from the MCB under cGMP conditions. An outline of the tests along with the results is included in [Appendix 2](#) (broad spectrum) and [Appendix 3](#) (specialized).

In summary, no adventitious agents were detected in MedImmune's MDCK cells.

3.3.3 Evaluation of Tumorigenic Potential of Intact MDCK Cells

The tumorigenic potential of MedImmune's MDCK cell line was evaluated using newborn athymic nude mice and adult athymic nude mice. Various doses (10^1 , 10^3 , 10^5 and 10^7 viable cells) of viable MDCK EOP cells, which were grown in MediV-105 SFM and represented

approximately 51 population doublings from the MCB, were inoculated subcutaneously into groups of both newborn and adult nude mice. All animals were observed for a period of six months, during which the animals were regularly checked for nodule development and clinical symptoms. As expected, HeLa cells which were used as positive controls, produced easily identifiable and palpable nodules at the site of inoculation within the second week of injection in over 90% of the positive control animals. These nodules progressed into large tumors that were confirmed through histopathological examination as HeLa cell carcinomas (tumors). In contrast, only one distal systemic tumor was observed in animals injected with MDCK cells and two observed in the negative control (PBS injected) group. The histiocytic sarcoma observed in the lungs, liver, and spleen of an adult nude mouse receiving 10^5 MDCK cells was further examined to determine whether it was of canine or murine origin. Tissue from this tumor was analyzed for the presence of canine protein by immunohistochemistry and demonstrated to be a murine tumor that did not express a canine epithelial protein. A second method was employed to detect canine short (130-150 bp) interspersed nuclear elements (SINE), and demonstrated that the tumor tissue did not contain canine DNA. Canine SINE are distributed throughout the genome, present in approximately every 5-8.3 kb of the canine genome, and represents 10% of the canine genome. This tumor was also tested for rodent SINE sequences and confirmed to be a murine tumor. Therefore, there were no canine tumors observed due to injection of intact MDCK cells into either the newborn or adult athymic nude mice. This demonstrates that MedImmune's cell line had a low potential to form tumors in either newborn athymic nude or adult athymic nude mice ([Table 3.3.3-1](#) and [Table 3.3.3-2](#)).

Table 3.3.3-1 Newborn Athymic Nude Mouse Tumorigenicity Study

Group	Number of Animals Injected	Tumors Observed at Site of Injection	Spontaneous Murine Tumors Observed in Other Locations	Conclusions
Negative control (DPBS)	43	0	0	No tumors observed as expected
Positive control (HeLa cells)	44	44	0	HeLa cell-derived carcinomas observed as expected (100% tumor formation rate)
10^1 MDCK cells	44	0	0	No MDCK-associated tumors detected
10^3 MDCK cells	44	0	0	No MDCK-associated tumors detected

Table 3.3.3-1 Newborn Athymic Nude Mouse Tumorigenicity Study

Group	Number of Animals Injected	Tumors Observed at Site of Injection	Spontaneous Murine Tumors Observed in Other Locations	Conclusions
10 ⁵ MDCK cells	39	0	0	No MDCK-associated tumors detected
10 ⁷ MDCK cells	44	0	0	No MDCK-associated tumors detected

Note: Duration of the in-life phase of the study was approximately 180 days

Table 3.3.3-2 Adult Athymic Nude Mouse Tumorigenicity Study

Group	Number of Animals Injected	Tumors Observed at Site of Injection	Spontaneous Murine Tumors Observed at Other Locations	Conclusions
Negative control (DPBS)	33	0	2 ^a	Two spontaneous tumors observed
Positive control (HeLa cells)	41	37	0	HeLa cell-derived carcinomas observed as expected (> 90% tumor formation rate)
10 ¹ MDCK cells	44	0	0	No MDCK-associated tumors detected
10 ³ MDCK cells	44	0	0	
10 ⁵ MDCK cells	44	0	1 ^b	
10 ⁷ MDCK cells	44	0	0	

Note: Duration of the in-life phase of the study was approximately 180 days

Test article tumor tissues were evaluated by immunohistochemistry for the presence of either canine ezrin or murine galectin-3

Tumor tissues were evaluated for the presence of canine DNA by PCR for canine SINE sequences; tissues negative for canine SINE were subsequently tested for rodent SINE sequences and confirmed to be rodent tumors

^a One lymphoma and one bronchiolo-alveolar adenoma were observed in two mice in the DPBS (saline) control group

^b A histiocytic sarcoma was observed in the 10⁵ MDCK cells group. This tumor was tested and found to be negative for the presence of canine protein and canine sequences by immunohistochemistry and SINE PCR, respectively, and was therefore a spontaneous murine tumor.

3.3.4 Evaluation of Oncogenic Potential of MDCK Cell Lysate and MDCK cell DNA

In addition to the above-described tumorigenicity studies that used intact MDCK cells, the potential for high doses of MDCK cellular DNA and MDCK lysates to induce tumor formation was evaluated. These studies were performed in newborn rodents (newborn athymic nude mice, newborn rats and newborn hamsters) by subcutaneously injecting each rodent with either lysate prepared from 10^7 viable MDCK EOP cells or 100 μg of cellular DNA (representing over 100,000 times more DNA than present in the vaccine) prepared from MDCK EOP cells. All animals were observed for a period of six months, during which the animals were regularly checked for nodule development and clinical symptoms. No tumors were observed at the site of injection. Three distal systemic tumors were observed in the test groups (MDCK cell lysate and MDCK cellular DNA) during histological examination. These tumors were deemed as spontaneous based on their sporadic and low frequency of occurrence in this study, their common occurrence in rodents (commonly observed spontaneously in untreated aged rodents), and in one case, the presence of similar tumors in the negative control groups (Chandra, 1992; Mahler, 1996; Nakashima, 1988; Boorman, 1990; Maronpot, 1999; Haseman, 1998; Pour, 1976). In addition, canine DNA was not detected in these tumors when tested by a sensitive PCR method using canine SINE primers. All tumors were shown to be of rodent origin with no evidence of canine DNA in these tissues. In conclusion, neither the MDCK cell lysate nor MDCK cellular DNA caused local or systemic tumors in any of the three animal species tested ([Table 3.3.4-1](#) and [Table 3.3.4-2](#)).

Table 3.3.4-1 Oncogenicity Study on Newborn Animals Using MDCK Cell Lysate

Animal Species	Group	Number of Animals Injected	Tumors Observed at Site of Injection	Spontaneous Rodent Tumors Observed at Other Locations	Conclusions
Newborn nude mouse	MDCK cell lysate (prepared from 10^7 cells)	45	0	0	No MDCK-induced tumors detected
	Negative control (DPBS)	45	0	0	
	Non-injected control	25	0	1 ^a	
Newborn hamster	MDCK cell lysate (prepared from 10^7 cells)	45	0	0	No MDCK-induced tumors detected
	Negative control (DPBS)	45	0	0	
	Non-injected control	25	0	0	
Newborn rat	MDCK cell lysate (prepared from 10^7 cells)	45	0	1 ^b	No MDCK-induced tumors detected
	Negative control (DPBS)	45	0	0	
	Non-injected control	25	0	0	

Note: Duration of the in-life phase of the study was approximately 180 days

^a Bronchiolo-alveolar adenoma in the lung

^b Hind leg carcinoma –This tumor was deemed as a spontaneous rodent tumor based on location and incidence rate; testing with canine SINE PCR primers demonstrated that it did not contain detectable canine DNA.

Table 3.3.4-2 Oncogenicity Study on Newborn Animals Using MDCK DNA

Animal Species	Group	Number of Animals Injected	Tumors Observed at Site of Injection	Spontaneous Rodent Tumors Observed at Other Location	Conclusions
Newborn nude mouse	MDCK DNA (100 µg)	45	0	1 ^a	No MDCK-induced tumors detected
	Negative control (DPBS)	44	0	0	
	Non-injected control	25	0	0	
Newborn hamster	MDCK DNA (100 µg)	45	0	1 ^b	No MDCK-induced tumors detected
	Negative control (DPBS)	45	0	0	
	Non-injected control	25	0	0	
Newborn rat	MDCK DNA (100 µg)	45	0	0	No MDCK-induced tumors detected
	Negative control (DPBS)	45	0	2 ^c	
	Non-injected control	25	0	0	

Note: Duration of the in-life phase of the study was approximately 180 days

^a Bronchiolo-alveolar adenoma in the lung – This tumor was deemed as a spontaneous rodent tumor based on location and incidence rate; testing with canine SINE PCR primers demonstrated that it did not contain detectable canine DNA.

^b Nephroblastoma – This tumor was deemed as a spontaneous rodent tumor based on location and incidence rate; testing with canine SINE PCR primers demonstrated that it did not contain detectable canine DNA.

^c Hepatocellular adenoma and skin hemangiosarcoma observed in two separate rats

3.4 Manufacturing Procedures Augment Product Safety by Removing Intact Cells and Reducing, MDCK DNA and MDCK Protein from the Vaccine

The cell culture-based manufacturing process for expansion of the cell substrate, production of the vaccine strains, and purification of bulk vaccine utilizes modern manufacturing technologies that comply with the current Good Manufacturing Practices of the US and international regulations to expand the cell substrate, produce the vaccine strain, and purify the bulk vaccine virus. During the manufacture of our seasonal and pandemic cell culture

manufactured vaccines, cell culture purity is ensured through maintaining sterility of culture media and the MDCK cell culture prior to introduction of the viral seed. In addition, fully qualified and characterized master viral seeds are used for manufacturing. Furthermore, a number of steps in the manufacturing process effectively eliminates the chance of introducing adventitious agents. Steps in the manufacturing process also ensure the complete removal of intact MDCK cells and reduce the amount and size of residual cellular DNA to extremely low levels. [Figure 3.4-1](#) shows an overview of the downstream manufacturing process.

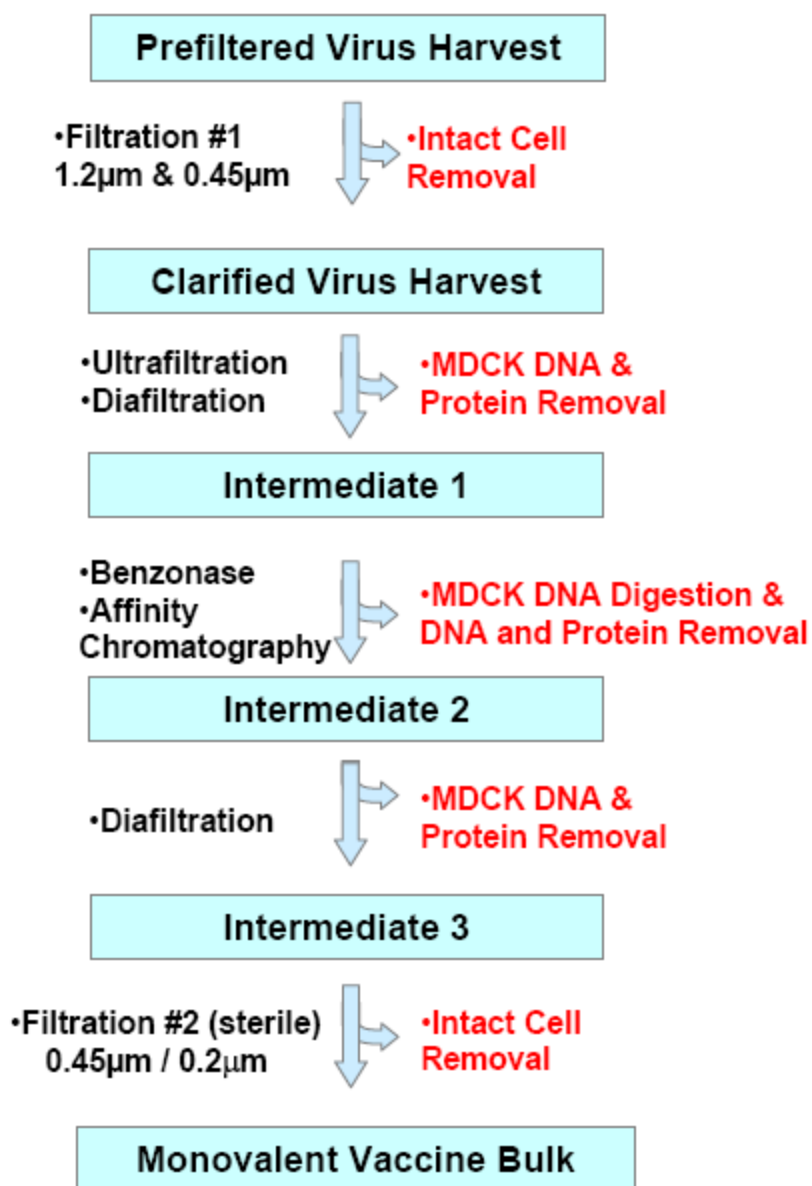


Figure 3.4-1 Overview of Downstream Manufacturing Process

3.4.1 Filtration Ensures the Removal of Intact MDCK Cells

Following infection of the cells, influenza virus replicates and lyses a large number of cells by virtue of its typically lytic nature in cell culture. After harvest of the bioreactor fluid, any remaining intact MDCK cells are removed by the downstream manufacturing process, which includes viral harvest clarification and sterile filtration. These filtration processes have been

validated by current industry standards, demonstrating that they are capable of efficiently removing microbial organisms that are significantly smaller than MDCK cells. By quantifying the number of microorganisms each filter can remove, this process can be shown to remove at least 21 log₁₀ cells from a solution, which is vastly greater than the number of cells present in a bioreactor even prior to the time of infection. At most, one cell could be expected to pass into the filtered material once every 100,000,000,000 times the process is run. All filters used in the downstream manufacturing process are integrity tested and must pass a bubble point test after filtration.

3.4.2 The Purification Process Removes Host Cell DNA and Host Cell Protein

Removal of host cell DNA is achieved through tangential flow filtration (TFF) and chromatography steps. During the TFF process step, DNA is removed from the vaccine intermediate based on the size differences between the virus and host cell DNA. The following step incorporates both Benzonase digestion and affinity chromatography. DNA is degraded through enzymatic action of the benzonase and is removed from the vaccine by virtue of its inability to bind to the affinity matrix. The combination of filtration, digestion and chromatography steps results in reducing the quantity and size of the residual MDCK DNA in the vaccine product. For the first clinical trial material, the quantity of residual MDCK cell DNA was approximately 0.1 ng/dose and the size of this DNA was reduced to a median size of approximately 450 bp in length with approximately 64% of the residual DNA being less than 500 bp in length and approximately 90% less than 1000 bp in length.

The combination of steps mentioned above used in the purification of the vaccine product resulted in a reduction of MDCK cell protein in the vaccine to 0.47 µg/dose for the clinical trial material intended for the first clinical study. A summary of the efficiency of the different purification steps with respect to removal of MDCK cellular DNA and MDCK host cell protein by the manufacturing process step is shown in [Table 3.4.2-1](#). The steps showing the greatest reduction in cellular DNA and host cell protein are highlighted in bold text.

Table 3.4.2-1 Summary of Host Cell DNA and Host Cell Protein

Process Step	HCD^a Remaining (accumulated %)	HCP^b Remaining (accumulated %)
Virus harvest	100.00	100.00
Clarified Virus Harvest	≤ 70.63	≤ 99.08
Ultrafiltration /Diafiltration	≤ 9.37	≤ 8.48
Affinity Chromatography	≤ 0.30	≤ 4.79
Diafiltration	≤ 0.47	≤ 4.62
Final Bulk	≤ 0.46	≤ 3.10

^a HCD: Host cell DNA^b HCP: Host cell protein

3.5 Routine Testing of the Bulk and Final Filled Vaccine Material

Each batch of monovalent bulk vaccine as well as the final vaccine product for the currently licensed egg produced vaccine undergoes comprehensive in vitro and in vivo testing to detect any potential adventitious agents. Each batch of monovalent bulk and vaccine product produced by cell culture will also be comprehensively tested to detect adventitious agents and will undergo further testing to measure residual quantities of MDCK DNA and protein. These tests will ensure the safety of the vaccine and demonstrate that the production and purification processes performed to the expected standards.

4 Risk Assessment of MDCK Cell Line for LAIV Production

As described in the previous sections, experimental safety data support the conclusion that MedImmune's MDCK cell line and its manufacturing process controls are capable of producing high quality, safe vaccines. To complement the safety data, we have assessed risks associated with defined components of the vaccine. Specifically, we have assessed three potential risks that might be associated with use of MDCK cells as a substrate for vaccine production: (a) tumorigenicity associated with intact cells (b) oncogenicity associated with MDCK DNA or occult viruses, and (c) infectivity associated with infectious nucleic acid components in the MDCK DNA.

4.1 Assessing Risk due to Intact Cells in the Vaccine

Experimental data described in previous sections indicate that MedImmune's MDCK cell substrate has a low tumorigenic potential when injected into test animals. It is important to note, however, that cells are removed from the bulk vaccine during the manufacturing process further ensuring that even cells of low tumorigenic potential are not present in the vaccine product. Experimental studies that validated the filtration steps demonstrated an overall clearance capability of $21 \log_{10}$ cells; this is approximately $11 \log_{10}$ times greater than the expected number of cells in the harvested fluids (Section 3.4.1). The capability of the filtration steps results in the probability of one person receiving a single MDCK cell as being less than 1 in 6.3 quadrillion (1 in $10^{15.8}$, Table 4.1-1). The manufacturing process, therefore, essentially eliminates the risk of intact cells entering the bulk vaccine product thus adding even further confidence to the low potential tumorigenicity associated with this MDCK cell line.

Table 4.1-1 Probability a Single Cell Could Be in a Dose

Theoretical starting cells per dose ^a	$10^{5.6}$
Cumulative cell removal	$10^{-21.4}$
Probability a single cell could be in a dose	$10^{-15.8}$

^a Assumes no cells are destroyed at the time of virus harvest

4.2 Assessing Risk due to Oncogenicity or Infectivity Resulting from Residual Host Cell DNA or Cellular Components

Experimental data described in previous sections indicate that high doses of DNA or cell lysates from MedImmune's MDCK line is not associated with oncogenicity when injected into experimental animals. These studies were performed to address concerns that MDCK DNA or cell components may have oncogenic potential due to oncoproteins, activated oncogenes, proviruses, latent infectious agent (e.g., retrovirus), or episomal DNA elements (e.g., papovavirus). There is consensus that cellular growth-promoting proteins (e.g., oncoproteins) are not a significant risk factor (Griffiths, 2001). To complement the results of oncogenicity testing (in which no evidence of oncogenicity was found associated with MDCK

cell DNA or cell components), we performed a risk assessment under a worst-case scenario, in which it was assumed that these components had some level of tumorigenic potential.

This risk assessment associated with the oncogenic potential of residual cellular components in the product was guided based on published regulatory guidance (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, CBER Draft Guidance 2006) and criteria and formula described by Sheng (2008) and Peden (2006). In brief, these investigators showed that tumors could be formed in nude mice that were co-injected with 12.5 µg each of two plasmids, each containing an activated oncogene (Figure 4.2-1). Each 12.5 µg of oncogene plasmid DNA is calculated to contain 1.63×10^{12} oncogene copies; two plasmid DNAs contain a total 3.26×10^{12} oncogene copies. Based on the canine genome size, 1 ng of residual MDCK cell DNA is the equivalent of approximately 190 diploid genomes or a total of 380 haploid genomes. By applying previously described calculations that takes into account the mass of oncogene DNA needed to elicit tumors in an animal model, the potential size of an oncogene, size of the canine genome and mass of canine DNA in the vaccine (assumed to be 1 ng for this calculation which is approximately 10 times the mass in the current clinical trial material), a safety factor is calculated of 5.88×10^9 (Appendix 4). This safety factor is equivalent to stating that over 5 billion doses would need to be distributed to deliver the mass of oncogenic DNA needed in the animal studies to elicit a tumor in one animal. By taking the inverse of this number, the risk in a single dose of vaccine would be 1.7×10^{-10} .

This safety factor calculation; however, misses a critical element of the nature of the MDCK DNA in the vaccine. Specifically, the DNA in the vaccine is degraded and has a median size of 450 bp, which is smaller than the average size of an oncogene (1,925 bp). In general, the larger the size of the DNA fragment, the more likely it would be to contain an intact oncogene. As the size of the DNA decreases, the likelihood of finding an intact oncogene also decreases. A series of equations predict the likelihood of identifying an intact oncogene that is dependent on the size of the fragmented DNA in the vaccine. In order to assess the degree of safety imparted by DNA degradation, a conservative assumption was made that the DNA was sheared to a length of only 1,925 bp, the average size of an intact oncogene. As shown in Appendix 4, shearing the DNA to this size resulted in the need to screen approximately 2,000 times more DNA to identify an intact oncogene. In order to calculate the safety factor for 1 ng of DNA sheared to a length of 1,925 bp, the safety factor needs to be increased by the additional factor of 1.9×10^3 , resulting in a more appropriate safety factor of

1.13×10^{13} or over 10^{13} doses would need to be distributed to equal the oncogene load needed to elicit a tumor in an animal model. This safety factor equates to the oncogenic risk due to degraded MDCK DNA in a single dose of vaccine of 8.9×10^{-14} .

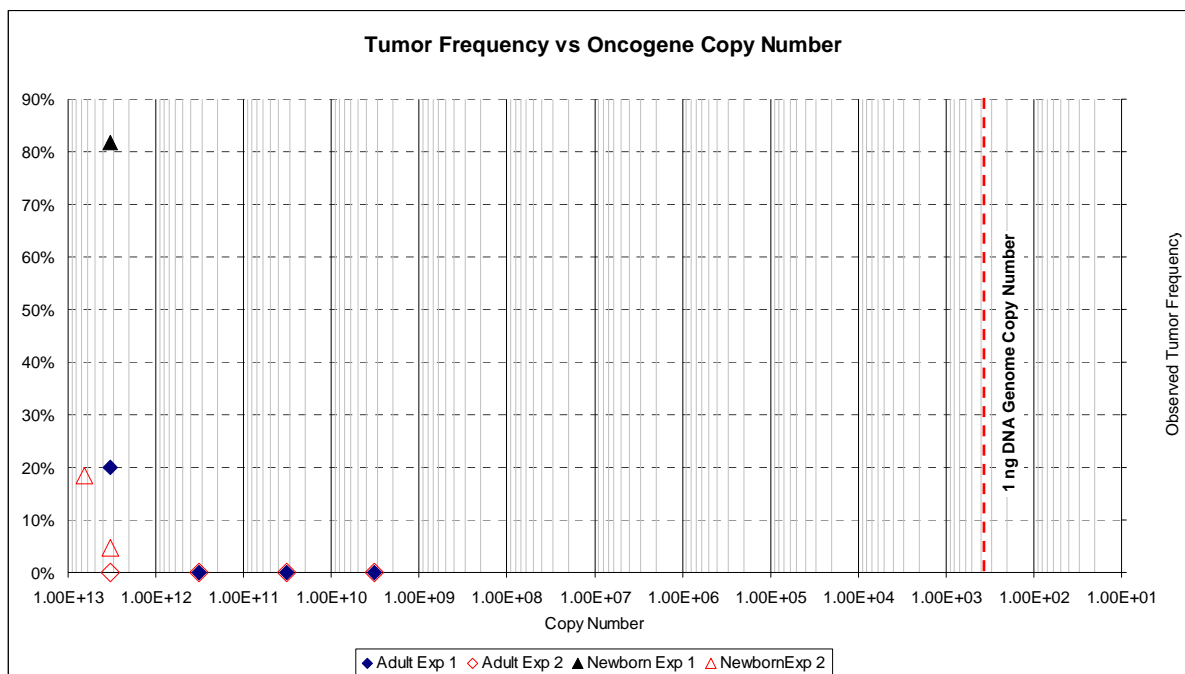


Figure 4.2-1 Relationship Between Tumor Formation and DNA Oncogene Copy Number From Published Studies (Sheng, 2008)

Another theoretical risk that may be associated with residual host cell DNA is the presence of a latent infectious viral genome in MDCK DNA. The infectivity risk assessment was modeled based on a published analysis by Peden (2006). In this model, HIV was used as a surrogate measure for latent DNA infectivity. When 150 ng of an HIV plasmid was degraded to a mean size of 650 bp, detectable infectivity was abolished. In order to calculate the safety factor for the potential of proviral infectivity in the vaccine, the amount of DNA in the vaccine (1 ng), size of the diploid canine genome, assumption of the size of a small canine retrovirus provirus (7.0 kb) and quantity of nucleic acid associated with infectivity that results in no infectivity following degradation (150 ng) were used (Appendix 4). Applying the calculation, 1 ng of host cell DNA would result in a safety factor of 1.03×10^8 , or the possibility of having one infectious provirus once in every one billion doses. On an individual basis, the risk in a single dose of vaccine would be 9.7×10^{-9} .

As an additional method to calculating the safety factor, an approach similar to the one described above for the oncogenicity risk assessment was applied. If the MDCK cellular DNA was degraded to a fragment size of 7,000 bp, then approximately 7,000 times more DNA would need to be evaluated to identify an intact provirus sequence (Appendix 4). These calculations would result in a safety factor calculation of 7.2×10^{11} . The experimental data further showed that linear provirus DNA was approximately 5 times less likely to result in infectivity compared to the plasmid cloned provirus used for the modeling purposes. Including this factor, a safety factor of 3.6×10^{12} , which is equivalent to the risk in a single dose of vaccine of 2.8×10^{-13} , would be achieved for the vaccine (Appendix 4).

The published experimental studies also demonstrated that DNA digestion could eliminate infectivity of the cloned HIV provirus in an experimental setting when the median size of the DNA was reduced to 650 bp by digestion. As shown through the release test results for the current clinical trial material, the manufacturing process reduced the MDCK DNA to a median size of approximately 450 bp in length with approximately 64% of the residual DNA being less than 500 bp in length. Additionally, approximately 90% of the residual DNA is less than 1000 bp in length, indicating the residual DNA is far below the size that could harbor an infectious provirus. These data would support that the MDCK DNA has been digested to a level that would destroy provirus infectivity. MedImmune is continuing to develop methods to reduce as well as assess and quantify this small fraction of larger DNA fragments.

4.3 Assessing Risk Due to Transmissible Spongiform Encephalopathy (TSE) and Bovine Spongiform Encephalopathy (BSE)

MedImmune has selected a cloned MDCK cell line as a cell substrate for influenza vaccine production. Although this cell line has been adapted to serum free media formulations, prior exposure to animal-derived raw materials including fetal bovine serum (FBS) may present a theoretical risk of introducing TSE/BSE agents into the manufacturing process. MedImmune strongly believes that its MDCK cell bank is free of BSE due to the following:

- 1) Canines appear to be refractory to TSE disease (Polymenidou, 2008; Diaz-San Segundo, 2006), and there are no reports of canine TSE disease in the literature;
- 2) MDCK cells are refractory to prion replication as reported by Polymenidou et al (2008);

- 3) Two rounds of limiting dilution cloning were performed on the MDCK cells obtained from ATCC at MedImmune to obtain a highly productive subclone. Reduction in the MDCK cell population to a single cell constrains the theoretical association of any TSE agent to a single cell;
- 4) Following receipt of MDCK cells from the ATCC, all subsequent cell passaging used fetal bovine serum (FBS) sourced from controlled herds in New Zealand, a geographic location that has not reported BSE to date (classified as GBR I);
- 5) Subsequent to limiting dilution cloning, the MDCK cell subclone was adapted to serum free media formulations and expanded to reach the vaccine production stage.

To further support the safety of the MDCK cell, we performed a risk assessment under a worst-case scenario, in which it was assumed that the MDCK cells had some level of exposure to TSE/BSE previous to the acquisition by MedImmune (see [Appendix 5](#)). In this analysis, it was assumed that the MDCK cells in general and the cloned MDCK cell (first isolated at passage 88) used for the preparation of the Master Cell Bank contained maximally 1 ID₅₀.

To reach the passage level for vaccine production, Passage 109, the original MDCK subclone would have undergone 24 passages. Assuming an average 5-fold increase in cell numbers at each passage, the number of cells should have expanded to approximately 5^{24} ($\sim 6.0 \times 10^{16}$) and the original one (1) ID₅₀ would have decreased to approximately 1.69×10^{-17} .

Assuming 20 million doses of vaccine are produced per vaccine lot, a further infectivity reduction due to the dilution of the drug substance to manufacture the drug product, there would be approximately 8.4×10^{-25} safety factor per dose per influenza strain.

4.4 Other Factors Contributing to a Risk Reduction

The route of vaccine administration is another likely factor that lowers risk associated with cellular DNA or other components present in MDCK produced LAIV. The ciliated epithelium of the respiratory tract rapidly clears the majority of nasally introduced foreign material thereby preventing it from entering the lungs. In order to further understand the clearance of residual host cell DNA following intranasal administration, a study was conducted in Sprague Dawley rats using purified MDCK DNA. The purified DNA was sonicated to a size comparable to that found in the current investigational vaccine material, where the median size of the residual DNA was approximately 450 bp. One hundred micrograms of the sheared DNA was administered intranasally (IN) or injected

intramuscularly (IM) to rats. Animals were euthanized at various time points (6, 24, 48, 72, 96, 168, 336 hours) and selected tissues were harvested (see [Appendix 6](#)). The presence of MDCK DNA was measured using the canine SINE quantitative PCR assay. Of the 14 tissue types examined, only four tissues were observed to contain detectable MDCK cell DNA. The average DNA amounts observed at the various time points post-inoculation are shown in [Figure 4.4-1](#). MDCK DNA was observed only in the respiratory tract (trachea and lungs) of IN inoculated animals, and in blood, muscle, and skin of IM inoculated animals. The amount of DNA present in these tissues varied significantly between the tissues with the highest level in the skin (IM) and muscle (IM) and lower levels in blood (IM) and trachea and lungs (IN). Not unexpectedly, the levels were highest at 6 hours post-inoculation for skin (IM) and muscle (IM) with the amount declining over the seven day study.

Based on the amount of DNA administered ($100\text{ }\mu\text{g} = 1.0 \times 10^8\text{ pg}$), by six hours post-intranasal administration only a small fraction (approximately 1.0×10^{-6}) of the input DNA was observed in the inoculated animals (3/4). The DNA persisted in 3/4 animals' lungs for at least two days, 0/4 animals on day three, 1/4 animals on day four, and 0/4 had detectable levels on day seven.

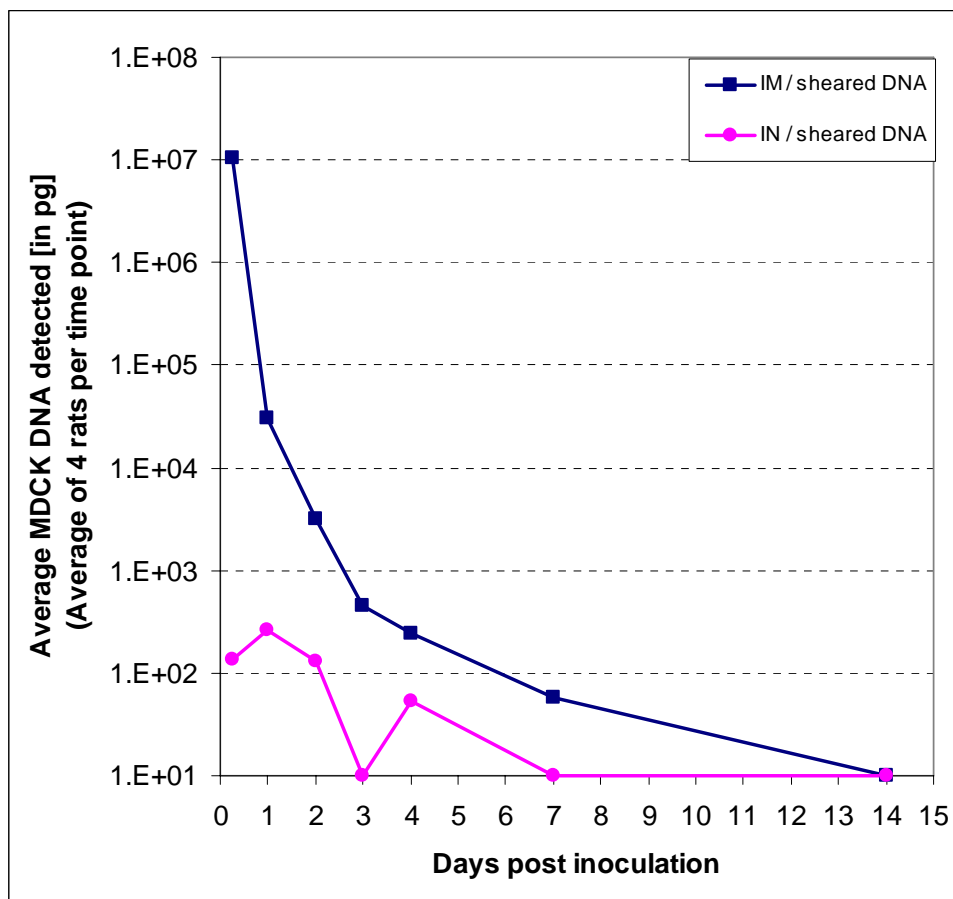


Figure 4.4-1 Log-Linear Plot of DNA Clearance in Rats Inoculated Intranasally or Intramuscularly With 100 Micrograms Sonicated MDCK Cellular DNA

Of the 14 different tissues harvested, only lungs and trachea, blood, skin, or muscle had detectable DNA (LLOQ, 0.005 pg). The average amount (pg) of DNA per whole tissue weight is plotted for all tissues with detectable DNA at any time post inoculation.

These results demonstrate that the bioavailability of sheared DNA (surrogate for process residual DNA in finished vaccine) is significantly diminished when introduced by intranasal inoculation. The observed clearance of approximately 1.0×10^{-6} would theoretically reduce the (less than) 1 ng of residual host cell DNA in the finished vaccine dose to less than 1.0×10^{-15} g (1 fg). To place this number in perspective, as noted above, 1 ng of canine genomic DNA is the equivalent of 190 diploid cells, thus 1 fg is the equivalent of 0.00019 cellular genome equivalents per dose.

4.5 Maintaining Safety and Robust Process Control in Routine Production

Many aspects of the production process for MDCK cell culture-produced LAIV assure the safety and quality of the bulk vaccine product. These aspects include use of highly tested and characterized materials (such as the MDCK cell substrate), use of aseptic controlled processes to manufacture the vaccine and extensive testing of each lot of bulk vaccine, (similar to the testing currently performed on each lot of commercially available, egg-produced FluMist vaccine bulk intermediates). The factors that contribute to the robust assurance of product safety are summarized as follows:

- The Master Virus Seeds (MVSs) used in the production of the MDCK cell culture-produced seasonal influenza vaccine for the first clinical study were the same MVSs that were used in the manufacture of the 2007-2008 commercial FluMist formulation. Production of MVSs for future clinical studies and eventual commercial production of cell culture-produced LAIV will use the plasmid rescue techniques, and the viral seeds will be amplified in MDCK cells instead of eggs or chicken cells. Plasmid rescue uses recombinant DNA technology to generate the seed viruses and eliminates the exposure of the vaccine strain to the wild type influenza virus and to any extraneous agent that might have contaminated the original wild type virus. The licensed plasmid rescue process currently used to manufacture commercial FluMist will be implemented for the Phase 2 seasonal cell culture influenza clinical study.
- The MCB and WCB have been produced under strict cGMP procedures and have been comprehensively tested and characterized demonstrating their high quality. These cell banks are maintained in highly controlled environments and are expected to be high quality substrates for vaccine production. In addition, the media used to grow and maintain the cells have been developed to eliminate all potential animal derived components (with the exception of sheep wool cholesterol). The media used for growth of the cells and production of the vaccine has been shown to support robust growth of MDCK cells.
- Manufacturing utilizes an aseptic, controlled, closely monitored and enclosed cell culture process. The process employs several steps to provide robust removal of host cells, cellular DNA and cellular proteins.
- A comprehensive in-process and release testing plan similar to that used for egg-produced, commercial FluMist monitors each production batch. Much of the testing occurs on the pre-filtered viral harvest in order to provide the greatest sensitivity of detecting any potential adventitious agent that may be present in the vaccine bulk material. This testing scheme is designed to detect any putative adventitious agent that could get amplified in the cell culture-produced viral manufacturing process, if introduced despite all other controls.

5 Conclusion: MedImmune's LAIV Produced in MDCK Cells is Safe for Use and Delivers an Important Public Health Solution for Seasonal and Pandemic Vaccines

In order to meet public health immunization needs, especially in the event of a pandemic outbreak, an alternative to the current embryonated chicken egg process is necessary for influenza vaccine production. MedImmune is developing both seasonal and pandemic influenza vaccines utilizing a MDCK cell culture substrate because it offers the following advantages over egg-based production:

- Better control of the production substrate through availability of high characterized banks of cells in the frozen inventory;
- More extensive cell substrate characterization and testing;
- Greater flexibility in production capacity to respond more rapidly to increased vaccine demand.

In order to address the risks of using a continuous cell line in the manufacturing process, MedImmune established and extensively characterized a uniform, serum free bank of MDCK cells. The results of these studies demonstrated:

- Adventitious agents were not detected following extensive testing;
- There was a very low propensity for intact cells to form tumors in animal models; additionally, the production technologies used are designed to eliminate intact cells in the product;
- DNA and lysates of the cells had no detectable oncogenic potential.

Licensure of a cell culture produced LAIV could provide additional benefits for both seasonal and pandemic influenza immunization due to the unique characteristics of LAIV-induced immune protection. The manufacturing platform will enable larger supplies of vaccine to be made available and is likely to be more reliable than egg produced vaccines. Improving the supply of influenza vaccine on a seasonal basis is a major component of pandemic preparedness. Additionally, the time and scale benefits of cell culture production make feasible the delivery of hundreds of millions of doses of vaccine within a short period of time.

Based on the MDCK cell testing and characterization results, the manufacturing processes and controls, and the risk assessments performed, MedImmune believes that seasonal and pandemic influenza vaccines produced using our MDCK cell culture production platform

meet the quality and safety standards necessary for clinical evaluation and continued product development.

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Appendix 1 Detection of Homologous Wild-type Challenge Viruses in Ferret Lung Tissues by EID50 Assay

Challenge Virus (FFU)	Treatment	HAI Log2 Titer at D21 post-inoculation	Log ₁₀ /ml (Mean ± SD) ^a	n/N
<i>wt</i> A/New Caledonia/20/99 5.5 Log ₁₀	Egg-based vaccine	3.2	< LOD	0/3
	MDCK cell-based Vaccine	4.6	< LOD	0/3
	Placebo	1.0	0.70 ± 0.3	2/4
<i>wt</i> A/Wisconsin/67/05 7.0 Log ₁₀	Egg-based vaccine	6.8	< LOD	0/5
	MDCK cell-based Vaccine	8.0	< LOD	0/5
	Placebo	1.0	0.54 ± 0.1	1/5
<i>wt</i> B/Malaysia/2506/04 5.5 Log ₁₀	Egg-based vaccine	5.3	< LOD	0/3
	MDCK cell-based vaccine	4.1	< LOD	0/3
	Placebo	1.0	2.06 ± 1.7	3/4

^a Limit of detection: 0.5 log₁₀ FFU/ml

Two dose vaccine study, immunogenicity evaluated after each dose by HAI titer, efficacy evaluated by inhibition of nasal viral replication after wild type homologous challenge

Appendix 2 Broad Spectrum Tests Performed on the MDCK MCB

Test Description	Test Result
Microbial Tests	
Sterility ^a	Pass
Bacteriostasis and Fungistasis	Pass
Mycoplasma ^a	Pass
Mycoplasma mastitis	Pass
Mycobacterium	Pass
Cell Culture Identity (Isoenzyme analysis) ^a	Pass
In vitro and In vivo Tests	
In vivo adventitious tests ^a	Pass
In vitro adventitious tests ^a	Pass
Bovine in vitro assay (9 CFR)	Pass
Porcine in vitro assay (9 CFR)	Pass
Equine in vitro assay (9 CFR)	Pass
In vitro assay for canine specific viruses	Pass
Antibody Production Tests	
Mouse antibody production test	Pass
Rat antibody production test	Pass
Hamster antibody production test	Pass
Occult Virus and Retrovirus Tests	
Induction assay for retroviruses (Infection assay -- 8 cell lines) ^b	Pass
Induction assay for retroviruses (Co-cultivation assay -- 5 cell lines) ^b	Pass
Induction assay for latent DNA viruses ^b	Pass
PERT assay	Pass
Co-culture with HUT-78 cells and detection by PERT	Pass
Co-culture with Raji cells and detection by PERT	Pass

Test Description	Test Result
Other	
TEM for viruses and retroviruses	Pass ^c
Karyotype analysis ^b	Pass ^d

^a Testing also performed on the WCB

^b Testing performed on EOP cells which are derived from the MCB

^c Normal cellular morphology observed; no identifiable virus-like particles nor other microbial agents detected or observed; no other structural abnormalities observed

^d Maintained the median chromosome number of 78; no aberrant chromosomes observed or detected and no significant differences between passages

Appendix 3 Specialized Tests Performed on the MDCK Master Cell Bank

Test Description	Test Result	Limit of Detection (No. of Copies)
Human Viruses		
PCR for AAV (Adeno-associated virus)	Pass	100
PCR for Human Parvovirus (B19)	Pass	100
PCR for CMV (Cytomegalovirus)	Pass	100
PCR for EBV (Epstein-Barr virus)	Pass	10
PCR for HAV (Hepatitis A virus)	Pass	100
PCR for HBV (Hepatitis B virus)	Pass	100
PCR for HCV (Hepatitis C virus)	Pass	100
PCR for HHV-6 (Human Herpesvirus)	Pass	100
PCR for HHV-7	Pass	100
PCR for HHV-8	Pass	100
PCR for HIV-1 and 2	Pass	100
PCR for BKV and JCV (Human Polyoma virus)	Pass	100
PCR for HPV (Human Papillomavirus)	Pass	1000
PCR for HTLV-1 and 2 (Human T-cell lymphotropic virus)	Pass	100
Simian Viruses		
PCR for SV-40 (Simian Virus -40)	Pass	100
PCR for SCMV (Simian cytomegalovirus)	Pass	100
PCR for SFV (Simian foamy virus)	Pass	100
PCR for SRV (Simian retrovirus)	Pass	100
PCR for SIV mac (Simian immunodeficiency virus)	Pass	100
PCR for STLV (Simian T-cell lymphotropic virus)	Pass	100

Test Description	Test Result	Limit of Detection (No. of Copies)
Canine Viruses		
Q Real time PCR assay for detection of canine Calicivirus	Pass	100
Q Real time PCR assay for detection of canine oral Papillomavirus	Pass	100
Q Real time PCR assay for detection of Canine Parvovirus -2	Pass	100
Q Real time PCR assay for detection of canine Papillomavirus Type 2	Pass	100
Q Real time PCR assay for detection of canine Papillomavirus Type 3	Pass	100
Other Viruses		
PCR assay for the detection of BPV-1 and 2 (Bovine Papilloma virus)	Pass	100
RT-PCR assay for the detection of PHEV (Porcine hemagglutinating encephalomyelitis virus)	Pass	100
PCR assay for Porcine circovirus type 1 and 2	Pass	100
qPCR assay for the detection of SVDV (Swine vesicular disease virus)	Pass	100
Xenotropic Murine Leukemia Virus	Ongoing	TBD
Bovine leukemia virus	Ongoing	TBD

TBD: To be determined

Appendix 4 Risk Assessment

Theoretical Oncogenicity of Process Residuals

Sheng et al (2008) demonstrated that two cellular oncogenes (activated human H-ras and c-myc) when inoculated together could induce sarcomas in two different mouse strains (NIH Swiss, C57BL/6). Tumor induction was observed at a high frequency in newborn mice, but at a reduced frequency in adults. In addition, tumors were observed only when both oncogenes were inoculated together, and only at the highest concentration tested (25 µg total plasmid DNA; where each oncogene was administered at 12.5 µg).

While Sheng et al (2008) examined two oncogenes; there are approximately 200 oncogenes that have been identified in various species. Zhou et al (2007) have examined the constellation of expressed human oncogenes found in various cancers using the SOURCE database. Restricting oncogene analysis to humans, a total of 81 oncogenes can be observed in 24 different tissues. The average oncoprotein coding sequence size was calculated to be $1,925 \pm 87$ bp.

For the risk analysis, the following assumptions were included to calculate a safety factor:

- The haploid genome size (GS) of the canine genome is 2.41×10^9 bp (Lindblad-Toh, 2005)
- Average oncogene size (OS) was assumed to be similar for dog and humans at 1,925 bp (Zhou, 2007), a conservative assumption as this size excludes intron sequences, and the intact gene sequences would be significantly larger.
- The oncogene amount (O_m) of DNA required for inducing an oncogenic event was assumed to be 4.7 µg.

This value (O_m) was extrapolated from the estimated size of the plasmid backbone (3186 bp) used in Sheng et al (2008) and assuming an average oncogene (1925 bp) is inserted into this backbone for a total size of 5,111 bp. Based on the total construct, the average oncogene would account for 0.377 of the construct. If 12.5 µg of the plasmid is required for each oncogene, as described by Sheng et al (2008) then the oncogene portion accounts for 4.7 µg ($12.5 \times 0.37 = O_m$).

- The amount of residual host cell DNA (hcDNA) is maximally less than 1 ng per dose, as measured in the CTM.

- Degradation of genomic DNA by benzonase is random and that reduction in the size of genomic DNA to the size of the average oncogene (1,925 bp) will reduce the frequency of an intact oncogene.
- An oncogenic event is probabilistic (eg, does not require a threshold) and requires at least two activated oncogenes for induction.

Safety Factor Calculation

The safety factor calculation used by Sheng et al (2008) used the following formula:

$$\text{Safety Factor} = \left[\frac{\frac{O_m}{[OS/GS]}}{hcDNA} \right] \quad (\text{Eq. 1})$$

Based on the defined assumptions, the genomic mass equivalent of a single oncogene is calculated from the oncogene amount (O_m) relative to the proportionality of the oncogene size to the genome size [OS/GS]. The genomic mass equivalent [$O_m / (OS/GS)$] is a measure of the oncogene equivalents present in the genome. This value is divided by the residual host cell DNA to obtain a single oncogene “safety factor” of 5.88×10^9 .

In this calculation, the genomic mass equivalent does not account for disruption of the oncogene sequence as the benzonase degraded DNA fragments approach the oncogene size (ie, 1,925 bp). To correct for the disruption of intact oncogenes, an additional safety factor was calculated using an algorithm that is routinely used for genomic library construction to assure that DNA molecular clones contain intact genes with specified probability for further study (Gardner, 1999; Kim, 2008).

In general, in a scenario where the DNA fragments are significantly larger than the target gene of interest, the probability of finding an intact gene is high; in the scenario where the DNA fragments are smaller than the target gene of interest, the gene sequences are distributed across multiple fragments and cannot be identified. The probability of identifying an intact gene can be calculated based on the size of the gene, the size of the fragments and the number of molecules (related to the mass of DNA) present in a sample. The number of clones (DNA molecules) that need to be screened, N_T , for a given probability of detecting a target gene

(oncogene) of interest, P , insert size (DNA fragment size), I , and genome size (canine), GS , can be given by the equation:

$$N_T = \frac{\ln(1-P)}{\ln\left(1 - \frac{I}{GS}\right)} \quad (\text{Eq. 2})$$

In the scenario where the DNA fragments approximate the size of the target gene of interest, the probability of finding an intact gene is lower compared to the scenario where the DNA fragments are larger than the target gene. The number of clones (DNA molecules) that need to be screened, N_W , to detect an intact target gene (intact oncogene) of interest where S is the target size (ie, 1,925 bp) can be given by the equation:

$$N_W = \frac{N_T}{\left(\frac{I - S + 1}{I}\right)} \quad (\text{Eq. 3})$$

The calculation for P (0.50, 0.90, and 0.99) and N_T and N_W using an average oncogene size of 1,925 bp is shown in the following table:

Table 1 **Probability of finding an intact oncogene in randomly degraded DNA based upon the DNA fragment size**

GS^a	I^b	S^c	$P[X > 0] =^d$	N_T^e	N_W^f
2.41×10^9	1925	1	0.500	8.7×10^5	8.7×10^5
2.41×10^9	1925	1925	0.500	8.7×10^5	1.7×10^9
2.41×10^9	1925	1	0.900	2.9×10^6	2.9×10^6
2.41×10^9	1925	1925	0.900	2.9×10^6	5.5×10^9
2.41×10^9	1925	1	0.990	5.8×10^6	5.8×10^6

Table 1 **Probability of finding an intact oncogene in randomly degraded DNA based upon the DNA fragment size**

GS^a	I^b	S^c	P[X > 0] =^d	N_T^e	N_W^f
2.41×10^9	1925	1925	0.990	5.8×10^6	1.1×10^{10}

^a Canine genome size bp^b DNA fragment size bp^c Oncogene size bp^d Probability of finding an intact oncogene in N molecular clones^e Number of molecular clones to find oncogene target sequence (distributed across multiple molecular clones)^f Number of molecular clones to find an intact oncogene sequence

The results of Equation 1 resulted in a safety factor of 5.88×10^9 based upon the presence of 1 ng of intact DNA in the vaccine. As shown in Table 1, where the DNA fragment size is large relative to the oncogene size (1 bp) then $N_T = N_W$; therefore, if the size of the DNA in the vaccine was genome size or very large, the safety factor would remain approximately 5.88×10^9 . The analytical data of the clinical trial material demonstrated a median size DNA of 450 bp in the vaccine. In order to calculate a conservative safety factor for sheared DNA, an assumption was made that the DNA in the vaccine was sheared to a size of 1,925 bp in length. If the DNA fragment is the same as the oncogene size (1,925 bp) then $N_W > N_T$ by 1.9×10^3 for any given probability and number of molecules. In other words, 1 ng of DNA sheared to 1,925 in length has fewer intact oncogenes (assuming an oncogene length of 1,925 bp) than unsheared DNA. In order to calculate the safety factor for 1 ng of DNA sheared to a length of 1,925 bp, the safety factor needs to be increased by the additional factor of 1.9×10^3 , resulting in a more appropriate safety factor of 1.13×10^{13} .

As reported by Sheng et al (2008), two oncogenes were required to cause an oncogenic effect. The “safety factor” is raised to a power of two to account for the assumption that two individual oncogenes are required to induce an oncogenic effect, and that this is probabilistic (eg, each oncogene induction is an independent event). Based on this calculation, the Safety Factor equals 1.28×10^{26} .

Additional Factors That May Increase the Safety Factor

It is worth noting that the following factors were not considered when the above oncogenicity safety factors were calculated: (1) loss of biological activity due to reduction of (oncogene) DNA size below the average oncogene size of 1925 bp through the vaccine purification process as described in Section 3.4 and (2) as the median size distribution of residual MDCK DNA is approximately 450 bp with approximately 64% of the DNA less than 500 bp in length and no detectable DNA above 1,000 bp, if biological activity is lost when DNA is degraded to below the average oncogene size then even the remote possibility of an oncogenic event calculated above from residual DNA vanishes.

Theoretical Infectivity of Process Residuals

Assumptions

Another theoretical risk that may be associated with residual hcDNA may be the presence of a latent infectious viral genome in MDCK DNA. Peden et al (2006) have studied this issue using HIV as a model. In their work, they reported that a cloned HIV genome (DNA in a plasmid) was infectious at 1 pg. Additionally, they found that hcDNA from HIV-infected cells was infectious at 2.5 µg.

Based on the HIV genome size of 10,000 bp and human diploid genome size of 6.0×10^9 bp, then a single provirus represents approximately 1.67×10^{-6} of the human diploid genome. Thus, infectivity of 2.5 µg hcDNA (HIV infected cell DNA), relative to the HIV genome frequency of 1.67×10^{-6} , is the infectious mass equivalent of 4.2×10^{-12} g (4.2 pg) of genomic DNA. Compared with the infectivity of 1 pg of HIV (DNA in a plasmid), the 4.2 pg represents a reduction in infectivity of about four-fold. Using a similar calculation but substituting the diploid canine genome size as 4.82×10^9 , and a 7.0 kb retrovirus (Goff, 2001), this retrovirus would represent approximately 1.45×10^{-6} of the dog diploid genome. If 2.5 µg canine hcDNA is assumed to have an infectivity similar to hcDNA containing an HIV provirus, a five-fold reduction in infectivity safety factor is obtained.

Peden et al (2006) also observed that infectivity could be reduced following endonuclease digestion of a mixture of cloned HIV (plasmid) and hcDNA. If hcDNA were digested to a mean size of 650 bp then complete loss of infectivity of 0.15 µg of cloned viral DNA was achieved. Using a similar calculation, a theoretical safety factor can be calculated for a canine retrovirus with the following assumptions:

- Viral genome size (VS) of 7.0 kb (Goff, 2001) representing a smaller retrovirus genome than HIV;
- A diploid canine genome size (GS) of 4.82×10^9 bp;
- A viral genome amount (V_m) of 0.15×10^{-6} μ g (analogous to 150 ng of cloned HIV) that can be completely inactivated when treated with an endonuclease to below 650 bp;
- Residual hcDNA present in the final dose as no greater than 1.0×10^{-9} g (1 ng)
- Degradation of genomic DNA by benzonase is random and that reduction in the size of genomic DNA to the size of the viral genome (7.0 kb) will reduce the frequency of an intact viral genome.

$$\text{Safety Factor} = \frac{\frac{V_m}{[VS/GS]}}{\text{hcDNA}} \quad (\text{Eq. 4})$$

Based on this calculation, a “safety factor” of 1.03×10^8 is derived. In this calculation the disruption of the viral genome as the benzonase degraded DNA fragments approach the genome size (ie, 7.0 Kb). To correct for the disruption of intact viral genomes, an additional safety factor was calculated using the same algorithm for oncogenicity (equations 2-4). If the DNA fragment is the same as the viral genome size (7000 bp) then there will be a reduction by $N_W > N_T$ to 7×10^3 . Incorporation of this reduction in intact oncogene into initial “safety factor” calculation (Eq. 1) increases the “safety factor” to 7.2×10^{11} .

If the five-fold reduction in infectivity between retrovirus infected hcDNA and cloned proviral DNA (plasmid) is included, the calculation for the Safety Factor equals 3.6×10^{12} or the equivalent of 3.6 trillion doses.

Safety Factor Calculation Due to Introduction of Infectious Virus DNA

It is worth noting that the theoretical safety factor calculated above is based on limited observational data and that any infectivity associated with process residuals is likely to be significantly less than calculated. For instance, the loss of cloned HIV (plasmid) infectivity was only assessed at 150 ng, and it is likely that this is scalable to higher DNA quantities with complete loss of infectivity due to disruption below the retrovirus genome size (eg, 7.0 kb).

As estimated from the vaccine purification process described in Section 3.4, the median size distribution of residual MDCK DNA is approximately 450 bp with approximately 64% of the DNA less than 500 bp in length and no detectable DNA above 1,000 bp. If biological infectivity is completely lost when DNA is degraded below the infectious genome size then even the remote possibility of an infectious event calculated above from residual DNA vanishes.

In addition, the theoretical safety factor calculation should be contrasted with the comprehensive extraneous agent testing of the MDCK MCB. Testing methods included in vivo and in vitro safety tests, latent virus induction studies, retroviruses by PERT assay, and over 36 different viruses by PCR amplification. Of particular note are in vitro studies where 1.0×10^6 or 1.0×10^7 MDCK cells were co-cultivated with various indicator cell lines or cell lysates derived from 1.0×10^7 cells.

Based on the sensitivity of HIV infected hcDNA, where 2.5 µg was found to be infectious, an equivalent number of MDCK cells containing this amount of diploid DNA is approximately 4.6×10^5 cells. The in vitro testing of up to 1.0×10^7 MDCK cells or cell lysates is the equivalent of a 20-fold excess over this theoretical infectivity sensitivity. While these tests are not direct tests of DNA infectivity, they do provide compelling evidence that the MDCK cell substrate does not harbor latent virus DNA.

Appendix 5 Assessment of TSE/BSE risk

An assessment of the theoretical TSE/BSE risk was performed using the following assumptions.

- 1) The theoretical infectious units present in the first MDCK clonal isolate was assumed to be maximally one (1) ID₅₀. Vorberg et al. (2004) have shown that NIH/3T3 fibroblasts are susceptible to mouse-adapted scrapie PrP^{Sc} when exposed to ($2 \times 10^{8.5}$ ID₅₀/g) infected brain homogenates in vitro. While not all of the cells appeared to be infected, of those clonal isolates examined the observed infectivity was calculated 2 cells per 1 ID₅₀.

It is improbable that the MDCK cells would have been exposed to any raw material during cell passaging outside of MedImmune that would have infectivity titers as high as that used in this study; thus, the resulting infectivity per cell is not unreasonable.

- 7) MDCK cells do not replicate prions and therefore any infectivity present in the original ATCC MDCK cells would decline based on passaging of the cells in culture medium that was free of any known TSE agent (FBS obtained from GBR I regions, or serum-free culture conditions).
- 8) MDCK cell clonal isolation occurred at passage 85, adaptation to serum free conditions occurred at passage 88, and the production cell substrate is used at passage 109. The increase in cell number at each passage is assumed to be five-fold (observed to be 5- to 10- fold) and the increase in cell number from passage 85 to 109 is 24 passages.
- 9) An average vaccine bulk lot (2,000 L) yields 20 million (2.0×10^7) doses of vaccine bulk (drug substance).

To reach the passage level for vaccine production, Passage 109, the original MDCK subclone would have undergone 24 passages. Assuming an average 5-fold increase in cell numbers at each passage, the number of cells should have expanded to approximately 5^{24} ($\sim 6.0 \times 10^{16}$) and the original one (1) ID₅₀ would have decreased to approximately 1.69×10^{-17} .

Assuming 20 million doses of vaccine are produced per vaccine lot, a further infectivity reduction due to the dilution of the drug substance to manufacture the drug product, there would be approximately 8.4×10^{-25} safety factor per dose per influenza strain.

Appendix 6 List of Tissues Examined in the MDCK DNA Biodistribution Study

Blood ^a
Intestine
Stomach
Pancreas
Spleen
Kidneys
Liver
Lung and trachea ^b
Thymus
Tongue
Nasal Cavity
Brain
Skin ^a
Muscle ^a

^a MDCK DNA found in IM animal group

^b MDCK DNA found in IN animal group

Animals were euthanized at 6, 24, 48, 72, 96, 168, and 336 hours