Formulation Development of Recombinant Adenovirus-based Vaccine for Newcastle Disease

Divya Srinivasan

Supervisor: Prof. Amine Kamen

Department of Biological & Biomedical Engineering

McGill University, Montreal

A thesis submitted to McGill University in partial fulfillment of the requirements of the degree of Master of Engineering

 August 2022

Contents

1.Abstract

Newcastle Disease has been causing much economic loss and food security issues in Sub-Saharan Africa. The risks associated with a zoonotic virus outbreak includes animal to human transmission. Vaccines against Newcastle Disease Virus have been in production in Africa, using chicken egg-based methods which are more expensive and have less throughput. There is a need to produce vaccines more efficiently. A recombinant adenovirus vectored NDV vaccine was developed in the earlier phase of the project. The goal of this research endeavor was to develop a more stable formulation for the developed Ad-CMV-F virus. An empirical screening test was performed to identify possible excipients and evaluated with $TCID_{50}$ assay to determine the infectious titers in formulation after stress testing the virus formulation in temperatures of 4ºC, 37 ºC, and 22 ºC. Excipients that showed a good result was further combined in formulations and compared against each other after exposure at 37 ºC for a week using $TCID_{50}$ assay and ddPCR. The stability of the virus that was purified with CsCl gradient ultracentrifugation was compared with the clarified product of tangential flow filtration, after exposure to a week of 37 ºC and performing $TCID_{50}$ assay. It was shown that trehalose 2% and polysorbate-80 in combination were effective as a formulation in maintaining the infectious titers.

Résumé

La maladie de Newcastle est à l'origine de nombreuses pertes économiques et de problèmes de sécurité alimentaire en Afrique subsaharienne. Les risques associés à une épidémie de virus zoonotique comprennent la transmission de l'animal à l'homme. Des vaccins contre le virus de la maladie de Newcastle ont été produits en Afrique, en utilisant des méthodes à base d'œufs de poule qui sont plus coûteuses et ont un rendement moindre. Il est nécessaire de produire des vaccins plus efficacement. Un vaccin NDV vectorisé par un adénovirus recombinant a été développé dans la phase précédente du projet. L'objectif de ce projet de recherche était de développer une formulation plus stable pour le virus Ad-CMV-F développé. Un test de criblage empirique a été effectué pour identifier les excipients possibles, et évalué avec le test TCID50 pour déterminer les titres infectieux dans la formulation après avoir testé la formulation du virus à des températures de 4ºC, 37ºC, et 22ºC. Les excipients qui ont donné un bon résultat ont été combinés dans des formulations et comparés les uns aux autres après une exposition à 37 ºC pendant une semaine en utilisant le test TCID50 et la ddPCR. La stabilité du virus qui a été purifié par ultracentrifugation en gradient de CsCl a été comparée au produit clarifié de la filtration à flux tangentiel, après exposition à une semaine à 37 ºC et réalisation du test TCID50. Il a été démontré que le tréhalose à 2 % et le polysorbate-80 en combinaison étaient efficaces en tant que formulation pour maintenir les titres infectieux.

2.Acknowledgments

First and foremost, I would like to express my deepest and sincere gratitude to my supervisor, Prof. Amine Kamen. He has been an immensely supportive and encouraging mentor and I am forever grateful to him for believing in me and giving me the opportunity to learn under his mentorship. I would also like to express my deepest gratitude to Dr. Omar Farnos Villar, who has guided me through every step of the project, taught me various laboratory techniques so patiently and allowing me to learn from his vast experience.

This thesis would not have been possible without my support system and no words can express my gratitude towards them – My husband Rakshith, my parents Revathy & Srinivasan, and my sister and brother-in-law Deepa & Vikram. They have always been my cheerleaders in every race in my life and I dedicate every success of mine to them.

I would also like to extend my gratitude towards my children, Rishir Rakshith, and Anoushka Rakshith and my niece Akshara, who have taught me to just have fun!

3.List of Figures and Tables

4. List of Abbreviations

Adv	Adenovirus
CMV	Cytomegalovirus
DoE	Design of Experiments
HN	Hemagglutinin Neuraminidase
HA	Hemagglutinin Antigen
MHC	Major Histocompatibility Complex
IPV	Inactivated Polio Virus
ND	Newcastle Disease
NDV	Newcastle Disease Virus
TEM	Transmission Electron Microscopy
Tg	Glass Transition Temperature
TFF	Tangential Flow Filtration

5.Introduction

5.1 History of Vaccine Development

Mankind has seen numerous pandemics during the course of history, with earliest records dating back to 430 BC in Athens, known as the Plague of the Athens. Since then, many communicable diseases have wiped out millions, destroyed empires, changed the course of history, and even led to changes in climate, as populations disappeared, and vegetation grew in previously ploughed land (1).

Smallpox, caused by the deadly variola virus, is thought to have been in existence in the world for at least 3000 years based on observations of Egyptian mummies with rashes like those caused by smallpox (2). It killed at least 300 million people from 1900s to 1977 before it was eradicated completely. Although the practice of variolation, which is the application of cowpox to a skin tear or by inhalation through the nose to bestow immunity to smallpox dates to 16th century China, Edward Jenner is credited with making the first vaccine in the world in 1796 by using cowpox injections to bestow immunity to smallpox and scientifically testing and encouraging vaccination (3). The word vaccine stems from the Latin word for cow, "*vacca"* (4). In the 19th century, Louis Pasteur, Robert Koch and Emil von Behring paved the way for research on immunology and immunotherapy. Robert Koch is recognized as a founder of bacteriology (5). Emil von Behring discovered serum therapy, which was in fact, using antibodies present in blood serum against diseases. In 1860s, Louis Pasteur proposed that micro-organisms can cause diseases in his Germ Theory (5). He studied fowl cholera, Anthrax, and in the process, discovered that weakened micro-organisms have a protective effect in animals. His significant research in the area led to the development of a Rabies vaccine (5).Vaccines have indeed played

the most crucial role in the entirety of medicines and interventions to human health, to have improved human health, drastically reduce susceptibility to diseases, eliminate physical disabilities caused by microbial agents and improve quality of life all over the world. (4) Numerous vaccines have been discovered since 1880 for various diseases, as shown in Figure 1 below.

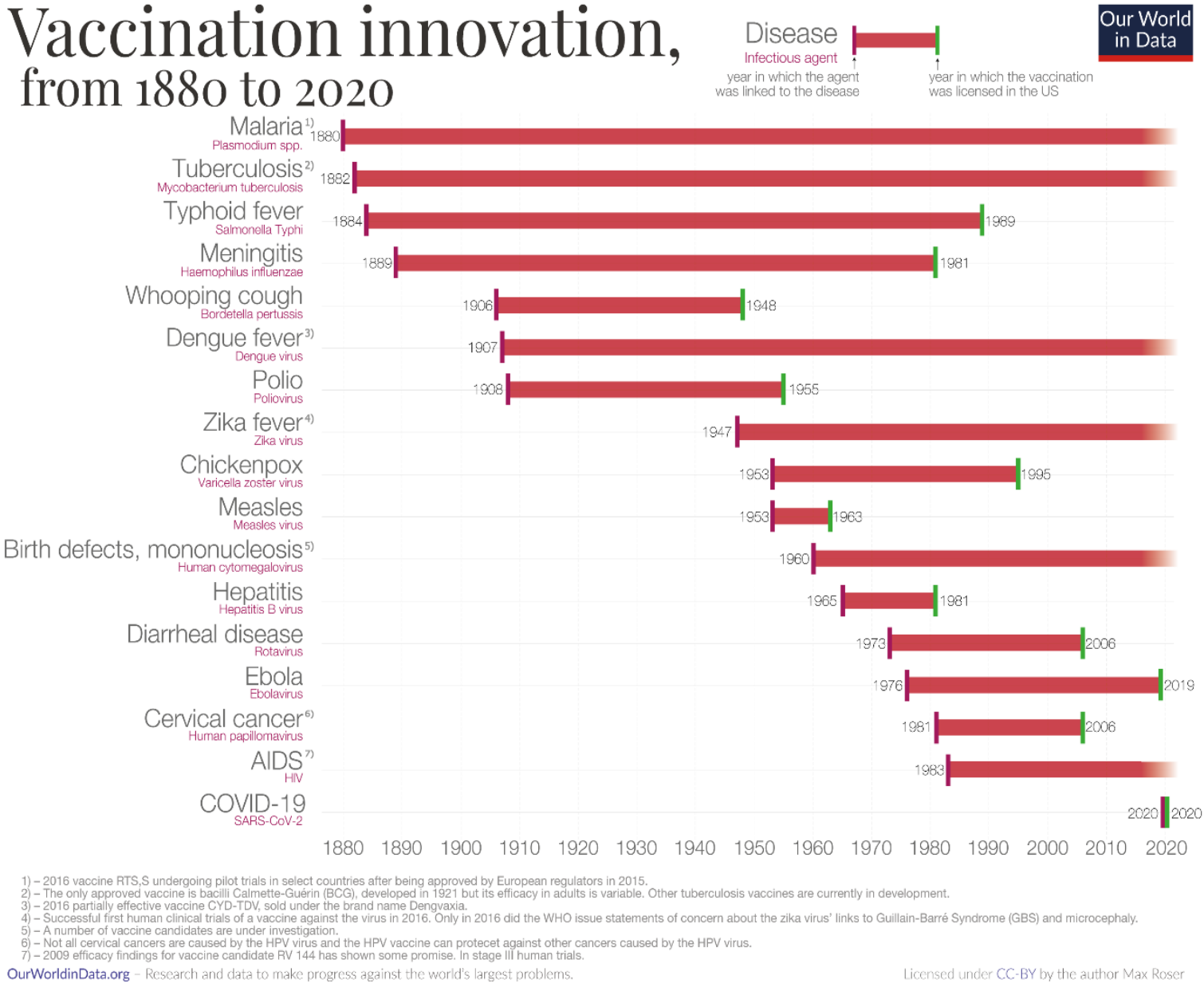

Figure 1 Vaccine Innovation from 1880 to 2020 (6) (Reproduced with Permission)

5.2 Cold-chain Vaccine Distribution

Despite the existence of vaccines for many diseases, the access to these vaccines remains uneven across different parts of the world. Apart from the cost of vaccines, the proper infrastructure to support cold chain storage from the point of manufacturing to the destination is not possible for many remote and underdeveloped regions in the world. Given that vaccines contain biological agents, and their function depends strongly on maintaining the integrity of their physical structure, they are naturally highly temperature sensitive. For example, the Hepatitis B vaccine remains stable for up to 4 years when maintained at a temperature of 2-8ºC, for only months when stored at 20°C to 25°C and for weeks when stored at 37°C and for days at 45°C (7). Every vaccine has a temperature range for storage and most of them in existence are between -70ºC to 8 ºC. A cold chain is the maintenance of a temperature-controlled supply chain from the point of manufacturing through the distribution route, to the point of vaccination. Power outages and unreliable power supply can disrupt the maintenance of a low temperature in underdeveloped regions, causing much vaccine wastage. In 2011, vaccines worth a total cost of 1.5 million USD were wasted because of the inability to maintain a cold temperature for storage in remote locations (8). One of the possible solutions to this problem would be developing more heat-stable vaccines that are more resistant to spoilage with changes in temperature. In some cases, a very low temperature can also lead to potency loss. For example, vaccines with aluminum adjuvants inactivate at low temperatures. In such cases, freeze indicators are generally used to monitor exposure to freezing temperatures. Further research and development on vaccine stability during distribution is being studied in the aspect of temperature variations, light exposure, and agitation and its effects on the maintenance of vaccine potency. Maintenance of vaccine potency from the stage of clinical development to commercial distribution is a prime challenge of vaccination programs.

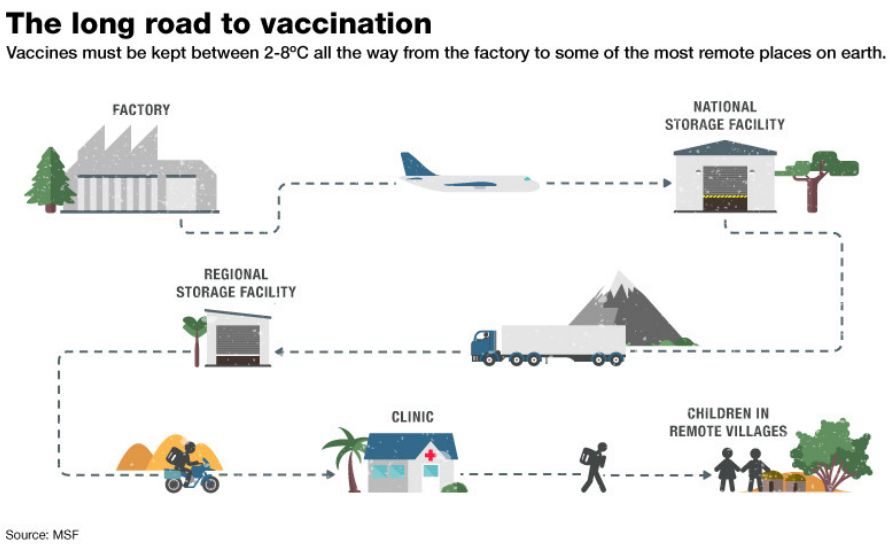

Figure 2 Cold Chain Distribution. Vaccine distribution involves many intermediate places of storage and maintenance of cold temperatures during transport to places with limited refrigeration capabilities. (9) (Reproduced with Permission)

5.3 Motivation for Research

Newcastle Disease is a highly pathogenic viral disease which affects mainly poultry. It has inflicted severe economic damage and food security issues in Sub-Saharan Africa where frequent outbreaks have been reported and greatly limited poultry production. Since main form of poultry farming in Sub-Saharan Africa has been traditional methods of husbandry farming, there are limited protocols in place to limit viral outbreaks and monitor farms. It has been challenging to keep Newcastle disease away from the poultry production chain. Moreover, large outbreaks of infectious zoonotic viruses increase the risks of an animal to human transmission. This could lead to a new disease outbreak among humans as well. Given that, it is vital to develop vaccines to tackle Newcastle Disease. The aim of this project is to develop a new more stabilizing formulation for the recombinant adenovirus-vectored NDV vaccine by investigating various excipients that can improve the thermal stability of the vaccine. Current vaccines employed for

the disease, which uses the egg-based production method increases the cost of production due to the import costs of pathogen-free eggs. Egg-based production method has limited production capacity compared to cell-based production processes which can scale to large volumes. It is also easier to control and optimize parameters and establish consistent product quality with cell-based technologies. Moreover, there are also limitations with the current vaccines for NDV. Firstly, current vaccines have caused virus shedding which led to healthy poultry being infected. Also, NDV strains used have been isolated too long ago and vaccines have not been updated for the current strains (10).This project has been motivated by the limitations of the current vaccines and the need to develop an efficient cell-based production platform for the NDV vaccine, with a more thermally stable formulation to reduce the needs of a cold-chain distribution in Sub-Saharan Africa where there are regions with limited infrastructure to support refrigeration. The new recombinant adenovirus-vectored vaccine which can be produced in bioreactor systems has a higher production capacity and can reduce the reliance on egg-based systems. Moreover, an improved formulation can reduce vaccine spoilage due to temperature variations and can minimize vaccine wastage. Reduced vaccine wastage and higher production capacity can increase the availability of the vaccines to more farmers and help reduce the cost of vaccines. This is highly beneficial as increased vaccination rates of poultry can reduce the chances of a viral outbreak and reduce the risks of a zoonotic virus transmission to humans. All of these benefits are the motivating factors for the research.

5.4 Impact of Research

This research, which is focused on developing improved formulation for an adenovirus-vectored vaccine, has broader benefits that will help tackle the current pandemic and future pandemics.

There are currently many vaccines against various viral diseases that have adenovirus as a vector. Numerous COVID-19 vaccines have adenovirus as the vector. CanSino Biological's Sinovac vaccine is an adenovirus-serotype 5 based vaccine. Gamaleya Institute's Sputnik V vaccine is based on adenovirus serotype 5 and 26. AstraZeneca's Vaxzervria is based on chimpanzee adenovirus. Janssen Pharmaceutical's Johnson & Johnson vaccine is based on adenovirus serotype 26. Apart from these, vaccines against HIV, Ebola, SARS, Rabies and SARS-Cov-2 have been developed using adenovirus as a vector. As such, improvements to the formulation of adenovirus-based vaccines can enable these lifesaving vaccines to reach remote areas, with less refrigeration capabilities and still maintain stability to a better extent than existing formulations. This can alleviate supply issues of vaccines, caused by vaccine spoilage, improve accessibility to underdeveloped regions, reduce overall cost of vaccination programs. This will in the future ensure more vaccine coverage in hard-to-reach communities with limited medical facilities, and prevent outbreaks from spreading at a fast rate, and reduce death rates.

6.Literature Review

6.1 Virus Structures

Viruses can be classified into DNA or RNA viruses, with single or double stranded DNA or RNA. The virus capsid is used to safeguard the genetic material from the environmental conditions of varying pH, temperature, and enzyme degradation. Viruses can be enveloped or non-enveloped. The envelope is a lipid bilayer which came from the host cell which was infected and formed through the budding off process where replicated virus particles become enveloped in the host's cell membrane parts. Most viruses fall under the structural classification of icosahedral or helical. Icosahedron is a geometrical structure with 20 equilateral triangles.

Viruses have genomes which are 7000-20000 base pairs long (11). The proteins are made up of capsid proteins which repeat multiple times, forming a tough structure that is hard to break.

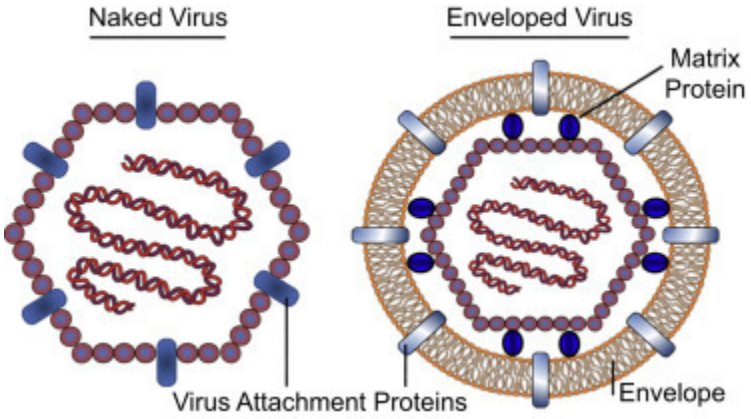

Figure 3 Virus Structure (11) Enveloped Virus has a lipid bilayer (right)enveloping capsid proteins. Non-enveloped proteins (left) lack a lipid bilayer. Viral DNA or RNA is contained within the capsid. (Reproduced with Permission)

The component which makes up most of the surface of viruses are called Glycans. These will determine the interaction that the virus has with the host cell through the pattern of glycans and the glycan binding receptors. Glycans will consist of complicated and vast branching of carbohydrates. The structural protein making up the virus will trigger the first immune responses in the host most of the time. Viral proteins can be classified into envelope proteins, spike proteins and membrane proteins. Glycoproteins on the surface will cling on to a cellular receptor, and this is followed by fusion and subsequent entry of the virion. The glycoprotein and receptor interactions facilitate the pattern recognition ability of the immune system (12).

6.2 Newcastle Disease Virus

Newcastle Disease Virus (NDV) belongs to the family of negative sense RNA viruses known as *Paramyxoviridae* (10). They are known to be of the serotype avian paramyxovirus serotype-1 (APMV-1). NDV is a pleomorphic enveloped virus, which is 200-300nm in size with a single stranded negative sense RNA genome. The genetic material contains six genes. Of the six genes

contained in the viral gene, genes encoding the fusion (F) protein and hemagglutinin-neuraminidase (HN) protein are very critical. Fusion protein allows for the fusion of the virus envelope with the host cell membrane. It is very important in determining the virulence of the ND virus. The HN protein allows for the binding of the virus with the host cell (10). HN has other functions as well. It helps with receptor recognition in the host cell, receptor removal, to prevent self-assembly, and interaction with F to allow fusion. HN is also responsible for the pathogenicity of the virus. The N protein has 489 amino acids and will form the nucleocapsid core with genomic RNA. P and L proteins will be found under the envelope and enables virus assembly and budding. L protein enables the production of viral mRNA and assists in genomic RNA replication. V protein and W protein are accessory proteins and present only in infected cells. V and P proteins have a role to play in the virulence of the ND virus. The matrix protein M controls viral RNA synthesis and helps to assemble the virion on the host cell membrane. NDV usually targets the epithelium cells by attaching to the sialic-acid containing compounds such as gangliosides and N-glycoproteins receptors using its surface glycoprotein (13).

6.3 Methodology of Immune Response to Vaccines

An infection with a microbe or vaccination contains protein units called antigens. These antigens will cause macrophages, T cells, B cells in the immune system to produce an immune response. Stimulation of immune cells results in the production of cytokines, which will determine the class of antibodies to be produced. An immune response is characterized by the breakdown of antigens into smaller fragments by macrophages. Major histocompatibility complex transports these fragments to the cell surface where they are exhibited. The exhibited fragments cause the B cells to secrete antibodies and prompt other immune responses (14). Antibodies in the bloodstream bind to antigens that stimulated its production. The binding of the antibodies

prevents the pathogens to bind to receptors on host cells. This also makes it easier for the phagocytic cells to destroy them (15), (16).

6.4 Types of Vaccines

Many different types of vaccines exist today. Vaccines can be broadly divided into nucleic acid-based vaccines such as mRNA, DNA vaccines, viral vectored vaccines, virus-like particles and recombinant protein subunit vaccines, live attenuated vaccines, and inactivated virus vaccines.

6.4.1 Nucleic Acid Based Vaccines

Nucleic acid vaccines use the genetic material of a pathogen, to invoke an immune response against the pathogen. The genetic material could be either DNA or RNA. The genetic material would stimulate the production of proteins specific to the pathogen and this would cause the immune system to recognize the protein produced as an antigen or a foreign body. The genetic material introduced causes the host cells to produce this foreign protein, which will result in an immune response. The main advantage of these vaccines is the simplicity of the manufacturing process, and the lack of any live viruses (17). Examples of nucleic acid-based vaccines include Pfizer-BioNTech ‘s and Moderna’s COVID-19 vaccine, which contains messenger RNA (mRNA).

6.4.2 Viral Vectored Vaccines

Viral vector vaccines are made up of a virus that is different from the virus that is being targeted and is harmless. The harmless virus enters the host cells to deliver information on producing antigens that are found in the target pathogen. The antigens trigger an immune response in the body to confer immunity. This will be explained in greater detail in the section 6.5 Adenovirus as a Viral Vector.

6.4.3 Virus-Like Particles

Virus-Like Particles (VLP) are made of multiple viral proteins and can mimic the organization and structure of the original virus which causes disease. However, these VLPs do not contain the viral genome. This enables immunity to be conferred by triggering the immune system without the risks associated with using live viruses (18).

6.4.4 Protein Subunit Vaccines

Subunit vaccines such as the Hepatitis B vaccine includes specific regions of the antigens that the T cells or antibodies would identify and attach to. There are less likely to cause severe reactions due to using only few specific antigens. Some protein subunit vaccines can also be classified as Virus-Like Particle vaccine (19). An example of a protein subunit vaccine is FLUBOK® QUADRIVALENT which is a vaccine against Influenza A and B strains. It contains hemagglutinin (HA) protein antigens which are produced by insect cell cultures.

6.4.5 Live Attenuated Vaccines

Measles, mumps, chickenpox vaccines contain weakened viruses that can replicate and induce an immune response without causing the actual disease. Viruses can be attenuated by environmental conditions that are harsh to weaken them, by genetic manipulation of the organism or by using naturally occurring strains that occur in related organisms but do not cause disease in humans (20).

6.4.6 Inactivated Virus Vaccines

Inactivated vaccines are produced by damaging a pathogenic agent with chemicals, heat or radiation. Such vaccines maintain stability much better. However, they regenerate a weaker immune response and hence booster shots are required regularly to maintain immunity (19).

Examples of inactivated vaccines include inactivated poliovirus (IPV), whole cell pertussis (DTaP) vaccine, and IMOVAX® rabies vaccine.

6.5 Adenovirus as a Viral Vector

Adenoviruses are non-enveloped viruses which are icosahedral. Their size ranges from 70-90nm. They contain 26-45kb size double stranded DNA. Adenoviruses have a core of capside and genome. The capsid is made up of proteins hexon, penton, fiber, IIIa, VIII and IX. The hexons are surface structural proteins which are the main targets for antibodies. These sites are key proteins which can be engineered to carry the antigen of interest for producing vaccines. Adenovirus-vectored vaccines contain a promoter sequence, gene encoding antigen and a poly-A tail. After intramuscular administration, muscle cells will be infected. Upon infection, the muscle cell will exhibit a processed antigen via MHC class I and produce a viral antigen which will subsequently activate the immune response by inciting the antigen-presenting cells. The genome of the adenovirus is well characterized and it is easy to manipulate making it an attractive candidate as vaccine vectors for numerous infectious diseases. There are 51 serotypes of human adenoviruses identified. These have been further classified into species from A to G based on their genetic sequences, pathobiology, and tumorigenicity and immunological properties (21). Large scale production of adenovirus generally utilizes human Embryonic Kidney cells (HEK293) and human embryonic retinal cells (PERC6) cell line as other mammalian cell lines face issues of scalability, especially for adherent type cells. (22) HEK-293 cells can be scaled up to 10000L stirred tank bioreactors and are well adapted to grow in suspension. Moreover, they have yields in the range of 10^9-10^{10} VP/mL (22). Adenoviruses used as vectors generally have their E1 and E3 regions in the viral genome deleted to prevent replication and to increase

transgene capacity (22). E1 genes must be present within cell line selected for the adenovirus to produce the E1 proteins. HEK293 allows E1 deleted adenoviruses to grow efficiently, but there is a risk of recombination to produce replication capable adenoviruses with HEK293, which have led to the development of PERC6 cells, which prevents recombination (23). Numerous steps are carried out after production to purify adenovirus produced. These steps are cell lysis and genomic DNA breakdown, clarification with dead end filters, ultracentrifugation or diafiltration, anion-exchange purification, gel filtration and finally sterile filtration (23). Adenovirus vaccines are not expensive to manufacture on a large scale and they do not require chicken eggs or involve labor and time-consuming processes needing enhanced biosafety facilities (24).Adenoviruses are currently also being used to develop vaccines against numerous other viruses such as HIV, Ebola, SARS, and Rabies. The development of adenoviruses as vectors for vaccines makes it imperative to develop formulations and processing techniques to improve their thermal stability.

6.6 Concept of Stability in Adenovirus Vaccines

The goal of vaccination stabilization methods is to ensure that the vaccines maintain their potency, titer, immunogenicity and activity throughout the transportation and storage duration despite accidental variations in external conditions until they are used, (25) and reduce the need for extreme cold temperature storage. Vaccine degradation can be broadly classified into mechanical and chemical degradation. Chemical degradation would involve the process of breaking or creation of new bonds to cause to cause chemically different species to arise. Physical degradation would comprise of changes that cause clumping, phase changes, or changes in structure and hence denaturation without any alteration to the chemical structure. Chemical degradation predominantly occurs by protein deamidation by removal of an amide group from a

protein, cleavage of peptide bonds without the presence of enzymes induced by acidic pH conditions, disulfide exchanges and incorrect disulfide bonds leading to altered conformation, protein oxidation, and temperature induced chemical degradation (26).

The effectiveness of vaccine is dependent on maintaining the integrity of the viral antigen and infectious titers. Antigenic structure and the correct steric presentation of the epitope, which is the site on the antigen which specifically binds to antibodies is crucial in determining the stability (27). The stability can be affected by factors such as temperature, pH, organic solvents, and process cycles of freezing and thawing, inactivating agents, free radical damage and light (25). The effect of these factors on the vaccine varies for different viruses.

To study the stabilization of vaccines, it is vital to consider the impact of the various environmental changes on the adenovirus.

$$N \rightarrow k_1 D$$
$$k_1 = A \exp(-Ea/RT)$$

Studies have shown that adenovirus degradation due to thermal stress follows the Arrhenius relationship between 4°C to 37°C. In the above equations, the degradation of the adenovirus, can be fitted to a pseudo first order reaction, with N representing the intact virus and D representing the disrupted virus concentrations. The rate constant k_1 is determined by the activation energy E_a and the temperature, T. Based on the Arrhenius relationship, the long-term storage effects on the vaccine can be predicted using short term studies (28).

The effect of temperature changes on the antigenic structure was studied by Rexroad et al (29). It was observed through circular dichroism spectroscopy, and TEM that an increase in temperature to 45°C changes the viral tertiary and secondary protein structure. Capsid integrity was observed to be lost at 45°C.Protein-protein interactions that holds the capsid structure together was lost, and at 50°C the capsid completely dissembled (29). pH has a significant impact on the virus

structure as well. A change in pH would cause conformational changes for surface coated proteins. This can cause aggregation and precipitation of viral particles. Rexroad et al also noted that the adenovirus serotype 2 is highly sensitive to pH outside the range of 5-7 and can undergo extensive aggregation (30).

Freezing has significant impact of virus structure as well. Freeze-drying vaccines creates strain on the viral integrity in several ways. Firstly, it might create a phase separation between various excipients in the formulation and reduce the protective feature of one or more excipients for the virus, leading to loss of viral activity by improper preservation. Another reason for the loss of viral activity is the creation of large ice-water interfaces because of ice crystallization leading to denaturation of surface proteins. The impact of freezing on protein structure has been a well-studied phenomena, and it can be expected that viral proteins would undergo similar strains. Cooling rates for freezing has an impact on the extent of denaturation and viral integrity. Cooling rates affect the size of the ice crystals formed. Mechanical damage to the virus therefore varies with the cooling rate. It was reported by Zhai et al that faster cooling methods created highest virus loss when comparing the viral infectivity of herpes-simplex virus after freeze-drying (31). The study was performed with an enveloped virus however and the impact of freeze drying on the morphology on adenovirus is still not very clear. It is important to consider phase changes during freezing and the stresses to viral protein structure.

6.7 Stabilization Theories in Vaccines

Vitrification, water replacement theory and preferential exclusion are the most widely studied mechanisms which enable preservation of vaccines (32).The water replacement theory states that during dehydration, sugar molecules will be able to form hydrogen bonds with the protein structures of the virus and provide stability through these chemical interactions. Alternatively, in

vitrification theory, virus structure is preserved as a glassy amorphous matrix. There will be limited mobility of the viral proteins in this structure, hence preventing aggregation and denaturation (25). In preferential exclusion theory, the water molecules in aqueous solution preferentially hydrate the protein surface by interacting with the polar groups on the protein surface. Upon freezing, the solute molecules are preferentially excluded from the vicinity of the protein surface. This increases the free energy of unfolding and keeps the protein in the native configuration (33).

Vaccine stability can be improved by improving formulation with the addition of cryoprotectants, buffers and other additives which contribute to maintaining stability through interaction with virus proteins. Freeze-drying of vaccines by selecting optimal process parameters resulting in a lyophilized form of vaccine also improves the stability of the vaccine.

6.8 Formulation Development of Vaccines

Vaccines are typically administered through one of the following methods- injection, intramuscular, subcutaneous, or intradermal. When considering the formulation of a vaccine, several factors would need to be evaluated. The reconstitution process, the stability of the final product, and the type of vaccine (e.g., the biological component of the vaccine) are key considerations for the formulation development.

Formulation of vaccines is a key factor in protecting vaccines from degradation. Careful selection of a cryoprotectant ensures viral proteins are protected from any changes in conformation and reduces aggregation of viruses.

The ability of the cryoprotectant to preserve viral proteins stems from its thermodynamic ability to shift equilibrium from unstable unfolded structure of protein to a stable native structure (8). This is attributed to a kinetic mechanism whereby the rate of inactivation is slowed down, and

viral proteins are thought to enter a glass phase which show high viscosity and limited mobility. This prevents damage to virus structures. A combination of several excipients is needed to improve the stability of the viral proteins through the process of freezing, drying and storage. The formulation should include a cryoprotectant, bulking agent, surfactant, and a buffer (33). Several sugars, sugar alcohols, metal ions, amino acids, surfactants, salts, and polymers have shown to be effective in improving the stability of vaccine formulations. Sugars are effective cryoprotectants which form hydrogen bonds with charged polar groups when water is removed to stabilize the viral protein structure as removal of water during drying process could inactivate proteins of the virus and reduce the efficacy of the vaccines.

6.8.1 Stability Data for Investigational Formulations of Vaccines

The table below summarizes the stability data for investigational vaccine formulations against various pathogens that are adenovirus vectored.

Table 1 Adenovirus-vectored Vaccine Formulations (34)

Vaccine	AERAS-402/ Crucell Ad35	Adenovirus type 5 (Ad5)	Adenovirus type 5 (Ad5)	Adenovirus type 5 (Ad5)	Ad5(MRKAd 5gag HIV)
Manufacturer/ Developer	Crucell, Aeras, CDC	NovaLabs, Oxford University	University of Michigan	University of Texas	Merck
Vaccine Type	Live virus vector	Live virus vector	Live virus vector	Live virus vector	Live virus vector
Formulation	Spray Dried	HydRIS(Air /filter-dried)	Lyophilized	Liquid	Liquid
Main Stabilizing Excipients	Mannitol, cyclo-dextrin, trehalose, dextran	Trehalose, Sucrose	Sucrose, Trehalose, Sorbitol, Gelatin	Mannitol, Sucrose, Pluronic F68	Sucrose, Ethanol, histidine, EDTA
Damage by Freeze Thaw			Resistant to freeze-thaw damage	Resistant to freeze-thaw damage	Resistant to freeze-thaw damage
2-8°C			150 days		≥ 24 months 7 years (est.)
25°C				9 days	13 days
37°C		≥15 months			
>37°C					
Reference	Jin et al 2010	Croyle et al 1998	Croyle et al 1998	Renteria et al 2010	Evans et al 2004

6.8.2 Excipients to Improve Thermal Stability of Vaccines

6.8.2.1 Sugar

Sugars preserve the hydration shell around proteins to maintain their structure. The glassy matrix hypothesis explains that sugars holding the proteins in a glassy matrix can prevent aggregation of proteins caused by reduced mobility of proteins.

Several sugars are known to be particularly effective as cryoprotectants. Evan et al have noted that sucrose effectively stabilizes Ad5 vaccines at 5wt/v% (35). Studies have also shown that when the weight percentage of sucrose is increased from 2 to 10%, there is an increase in thermal stability of Ad5 (36). Stewart at al have also noted that formulations with sucrose can maintain the infectivity of the adenovirus-based vaccines for up to 6 weeks at 40°C (37). Sucrose with 75mM of NaCl has been shown to be more resistant to freeze thaw damage (16). Other sugars which are known to be effective in stabilizing proteins are trehalose, inulin and dextran. Disaccharides such as sucrose, maltose, trehalose are known to be better as lyoprotectants than monosaccharides. This may be due to their higher glass transition temperature (Tg), although their bigger structure may increase steric hindrance impeding hydrogen bonding capabilities with the proteins. Hydrogen bonding and Tg should be considered together when choosing a sugar for stabilization (33). Sucrose and trehalose have numerous differences in their physicochemical properties. For example, trehalose has a greater tendency to phase separate when compared to sucrose but will form a dense hydrogen bonding network with proteins and with other trehalose molecules to protect proteins (33). Trehalose has been used for commercially available drugs such as, Herceptin®, Avastin®, Lucentis®, and Advate® (67). Currently, trehalose is being extensively researched as an excipient for Influenza vaccines (68). The determination of the sugar which is best suited for formulation of the vaccine should be determined on a case-by-case basis.

6.8.2.2 Sugar Alcohols

Sugar alcohols such as mannitol, sorbitol and xylitol are known to have stabilizing effect on proteins too. The mechanism through which they stabilize proteins is preferential exclusion. However, there are limitations for using them in the freeze-drying process of vaccines. While

sorbitol has a low collapse temperature, which is the maximum temperature a product can withstand during the freeze-drying process without collapsing, mannitol crystallizes inconsistently, and releases water due to dehydration while in storage impacting its stability (33).

6.8.2.3 Surfactants

Another excipient which is used in vaccine formulation is surfactants. Surfactant such as polysorbate-80 is used to stabilize viral proteins to prevent aggregation and denaturation which are prone to occur at interfaces. This is achieved by surfactant molecules which bind to proteins through hydrophobic interactions which prevent protein-protein interactions which can result in aggregation (33).

6.8.2.4 Amino Acids

Amino acids can act as secondary stabilizing agents which enhance the function of sugars as cryoprotectants (33). The amino acids most used for this purpose are histidine and arginine and L-glutamate. Histidine can act as a buffer and prevent pH changes, and act as an antioxidant. (38). Arginine can act prevent protein-protein interaction by hydrophobic interactions and reduce aggregation and stabilize virus structures (38). However, it lowers the collapse temperature during freeze drying and would reduce the maximum temperature that can be used in the drying process (33). L-glutamate can stabilize proteins by having protein specific interactions (38). Amino acids are also capable of stabilizing proteins in the solid state through hydrogen bonding and ion dipole interactions (38).

6.9 Existing Formulations for Adenovirus Vaccines

Recombinant adenoviruses are currently used for some Covid-19 vaccines. The Johnson & Johnson Janssen COVID-19 vaccine utilizes adenovirus serotype 26 and comprises of citric acid

monohydrate as an acid, trisodium dihydrate as a salt, 2-hydroxypropyl-B-cyclodextrin (HBCD) as a sugar and polysorbate-80 as a surfactant (39).The AstraZeneca COVID-19 vaccine utilizes the chimpanzee adenovirus and its excipients are histidine, L-histidine hydrochloride monohydrate, magnesium chloride hexahydrate, polysorbate 80, ethanol, sucrose, sodium chloride, disodium edetate dihydrate and water (40). Sputnik V, another COVID-19 vaccine currently approved for use in many countries, utilizes adenovirus serotype 26 and serotype 5, with the first dose containing Ad26 and a booster given 21 days apart, with Ad5. It contains Tris-(hydroxymethyl)a-aminomethane, sodium chloride, sucrose, magnesium chloride hexahydrate, Disodium EDTA dihydrate, polysorbate 80, ethanol and water (41). The Ebola vaccine by Johnson & Johnson Janssen, which consists of recombinant adenovirus 26, is formulated with disodium edetate, ethanol, histidine hydrocholoride monohydrate, polysorbate-80, sodium chloride, sucrose, sodium hydroxide and water (32).

6.10 Research on Adenovirus Formulations

Croyle et. al reported in 2001 that PBS buffered adenovirus formulations with mixtures of sucrose, mannitol, and surfactant showed negligible loss of titer for 35 days at 4ºC for 1 year, and formulations of sucrose with cyclodextrin were stable for 2 years at -20ºC (42).

Evans et.al 2004 established an adenovirus formulation, now widely utilized for adenovirus storage, consisting of Tris, NaCl, $MgCl_2$, EDTA and ethanol and polysorbate-80 (35). Since then, there has been several papers on the recent developments in adenovirus formulations research.

Pellicia et.al indicate that at room temperature, the adenovirus lifetime in 0.3M sucrose is 30 times greater than in PBS (43). Pellicia et al describes that at 37ºC the virus lifetime was 8 times greater than in PBS with 0.6M sucrose (43). The study also tested other compounds for

stabilization effects on Ad5. Glycerol was reported to be destabilizing while di- and tri-saccharides were shown to have better stability. When Ad5 was stored in various excipients at 37ºC for 5 days and subsequently analyzed for infectivity based on % cells infected, raffinose and sucrose at 40%w/w showed better results compared to glycerol, glucose and maltose at 40%w/w (43).

Patents on latest adenovirus formulation by Janssen Vaccines & Prevention B.V indicate that a combination of a citrate buffer with concentration of 5mM-30mM, hydroxypropyl-beta-cyclodextrin (HBCD) between 1-10% w/w, NaCl between 20-200mM, and polysorbate-80 between 0.01% w/w and 0.05% w/w show good results on prolonged adenovirus stability at 2-8ºC (44). In another patent by Arecor Limited, adenovirus aqueous formulation containing at least one anionic polymer, from the list of dextran sulfates, carboxymethyl cellulose, polyglutamate, polyaspartate, salt forms like sodium salts at a concentration of 0.5mg/mL to 10mg/mL, was shown to maintain infectivity of high levels, when stored from 2-8ºC for 24 months or 3 months at 25ºC (45). Another patent for adenovirus formulation indicates that the use of polyols such as polyethylene glycol, propylene glycol, sorbitol, mannitol, together with DMSO are shown to be effective in maintaining adenoviral titers (46).

Reinauer et. al carried out an algorithm-based adenovirus liquid formulation using DoE for preselection to screen excipients from a database and subsequently study amino acid excipients in a challenge condition of 37°C for 2 and 3 weeks of storage and 24 months of storage at 5°C (47). Met showed significant protective effect after 14 days, and Ala, a slight positive effect, whereas Trp, Lys-HCl, His, Glu, Gly did not show any positive effect (47).

7.Materials & Methods

7.1 Recombinant Adenovirus Production & Purification

7.1.1 Recombinant Adenovirus Stocks

Recombinant adenovirus virus stocks with the F protein gene and the human CMV promoter, were obtained from the earlier experiments done by other researchers in the research group to generate more Ad-F-CMV virus needed for the formulation development aspect of the project. For determining the genetic sequence of the NDV LaSota strain which was utilized for the NDV-vaccine, RT-PCR of a 749-bp region of the fusion protein with cleavage site was performed. The F-gene segment was amplified using the primers 5′ -GGAATTGTGGTAACAGGAGACAAAG-3′ and 5′ -ATATTATTGAGGTTCCCGACTGAGG-3′ . The F-gene sequence and the list of primers used is given in the section 13. Appendix. The product of PCR was viewed on 0.8% agarose gels and sequenced. DNA sequencing was carried out by using the Sanger Method and sequences of DNA were studied using BLAST alignments and compared against NDV isolates from GenBank. The NDV/Debre/zeit/2018(MN909678) sequence was used in conjunction with APMV-1/Ethiopia/13VIR3936-27/2012 for completing the fusion protein gene of length 1622 bp to construct the NDV adenoviral vaccine vectors.

The sequences of F gene of the Newcastle Disease Virus were produced chemically (Genscript Biotech, Piscataway, NJ, USA) and cloned into Kpn I/HindIII sites of the vector pShuttle-CMV adenovirus transfer vector pShuttle-Cytomegalovirus (CMV) (Agilent Technologies,Saint-Laurent,QC,Canada) to generate adenovirus serotype 5 which was replication deficient by deleting the E1 and E3 region. The vectors were transformed in the BJ5184 *E-coli* strain containing pAdEasy plasmid (Agilent Technologies,Saint-Laurent,QC,Canada).The transfer vector and plasmids were confirmed by restriction endonuclease analysis and sequencing. (New

England Biolabs,Whitby, ON, Canada). Primary stocks of viral stocks were obtained after transfection into HEK293A cells.

7.1.2 Cell lines Used for Virus Production

HEK293SF cell line, derived from HEK293 cells are adapted to thrive in suspension culture and serum-free media. HEK293SF were derived from Good Manufacturing Practices (GMP) master cell banks. Cells were grown in in vented cap shake flasks (Corning, Tewksbury, MD, USA) and passaged twice per week, diluting to 2.5 x 10^5 viable cells per mL in fresh medium. The media used for cell growth was either HyClone HyCell TransFx-H medium (GE Healthcare, Chicago, IL, USA) or Xell AG HEK-GM with 6mM GlutaMax Supplement (Fisher Scientific, Saint-Laurent, QC, Canada). Cell growth and viability was examined by using Vi-CELL-XR Cell Viability Analyzer (Beckman Coulter, Montreal, QC, Canada) using live density determination with 0.2% trypan blue exclusion dye.

7.1.3 Virus Production in Bioreactors

The production of the recombinant adenovirus was done in 1-L and 3-L controlled bioreactors (Applikon Biotechnologies Delft, The Netherlands) in fed-batch mode. The bioreactors were provided with a single marine impeller, pH sensor, temperature sensor, and dissolved oxygen DO sensor, a capacitance probe to monitor cellular biomass and a micro sparger with 100um pore size. The 1-L bioreactor had a working volume of 0.75L and the 3-L bioreactor had a working volume of 2.7-L. The bioreactors were controlled with the Applikon my-control controller (Applikon Biotechnologies Delft, The Netherlands) to control pH, temperature, DO and stirrer and monitor capacitance. HEK293SF cells were grown in 125 mL shake flasks, with 25mL working volume, and placed on an orbital shaker platform (Infor's HT, Montreal, QC, Canada) at 110 revolutions per minute (rpm) at 80% humidity, 5% CO_2 and 37ºC. Cells were seeded in the

bioreactors at 0.38-0.66 x 10^6 cells/mL. Media used was HEK-GM (Xell, AG, Bielefeld, Germany). Once cells reached the density of 3 x 10^6 cells/mL, they were infected with Ad-CMV-F, at MOI of 1. DO concentration in the bioreactor was maintained at 40% by surface aeration of 5 or 12.5mL/min. pH was maintained at 7.15 and injection of CO2 into the headspace and addition of NaHCO3 (90g/L) (MilliporeSigma, Oakville, Ontario, Canada). Agitation was kept at 100rpm. When the cell viability drops to 70-80%, harvest of cells were done.

Figure 4 Virus Production in Bioreactor

Figure 5 Apparatus and Bioreactor used For Virus Production

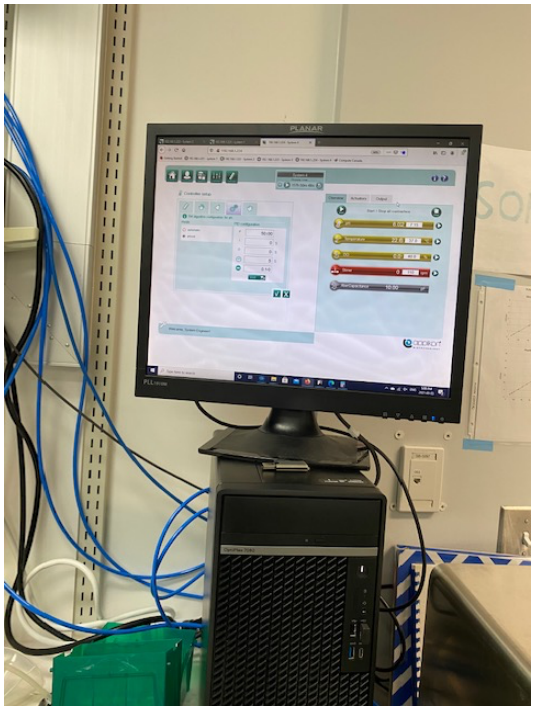

Figure 6 Control Panel Applikon

Figure 7 Controller

7.1.4 Harvest of Virus

Once the cells are ready for harvest, the cells in cell culture media were collected separately. The suspension was allowed to undergo centrifugation at 400 x g for 15 minutes at 4°C. The obtained dense cell material was allowed to be resuspended in a volume which is 1/5 of the original volume, discarding some supernatant. The next step of the process was to perform cell lysis. To achieve cell lysis, the concentrated cell solution was allowed to undergo 3 cycles of freeze and thaw. Freezing was achieved at -80°C and thawing was done at 37°C. Once freeze thaw was complete, the cell culture solution was centrifuged at 4500 x g for 15 minutes. The resulting supernatant contained adenoviruses and the cell debris was collected at the bottom of the centrifuge container. The supernatant would be poured into a new sterile glass bottle and used for further processing, discarding the cell debris.

7.1.5 Concentration of Virus

To concentrate the cells after cell disruption, the supernatant was poured into 50-mL particle filter tubes with a molecular weight cut-off of 30000 Daltons. The centrifuge tubes were centrifuged at 4000rpm for 20 minutes at 4°C. The liquid collected at the bottom of the centrifuge tubes was discarded. The fluid collected above the filter was recovered for the next step in virus purification.

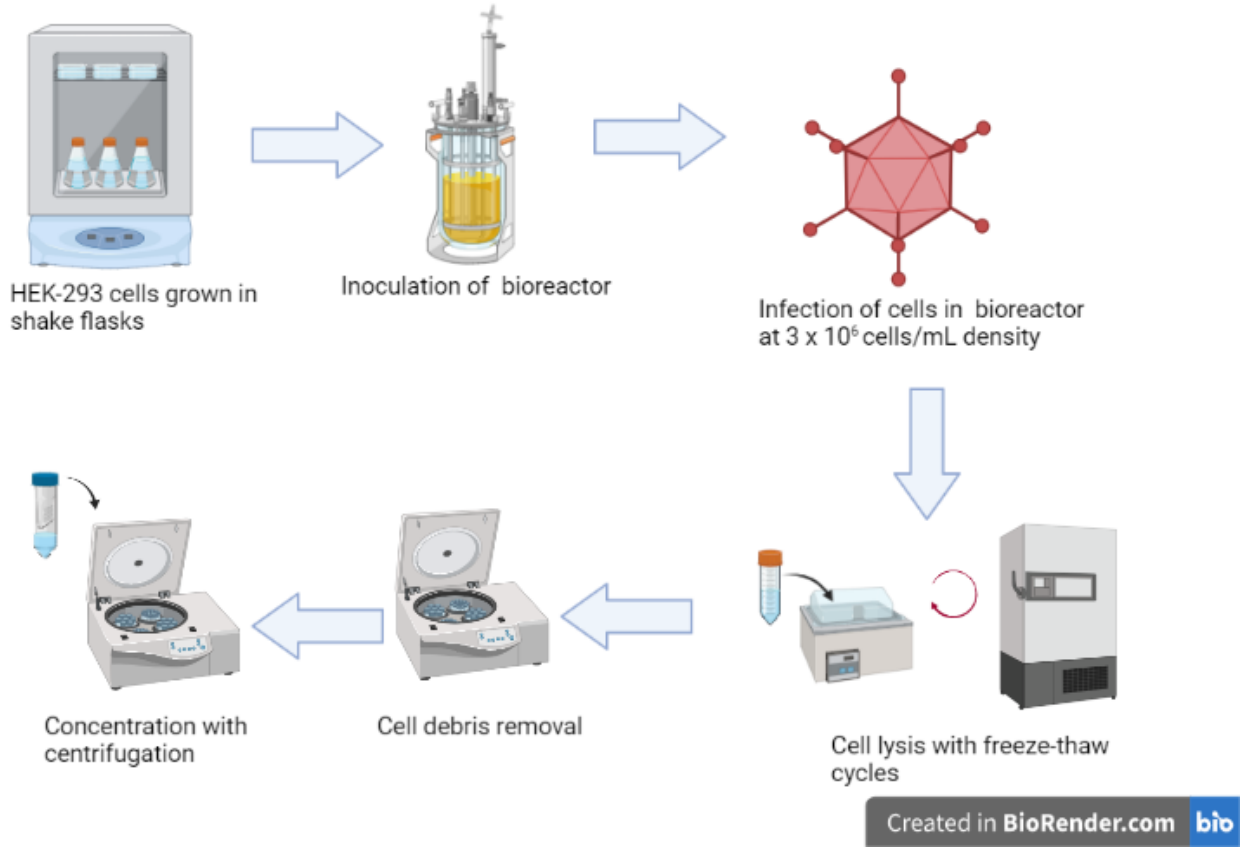

Figure 8 Steps of Virus Production and Concentration Before Ultracentrifugation (48)

7.1.6 CsCl Purification

To perform CsCl ultracentrifugation, 20mL of the concentrated cell culture lysate supernatant what was obtained from previous step was transferred to am ultracentrifugation tube containing a CsCl gradient. The CsCl gradient was formed by using CsCl with two different densities. The

1.4” CsCl solution was prepared using 53 g of CsCl with 87 mL10 mM Tris-HCl adjusted to pH 7.9. The 1.2” CsCl solution was prepared by adding 26.8 g CsCl with 92 mL 10 mM Tris-HCl adjusted to pH 7.9. 8mL of the 1.4” CsCl solution was added to a centrifuge tube (Ultra-Clear Beckman Centrifuge Tubes 25x 89mm) and layered with 6mL of the 1.2 CsCl solution. 20mL of the cell culture lysate was added slowly to not disrupt the layers. The centrifuge tubes were then balanced to be within 100mg of mass. They were then ultracentrifuged at 28,500 rpm (100,000×g) at 4°C for 1 h and 30 min in a Beckman SW32 Tirotor. After centrifugation, two bands were seen. The band that was seen closer to the lowest point of the tube lowest contains intact viral particles. An 18-gauge needle was used to puncture the centrifuge tube to extract the virus band. Dialysis was carried out by using Slide-A-Lyzer™G2 Dialysis Cassettes (300 kDa cutoff, 15 mL capacity) against 2 rounds of PBS buffer. The purified adenovirus was then frozen at -80°C with PBS buffer until formulation in the subsequent step.

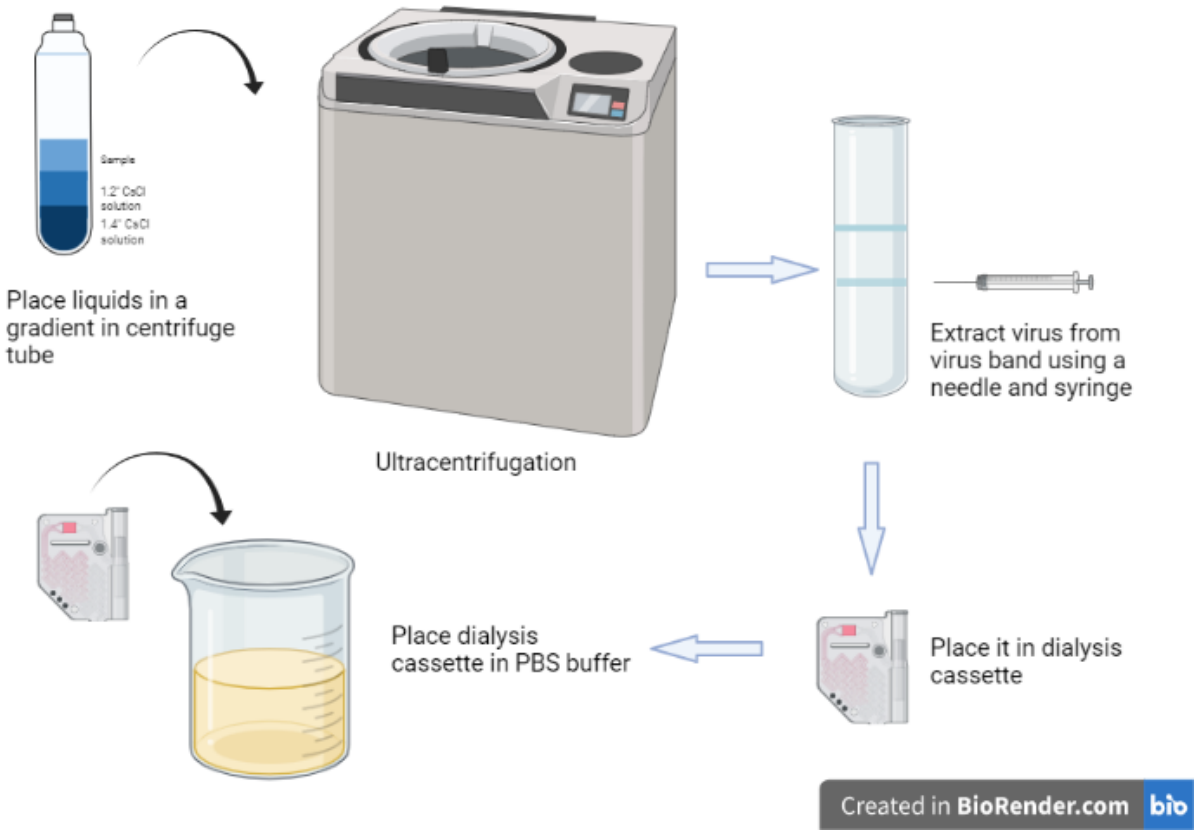

Figure 9 Ultracentrifugation to Extract Purified Virus (48)

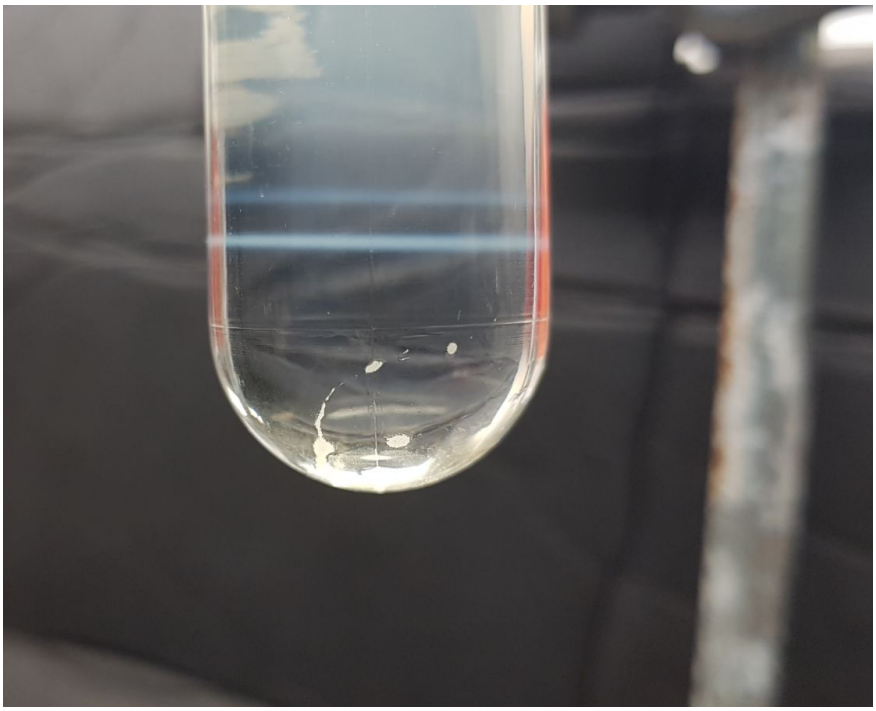

Figure 10 Virus Band after Ultracentrifugation

7.1.7 Tangential Flow Filtration

An alternative to CsCl purification step that used was tangential flow filtration to clarify virus supernatant after harvest step. Tangential flow filtration is pressure driven whereby the flux through the membrane is dependent on the transmembrane pressure, total membrane resistance and fluid viscosity. The transmembrane pressure will be the factor which generates the difference in pressure between the inflow and outflow sides. The tangential flow filtration process is shown as below.

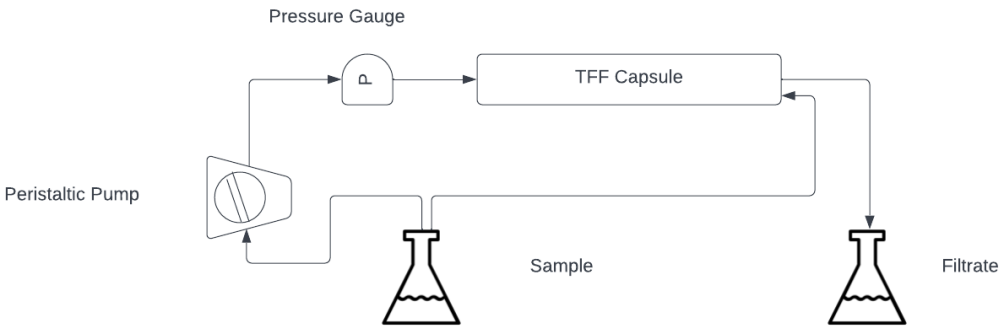

Figure 11 Tangential Flow Filtration

The product of the tangential flow filtration was stored at -80ºC after purification.

7.2 Single Excipient Formulation Development

The first step in formulation development was the pre-screening of multiple excipients to determine the most protective excipients that could maintain the virus infectivity after exposure to various temperatures. For this step, formulation solutions with single excipients were prepared at 10x concentration. 9 parts of PBS with the purified virus, with viral titers estimated to be in the range of 10^8- 10^9 VP/mL, was mixed with 1 part formulation solution. The viral titer at the end of the CsCl purification was estimated to be 10^{10}-10^{12} VP/mL. 10 mL of the extracted virus was diluted with 990 mL of PBS, to prepare a solution of ~10^9 VP/mL. The excipients prepared are shown in the list below. The negative control was the virus in PBS solution without any excipient. The weight percentage of each of these components were determined from Patel et.al. (49).

Table 2 Excipients and the Concentration of Each Excipient

Excipient	**Concentration Final**	**Manufacturer**
Sucrose	2%, 5% w/w	Sigma-Aldrich SKU: S9378-1KG
Trehalose dihydrate	2%, 5% w/w	Sigma-Aldrich SKU: Y0001172
Mannitol	3% w/w	Sigma-Aldrich SKU: PHR1007
Sorbitol	3% w/w	Sigma-Aldrich SKU: PHR1006
Polysorbate-80	0.5% w/w	Sigma-Aldrich SKU: 59924-100G-F-
Pluronic F-127	0.5% ,1% w/w	Sigma-Aldrich SKU: P2443
Arginine	10mM	Sigma-Aldrich SKU: A5006-100G
L-histidine	10mM	Sigma-Aldrich SKU: H3911-100G

7.3 Stress Tests

7.3.1 Stress Test 1: Screening of Excipients

Figure 12 Process of Assessing Effectivity of Each Excipient

Once the virus was placed in the various formulation solutions, each formulation solution was split into different vials. Each vial and its replicate were placed at one of the three temperatures for a duration of 1 week. The storage temperatures were 37ºC, room temperature of 22ºC, and 4ºC. At the end the time duration, all the vials were frozen at -80ºC until they were thawed for the $TCID_{50}$ infectivity assays. The storage temperature of -80ºC was considered the optimum temperature for invariable viral titer for the storage period of 1 week.

7.3.2 Mixed Excipient Formulation Development

In the second step of formulation development, excipients which showed a better result in preserving the viral titers across all the 4 temperatures were selected. A combination of two or three excipients were then prepared as a formulation, to evaluate the synergistic effect of multiple excipients with the virus. Four different formulations were prepared along with the traditional formulation used to preserve adenovirus. The traditional formulation would act as an indicator to show the effectiveness of the new formulations against the existing formulation for adenovirus. Formulation A consisted of trehalose 2% w/w + polysorbate-80% at 0.5% w/w. This formulation aimed to study the synergistic effect of a surfactant and sugar in protecting the virus. Formulation B consisted of trehalose 2% w/w + pluronic F-127 at 1%. This aimed to compare effect of the different surfactants (polysorbate-80 and pluronic F-127) in acting together with the sugar to preserve virus. The third formulation was trehalose 2% w/w + sorbitol 1% w/w + polysorbate-80 0.5%w/w. This formulation intended to study the effect of having both sugar and sugar alcohol together with polysorbate-80 as a surfactant. Formulation D was trehalose 2%w/w+ sorbitol 1%w/w + pluronic F-127 at 1%w/w. This formulation was used to compare the effectiveness of polysorbate-80 and pluronic F-127 in working with sugar and sugar alcohol. The last formulation was Formulation O, which was the original formulation for adenovirus that is

widely used and available in literature. This formulation consisted of NaCl 0.1mol/L + sucrose 2%w/w + tris 0.01mol/L +$MgCl_2$ 2mM. All the formulations were prepared with the same batch of virus production, and the initial titer was 1.87 x 10^{10} VP/mL.

7.3.4 Stress Test 2: Mixed Excipient Formulations Stored at 37°C

To test the 5 formulations prepared in the previous step, formulations were prepared at 10x the final concentration. 1 part of the formulation was added to 9 parts of virus + PBS. The resulting virus + formulation was then split into vials. Each prepared formulation with virus was then split into 5 replicates. The vials were placed at 37ºC for a week to assess the effectiveness of the formulation in preserving virus titers. To assess the most promising formulation, $TCID_{50}$ Assays were performed as described in section .

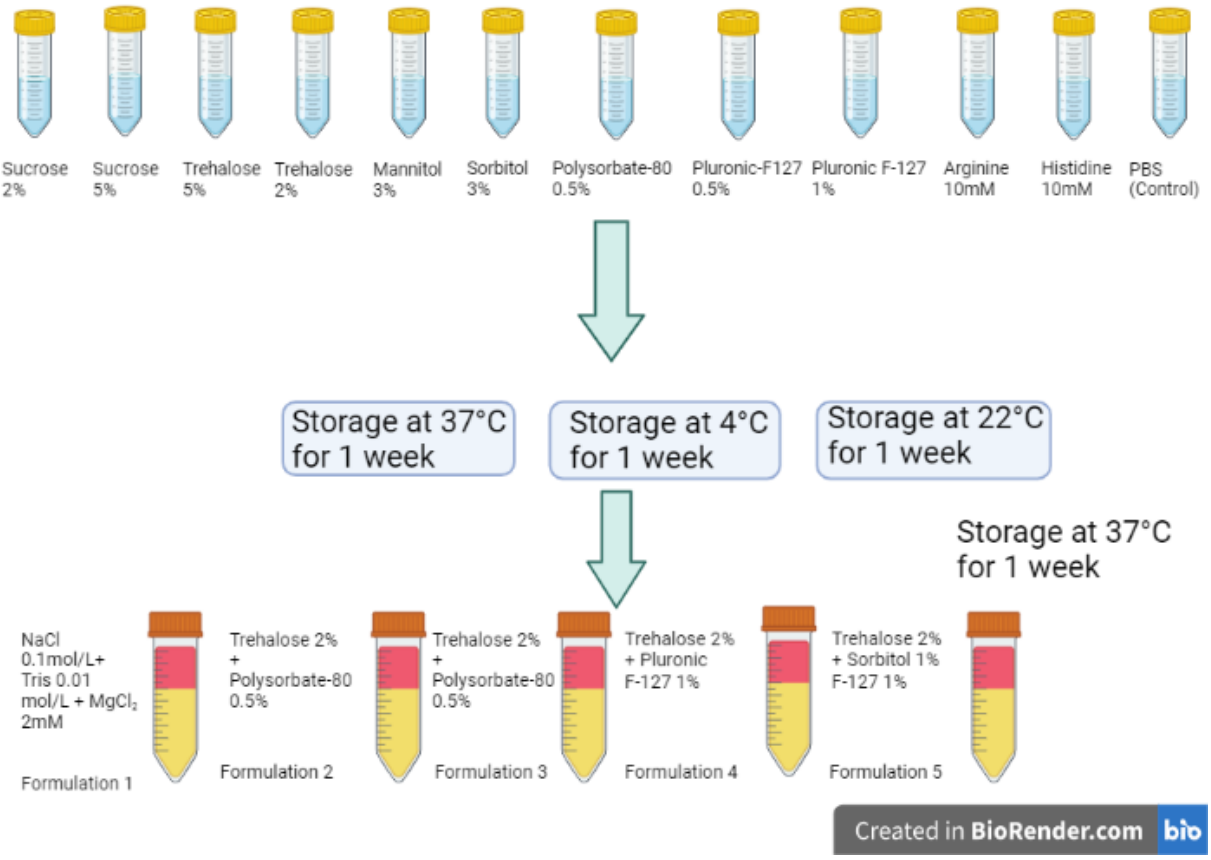

Figure 13 Formulation Selection (48)

7.3.5 Stress Tests 3

The most effective formulation was then used to prepare the virus obtained from clarification in the tangential flow filtration step. 5 replicates, each with virus obtained from tangential flow filtration was placed at 37ºC for a week. After one week, the samples were removed to be tested with $TCID_{50}$ Assay for infectious titers. The procedure of the assay was similar to what is described in section . The initial titer of the TFF product was 8.27E+10 VP/mL and for ultracentrifugation was 1.87E+10 VP/mL. There were 5 replicates of each method of processing.

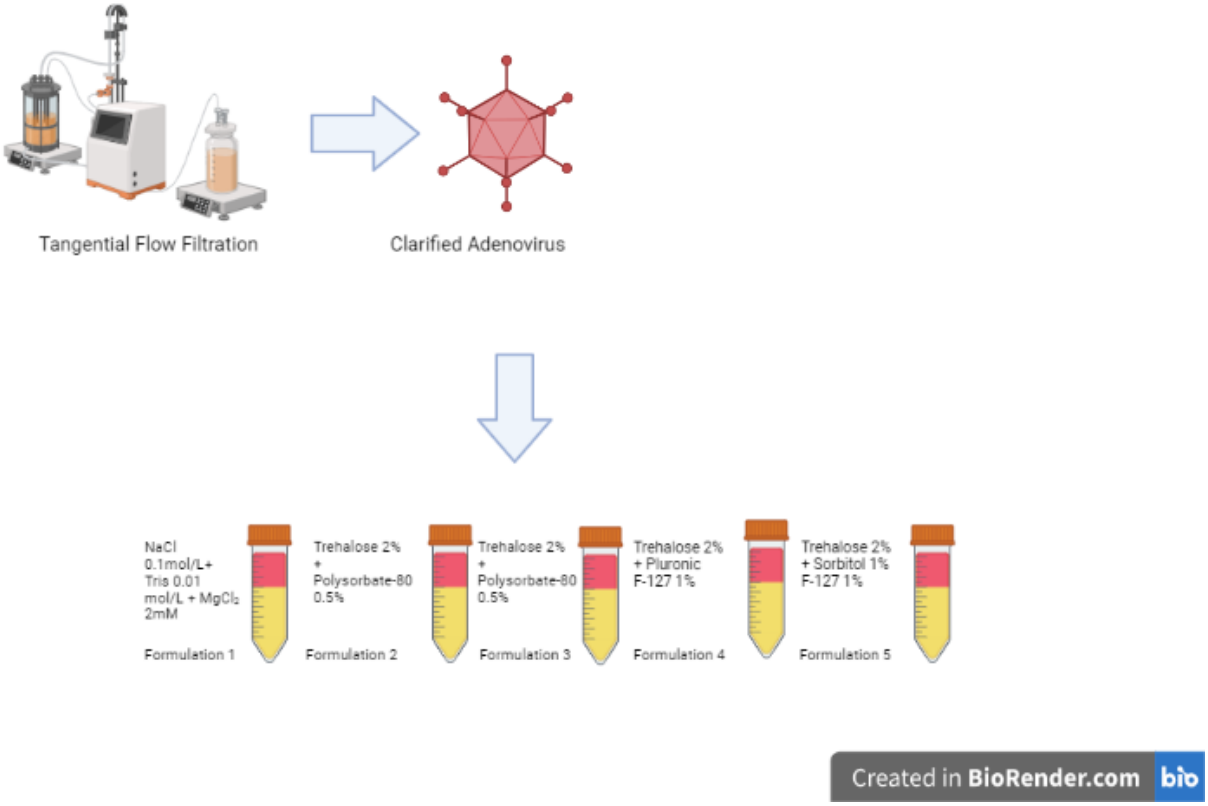

Figure 14 Stress Test for Tangential Flow Filtration (48)

7.4 Analytical Methods

7.4.1 $TCID_{50}$ Assays

The sample vials were removed from stress test storage temperatures and stored at -80°C until they were utilized for $TCID_{50}$ Assays. Prior to performing the $TCID_{50}$ Assay for adenovirus, HEK-293 cells were expanded in two or three T-75 or T-175 flasks in Growth Medium, consisting of 1% Pen/Strep antibiotic, 10% FBS, and DMEM. The quantity of cells needed depended on the number of 96 well plates that were to be plated on a particular day. Each plate had 2 million cells and 96 wells. On the day of preparation of plates, each of the 96 well plates were seeded with 100 µL of Virus Growth Medium per well at 0.02 x 10^6 cells/mL. The virus growth medium contained 1% Pen/Strep antibiotic, 2% FBS and DMEM. The plates were incubated at 37ºC and 5% CO_2 for 24 hours before infection. After 24 hours, cells were over

80% confluent in each well. The next step in the process was serial virus dilution. In 1.5mL microcentrifuge tube, 10-fold dilution of the sample was prepared, by using 100µL of sample and 900µL of virus growth medium. Each sample belonged to one of the vials that was subjected to a temperature condition previous step. Once prepared, diluted samples were vortexed to ensure homogeneity. Following this, a 96 well deep well plate was filled with virus growth medium. Each well contained 900µL of virus growth medium. The first column (8 wells) of the deep well plate was loaded with 100µL each diluted sample. Using a multichannel pipette, the fluid from each of the well in the first column was extracted, well mixed, and 100 µL of solution was transferred to the next column. This was repeated all the way until the transfer of the solution was stopped one before the last column on the deep well plate. The last column was a virus free culture, acting as a negative control. Following this, using the multichannel pipette again, virus was extracted from each row (12 wells serially diluted) and transferred to rows of wells on a 96 well plate seeded with HEK-293 from the earlier step. The plates were then incubated for 10 days at 37°C, and 5% CO_2. On the 10^{th} day post infection, each of the 96 well plates were read under the microscope. Each well was assessed for cytopathic effect. The number of cells in each column showing cytopathic effect was noted down. The infectious titer of virus present in each sample was calculated based on the Reed-Muench Method. In this method, the proportionate distance (PD) is calculated as

$$PD = \frac{\%\ positive\ above\ 50\% - 50\%}{\%\ positive\ above\ 50\% - \%\ postive\ below\ 50\%}$$

Then, $logID_{50} = -6 + PD \times (-1)$

$$ID_{50} = 10^{(-6+PD(-1)}$$

This would give the dilution that would infect 50% of the test units inoculated. The infectious titer would then be the reciprocal of the value (50). Based on the dilution factor of the initial

sample, the virus titer of the sample was found. In the prepared samples for $TCID_{50}$ Assay, the samples were diluted by using 1 part sample and 9 parts virus growth media.

7.4.2 Polymerase Chain Reaction (PCR), SDS-PAGE, digital droplet Polymerase Chain Reaction (ddPCR)

PCR was performed on samples containing virus placed in Formulation 2 and 5 to identify the primers needed for the amplification of the viral genome and to confirm the identity of the adenoviral vectors and validate that the samples contained the viral genome with the right genetic make-up. This was performed by first isolation of the viral DNA by using High Pure Viral Nucleic Acid Kit by Roche. Once the viral DNA had been isolated, the samples were allowed to undergo PCR amplification and subsequent SDS-PAGE electrophoresis to identify the primers needed for ddPCR in the next step.

For the quantification of viral genome via digital droplet PCR the following steps were followed. Firstly, 2 µM of Primer Mix was prepared by adding 96 µL of Milli-Q water in 2m-L centrifuge tube. 2 µL of 100 µM Forward Primer and 2 µL of 100 µM Reverse Primer was added and vortexed and stored at -20C. In order to prepare the samples, a 96 well plate was used to make 3 dilutions of the viral DNA samples at $10^{2,}10^{3}$,10^{4}. 99 µL of Milli-Q water was added to the first well for the first sample. 90 µL of Milli-Q water to the 2^{nd}, 3^{rd} and 4^{th} wells for sample 1. On the adjacent column in the 96 well plate, the same quantity of Milli-Q was added as before for sample 2. To the first well in each column, 1 µL of the viral DNA was added to make up for a total of 100 µL. It was mixed it by pipetting up and down 30 to 40x.10 µL was transferred from the first well to the second well, and mixed very well before transferring by pipetting up and down 30 to 40x.

Next 10 µL from the second well was transferred to the third well, and mixed 30 to 50x before transfer.

Next a Master Mix was prepared by adding EvaGreen Supermix (11 µL), 2 µM Primer Mix (1.1 µL), and 4.4 µL Milli-Q water per reaction. This mix was vortexed and kept on ice. 16.5 µL of the Master Mix was then placed in each tube of the 8-tube strip on the PCR cooler. The caps were labelled with the names of the samples (Formulation 1,2,3,4,5). 5.5 µL of the diluted sample in the tubes with the Master Mix. 5.5 µL of the positive control (final concentration, 100 to 1000 copies/µL) in the tubes for positive control was used and 5.5 µL of water in the tubes for negative control was used. In order to generate droplets, the cartridges were placed on the holder. 20 µL of the samples were transferred from the 8-tube strip to the middle row of the cartridge using the multichannel pipette. The bottom row was filled with oil (oil for EvaGreen). The gasket was put on and placed in the droplet generator. Once droplets were generated, they were transferred from the first row to the first column on the PCR plate on PCR cooler. The position of each sample was noted. Foil was used to seal the wells and placed on the Thermal Cycler. The pre-set program was selected for the amplification process.

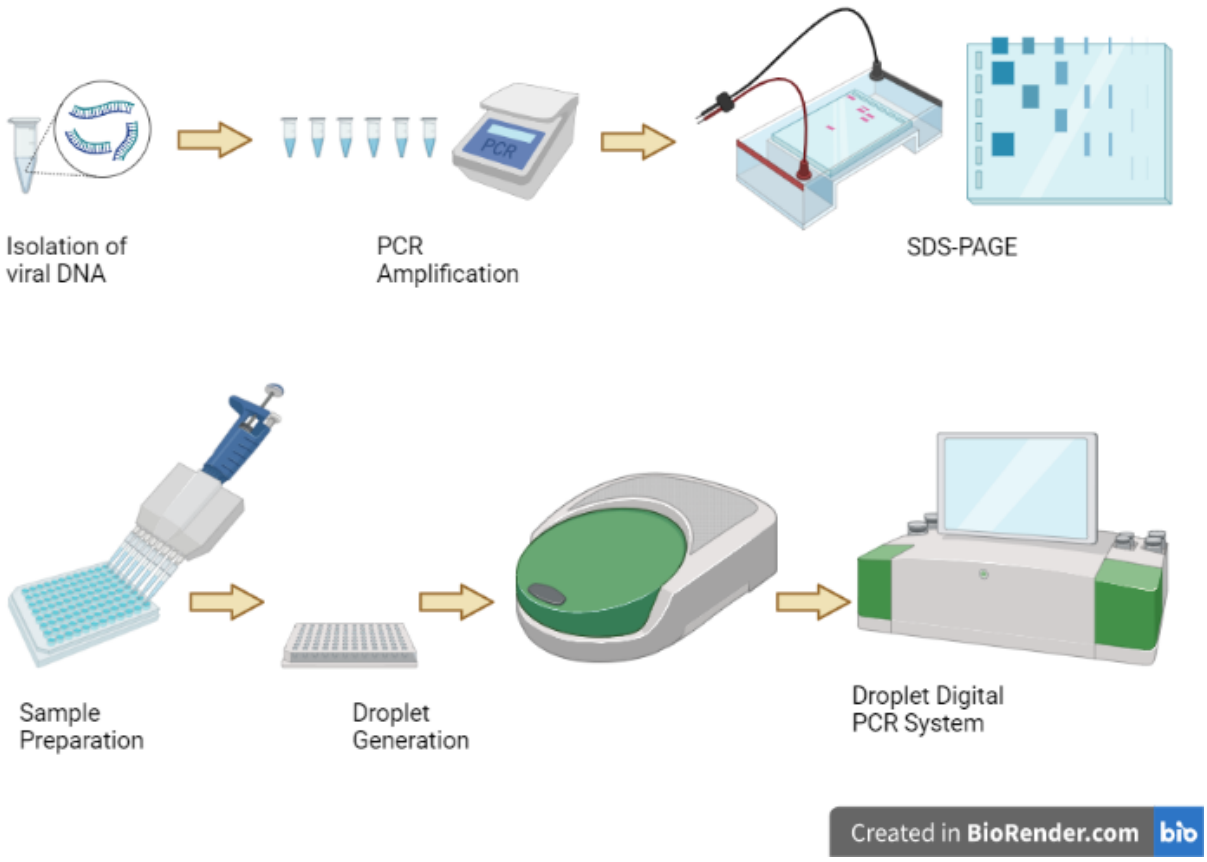

Figure 15 PCR and ddPCR (48)

8.Results

8.1 Screening of Individual Excipients in the Formulation of the Ad-CMV-F Adenoviral Vector under Thermal Stress Treatment and Storage Conditions

Twelve formulations were prepared with 9 different excipients as described in Materials and Methods, including pluronic F-127, trehalose, and sucrose which were evaluated at 2 different weight percentages and 2 replicates for each formulation. The objective of this initial screening was to identify those excipients, and the weight percentages at which they could provide increased stability to the Ad-CMV-F adenoviral vector in a liquid formulation. The excipients were chosen based on different categories of substances that have shown potential or proved effectiveness protecting and stabilizing viral structures in previous research. The initial screening experiment with various excipients during thermal stress conditions of storage were

conducted at three different temperatures for a duration of 1 week and the results are shown in the following section . The Ad-CMV-F vector was diluted in Phosphate Saline Buffer (PBS) to a working concentration of 5 x 10^9 IVP /mL for all excipients except Trehalose, for which the infectious viral titer was at 1 x 10^{10} IVP/mL.

Figure 16 shows the log decrease of viral titer at room temperature of 23°C in the presence of the excipients assayed. Sucrose 2%, sucrose 5%, trehalose 2% and trehalose 5% were compared to determine the most protective sugar in maintaining the virus titers. The use of sucrose 5% was less effective compared to sucrose 2%, with sucrose 2% allowing a titer log loss of 1.732 versus 2.190 with sucrose 5%. Trehalose 2% was much more effective, showing only a log decease of 0.464. Trehalose 5% was the most protective condition with no loss of infectious titer. When comparing the sugar alcohols, both mannitol 3% and sorbitol 3% were also effective to some extent. Sorbitol 3% was more effective than mannitol, showing a titer log loss of 1.660. When comparing the protective ability of the surfactants, pluronic F-127 at 0.5% was the less effective with a log loss of 3.460 at 0.5% and 2.290 at 1%, compared to polysorbate-80 at 0.5% which stabilize the viral vector to a log loss in titer of 1.706. Pluronic F-127 at 1% was more effective than the use of PBS without additive.

The two protein excipients evaluated allowed the highest log loss in infectious viral titer, with histidine at 10mM concentration being the least effective excipient, with a log decrease of 4.262. Arginine was similarly poorly effective ,with a log loss in viral titer of 3.444 at 10mM

concentration. With PBS alone, a log loss of 2.496 units were observed.

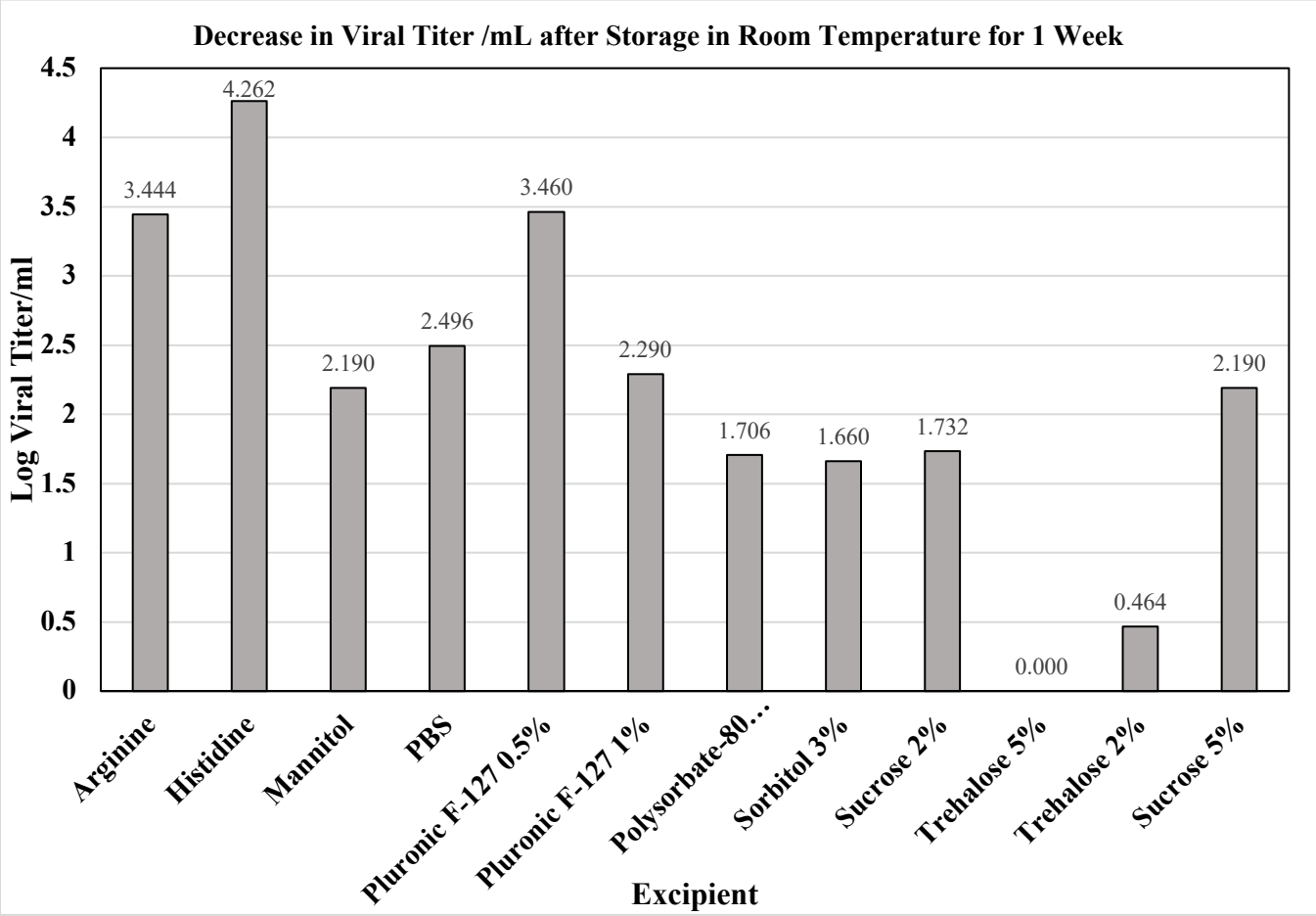

Figure 16 Log decrease of Infectious Viral Titer for the Adenoviral vector Ad-CMV-F. The formulations were kept at Room Temperature for 1 Week. Sample containing only PBS and Ad-CMV-F without any additional excipient acted as a control.

Figure 17 shows the logarithmic decrease in viral titers/mL during storage temperature of 37°C for 1 week. As seen from the graph, trehalose 5% was more effective than Sucrose 2%. The most effective excipient was trehalose, in which the Ad-CMV-F adenoviral suffered only a decrease in titer of 0.415 log units.

Arginine 10mM was the less effective excipient, with a high log loss of titer at 4.023. With the exception of histidine 10mM (3.491), all other excipients had some protective effect (compared

with PBS alone), with a viral titer decrease below 3.358. Mannitol 3% and sorbitol 3% had a similar virus infectivity protective effect, with sorbitol 3% being slightly more effective than mannitol. Pluronic F-127 at 0.5%,showed to be less effective compared to pluronic F-127 at 1% . Polysorbate-80 0.5% had a similar effect as pluronic F-127 0.5% .

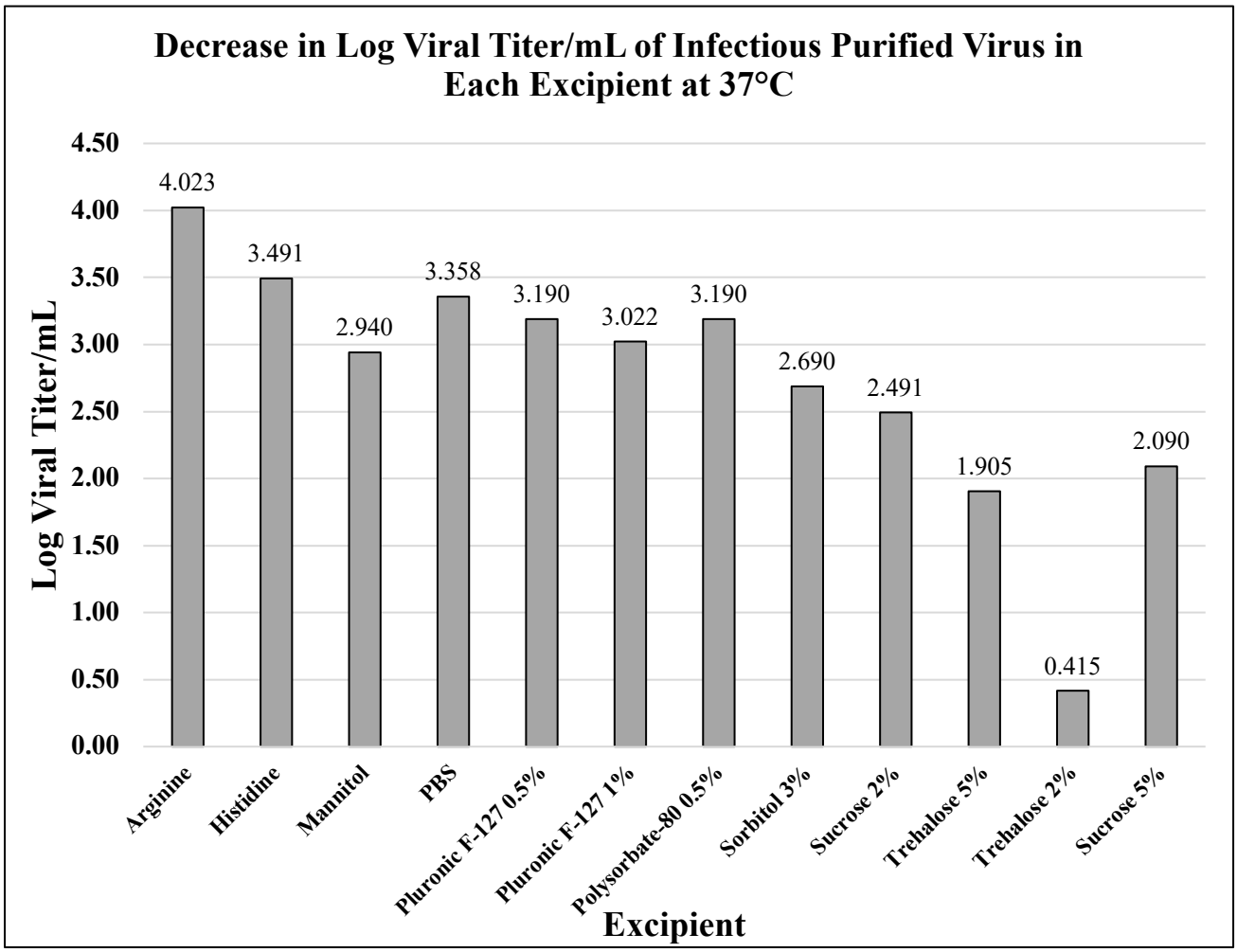

Figure 17 Decrease in Log Viral Titer/mL of Infectious Purified Virus in Each Excipient at 37°C. The samples were stored at 37°C for a week. Sample containing only PBS and Ad-CMV-F without any additional excipient acted as a control.

An additional experiment was conducted to evaluate the effect of the single excipients on the formulations stored at 4°C as shown in Figure 18. Sucrose 2% was less effective compared to sucrose 5% . Trehalose 2% and trehalose 5% were again more protective than minimal infectious titer loss during storage (0.194 and 0.998 respectively).

Arginine 10mM and histidine 10mM showed the lowest ability to keep maintain the original titers, accounting for a decrease in titers similar to PBS alone. All other excipients showed greater beneficial effect on the virus stability, with sorbitol 3% being better and mannitol 3% and pluronic F-127 1% less effective with a titer loss of 1.983 compared to pluronic F-127 0.5% (decrease of 1.592 log). Polysorbate-80 0.5% was the most protective surfactant with a titer loss of 1.065 log units.

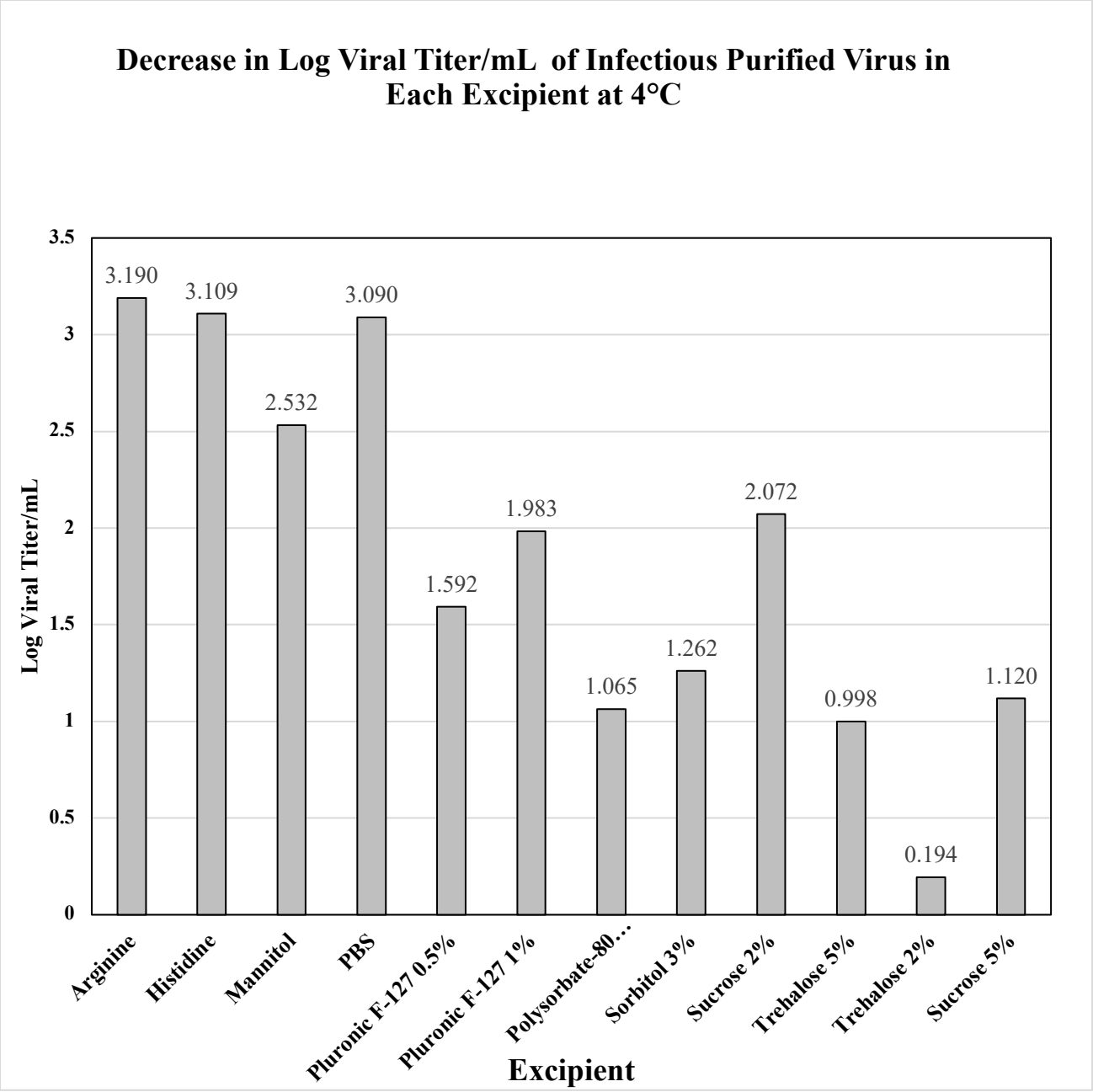

Figure 18 Decrease in Log Viral Titer/mL of Infectious Purified Virus in Each Excipient at 4ºC. The samples were stored at 4ºC for a week. Sample containing only PBS and Ad-CMV-F without any additional excipient acted as a control.

8.2 Evaluation of Combinations of Excipients in the Formulation of the Ad-CMV-F Adenoviral Vectors

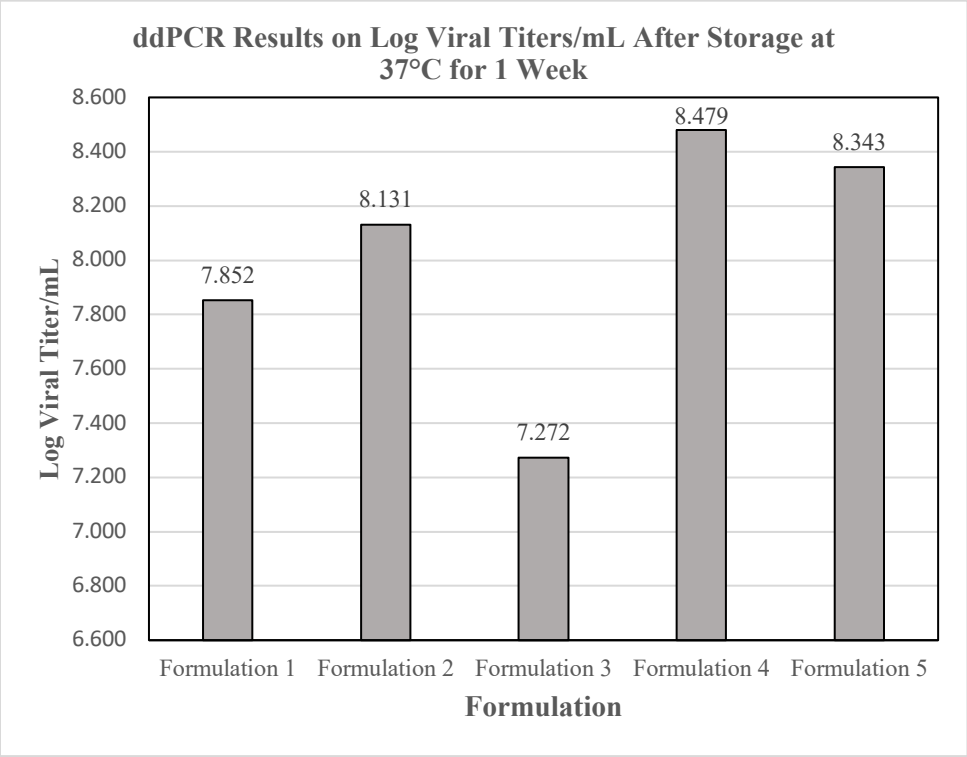

Figure 19 Quantification by ddPCR of Total Viral Particles (Viral Genomes/mL) After Evaluation of Various Combinations of Excipient (Formulations 1-5) used in an Accelerated Stability Assessment at 37 °C. Ad-CMV-F stored in classical Formulation 1 acted as a control.

Formulation 1 : NaCl 0.1mol/L + Sucrose 2% + Tris 0.01mol/L +$MgCl_2$ 2mM
Formulation 2: Trehalose 2% + Polysorbate-80 0.5 %
Formulation 3: Trehalose 2% + Pluronic F-127 1%
Formulation 4: Trehalose 2% + Sorbitol 1% + Polysorbate-80 0.5%
Formulation 5: Trehalose 2% + Sorbitol 1% + Pluronic F-127 1%

8.3 Determination of Total Viral Particles Titer

Quantification of total adenoviral particles titer was also conducted. Figure 19 shows the ddPCR quantification results for the total viral particles (expressed as viral genomes per mL) in the 5

formulations assayed after storage at 37°C for 1 week. Formulation 1 is the final adenovirus formulation routinely used in adenovirus preparations. According to the total virus titers, the Formulations 2,4,5, with the addition of excipients, were effective in maintaining the virus stability. VGs/mL were not too dissimilar in all the formulation assayed, with and without excipients. The classical formulation accounted for a log titer of 7.852 whereas Formulation 2,4,5 showed titers of 8.131,8.479 and 8.343, respectively. Formulation 3 was apparently less effective with a log titer of 7.272. Although absolute quantification of genomic adenoviral DNA is used for calculation of total viral particles, it is noteworthy that results from the infectivity assay demonstrated a high performance on the stability of the Ad-CMV-F for Formulation 2. This will be discussed further in the Discussion section.

8.4 Determination of Infectious Viral Particles Titer

Figure 20 Log Viral Titer for Formulations with Combined Excipients shows the effect on the infectious viral titers when evaluating a synergic effect of excipient combinations, using phosphate buffered formulations containing the Ad-CMV-F adenoviral vector. The adenoviral samples were analyzed in $TCID_{50}$ assays for quantification of the infectious titer after a thermal stress treatment at 37°C for one week. Each assessment was conducted with five replicates. The

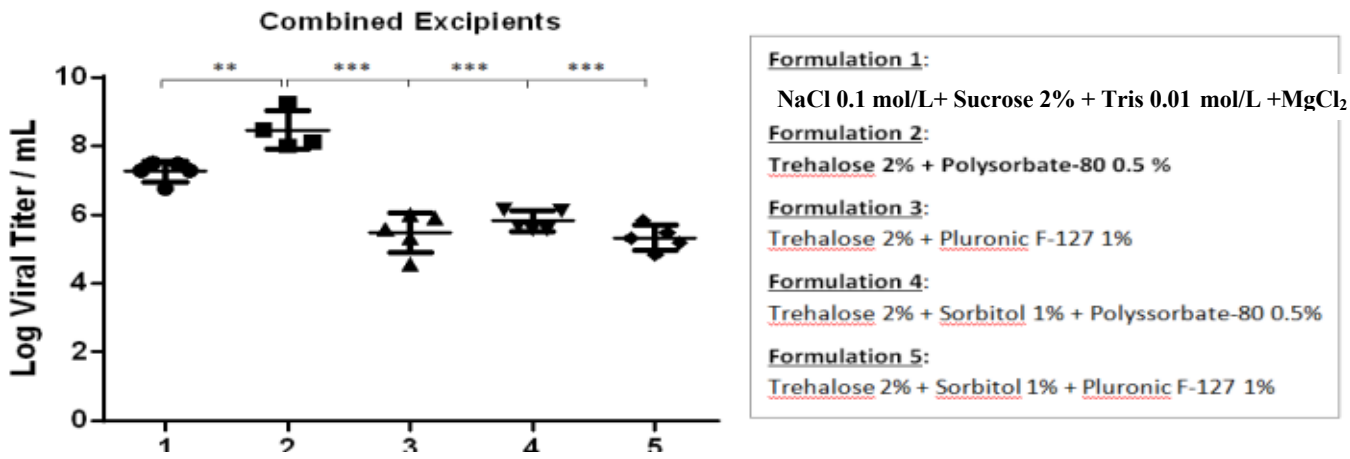

Figure 20 Log Viral Titer for Formulations with Combined Excipients

Formulation 2, containing trehalose 2% + polysorbate-80 0.5 % was the most effective one in maintaining the infectivity of the virus. An analysis of variance (ANOVA) was employed to compare the different outcomes and mean values from the different replicated were compared using the Tukey's Multiple Comparison test. The tests were conducted using the statistical software GraphPad Prism v6.0. Statistically significant differences ($p < 0.001$) are represented with three asterisks. The error bars in both senses represent the standard deviations.

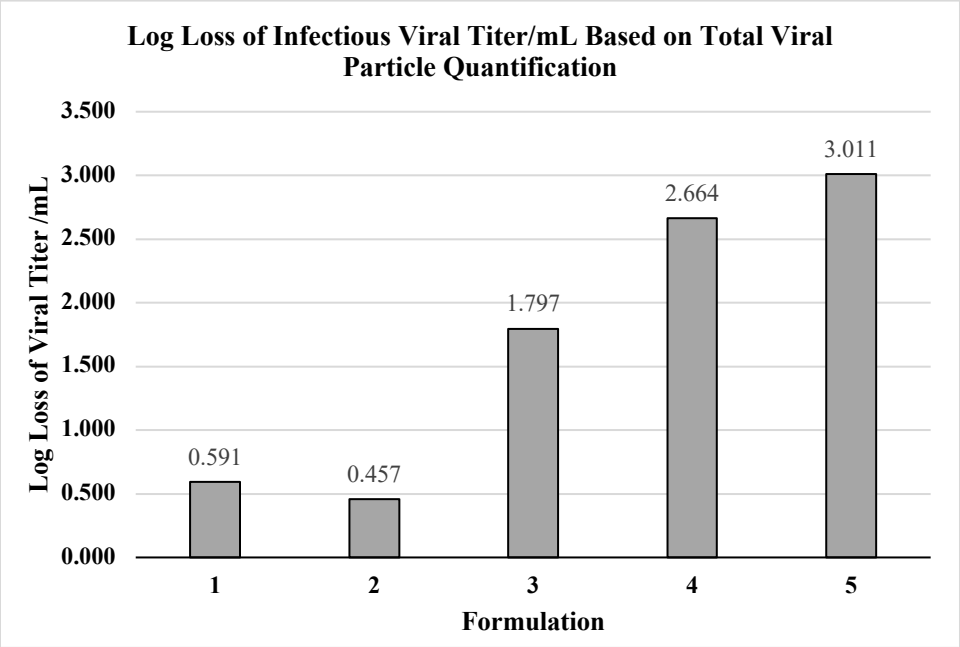

Figure 21 Log Loss of Infectious Viral Titer/mL Based on Total Viral Particle Quantification

Figure 21 shows the log loss of Infectious Viral Particles (IVP) deduced by considering the difference between the total viral particles quantified by ddPCR and the results of the $TCID_{50}$ Assay. Negative loss of titer is considered to show no log loss of viral titer. The results show that the classical formulation of NaCl 0.1 mol/L+ sucrose 2%+Tris 0.01 mol/L+$MgCl_2$ and Formulation 2 with trehalose 2% and polysorbate-80 0.5% was the most effective in maintaining the infectivity of the viral titers. Formulation 2 had a much lower loss of viral titer on average compared to the classical formulation. Statistical analysis of ANOVA conducted using Jamovi

software indicated a p-value of <0.001 indicating a significant difference between the various samples. Tukey post-hoc test comparing each formulation against every other indicated that Formulation 2 had a significantly lower log loss of viral titer against Formulation 3, 4, and 5 and a less significantly lower difference compared to the classical Formulation 1. This shows that Formulation 2 is as effective as the classical formulation in preserving the infectivity of the virus.

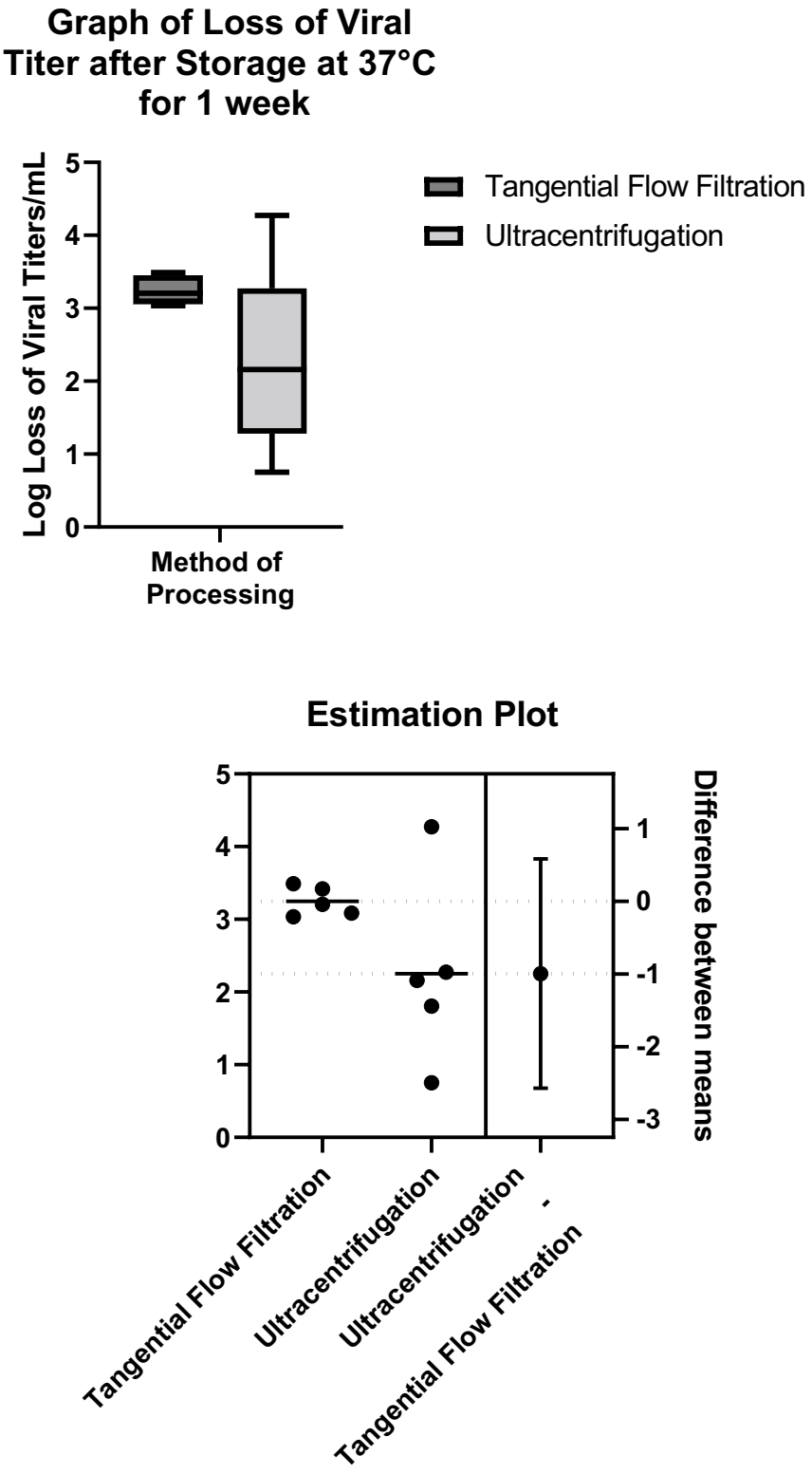

Figure 22 $TCID_{50}$ Assay Results for Different Methods of Processing for Formulation 2: Trehalose 2% and Polysorbate-80 0.5%

8.5 Analysis of Stabilizers in Ad-CMV-F Adenoviral Vector Formulated after Purification in CsCl Chloride Gradients or Contained Virus Clarified in Culture Supernatant

Figure 22 TCID50 Assay Results for Different Methods of Processing shows the log loss of viral titer when the virus obtained from Tangential Flow Filtration (TFF) and ultracentrifugation is placed in 37ºC for a period of 1 week in Formulation 2. The difference between the mean of tangential flow filtration and ultracentrifugation is -0.9940 ±0.5785.The 95% confidence interval difference between the mean is -2.571±0.5830. The Welch t-test was conducted using the statistical software GraphPad Prism v6.0. The Welch t-test statistical analysis of the results yielded $p=0.1575$, $p > 0.05$. Therefore, result is not statistically significant to reject the null hypothesis that the mean of the two groups is same.

9.Discussion

Newcastle Disease has been causing severe economic losses and affecting poultry production in Sub-Saharan Africa. The goal of this project was to develop an stable adenovirus-vectored vaccine formulation that can reduce the dependence on the cold-chain distribution system to maintain the vaccine stability in locations without the infrastructure to support deep refrigeration. The recombinant adenovirus vectored vaccine (Ad-CMV-F) against NDV that has been developed in the earlier phase of the project, was produced in bench-top 1-L and 3-L bioreactors clarified by tangential flow filtration and alternatively purified by ultracentrifugation with CsCl gradient. A pre-screening empirical method was used in Phase 1 of the project, whereby several excipients were selected from the known categories of protective excipients such as disaccharides, polyols, non-ionic surfactants, and proteins. Trehalose, sucrose at 2w/w% and 5% w/w, pluronic F-127 at 0.5% w/w and 1% w/w, sorbitol and mannitol at 3% and arginine and histidine at 10mM were the excipients tested.

The first set of results from the prescreening tests comprised 2 replicates for each excipient. Additional replicates for each excipient at this early stage were not considered due to the large number of $TCID_{50}$ assays involved in such screening . The objective of the screening test was to identify the excipients able to confer increased stability to the adenoviral vector produced, during storage at different temperatures. In all cases, the decrease in infectious titers were analyzed and the excipients able to maintain the original viral titers by a higher extent were selected for further evaluations. Key insights from this experiment were necessary to formulate the multi-component formulations in Phase 2.

First, arginine 10mM and histidine 10mM were consistently less protective than using PBS alone, for the 3 temperatures of storage. This suggested a detrimental effect on the stability of the viral proteins necessary to maintain infectivity by affecting the antigenic structure. The infectivity of the virus depends on the maintaining the antigenic structure and the correct steric presentation of the epitope. An establishment of unfavorable electrostatic and co-ordination bonds with amino acid side chains and peptide bonds between viral proteins and arginine and histidine, could be promoting aggregation and destabilize the virus, causing decreased infectivity of the virus. Ohtake et al indicates that unfavorable protein interactions with excipients, and subsequent free energy increase before aggregation then thermodynamically favors aggregation. The aggregating effects could be causing the loss of infectivity that is seen for histidine and arginine (51).

The screening results consistently showed that trehalose 2%, trehalose 5% were superior in maintaining the viral infectivity compared to other excipients. Trehalose 2% was the most effective sugar protectant in all the 3 temperatures studied. The method of stabilization of trehalose is still being studied and is thought to be due to the preferential exclusion model,

creating a barrier between protein molecules to prevent their aggregation and denaturation (52). Trehalose results in a large amount of water near the protein surface, limiting the protein's ability to denature. Previous studies have shown that trehalose increases the denaturation temperature of proteins. The denaturation temperature of RNase, for instance, increased from 40.9°C to 50.9°C in the presence of 1M trehalose (53). The addition of 1M trehalose also increased the surface tension by 1.69 dyne/cm (53). Trehalose also has greater conformational flexibility compared to sucrose (54) which may cause better ability to create stabilizing bonds to prevent denaturation. Although sucrose and trehalose are both disaccharides and have the same chemical formula, their geometric structure has several differences. Sucrose consists of a glucose and a fructose ring which is linked by a glycosidic bond, whereas trehalose consists of two rings connected by a glycosidic bond.

Previous research findings have shown that trehalose is more effective compared to sucrose against thermal stress (55) and is better for stabilizing biological molecules (56).Magazu et al's paper concluded that the difference in stabilizing effects is due to stronger hydrogen bonds with water using experimental techniques such as quasi-elastic neutron scattering (QENS) measurements (57). Olsson et al compared the structure of sucrose and trehalose in aqueous solution using several techniques such as neutron and x-ray diffraction together with empirical potential structure refinement (ESPR) modeling (58). The results of this study showed that trehalose has a stronger perturbing effect on water due to its structure being more bulk water than sucrose. Moreover, the overall radius of gyration was shown to be larger for trehalose than sucrose, leading to water molecules interacting differently with the different atomic sites of both the sugars. This could answer why trehalose stabilizes biological molecules better than sucrose. The stabilizing effect of sugars depend on the extent of hydrogen bonding and the subsequent

reduction of protein mobility. Smaller sugar molecules are more favorable to reduce local mobility through higher number of interactions but larger molecules favor vitrification which is holding the protein immobilized in a rigid glassy matrix. The hydroxyl groups in the sugar alcohol form hydrogen bonds with the protein, and subsequently replacing hydrogen bonds between water and protein. The smaller size of the sugar alcohols mannitol and sorbitol compared to the larger trehalose and sucrose, could possibly cause the sugar alcohols to stabilize the viral proteins by water replacement rather than vitrification, and the disaccharides are stabilizing the protein by vitrification process rather than water replacement and hydrogen bonding, leading to differences in the stabilizing effects.

Among the non-ionic surfactants, pluronic F-127 1% provided better protection to the virus than pluronic F-127 at 0.5% in 2 out of 3 temperatures. Pluronic F-127 is a poloxamer with superior abilities to protect proteins and prevent denaturation in previous studies, where a 14% w/w PF-127 solution greatly reduced the capability of a denaturant to affect the stability of a test enzyme (59). The protective effects of the PF-127 stems from its ability to mutually exclude hydrophobic residues from aqueous medium and prevent aggregation. Polysorbate-80 showed a log loss of titer of 1.07-3.19 across the three temperatures. The protective nature of the polysorbate-80 as a nonionic surfactant arises from its ability to reduce surface stresses for the viral proteins and reduce the aggregation. Previous study by Perez et al (60) showed that polysorbate-80 was able to reduce the surface tension experienced by proteins and increase stabilization. The stabilization principle can be explained by both interfacial competition and surfactant-protein complexation (61). The interaction of the protein with the surfactant is more thermodynamically favored than the protein-protein interaction which is determined by mass-action kinetics. Also, the attachment of the surfactant with protein at the exposed hydrophobic regions would increase the protein's

colloidal stability and leading to thermodynamic stability. The protective effect of polysorbate-80 at 0.5% w/w and pluronic F-127 at 0.5% w/w was very similar. Future studies could incorporate stress tests with longer storage duration of ~ 4-6 months to compare the protective effects of both excipients.

The next phase of the project was to study the combined effects of two or more excipients. The result of the experiment showed that Formulation 2 : trehalose 2% + polysorbate-80 0.5 % is as protective in maintaining both the infectious titers and the viral integrity as the classical formulation of NaCl 0.1mol/L + sucrose 2% + Tris 0.01mol/L +$MgCl_2$ 2mM used. The largest loss of viral titer was for Formulation 5 : trehalose 2% + sorbitol 1% + pluronic F-127 1% which had a loss of 3 log units.

The ddPCR results, quantified the number of intact viral genome copies, suggesting a similar number of intact viral particles, with slight differences in the total viral particles, with the overall range being between 7.3-8.5 log units/mL. This result showed that Formulation 4: trehalose 2% + sorbitol 1% + polysorbate-80 0.5% was most effective in maintaining the most amount of viral particles intact. Comparison with the $TCID_{50}$ Assay results provide a better understanding of the nature of the excipients in maintaining the infectivity of the virus. When comparing against $TCID_{50}$ assay results, the combined effects of having sorbitol 1% and polysorbate-80 0.5% in Formulation 4 did not have a better effect on maintaining the infectivity compared to having trehalose and polysorbate-80 alone in Formulation 2 : trehalose 2% + polysorbate-80 0.5 %. This shows that although more virus particles maintained stability in Formulation 4, there were other factors that influenced the drop in infectious titers in Formulation 4.

Conformational changes in the viral proteins which allow the virus to enter the host cells could be causing the drop in infectious titers, although capsid remains intact in Formulation 4. The

presence of the sugar alcohol sorbitol, with additional hydroxyl groups seem to reduce the stability of infection-enabling viral proteins in the presence of polysorbate-80. The mechanism of virus stabilization is a complex process with multiple excipients, which have different mechanisms and thermodynamic effects on stabilizing viruses. In view of the complexity of identifying favorable multi-component excipients, the empirical method may indicate effects on the stability for the concentrations studied but further studies would need to be done to carefully optimize the concentration of each excipient in order to achieve optimum stability from addition of each excipient.

The combination of having a sugar with a surfactant was studied in Formulation 4 and 5. Both these formulations showed the highest log loss units for viral titer. Kubbutat et al studied the greater the stability of the foam, the better it will help preserve proteins, which is especially critical in the pharmaceutical formulations containing polysorbate-80 with sugars (64). When sorbitol was added to polysorbate-80 and the resulting surface tension was assessed, it could be seen that sorbitol interacted significantly with polysorbate-80, unlike other sugars like maltose, which did not interact at all any sugar concentration. Interaction of various sugar and sugar alcohols with polysorabate-80 creates stable foams. Sorbitol interacts with the surfactant through the formation of hydrogen bonds with the ethyl oxide groups in the surfactant. The interaction increased the surface pressure and supports foam stability. It was suggested that the addition of sorbitol increases the concentration of surfactant at the air-water surface. Moreover, the chemical potential of the surfactant is affected by the addition of the sorbitol, and the shape of the micelles it forms is also affected (62). The exact nature of the interactions, and the impact on stabilizing viral proteins is not clearly understood yet. Contrary to the stabilizing effect of polysorbate-80 and sorbitol which was discussed by Kubbutat et al (62), this work, shows there is an increased

log loss units of infectious titers for formulations with both sugar alcohol and surfactant in the formulation. Infectivity of the virus is affected by the stability of the proteins which are vital to cause infection in host cells. The presence of both sugar, sugar alcohol and a surfactant seemed to have cause a negative effect on the stability of the formulation. A similar result is seen in Formulation 5: Trehalose 2% + Sorbitol 1% + Pluronic F-127 1% which had a high infectious titer loss based on the $TCID_{50}$ Assay. It is also possible that the ratio of surfactant to sugar in the formulation is not optimized to improve the overall synergistic stabilizing effect of the sugar and the surfactant.

The stress test comparing the loss of viral titer for virus obtained through clarification through tangential filtration and purification with CsCl ultracentrifugation shows that Formulation 2 was able to retain more infectious virus titer for the CsCl purified virus product. Apart from the processing methodology, the presence of higher level of impurities in the clarified virus product such as empty capsids, cellular debris, broken/dissembled virus particles, virus-DNA aggregates in the TFF product compared to the product obtained after CsCl ultracentrifugation is another difference between the samples. Generally, purified viruses are more vulnerable to loss of stability compared to viruses stored with supernatant (63). The presence of the cellular debris has cryoprotective effect on the viruses. Given that, the TFF product should be retaining higher infectivity compared to the CsCl purified viruses. However, in stress test 3, the tangential flow filtration product and CsCl ultracentrifugation product which were formulated with Formulation 2 resulted in the $TCID_{50}$ Assay showing that the former had a lower titer compared to the latter after 1 week of storage at 37°C.

It is interesting to note that viruses will face physical stresses during the process of purification or concentration. The exposure to the processing conditions could make the virus lose stability, cause aggregation and precipitation and subsequent loss of infectivity.

A possible explanation for the TFF product showing a great loss of infectivity could be due to shear forces experienced by the virus during membrane filtration and recirculation. Besnard et al note that viruses which are more sensitive to shear forces can be easily damaged during the membrane filtration step and result in loss of infectivity (64). Besnard et al discuss that the shear tolerability of the viruses can be dependent on the capsid size, thickness and geometry. Therefore, it is suggested that the TFF process should be carefully designed to minimize loss of viral integrity by having a low cross flow, moderate transmembrane pressure and short processing time (65).

CsCl ultracentrifugation on the contrary allows the acceleration of liquid samples while keeping the shear forces to a minimum. There are rotational forces acting on the samples during acceleration and deceleration, but they are achieved gradually (66). Given that, the reduced shear forces on the viral particles could be causing the CsCl purified virus particles to retain their infectivity better compared to those purified by tangential flow filtration.

Overall, the result of this study shows that trehalose and polysorbate-80 in combination can be very effective in stabilizing the recombinant NDV vaccine. These excipient combinations can be also investigated in various adenovirus-vectored vaccine formulations to improve their stability such as the AstraZeneca Vaxzervria COVID-19 vaccine and the Johnson & Johnson Ebola vaccine. In broader applications, trehalose and polysorbate-80 combination effect can be studied in spray-dried or lyophilized formulations as well to study the impact on maintaining vaccine stability during storage.

10. Conclusion and Perspectives

Many licensed vaccines are adenovirus-based vaccines and improving the formulation for adenovirus vaccines have immense potential to improve vaccine stability issues for many other vaccines as well. The COVID-19 pandemic has highlighted the importance of vaccines in a global health crisis and research to improve vaccine stability is critical to help improve vaccine availability to millions of people in remote regions.

With regards to vaccine formulation development, there is a need to explore more excipients, and their study their effect on stabilizing viruses at a molecular level. Future studies could also utilize molecular dynamics simulations in order to study the thermodynamics of protein stabilization with excipients. Moreover, experimental studies could focus on X-ray diffraction, Neutron Diffraction, ESPR Modelling to understand the impacts of each excipient at the molecular level. Moreover, Nuclear Magnetic Resonance (NMR) Spectroscopy has numerous benefits for studying viral proteins in solution, to understand binding interactions, protein dynamics and kinetics to track structural changes real time. Also, changes in binding interactions can be easily monitored with changes in chemical shifts. With the help of these simulations and experimental studies, a clearer understanding of the stabilization process is likely to be achieved.

Also, this study focused on the effects of various excipients for short term storage. Studies to determine the effectiveness of these formulations for longer duration of storage is useful to understand the effectiveness of the excipients as well. Other stresses like effect of agitation, thermal cycling, which is likely to occur during transportation, will allow vaccine formulation to experience stresses similar to that experienced during vaccine distribution.

The result of this work has shown that trehalose and polysorbate-80 have good potential to be used in adenovirus formulations as excipients. It will be vital to direct future research towards

studying the stability of lyophilized and spray-dried formulations with trehalose and polysorbate-80, and the effect of these excipients on the cake appearance for the lyophilized product and the ability to maintain infectivity after lyophilization and rehydration of dried product.

In order to advance the research of adenoviral vaccine formulation, a multi-pronged approach is necessary. The multi-pronged approach would involve studying excipient-viral interactions at a molecular level using advanced experimental techniques, molecular dynamics simulations to screen excipients from a large databank of possible excipients, short-term and long-term stability studies, and stress-tests involving a greater number of stresses other than thermal stress.

11.Summary

In this project, recombinant Ad-CMV-F against the Newcastle Disease Virus infection was produced in bench-scale bioreactors. The primary goal of the project was to develop a formulation for adenovirus vector, which enables the virus to maintain stability better and reduce loss of infectious viral titers. An empirical testing method was followed, whereby several excipients were tested in single component formulations to study their influence on maintaining the stability against exposure of the virus + formulation to 3 different temperatures of 4ºC, 37ºC, and 22ºC. Trehalose 2% (sugar), sorbitol 3% (sugar alcohol) and polysorbate-80 0.5%, and pluronic F-127 1% were more effective than other excipients in maintaining the infectious titers. Combinations of the more protective excipients (determined with $TCID_{50}$ assay) with the virus were tested against 1 week exposure to 37 ºC. Formulation 2 with trehalose 2% and polysorbate-80 0.5% were the most effective, with statistically significant results. Total virus particle

quantification was performed with ddPCR. Formulation 4 with trehalose 2%, sorbitol 1% and polysorbate-80 0.5% showed the highest viral titer, although it did not show a high infectious titer as Formulation 2. A comparison between the tangential flow filtration product and CsCl ultracentrifugation in retaining the virus infectivity was studied as well. Tangential flow filtration product showed a lower infectious viral titer after 37 ºC exposure for 1 week compared to CsCl ultracentrifugation purified product but a Welch t-test of the comparison showed that the difference was not statistically significant. Future direction of research includes increasing replicates, performing long term storage stability studies, examining viral protein-excipient interactions at a molecular level and studying the impact of other types of stresses such as agitation and thermal cycling.

12.References

1. Editors, History.com. Pandemics That Changed History. [Online] February 20, 2022. https://www.history.com/topics/middle-ages/pandemics-timeline.
2. CDC. History of Smallpox. [Online] Feb 2021. https://www.cdc.gov/smallpox/history/history.html.
3. Louten, Jennifer. Poxviruses . Essential Human Virology. s.l. : Academic Press, 2016.
4. Greta Keenan. A brief history of vaccines and how they changed the world. World Economic Forum. [Online] Apr 9, 2020. [Cited: Feb 21, 2022.] https://www.weforum.org/agenda/2020/04/how-vaccines-changed-the-world/.
5. Institute, Science History. Science History Institute . [Online] December 14, 2017. [Cited: March 02, 2022.] https://www.sciencehistory.org/historical-profile/louis-pasteur.
6. Samantha Vanderslott, Bernadeta Dadonaite, Max Roser. https://ourworldindata.org/vaccination. Our World in Data . [Online] December 2019. [Cited: February 21, 2022 .] https://ourworldindata.org/vaccination.
7. Qian Ren, MD, Hongyan Xiong, MD, Yafei Li, MD, Rufu Xu, PhD, and Caizhong Zhu, MD. Evaluation of an Outside-the-Cold-Chain Vaccine Delivery Strategy in Remote Regions of Western China. s.l. : Public Health Rep, 2009, Vol. 124.
8. Mukami Mungai Reaching Remote Villages in Kenya :“We’ll have to go to them” Gavi Vaccines Work. [Online] Jul 6, 2021. [Cited: Jul 6, 2022.] https://www.gavi.org/vaccineswork/reaching-remote-villages-kenya-we-have-go-them

9. Jackson Thomas, Gregory Peterson,Mark Naunton, Sam Kosari,Yap Boum. Over half of vaccines are wasted globally for these simple reasons. World Economic Forum. [Online] July 24, 2018. [Cited: March 02, 2022.] https://www.weforum.org/agenda/2018/07/the-biggest-hurdle-to-universal-vaccination-might-just-be-a-fridge.
10. Omar Farnós, Esayas Gelaye, Khaled Trabelsi, Alice Bernier, Kumar Subramani,Héla Kallel, Martha Yami and Amine A. Kamen. Establishing a Robust Manufacturing Platform forRecombinant Veterinary Vaccines:An Adenovirus-Vector Vaccine to Control NewcastleDisease Virus Infections of Poultry inSub-Saharan Africa. s.l. : vaccines , 2020.
11. Louten, Jennifer. Virus Structure and Classification. Essential Human Virology . 2016.
12. Mukhopadhyay, Nilotpal Banerjee and Sumi.Viral glycoproteins: biological role and application in diagnosis., s.l. : Virusdisease, 2016, Vol. 27.
13. Ketan Ganar.Moushumee Das, Sugandha Sinha ,Sachin Kumar. Newcastle disease virus: Current status and our understanding. s.l. : Virus Research, 2014, Vol. 184.
14. Vaccine Safety Forum . s.l. : The National Academic Press , 1997.
15. Bruce Alberts, Alexander Johnson, Julian Lewis, Martin Raff, Keith Roberts, and Peter Walter. Molecular Biology of the Cell .. s.l. : Garland Science , 2002.
16. Alberts B, Johnson A, Lewis J, et al Development of vaccine formulations: past, present, and future. Carmine D'Amico, Flavia Fontana, Ruoyu Cheng & Hélder A. Santos. s.l. : Drug Delivery and Translational Research , 2021, Vol. 11.
17. Gavi. What are nucleic acid vaccines and how could they be turned against COVID-19? [Online] [Cited: Mar 2022, 3.]
18. António Roldão 1, Maria Candida M Mellado, Leda R Castilho, Manuel J T Carrondo, Paula M Alves Virus-like particles in vaccine development. , s.l. : Expert Rev Vaccines , 2010, Vol. 9.
19.Clem Angela S. 1Fundamentals of Vaccine Immunology., s.l. : J Glob Infect Dis, 2011, Vol. 3.
20. Emory University. What is Live Attenuated Vaccines ? [Online] [Cited: March 03, 2022.]
21.A.E.Kajon, J.B.W,K.R Spindler, Adenoviruses . s.l. : Elsevier Reference Collection, 2019.
22.David Sharon, A.K. Advancements in the design and scalable production of viral gene transfer vectors. s.l. : Wiley Biotechnology Engineering , 2018, Vol. 115.
23. Jort Vellinga, J.P.S, Agnieszka Lipiec,Dragomira Majhen, Angelique Lemckert and P.I Mark van Ooij, Christopher Yallop, Jerome Custers and Menzo Havenga Challenges in Manufacturing Adenoviral Vectors for Global Vaccine Product Deployment . s.l. : Human Gene Therapy, 2014, Vol. 25.
24. Sai.V.Vemula, S.K.M.Production of adenovirus vectors and their use as a delivery system for influenxa vaccines . s.l. : Expert Opin Biol Ther, 2011.
25. L.J.J Hansena, R.D.b.,C.Vervaet c, J.P Remonc, T.R.M De Beer. Freeze drying of Vaccines : A Review. s.l. : Vaccine, 2015, Vol. 33, pp. 5507-5519.
26. Manvi Hsija, LIlian Li,Nausheen Rahman, Salvador F Ausar.Forced degradation studies: an essential tool. s.l. : Vaccine: Development and Therapy, 2013, Vol. 3.
27. J.P. Factors affecting stability of viral vaccines. s.l. : Dev Biol Stand, 1996, Vol. 87.
28. Peter M. Ihnata, Gary Vellekampa, Linda J. Obenauer-Kutner,Jenny Duanb,Michelle A. Hanb,Leonore C. Witchey-Lakshmananb, Michael J. Grace.Comparative thermal stabilities of recombinant adenoviruses and hexon protein. s.l. : Biochimica et Biophysica Acta, 2005, Vol. 1726.
29. Jason Rexroad, C.M.V,Anthony P Green,Timothy D.Kirkstead.Structural Stability of Adenovirus Type 5. s.l. : Journal of Pharmaceutical Sciences, 2002, Vol. 92.

30. J.Rexroad Thermal stability of adenovirus type 2 asa function of pH. s.l. : Journal of Pharmaceutical Sciences , 2006, Vol. 95.
31. Suling Zhai 1, Raino K Hansen, Richard Taylor, Jeremy N Skepper, Raquel Sanches, Nigel K H Slater. Effect of freezing rates and excipients on the infectivity of a live viral vaccine during lyophilization. s.l. : Biotechnol Prog , 2004.
32.Steven P.Toniolo, S.A. Ahmand Mahmood,Cecile Fradin, Brian D Lichty,Matthew S Miller, Zhou Xing, Emily D Cranston, Michael R. Thompson Excipient Selection for thermal stable enveloped and non enveloped viral. s.l. : International Journal of Pharmaceutics, 2019.
33. Seema Thakral J.S, Bhushan Munjal,Raj Suryanarayanan Stabilizers and their interaction with formulation components in frozen and freeze dried protein formulations. s.l. : Advanced Drug Delivery Reviews , 2021.
34. Summary of Investigational Formulations of Vaccines . [Online] Working in Tandem, Ltd., for the PATH Vaccine and Pharmaceutical Technologies Group., May 9, 2012. https://path.azureedge.net/media/documents/TS_vaccine_stability_table_invest.pdf.
35. Evans, R.K.e.a., Development of stable liquid formulations for adenovirus-based vaccines. s.l. : J.Pharm Sci, 2004.
36. Nedim E Altaras, John G Aunins,Robert K. Evans, Amine Kamen, John O Konz, J.J.W. Production and Formualation of Adenovirus Vectors in Gene Therapy and Gene Delivery Systems. Advances in Biochemical Engineering/Biotechnology. s.l. : Springer, 2005.
37. Stewart.M, W.S, J.Drew. Use of adenovirus as a model system to illustrate a simple method of using standard equipment and inexpensive excipients to remove live virus dependence on the cold chain. s.l. : Vaccine, 2014.
38. Ankur Ajmera, R.S Stabilisation of Proteins via mixture of amino acids during spray drying. s.l. : International Journal of Pharmaceutics, 2014, Vol. 463.
39. C.Ravell, J.Hackensack. s.l. : Meridian Health , 2021.
40. Nicholas G. Kounis, Ioanna Koniari, Cesare de Gregorio, Dimitris Velissaris, Konstantinos Petalas,Aikaterini Brinia, Stelios F. Assimakopoulos, Christos Gogos, Sophia N. Kouni, George N. Kounis,Gian Franco Calogiuri,and Ming-Yow Hung. Allergic Reactions to Current Available COVID-19 Vaccinations:Pathophysiology,Casuality and Therapeutic Considerations.s.l. 2021 : Vaccines.
41. Sputnik V Vaccine. Precision Vaccinations . [Online] 2021.
42. MA Croyle, X Cheng & JM Wilson.Development of formulations that enhance physical stability of viral vectors for gene therapy. s.l. : Gene Therapy, 2001, Vol. 8.
43. Maria Pelliccia, *† Patrizia Andreozzi,,*‡ Jayson Paulose,* Marco D'Alicarnasso,* Valeria Cagno,,§ Manuela Donalisio, Andrea Civra, Rebecca M. Broeckel, Nicole Haese,Paulo Jacob Silva, Randy P. Carney,,‖ Varpu Marjomäki, Daniel N.Additives for vaccine storage to improve thermal stability of adenoviruses from hours to months. s.l. : Nature Communications, 2016, Vol. 7.
44. Adriaansen, Janik. Adenovirus formulations. 9,974,737 September 16, 2014.
45. Jan Jezek, Angela Buckler. Stable Aqueous Formulations of Adenovirus Vectors. 20160199496 July 14, 2016.
46. Zheng Wu, Shuyuan Zhang. Liquid adenovirus formulations. 7,888,096 June 26, 2007.
47. Eva B. Reinauer, Stella S. Grosso, Stefan R. Henz, Julia A. Rabas, Carina Rodenstein. Algorithm-Based Liquid Formulation Development Including a DoE Concept Predicts Long-Term Viral Vector Stability. s.l. : Journal of Pharmaceutical Sciences, 2020, Vol. 109.

48. Biorender . Biorender . [Online]
https://app.biorender.com/illustrations/62485e6f73304547a4698a65.
49. Ashaben Patel, Steven M.Erb,Linda Strange,Ravi.S.Shukla, Ozan S.Kumru,Lee Smith,Paul Nelson, Sangeeta B. Joshi, Jill A Livengood,David B.Volkin.Combined semi-empirical screening and design of experiments (DOE). s.l. : Vaccines , 2018, Vol. 36.
50. Chengfeng Lei,Jian Yang, Jia Hu, and Xiulian Sun. On the Calculation of TCID50 for Quantitation of Virus Infectivity. s.l. : Virol Sin, 2021, Vol. 36.
51. Satoshi Ohtake, Yoshiko Kita,Tsutomu Arakawa. Interactions of formulation excipients with proteins in solution and in the dried state. s.l. : Advanced Drug Delivery Reviews , 2011.
52. Christoffer Olsson, Helen Jansson and Jan Swenson.The Role of Trehalose for the Stabilization . s.l. : The Journal of Physical Chemistry, 2016, Vol. 120.
53. Lin T.Y, Timasheff S.N. On the role of surface tension in the stabilization of globular proteins. s.l. : Protein Sci, 1996, Vol. 5.
54. Leslie S.B, Israeli E,Lighthart B,Crowe J.H,Crowe L.M.Trehalose and sucrose protect both membranes and proteins in intact bacteria during drying. s.l. : Appl Environ Microbiol, 1995.
55.G., Bellavia, et al Protein Thermal Denaturation and Matrix Glass Transition in Different Protein−Trehalose−Water Systems . s.l. :J Phys Chem B 2011. 115
56. K., Jain N. and I., Roy Effect of trehalose on protein structure. s.l. : Protein Sci, 2008, Vol. 18.
57. S., Magazu, et al. Quasielastic neutron scattering study on disaccharide aqueous solutions.s.l. : Phys. B, 2001, Vol. 301.
58. Swenson, Christoffer Olsson* and Jan. Structural Comparison between Sucrose and Trehalose in Aqueous Solution. s.l. : J Phys Chem B. , 2020, Vol. 124.
59. Wout, Z. G., E. A. Pec, and T. P. Biological Activity of Urease Formulated in Poloxamer 407 After Intraperitoneal Injection into the Rat . s.l. : Journal of Pharmaceutical Sciences, 1992, Vol. 81.
60. Perez-Gramateges, M.Ruiz-Pena, R. Oropesa-Nunez,T.Pons, S.R.W Louro. Physico-chemical studies of molecular interactions between non-ionic surfactants and bovine serum albumin. A. s.l. : Colloids Surf B-Biointerfaces , 2010, Vol. 75.
61. Tarik.A.Khan, Hanns-Christian Mahler, Ravuri S.K Kishore. Key interactions of surfactants in therapeutic protein formulations:. s.l. : European JOurnal of Pharmaceutics and Biopharmaceutics , 2015, Vol. 97.
62. Peter Kubbutat, Ulrich Kulozik Interactions of sugar alcohol, di-saccharides and polysaccharides with polysorbate 80 as surfactant in the stabilization of foams.. s.l. : Colloids and Surfaces A: Physicochemical and Engineering Aspects, 2021, Vol. Volume 616.
63. Hasnaa Jorio, Rosa Tran, Amine Kamen Stability of Serum-Free and Purified Baculovirus Stocks under Various Storage Conditions. Montreal : Biotechnol. Prog., 2006, Vol. 22.
64. Lise Besnard, Virginie Fabre, Michael Fettig, Elina Gousseinov, Yasuhiro Kawakami, Nicolas Laroudie,Claire Scanlan, Priyabrata Pattnaik Clarification of Vaccines: An overview of filter based technology trends and best practices . s.l. : Biotechnology Advances , 2016, Vol. 34.
65. Loewe D, Grein TA, Dieken H, Weidner T, Salzig D, Czermak P. Tangential Flow Filtration for the Concentration of Oncolytic Measles Virus: The Influence of Filter Properties and the Cell Culture Medium. s.l. : Membranes (Basel), 2019
66. Janice E. Lawrence, Grieg F.Steward Purification of Viruses by Centrifugation.. s.l. : Manual of Aquatic Viral Ecology, 2010.

67.Satoshi Ohtake, Y.John Wang Trehalose: Current Use and Future Applications.s.l : Journal of Pharmaceutical Sciences, 2011,Vol.100
68. Yeu-Chun Kim 1, Fu-Shi Quan, Jae-Min Song, Aswani Vunnava, Dae-Goon Yoo, Kyoung-Mi Park, Richard W Compans, Sang-Moo Kang, Mark R Prausnitz, *Influenza immunization with trehalose-stabilized virus-like particle vaccine using microneedles* s.l. : Procedia Vaccinol, 2010
69. He TC, Zhou S, da Costa LT, Yu J, Kinzler KW, Vogelstein B. *A simplified system for generating recombinant adenoviruses* Proc Natl Acad Sci U S A. 1998 Mar 3. 95(5):2509-14. 10.1073/pnas.95.5.2509

13. Appendix

Nucleotide sequences used for the F gene(10):

ATGGGCTCCAGATCTTCTACCAAAATCCCAGCACCTGTGACGCTGACTGCCCAAATTATGTTGATATTG
AGCTGTATCTGTCCGACAAACCCTCTTGACGGCAGGTCTCTTGCAGCTGCAGGAATTGTGGTAACAGG
AGACAAAGCAGTTAATATATACACCTCATCTCAGACGGGATCAATCATAGTCAAGTTGCTCCCAAACA
TGCCCAAAGATAAAGAGGCATGTGCAAAAGCCCCATTAGAAGCATACAACAGAACACTGACCGCTTT
ACTCACCCCTCTCGGTGACTCTATCCGCAGGATACAGGGGTCCGTATCCAAGTCAGGAGGAGGAAGA
CAAGGACGCTTAGTAGGTGCCATTATTGGCAGTGTAGCTCTCGGGGTCGCAACATCAGCACAGATAAC
AGCAGCTGCAGCCTTAATACAAGCCAACCAGAATGCTGCTAACATCCTTCGGCTTAAGGAGAGCATTG
CTGCCACCAATGAAGCTGTGCATGAAGTCACTGACGGATTATCACAACTAGCTGTGGCAGTTGGTAAG
ATGCAGCAGTTTATTAATGACCAATTCAATAATACGGCGCGGGAACTGGACTGTATGAAAATTACCCA
ACAAGTCGGTGTAGAACTCAACCTATACCTAACTGAACTGACTACAGTGTTTGGGCCGCAAATCACCT
CCCCTGCCCTAACTCAGCTGACTATCCAGGCGCTTTATAATTTAGCTGGTGGCAATATGGATTACTTGC
TGACTAGGTTAGGCGTGGGGAACAATCAACTTAGCTCATTAATAAGTAGTGGCTTGATCACAGGCCAT
CCTATACTGTATGACTCACAAACTCAGCTCCTGGGCATACAAGTAAATTTACCCTCAGTCGGGAACCT
CAATAATATGCGTGCCACCTACTTGGAGACTATATCCGTAAGCACAACCAAGGGGTTTGCCTCAGCAC
TTGTCCCAAAGGTAGTGACGCAAGTCGGCTCCGTGATAGAAGAACTCGACACTTCACACTGTATAGAG
TCTGAACTGGATTTATATTGTACAAGAATAGTGACATTCCCCATGTCCCCTGGCATTTATTCCTGTC TGA
GCGGCAACACATCGGCTTGCATGTACTCAAAGACTGAAGGTGCACTCACTACGCCATATATGACCCTC
AAAGGCTCAGTTATTGCCAATTGCAAAATAACAACATGTAGGTGCGCAGACCCCCCAAGTATCATATC
GCAAAACTATGGAGAAGCTGTGTCTCTGATAGATAGACATTCATGCAATGTCCTGTCACTAGATGGGA
TAACTCTGAGGCTCAGTGGGGAGTTTGATGTGACTTATCAAAAGAATATCTCAATACTAGAATCTCAA
GTCATCGTGACCGGCAATCTTGATATATCAACCGAGCTAGGAAATGTCAACAATTCAATAAGCAGTGC
CCTAGGTAAGTTAGCGGAGAGTAACAGTAAACTAGACCAAGTCAATGTCAAACTGACCAGCACATCT
GCACTCATTACCTATATTGCTCTAACCACCATATCTCTTGTCTCCGGTATACTTAGCCTGGGTTTGGCGT
GCTACCTAATGTACAAACAAAAAGCACAACAAAAGACCTTAATATGGCTTGGGAATAATACCCTTGA
TCAGATGAGAGCTACTACAAGGACATGA

Oligonucleotide primers for F gene :

Table 3 Oligonucleotide Primers for F gene(10) (Reproduced with Permission)

F gene	Tm (℃)	Product Size (bp)
F- TTAGCTGGTGGCAATATGGA	59.1	221
R- AACCCCTTGGTTGTGCTTAC	59.0	
F- TGTTGATATTGAGCTGTATCTGTCC	59.6	204
R- GTGTTCTGTTGTATGCTTCTAATGG	59.2	
F- ATTGCTCTAACCACCATATCTCTTG	59.9	150
R- TCATGTCCTTGTAGTAGCTCTCATC	59.0	

Map of the pShuttle-CMV :

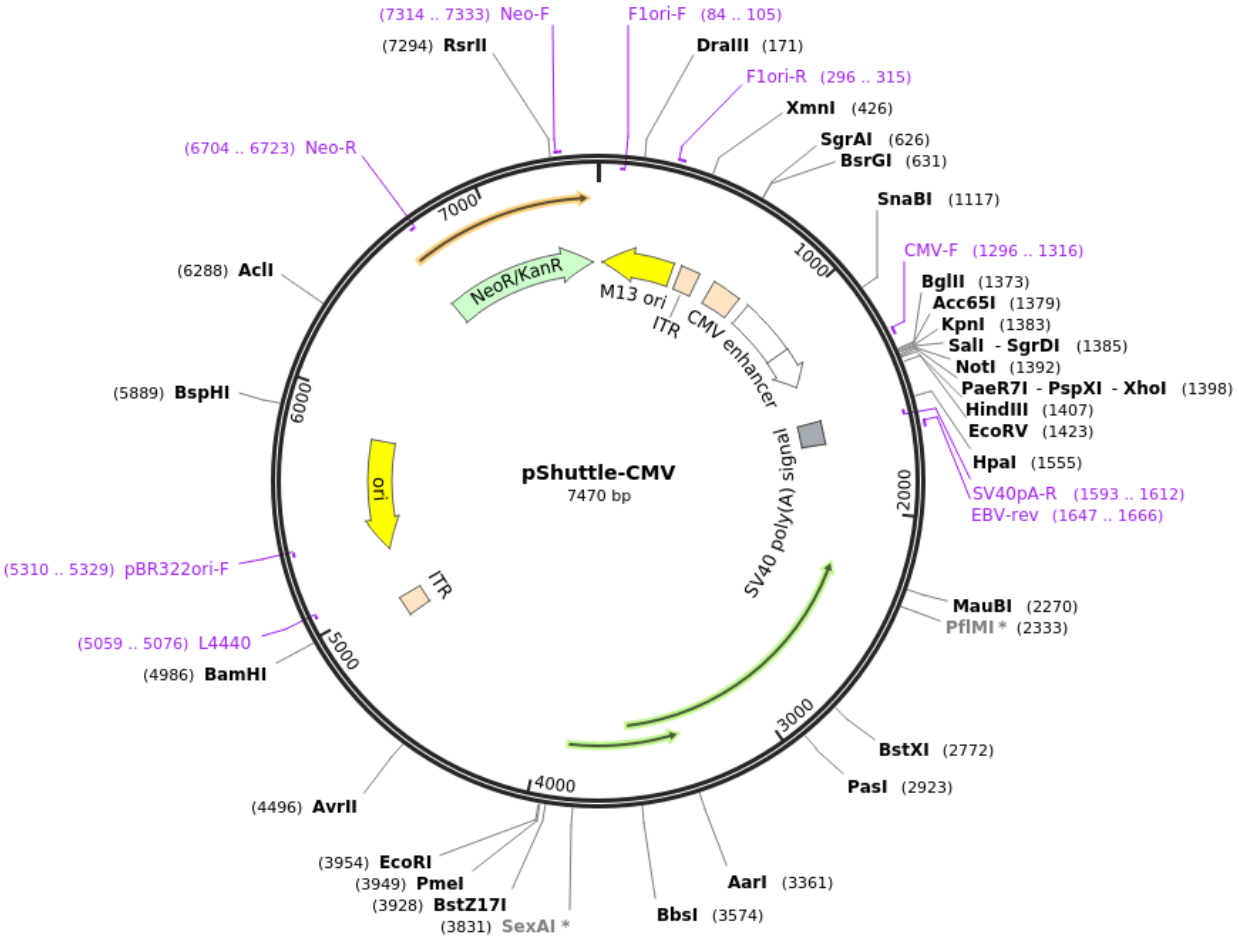

Figure 23 pShuttle-CMV Map