

Development and Characterization of a Modified Vaccinia Ankara Vaccine Candidate Expressing the SARS-CoV-2 Spike Glycoprotein.

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“Owaqala umsebenzi omuhle kimi uyakuwufeza kuze kube lusuka luka Kristu Jesu”

Philippians 1:6 Zulu Ibhayibeli Elingcwele

LIST OF ABBREVIATIONS

µg	Microgram
µl	Microlitre
µm	Micrometre
ACE2	Angiotensin-converting enzyme 2
Ad5, 26	Adenovirus serotype 5, 26
ATCC	American Type Culture Collection
BB	Binding buffer
BCG	Bacillus Calmette-Guerin
BHK21	Baby hamster kidney 21 cells
bp	Base pair
BSA	Bovine serum albumin
CDC	Centers for Disease Control and Prevention
ChAd	Chimpanzee adenoviruses
CMV	Cytomegalovirus
Con A	Concanavalin A
COVID-19	Coronavirus disease 2019
CPXV	Cowpox virus
CTD	C-terminal domain
CTL	Cytotoxic T lymphocyte
DMEM	Dulbecco's Modified Eagle Medium
DMSO	Dimethyl sulfoxide
DNA	Deoxyribonucleic acid
<i>E. coli</i>	<i>Escherichia coli</i>
EB	Elution buffer
ELISA	Enzyme-Linked Immunosorbent Assay
ELISPOT	Enzyme-Linked Immune Absorbent Spot

ETR	Endotoxin removal buffer
FCS	Fetal calf serum
FDA	Food and Drug Administration
ffu	Fluorescent forming unit
g	Gram or gravitational force
GFP	Green fluorescent protein
gp160	Glycoprotein 160
HCoV	Human coronaviruses
HEK293	Human embryo kidney cells
HIV	Human immuno-deficiency virus
HPCoV	Highly pathogenic coronaviruses
HRP	Horseradish peroxidase
HSPV	Horsepox
IBV	Infectious bronchitis virus
IFN- γ	Interferon gamma
IgG	Immunoglobulin G
IL-2, 5	Interleukin-2, 5
kb	Kilobase
kbp	Kilo-base pairs
kDA	Kilodalton
LB	Luria broth media
MERS	Middle Eastern Respiratory Syndrome
mg	Milligram
MHV	Mouse hepatitis virus
ml	Millilitre
MOI	Multiplicity of infection
mRNA	Messenger ribonucleic acid
MVA	Modified Vaccinia Virus Ankara

MW	Molecular weight
ng	Nanograms
NTD	N-terminal domain
ORFs	Open reading frames
PAGE	Polyacrylamide gel electrophoresis
PBS	Phosphate buffer saline
PCR	Polymerase chain reaction
pfu	Plaque forming units
PLWH	People living with HIV
PVDF	Polyvinylidene fluoride
RBD	Receptor binding domain
RK13	Rabbit kidney cells
rMVA	Recombinant MVA
RNA	Ribonucleic acid
rpm	Revolutions per minute
RPMI	Rosewell Park Memorial Institute medium
RT-PCR	Reverse transcription polymerase chain reaction
SARS	Severe Acute Respiratory Syndrome
SARS-CoV-2	Severe Acute Respiratory Syndrome Coronavirus 2
SDS	Sodium dodecyl sulphate
sfu	Spot forming unit
TBE	Tris-borate-EDTA
T-cell	T-lymphocyte
TCID ₅₀	Median Tissue Culture Infectious Dose
TPA	Tissue plasminogen activator
UV	Ultraviolet
V	Volt
VACV	Vaccinia virus

VLPs	Virus-like particles
WB	Western blot
WHO	World Health Organization
wt	Wildtype
wtMVA	Wildtype Modified Vaccinia Ankara

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ABSTRACT

Coronavirus disease 2019 (COVID-19) is caused by severe acute respiratory syndrome coronavirus 2, SARS-CoV-2. Given the ongoing COVID-19 pandemic and the continued evolution of the virus to escape host immunity, new vaccines and refinement of first-generation vaccines to improve protection against SARS-CoV-2 variants of concern is vital. In Africa, the cost of vaccine manufacturing as well as the scarcity in resources for storage and distribution have all contributed to the inequitable access to vaccines and heavy reliance on donations. Modified Vaccinia Virus Ankara (MVA) is a low-cost production vector platform which is suitable in this context. This project falls into a bigger study where our group compared different vector platforms, including MVA. The project serves as a proof of concept that this platform can be used to produce vaccines encompassing different variants of SARS-CoV-2 as they emerge. The most recent variant, Omicron, has proven to be highly immune evasive and demonstrates this need well. As the virus mutates, the variants of concern each present with differing characteristics and subsequently differ in immunogenicity and pathogenicity. Sub-Saharan Africa has been ransacked by the pandemic, resulting in loss of lives and livelihoods; the effects of which will undoubtedly be felt for decades to come. This study had two aims:

1. The development of a candidate vaccine, MVA-SARS-CoV2- Δ TM by using the widely used MVA platform and poxvirus recombinant vaccine strategies used in our research group,
2. The testing of this vaccine's immunogenicity in mice.

The MVA-based vaccine was constructed by infection of BHK21 cells with wildtype MVA, and transfection with transfer vector pMVA-FNK2. The transfer vector contains a truncated form of the SARS-CoV-2 spike glycoprotein gene, the vaccinia virus host-range gene K1L and reporter gene eGFP, flanked by gene sequences to allow homologous recombination into MVA. The presence of the K1L gene in the recombinant virus allowed for selection by passaging in RK13 cells, which were not permissive for the parent MVA. Following the potentially successful isolation, the recombinant MVA-SARS-CoV2- Δ TM was validated and characterized by PCR, Sanger sequencing, Western blot analysis and immunofluorescence, which all confirmed the presence and expression of the spike protein. Large scale propagation of the vaccine was done in

RK13 cells, and the stock was titrated to yield a titre of 3.9×10^6 ffu/ml. Balb/c mice were inoculated three times with MVA-SARS-CoV2-SΔTM at a dose of 3×10^5 to assess immunogenicity of the vaccine. Results from the immunogenicity assessments demonstrated that the vaccine induced T-cell responses shown by an enzyme-linked immunosorbent spot (ELISpot) assay. An enzyme-linked immunosorbent assay (ELISA) confirmed the ability of MVA-SARS-CoV2-SΔTM to induce increasing titres of binding antibodies in mice over a period of 56 days. However, the vaccine did not induce neutralizing antibodies against a matched SARS-CoV-2 pseudovirus, highlighting the need for further refinement of the vaccine.

In conclusion, recombinant MVA expressing a truncated SARS-CoV-2 spike protein was successfully constructed and tested for immunogenicity in mice. The candidate vaccine induced good T-cell responses and binding antibodies, but not neutralizing antibodies. This study provides additional evidence that MVA can be used as a platform for a SARS-CoV-2 vaccine, as has been previously demonstrated by others, and this could potentially be adapted for emerging variants of concern. This work also allows for the direct comparison of the MVA platform to other platforms employed by our group (DNA and plant-based subunit) as SARS-CoV-2 vaccines.

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CHAPTER 1

LITERATURE REVIEW

1.1 Emergence of SARS-CoV-2

In December 2019, a cluster of respiratory-related outbreaks occurred in Wuhan city, China, due to a novel coronavirus. The spread of the SARS-CoV-2 virus can be traced back to the Chinese New Year in China [1], a period which marks a period of mass movement, celebration gatherings and the heightened use of wet markets where food and live animals are sold. Bats have been identified as the most likely origin of the virus based on phylogenetic sequencing analysis that showed a 96.1% similarity to a bat coronavirus, RaTG13, found in Yunnan, a province in the south of China [2]. Further research on bats in south east Asia has revealed that closer relatives of the SARS-CoV-2 virus are circulating in bats [3]. These viruses are shown to have almost identical receptor binding domains which the virus uses to facilitate entry into the host cell. Together, these findings support the theory that the virus probably originated in bats, although this is still an area of ongoing research. Moreover, the close relation to the MERS-CoV and SARS-CoV viruses, which both have been shown to have originated in bats, provides strong evidence for this case [4]. Chinese traditional medicine and common Chinese practices of consuming foods from animals such as raccoons, civet cats and bats, coupled with the densely populated wet markets that see animals and humans being crowded in small spaces, having close contact has made room for the spread of new diseases [5], [6], [7] and are factors that contributed to the Wuhan epidemic. Pathogens, including viruses, get exposed to a new environment, move from animal excrement such as that of bats, which subsequently facilitates the species jump from animals to humans [6].

For a while, Wuhan was incorrectly identified as the origin of the virus. Wuhan is a city in the Chinese province of Hubei. With a population of about 11 million and connection to mass domestic and international travel, the virus was easily and unknowingly transported by asymptomatic hosts [8]. Reports have indicated that albeit in small clusters of cases, the virus was likely already present in China [9], and Europe [10] months before the Wuhan epidemic in December 2019. The virus lay dormant for months before it was awakened due to favourable ecological conditions in the Wuhan wet market, leading to the epidemic. It is speculated that the transition from epidemic to pandemic did not happen before Wuhan because of two reasons; the lack of close human contact

in large numbers which disallowed the spread, and the nature of the virus itself – the ancestral SARS-CoV-2 strain had a low transmission ability [11]. The first confirmed cluster of cases was in late December 2019, and this quickly rose to 7818 confirmed cases by the end of January 2020 [12]. The most common symptoms of this virus were high fevers and respiratory distress with patients reporting difficulties with breathing, and in severe cases resulting in death due to septic shock, multiple organ failure or respiratory failure [13], [14]. By 3 March 2020, the COVID-19 disease had been responsible for a total of 80,151 confirmed cases in Mainland China whilst 72 countries had reported a total of over 10 000 additional cases [15]. By the 23rd of March 2020, there were over 300,000 infections worldwide and over 13 000 deaths [16]. The World Health Organization declared this a pandemic, due to the alarming severity and rapid spread of the disease. With an initial reproductive number (R_0) estimated to range between 1.5 – 3.5 the spread from China to the rest of the globe was unprecedented, with the WHO categorizing the risk assessment in China as “very high” and “high” globally in January, and eventually declaring it a pandemic on the 11th of March 2020. To put the R_0 number into perspective; the Ebola virus had an R_0 of 1.51 – 2.53, SARS was 2.75, and MERS was 1 [17]. With the virus frequently mutating, the reproductive number has increased exponentially to a high above 5, rendering SARS-CoV-2 the most transmissible pathogenic human coronavirus [18].

Global cases of SARS-CoV-2 have continued to increase exponentially, and deaths have followed suit. At the time of compiling this review (in November 2021), almost 2 years after the first case, the global number of cumulative cases was approximated to be 244 million with over 182 countries having cases (Figure 1.1) and the number of deaths was estimated to be 4.95 million [19]. Africa has seen at least 8 650 000 cases and over 221 000 deaths to date [20]. In South Africa, the number of cumulative cases was reported to be 2.92 million with 88 925 deaths in mid-November 2021 [19].

1.1.1 SARS-CoV-2 in Africa

The COVID-19 pandemic has had a devastating global impact which has been exacerbated in Africa. The lack of infrastructure has had a widespread and deepening effect spanning from challenges in testing and diagnosing facilities, burdened hospitals resulting in difficulties in providing adequate healthcare to people showing severe COVID-19 symptoms, and finally the lack of vaccine manufacturing and production factories to facilitate equitable vaccination of the

population against the virus. Collectively these factors have contributed to the severity of the pandemic in Africa. Access to vaccines is extremely limited due to various reasons including sparse manufacturing capacity and the limited resources to enable purchasing of vaccine doses. The limitation in vaccine manufacturing infrastructure is the main challenge. For the largest continent with a population of 1.2 billion which constitutes 15% of the global population, there are less than ten vaccine manufactures [21]. The disproportionate ratio of people to vaccine manufacturing facilities not only leaves the continent behind but further forces a reliance on high-income countries and charitable initiatives like the COVAX initiative [22] for vaccine donation, a process which has seen Africa struggling to vaccinate efficiently. This has left the continent vulnerable and susceptible to several waves of infections, driven by various variants [23]. Efforts to boost vaccine manufacturing in Africa are therefore paramount now more than ever.

There are several factors that should be considered when deciding on the most effective and suitable vaccine for Africa. Costs of vaccines are a significant one. The pandemic worsened an already struggling African economy, with an estimated 40 million people anticipated to be pushed into extreme poverty as a result of COVID-19 [24], [25]. With the continent seeing its first recession in 25 years, there are pressing areas of financial need such as cash-transfer schemes which are designed to mitigate the effects of economic loss. Channelling of funds towards vaccines is near impossible whilst the monopolizing of vaccines in richer countries has contributed to increasing this cost five-fold. Oxfam International estimates the cost of production of a single dose of the Pfizer-BioNTech vaccine to be \$1.20 whilst COVAX is said to have paid on average \$5.20 per dose purchased [26] – which, when put into perspective of a continent where almost 40% of the population survives on less than \$1.90 [27] a day shows the extent of challenges in the cost incurred by charitable organizations such as COVAX when securing vaccines to donate to African countries. Additionally, vaccine hoarding by rich countries provided an additional challenge making the process of securing vaccines for poorer countries significantly more difficult. Cold chain is another important consideration when choosing vaccines to acquire and distribute across Africa. The process from storage of vaccine raw materials all the way through to transportation, storage, and delivery of vaccine doses to individuals requires careful logistical planning and availability of resources. For Africa, the network of cold rooms, freezers and cooler boxes is yet another expensive and limited resource. Most of the currently approved COVID-19 vaccines need to be stored at 2-9° C, whilst the Pfizer vaccine needs a storage of about -15 to -25° C [28]. In areas

where there is no access to electricity, this is exceptionally challenging. An additional factor that must be considered is the collision of epidemics in Africa. The disease burden on Africa is huge due to competing diseases such as HIV, Tuberculosis, Ebola, and malaria, amongst others [29][30], [31]. HIV is still a public health burden in Africa with many countries still struggling to get the epidemic under control. Sub-Saharan hosts almost 70% of people living with HIV globally [32] and therefore most health resources are structured around this problem. Diversion of research, funds, and facilities from HIV to COVID-19 vaccine research has been necessary albeit strenuous on both diseases. The reality is that there are not enough resources to cater for all the public health issues in Africa. Ideally, a low-cost, single dose vaccine that does not require cold chain storage would be best suited for the continent. However, there are pros and cons to each vaccine platform which need to be weighed to inform the decision-making process.

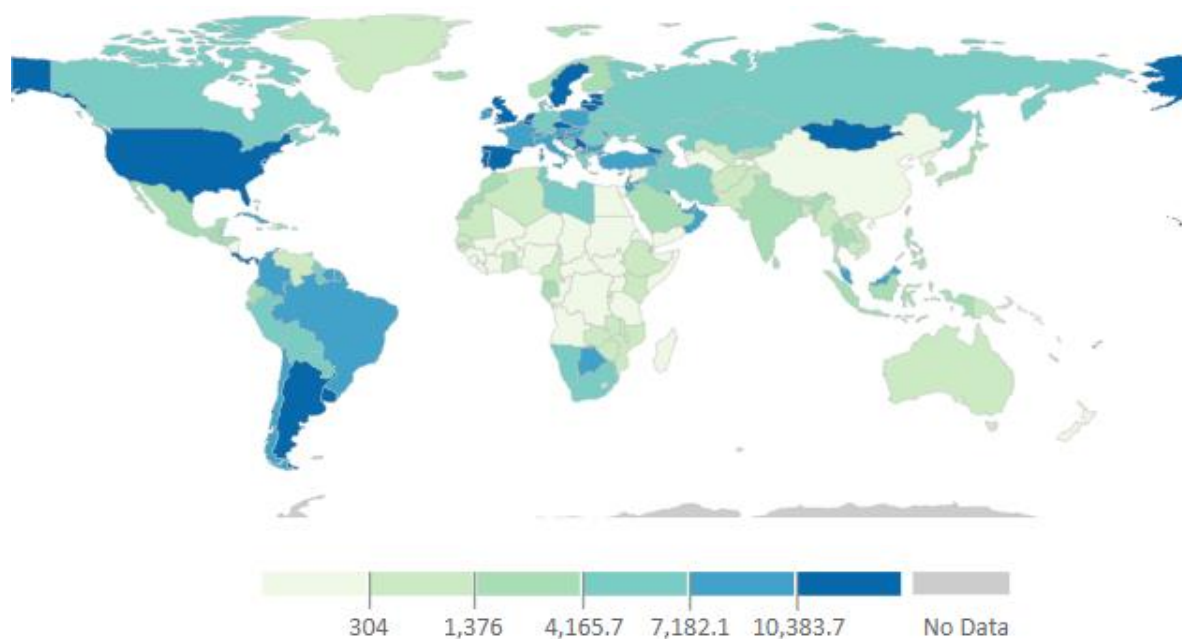


Figure 1.1: Global cumulative cases of COVID-19 reported per 100,000 population. Figure taken from Centers for Disease Control and Prevention, November 2021.

1.2 Coronavirus diversity and impact on human health

Seven coronaviruses infect humans. Of these, four are species of zoonotic coronavirus (human coronaviruses also known as HCoVs) namely 229E, OC43, NL63, and HKU1. They were not thought to be dangerous to humans until more pathogenic coronaviruses, known as highly

pathogenic coronaviruses (HPCoVs) were identified [33]. HPCoVs are also zoonotic and have caused more severe diseases in humans; namely Severe Acute Respiratory Syndrome (SARS), Middle East Respiratory Syndrome (MERS), and the novel Coronavirus disease in 2019 (COVID-19) [34].

The primary reservoir for all these zoonotic coronaviruses has been identified as bats, although other animals are suspected to be involved as intermediary hosts such as civets for SARS and camels for MERS [35]. With the exception of HKU1 and OC43 which likely originated in rodents, most human coronaviruses are believed to have originated in bats [35] and have an intermediary domestic animal host which could have played a role in transmitting the virus from the original primary host to humans. Intermediate hosts have previously ranged from dromedary camels for MERS-CoV [36], to livestock for OC43 [37], whilst for some coronaviruses, intermediate hosts are unknown [33].

Despite having RNA polymerase proof-reading ability, much diversity has been observed in SARS-CoV-2. This diversity has led to the generation of new strains and diverse genotypes of different species which are able to easily adapt to new hosts and ecological niches. The infection of a wide variety of animals with different coronaviruses has allowed us to better understand this family. Numerous studies have identified bats and birds as the species with an astonishingly high diversity of coronaviruses which may be because of their diversity, ability to fly, environmental pressures, and habits of roosting and flocking. As the number of coronaviruses increases over time, more and more closely related coronaviruses with origins in distantly related animals have emerged, and this could be due to the viruses' ability to cross host species barriers [38].

The impact of coronaviruses in humans is multifaceted. Typically, the upper respiratory tract and gastrointestinal tract are affected resulting in symptoms ranging from a common cold to severe pneumonia and bronchitis [39]. In animals, coronaviruses generally cause respiratory and gastrointestinal diseases, except for infectious bronchitis virus (IBV) which caused avian infectious bronchitis in chickens, mouse hepatitis virus (MHV) which causes progressive demyelinating encephalitis in mice and canine respiratory coronavirus (CRCoV) which caused respiratory disease in dogs [40].

The main difference between endemic human coronaviruses and the highly pathogenic human coronaviruses is the extent and severity to which they impact human health. Coronaviruses are transmitted by respiratory aerosols to produce mild upper respiratory infections and typically cause a common cold in humans. More specifically, the four HCoV cause mild disease in humans, whilst the highly pathogenic human coronaviruses can cause severe disease, and ultimately death.

1.2.1 Endemic human coronaviruses

All four endemic human coronaviruses have been reported to cause severe lower respiratory tract infections in elderly adults and young children with underlying or preexisting comorbidities. HCoV-229E and HCoV-OC43 were first identified over 50 years ago and shown to mainly be responsible for common colds. HCoV-NL63 and HCoV-HKU1 were more recently identified and reported to cause mild respiratory tract infection [41]. Zhang *et al.*, observed the epidemiological characteristics of humans with respiratory infection symptoms over a period of five years by taking nasal and throat swabs from adults and children presenting with respiratory infections and fever [41]. Tests for HCoVs revealed evidence that 229E (16.67%), OC43 (60.20%), NL63 (14.97%) and HKU1 (7.82%) were generally prevalent in the Guangzhou population during 2010-2015, although prevalence was higher in winter. Children under the age of 3 and adults above 50 years old were shown to be high risk, whilst 34.69% of the test group was already infected with common respiratory viruses such as influenza. Consistent with this, it has been shown that most humans are likely to be infected for the first time with at least one of the four HCoVs in early childhood years increasing and plateauing at 6 years of age [42]. Human coronavirus infection presents as a wide spectrum of respiratory symptoms such as pneumonia and bronchiolitis [43].

1.2.2 Highly pathogenic human coronaviruses

Knowledge of previous highly pathogenic human coronaviruses has allowed for the easier, quicker, and better understanding of the novel SARS-CoV-2 virus.

The first case of severe acute respiratory syndrome (SARS) was reported in Fushan, China in November 2002 [44]. By February 2003, over 300 cases were reported and eventually by the time the WHO declared the pandemic over in July of 2003 [45], 774 deaths in over 27 countries had been reported [44]. This virus had a case fatality rate of 15% [46]. A new coronavirus, SARS-CoV, that was phylogenetically different from previously known coronaviruses was identified as

the etiological agent causing SARS [47]. With a genetic structure typical of coronaviruses, the virus had spike, envelope, membrane, and nucleocapsid genes [48]. Patients infected with this highly infectious virus typically presented with a fever, followed by a cough, and eventually suffered respiratory distress in severe cases [49].

The start of the second human life-threatening coronavirus, was marked by the death of a Saudi Arabian man in June 2012, from acute pneumonia and renal failure. These symptoms had a similarity to the SARS outbreak, were quickly identified in another individual two days later, and were subsequently identified as the Middle East respiratory syndrome (MERS) [50], [51]. By September 2012, cases were reported in the United Kingdom, and eventually the virus spread globally. In April 2013, MERS-CoV had infected 1728 people, resulting in 624 deaths in 27 countries MERS-CoV, the etiological agent responsible for MERS, is a 26-33 kb [52] relative of bat coronaviruses, HKU4 and HKU5 [51]. Genomic sequencing showed that although belonging to the same Betacoronavirus family as SARS-CoV, this virus came from a different lineage, C and used the dipeptidyl peptidase 4 (DPP4) receptor as opposed to the angiotensin-converting enzyme 2 (ACE2) receptor to enter human cells [53]. MERS patients typically presented with acute respiratory syndrome and kidney failure [50] and in extreme cases this disease led to death. MERS had a high case fatality rate of 32.7% [54].

The latest coronavirus to be transmitted from animals to humans is the SARS-CoV-2 virus, named so because of the 79% genetic similarity to SARS first discovered in 2002 [55]. Genetic sequencing analysis of the virus showed it to be in the same Betacoronavirus lineage as SARS-CoV, lineage B [56], [57]. This virus has been proven to be less deadly as compared to MERS with a case fatality ratio ranging from 28.9% in Yemen to 1% in countries like Singapore and Qatar, [58] but far more transmissible.

1.3 Diagnosis of SARS-CoV-2 infection

The clinical characteristics of COVID-19 are cough, fever, fatigue, and a wide array of symptoms that have evolved as the virus mutates into various variants – these include vomiting, abdominal pains, loss of smell and taste, and more recently, with the Delta variant, congestion [2]. Omicron however, presented with milder symptoms. It was largely speculated that this could be due to the pre-existing immunity in the population based on previously circulating variants [59]. Some patients have reported neurological symptoms such as headaches, myalgia and impaired

consciousness [60]. These neurological symptoms serve as further proof that coronaviruses infect not only the respiratory tract but the brain too [61]. Clinical characteristics are a primary diagnostic method for infection with SARS-CoV-2 and are used as a screening tool to determine a necessity for testing.

The call for rapid diagnosis of SARS-CoV-2 arose due to the urgent need to contain a highly transmissible virus. Isolation, whereby a positive and symptomatic case is separated from people for a period of 7-10 days until symptoms subside, is one way that has been employed to try and contain the spread. A second method has been quarantining, where a potentially positive person is separated from people as a safety measure in case symptoms do develop. However, since a significant portion of positive COVID-19 cases are asymptomatic, this separation of contagious people has been challenging. Furthermore, pre-symptomatic cases are likely to be highly infectious as viral loads are high before the onset of symptoms [62]. The efforts to contain this virus, have had to be strategic and widespread. Various methods of diagnosis have had to be employed to diagnose early viral infection such as PCR and rapid antigen tests. Countries have had to decide on the most efficient way of testing based on test sensitivity and costs associated with each diagnostic method. At the onset of the pandemic, wealthy countries traced suspected positive cases by widespread testing. In low-income countries this was difficult to implement so basic contact tracing was employed. As the pandemic progressed this became challenging for low-income countries such as Ethiopia, where the diagnostic laboratories encountered backlogs and great difficulty in the turnaround time for results, thus making measures such as quarantine and self-isolation impossible to implement [63]. In South Africa, for example, suspected cases are currently only tested if there has been evidence of close contact with a positive case or the onset of symptoms consistent with COVID-19. At the University of Cape Town, the notion of “close contact” is further defined to be within 1.5 m distance for a period longer than 15 minutes in an enclosed area. Wearing of personal protective equipment is also considered [64]. Self-isolation is mandated for symptomatic disease or confirmed infection in the absence of symptoms.

For most countries, upon presentation with the typical symptoms associated with the virus and a history of travel or exposure to a positive case [65], respiratory samples are collected either in the form of a swab from the nasopharyngeal passage, sputum, or the passage of a bronchoscope through the mouth and nose. The sample is used for either molecular diagnosis, RT-PCR which is

currently the most reliable way to test for infection with SARS-CoV-2 [66] or rapid diagnosis. In rapid diagnosis a rapid antigen detection test is used, although the latter has been shown to be less reliable and thus used as a secondary test to the PCR by reverse transcriptase [67]. Evaluation of the rapid antigen detection test in hospitalized children revealed that this method detected the nucleocapsid in about 80% of PCR positive individuals and was most useful during the early days of disease [68]. In the United Kingdom, rapid antigen tests were used to control an outbreak [69]. This mass testing strategy capitalized on the high viral load in pre-symptomatic individuals with the aim of detecting infection with SARS-CoV-2 in its early stages before one could transmit it. This also targeted asymptomatic cases. Other countries such as Slovakia have also quelled infections by 60% by rolling out rapid antigen tests for self-testing [70]. Chest imaging is also used to identify patterns of abnormality such as plaques and hazy gray areas [71], [65]. Similarly, to the rapid antigen test, this diagnostic method is employed in addition to PCR. An additional diagnostic measure used is that for unvaccinated individuals, presentation with a positive serology test could confirm that one has previously had COVID-19.

More recently, several machine learning and artificial intelligence innovations are being developed to diagnose COVID-19 [72], [73] using computational methods leveraging off existing patient datasets. This technology would greatly fast track the diagnosis, subsequently preventing the spread and effects of SARS-CoV-2.

1.4 Impact of the SARS-CoV-2 pandemic on Sub-Saharan Africa

The repercussions of the rapid spread of the SARS-CoV-2 virus led to social and economic meltdowns throughout the world. At the time of compiling this review, the virus had resulted in nationwide lockdowns in most countries on the globe [74], with disadvantaged regions such as Sub-Saharan Africa experiencing an increased and intensified burden on healthcare, amongst other vital resources. The implementation of non-pharmaceutical interventions such as lockdowns, wearing of masks, hand washing, and social distancing was necessary due to the exposure of a naïve population to the novel SARS-CoV-2 virus. These were all in an effort to suppress virus transmission and to prevent the hospitals from reaching capacity. Although necessary, these interventions were devastatingly costly and challenging in Sub-Saharan Africa. The reality is that

many homes have no access to running water for hand washing, a large percentage of the population dwells in shanty towns and overcrowded townships make social distancing difficult. Furthermore, the shutting down of many small businesses has made access to funds for purchasing of masks, or vitamins and healthy food merely impossible for many [75]. Economically, an estimated 31.8 million people were thrown into food deprivation because of a mere 8 weeklong lockdown, a figure which was projected to increase to 77 million people after several continuous lockdowns [76]. With already dwindling social protection programs available, governments were forced to implement cash transfer schemes which could only help a small portion of the Sub-Saharan population.

The virus impacted countries differently and influenced the mortality rate in each country differently. Previous research has placed Africa as the continent with the most vulnerable population to die from infectious diseases, a factor which predicted extremely high COVID-19 infections and death at the start of the pandemic [77], [78]. Surprisingly, as the pandemic progressed, Africa, in comparison to other continents, has been found to have lower infection and mortality rates [79]. These differences could be attributed to reporting inaccuracy caused by inefficient contact tracing, low testing rates, and therefore low cases reported. However, the presence of sunlight and high Vitamin D absorption due to the warm African weather has also been cited as a possible explanation if the reduced infection rate is real [80], [81]. Africa's more youthful population has also been suggested as a possible reason for lower cases [81] – as increased age has been reported as a risk factor. Additionally, differences in genetic predisposition have also been investigated as a risk factor. Inheritance of a Neanderthal haplotype was shown to contribute to increased risk of severe COVID-19 [82]. This was mapped globally and reported to be prevalent in Europe and South Asia, but almost completely undetected in Africa. Risk factors differ amongst countries, and this has a considerable effect on the impact of COVID-19 on populations.

Similarly to the rest of the world, age, presence of comorbidities, and sex have all been shown to increase susceptibility to severe disease and a negative outcome in Africa [83]. Although a young continent, a cohort study in South Africa confirmed that increasing age correlated with a higher risk of death after COVID-19 hospitalization and was the strongest predictor for death [84]. These findings were further supported by a similar study in the Western Cape province of South Africa which sought to investigate the role of HIV and Tuberculosis, as well as age, sex, and

comorbidities [85]. Male sex was also found to be a risk factor, as well as comorbidities such as hypertension, diabetes, chronic cardiac, renal, and pulmonary diseases [84], [85].

Unique to the African continent are risk factors such as high population density, extreme poverty, and scarcity of hospital resources and personnel [86], [87]. An additional risk factor that is unique to the African continent is the high prevalence of diseases such as HIV, Tuberculosis, and malaria, amongst many others. In Sub-Saharan Africa, the region that is host to the highest HIV prevalence rate globally [88], there was high concern on how coinfection would impact the continent. HIV suppresses the immune system resulting in immunocompromised individuals; therefore, it is expected that people living with HIV (PLWH) were at high risk for contracting SARS-CoV-2. Tesoriero *et al.*, conducted a cohort study in New York which compared the COVID-19 diagnosis rate and clinical outcomes of PLWH with an undiagnosed group. Results from this study showed no significant difference in the diagnosis rate but large difference in clinical outcomes in PLWH – with more severe cases and deaths seen in this group [89]. Supporting studies highlighted that a low CD4 count and lack of antiretroviral treatment in PLWH and individuals with tuberculosis is associated with a greater risk for severe disease and mortality upon infection with SARS-CoV-2 [90], [85].

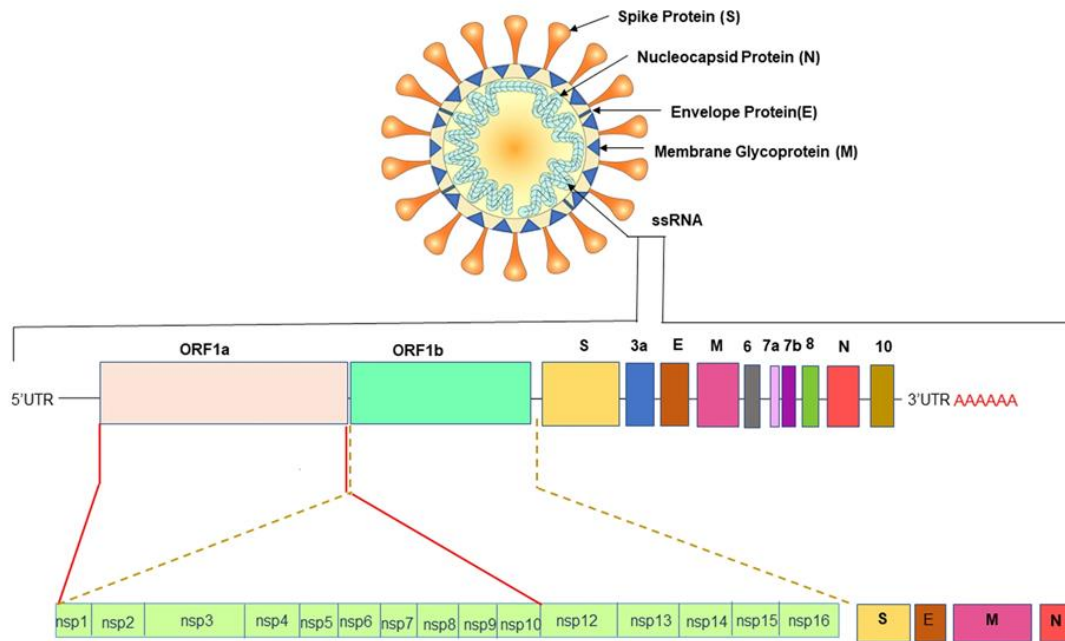
Another study investigated the impact of COVID-19 on HIV, Tuberculosis and malaria in low-income countries. It was simulated that the strain and pressure on the healthcare systems could contribute to increased loss of life. This manifested as a lack of access to antiretroviral treatment in HIV. It was further described that the delayed diagnosis due to the similarity of symptoms between COVID-19 and Tuberculosis were an additional strain. Finally, the interruption in net protection implementation systems in malaria due to diversion of attention towards the COVID-19 pandemic were simulated to be a contributing factor [91].

1.5 SARS-CoV-2 virus structure and genetic organization

Coronaviruses, named for the spike-like glycoprotein crown on their surface [92] which the viruses use to attach to human cells, manifest differently. Some can cause pathogenesis in animals and humans in the lungs, the central nervous system and the liver [93] whilst others do not cause severe disease. First identified in 1968 [94], coronaviruses are the largest known RNA viruses with genomes spanning 27 to 33 kb [92]. These viruses are single stranded, enveloped and have a large

genome consisting of open reading frames (ORFs) on the 5' end encoding for replication proteins, whilst the 3' end consists of genes encoding the structural proteins – spike protein (S), membrane (M), envelope (E) and the nucleocapsid (N) [95] (Figure 1.2). The spike glycoprotein has been identified as the protein that exhibits the most diversity amongst coronaviruses, particularly the S1 subunit of the protein. The spike is the determinant of the virus' broad host range as it initiates infection by interacting with the host receptor [96].

I.



II.

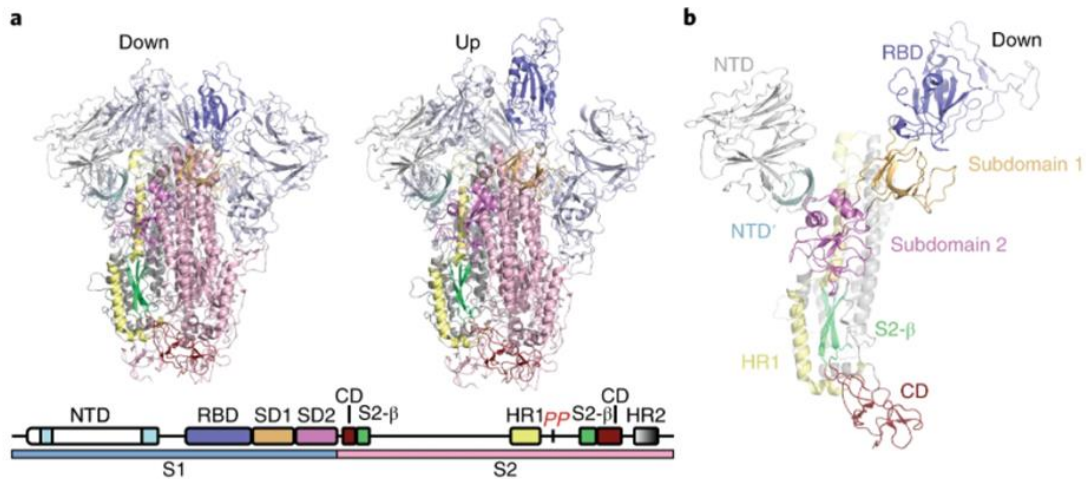


Figure 1.2: Schematic of SARS-CoV-2 genomic structure. I) The genome is approximately 29 903 nucleotides long. Structural proteins are shown; M (Membrane protein), N (Nucleocapsid protein), S (Spike protein) and E (Envelope protein). The RNA is also shown (ssRNA). Open Reading Frames ORF1a and ORF1b encode non-structural proteins (nsp). Figure taken from Rastogi, M., Pandey, N., Shukla, A. *et al*, *Nature*, 2020 [97] II) a) The spike glycoprotein in the “down” and “up” conformations showing the exposed receptor binding domain; b) Domains of the spike glycoprotein; NTD = N-terminal domain, RBD = Receptor binding domain, HR1 = Heptad repeat region

1, CD = Connector domain, S2- β = S2 subunit β -sheet motif. Figure taken from Henderson *et al.*, Nature Structural & Molecular Biology, 2020 [98]

SARS-CoV-2, like all coronaviruses has a spike glycoprotein (S) which it uses to infect the host cell through a complex process that involves receptor binding and proteolytic cleavage to facilitate fusion of the viral and host cell membranes [96]. It is therefore of paramount importance to understand the structure of the S protein for vaccine development. The S protein, synthesized as a precursor of ~1,300 amino acids, is cleaved into two subunits, S1 and S2, which are responsible for receptor recognition and membrane fusion. It becomes a trimer upon folding and forms a crown on the viral surface [96]. S1, the part of the S protein that is the most diverse amongst coronaviruses and is responsible for receptor recognition, is divided further into the C and N terminal domains (CTD and NTD). The receptor binding domain (RBD) is where the SARS-CoV and SARS-CoV-2 viruses interact with the human angiotensin-converting enzyme [99]. The spike glycoprotein is a trimeric class 1 fusion protein, like that of the influenza, HIV and Ebola viruses [100].

To trigger viral fusion, the S1 subunit binds to a host cell receptor, thus kickstarting the subsequent structural rearrangements which result in fusion of the viral membrane to the host cell membrane. The process is in specific molecular steps; firstly, S1 recognizes and binds to ACE2, the host cell receptor, thus destabilizing the prefusion trimer. This leads to cleavage of the RBD by the protein furin [101] and allows the S2 subunit to transform into a stable post fusion state. When the RBD undergoes conformational changes this either exposes or hides the receptor binding determinants. These two states of receptors are called “down” and “up” states where “down” refers to the inaccessibility of a receptor and the “up” conformation refers to the receptor accessible state [102]. The extensive glycosylation of the spike is also fundamental in facilitating folding and stabilization of the protein [103].

The indispensable function of the spike glycoprotein makes it a target for antibody mediated neutralization of the virus [100] and understanding the structure of the SARS-CoV-2 S protein presents opportunities for vaccine development. Furthermore, the SARS-CoV-2 spike glycoprotein structure is metastable which requires careful consideration when designing a vaccine [104].

A crucial role is played by the interaction of the SARS-CoV-2 S protein with the host cell angiotensin-converting enzyme 2 (ACE2) receptor as this serves as the entry point of SARS-CoV-2 into a cell. Overall, the structures of SARS-CoV and SARS-CoV-2 are very similar. However, Wrapp *et al.*, used surface plasma resonance to quantify and compare the binding affinity of the two coronaviruses to ACE2 [104]. Results showed that the binding affinity of SARS-CoV-2 is ~10- to 20-fold higher than that of SARS-CoV, which may explain the ease of human-to-human transmission that has been observed in SARS-CoV-2 thus far.

It has been shown that the C-terminal domain (CTD) of SARS-CoV-2 forms more atomic interactions with the human ACE2, which is in correlation with data that shows higher receptor binding in the novel virus [99]. In a study which revealed the S1 CTD of SARS-CoV-2 as the key binding area for ACE2, it was shown that monoclonal antibodies and murine polyclonal antisera against SARS-S1/RBD were unable to bind SARS-CoV-2 [35], [99], [102] which suggests that pre-existing immunity to SARS-CoV does not offer protection against SARS-CoV-2.

There are several regions of the SARS-CoV-2 spike that have been identified as regions of vulnerability to antibodies which are ideal for therapeutics. By isolating and profiling monoclonal antibodies from B-cells of individuals infected in the early days of the pandemic, it was revealed that the receptor binding domain is the major region of vulnerability [105]. The N-terminal domain of the spike protein has also been found to be highly recognized by antibodies, although this was secondary to the RBD [106].

As the globe sees several waves of high prevalence of infections and deaths driven by different variants of the virus, vaccine development continues to be an ongoing feat.

1.6 SARS-CoV-2 variants of concern and their impact on vaccines

Mutations of the SARS-CoV-2 virus can have a crucial effect on viral transmissibility, natural immunity and subsequently vaccine efficacy [107], [108], [109]. RNA viruses commonly mutate during a process of error-prone replication, which results in genetic changes that potentially enhance the virus' fitness [110]. Coronaviruses however, have RNA proofreading functionality which prevents them from mutating at a rate as fast as other RNA viruses. Recombination events

between different already circulating variants pose an additional challenge, as this could possibly result in a virus that has enhanced transmissibility and pathogenicity [111].

There are three types of variants: variants of interest, variants of concern and variants of high consequence [112]. Variants of concern are classified as such when there is evidence of high transmissibility, threat to evasion of neutralizing antibodies, and evidence of causing an increase in disease severity. These types of variants additionally have decreased response to public health measures such as vaccines, diagnostics, and other therapeutics. According to the World Health Organization, global genomic surveillance efforts have identified five variants of concern namely the Alpha (B.1.1.7), Beta (B.1.351), Gamma (P.1) and Delta (B.1.617.2) which were first identified and documented in the United Kingdom (September 2020), South Africa (May 2020), Brazil (November 2020) and India (October 2020) respectively [113]. As of late November 2021, a new variant, Omicron (B.1.1.529) was first reported by South African scientists [114].

The Alpha variant is characterized by three major mutations: a change in the receptor binding domain of N501Y [115], which increases the virus' binding affinity to the ACE2 receptor thus increasing transmission [116]; two amino acid deletions in the spike at positions 69 and 70 which result in decreased detection in diagnostics [115], and increased infectivity as compared to wildtype spike [117], [118]; and a point mutation P681R in the spike S1/S2 cleavage site which increases proteolytic processing of the spike [119].

An identical N501Y mutation is shared by the Beta variant, first described in South Africa [120], in addition to several other mutations which together contributed to the variant evading immunity [121] and having approximately 50% increased transmissibility. Driving a second and larger wave of infections in South Africa, the Beta variant was shown to have key mutations in the receptor binding domain (K417N, E484K and the previously mentioned N501Y) which provided a challenge to the safety and efficacy of vaccines as demonstrated in a study where participants were given the FDA approved ChAdOx1 nCoV19 vaccine in a two dose regimen which did not show any protection against mild to moderate COVID-19 because of the Beta variant [122].

The Gamma variant that drove an increase in infections, hospital admissions and deaths in Brazil was found to carry the same key mutations as the Beta variant, hence the increased binding affinity and subsequent transmissibility of the virus strain [123]. Wang *et al.*, investigated the neutralizing antibody response to the Gamma variant whereby it was shown that the variant is resistant to

monoclonal antibody therapy and to a greater degree more resistant to convalescent plasma and vaccine sera [124].

Owing to a second wave of infections in India, enhanced sequencing efforts revealed a new circulating variant of concern, named Delta. This variant has three subtypes, B1.617.1, B1.617.2 and B1.617.3, and has receptor binding domain mutations L452R, T478K, E484Q, D614G and P681R. The first three mutations are responsible for greater binding affinity to the ACE2 receptor, whilst the P681R mutation is at the furin cleavage site and is responsible for improved transmissibility [125]. Further investigations into the variant revealed that it is six-fold less sensitive to antibodies from recovered individuals, and eight-fold less sensitive to vaccine sera [126], [127]. The same study showed that the Delta variant had a replication advantage as compared to the Alpha variant, which explains the exponential increase in cases and spread to at least 90 countries. A threat to vaccine effectiveness caused by the Delta variant was shown through assessment of vaccine breakthrough infections in healthcare workers after vaccination with ChaAdOx1 [126]. The same vaccine was tested against the Alpha variant and found to have decreased efficacy as evidenced by the low titres of neutralizing antibodies [128]. BNT162b2, a mRNA vaccine, also demonstrated lower effectiveness against the Alpha and Beta variants with the percentage effectiveness dropping from 95% to 85.9% and 70% against the Alpha and Beta variants respectively [129].

A doubling of positive cases in the Gauteng province of South Africa prompted increased genomic surveillance which resulted in the identification of the Omicron variant [130]. The variant was shown to have about 30 mutations in the key receptor binding domain region of the spike protein, which has implications for increased transmissibility, immune evasion, and difference in clinical outcomes. The investigation on all of these is ongoing, as more Omicron cases are being identified globally. In the midst of a fairly vaccinated world, the most pressing concern is that of immune evasion. The emergence of SARS-CoV-2 variants continues to present a challenge to attaining population immunity and warrants global efforts towards production of vaccines that offer immune protection against a continuously mutating virus.

1.7 Potential correlates of protective immunity against COVID-19

The identification of indicators of protective immunity against the SARS-CoV-2 virus is an important factor for vaccine development. When the virus infects and deposits RNA within the host cell, the innate immune response is activated. B cells produce binding and neutralizing antibodies whilst T cells are triggered to kill virus infected cells. There are several factors that need to be considered when identifying correlates of protection. One of these is that, although the majority of infected individuals will produce antibodies, in some cases, there is no seroconversion [131]. Additionally, the immune response has been shown to wane over time [132]. Cellular responses are also not fully understood. There is a possibility of cross-reactive T cell responses to common cold causing coronaviruses which might work advantageously to clearing SARS-CoV-2 [133]. Evaluation of peptides has also revealed that T cell responses are broad and span across several SARS-CoV-2 proteins such as the spike, the membrane and the nucleocapsid [133]. To understand correlates of protection, a distinction must be made between the roles of neutralizing antibodies and T cells. The latter prevents disease and severe disease whilst the former prevents infection. Clues on the likelihood of neutralizing antibodies as immune correlates of protection against infection were given by the identification of high titres of neutralizing antibodies in individuals who had recovered from COVID-19 [134].

Neutralizing antibodies have been shown to be the likely key correlate of protection in infection, in several studies. The use of neutralizing antibody serum in both rhesus macaques and human subjects has proven to offer a degree of protection against severe SARS-CoV-2 [135], [136], [137]. This information provided a clue towards the role of neutralizing antibodies as a correlate of protection. Khoury *et al.*, confirmed neutralizing antibodies as a correlate of protection, and further used a model to calculate the protective neutralization titre of seven emergency approved vaccines [134]. In support of these findings, evidence is provided by Earle *et al.*, towards antibodies as correlates of protective immunity. This study identified a robust correlation of high neutralizing and binding antibody titres with high vaccine efficacy of seven vaccines in Phase III trials [138]. Both studies investigated vaccines developed with different platforms such as nucleic acids, adenoviruses, protein subunits and inactivated viruses.

T-cells have also been identified as possible correlates of protective immunity against SARS-CoV-2. Assessment of the adaptive arm of immunity in humans showed that CD4⁺ cell responses to the

spike glycoprotein were robust, with the membrane, nucleocapsid and spike accounting for up to 27% of the CD4⁺ response. CD8⁺ T cells showed recognition for the M and S proteins. It was additionally found that there was a high percentage (40 – 60%) of CD4⁺ and CD8⁺ T cells in SARS-CoV-2 immunologically naïve individuals, which suggested cross protection from endemic “common cold” causing coronaviruses [133]. The role of T cells as correlates of protection was further supported by a study where convalescent rhesus macaques with depleted CD8⁺ T cells were challenged with SARS-CoV-2, and had lower protection as compared to the non-depleted group [136]. Knowledge of neutralizing antibodies and T cells as indicators of protective immunity is important to consider in the development of future vaccines, especially as the virus continues to evolve.

1.8 Vaccine platforms for immunization against COVID-19

The magnitude of the COVID-19 pandemic has called for the rapid development of over 100 vaccine candidates which have advanced into clinical testing [139]. The ancestral SARS-CoV-2 virus has an average reproductive number of 2.44 [140], the Delta variant of the virus has a higher reproductive number of 5.08 [141] whilst the most recently identified Omicron variant has been estimated to have a reproductive number that is 4.2 times that of the Delta variant [142]. This unprecedented transmissibility has resulted in ongoing waves of infections [108] which make the need for effective vaccines more urgent. The speed at which vaccine candidates have been developed against SARS-CoV-2 has been unprecedented, driven by the global spread of the virus, accompanying economic and social impact. Various technologies have been explored with several vaccines gaining FDA emergency use approval (Table 1.1) and some being licensed.

Development of vaccines is a sequential process from initial development in the laboratory, through to licensure and approval for use in mass immunizations (Figure 1.3) [143], [144]. In the preclinical laboratory step, antigens are identified and selected for use in developing a vaccine using a platform for delivery. Having developed the vaccine, animal studies are conducted before those data can be used to gain approval from a regulatory body such as the FDA. Phase I trials follow, where a small group of healthy humans is immunized and assessed for response. The appropriate dose and safety are also assessed. From Phase I to III, the number of humans increases from hundreds to thousands whilst safety is continuously monitored. In the Phase III trials, the

vaccine is evaluated for efficacy, and eventually reviewed, approved and manufactured at a large scale. The monitoring of the safety and efficacy continues post manufacturing during widespread vaccination, by recording side effects in immunized populations. In the instance of a global pandemic, this entire process can be shortened, as was the case with the COVID-19 pandemic. Phases I and II can overlap, and the vaccine can be approved for emergency use before full licensing.

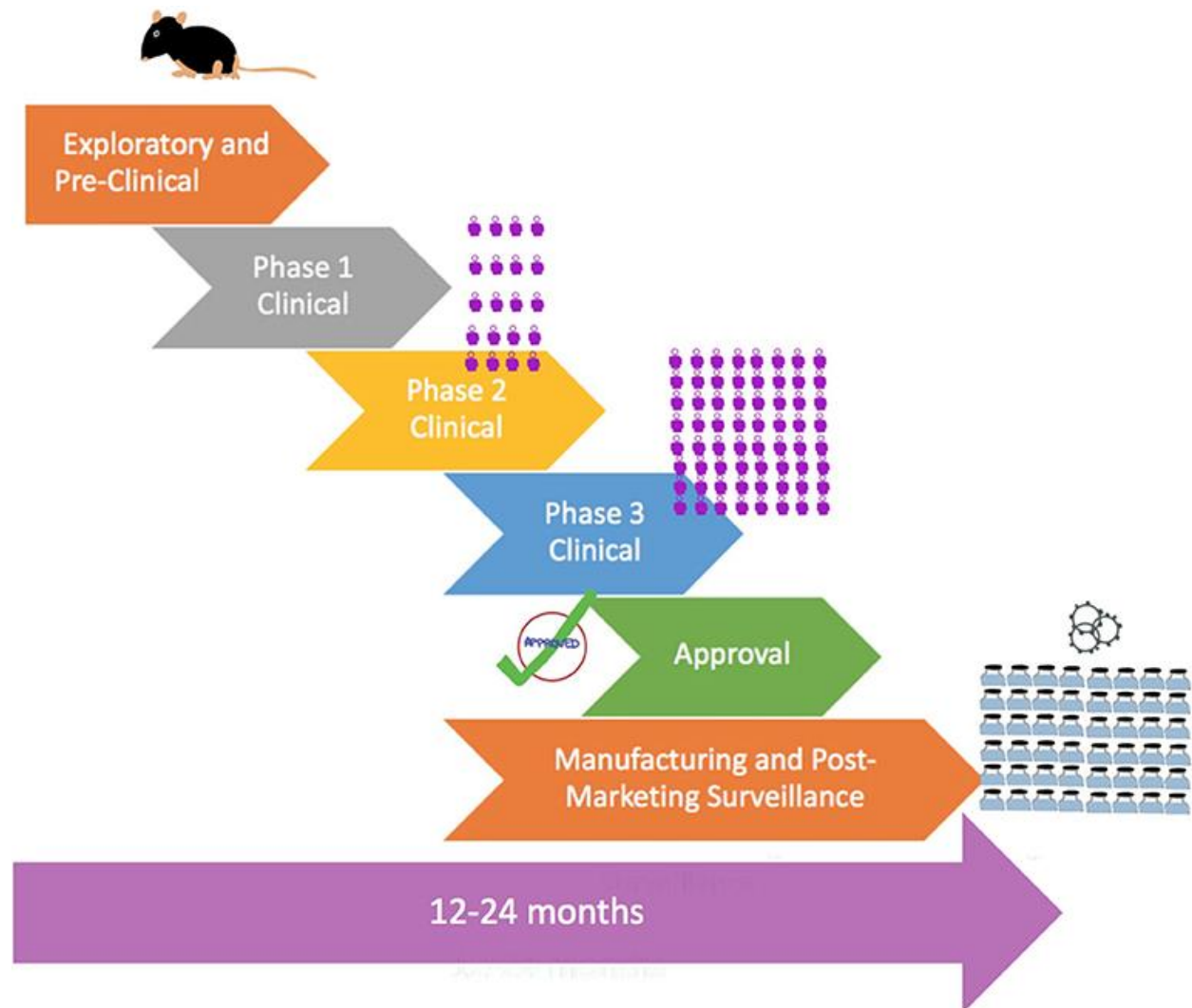


Figure 1.3: Stages and timeline of COVID-19 vaccines development. Vaccines are first developed in a pre-clinical laboratory setting, before moving on to clinical trials (Phase 1 – Phase 3) where they are tested in increasing numbers of humans and eventually approved for manufacturing. Post-marketing surveillance continues once the vaccine has been used for widespread vaccination. In a pandemic the steps of the flowchart can overlap to fast track the process. Figure taken from *Sharma et al.*, *Frontiers in Immunology*, 2020 [143]

As of October 2021, only 38.6% of the world’s population has been fully vaccinated, 49.7% has had at least one dose, and a total of 7 billion doses have been administered [145]. According to the Africa CDC, a meagre 5.58% of the African population had been fully vaccinated in October 2021 [146]. In January 2022, 10.99% of the population has been fully vaccinated [146]. There is a huge disparity in vaccinations efforts between countries globally (Figure 1.4), evidenced by the creation of coalitions such as COVAX which are tasked with ensuring the equitable distribution of vaccines across the globe. Low-income countries such as those in Africa received vaccine doses after high-income countries had fully vaccinated a considerable percentage of their populations. The COVAX initiative undertook to provide vaccinations to 42 African countries, however, this has not been as successful as anticipated [147]. Amidst the emergence of new variants threatening vaccine efficacy, vaccine hesitancy, and the limitations in global manufacturing capacity, Africa continues to struggle with returning to “normalcy”. Collectively, the world continues to wade through several waves of high infections and periods of lockdowns.

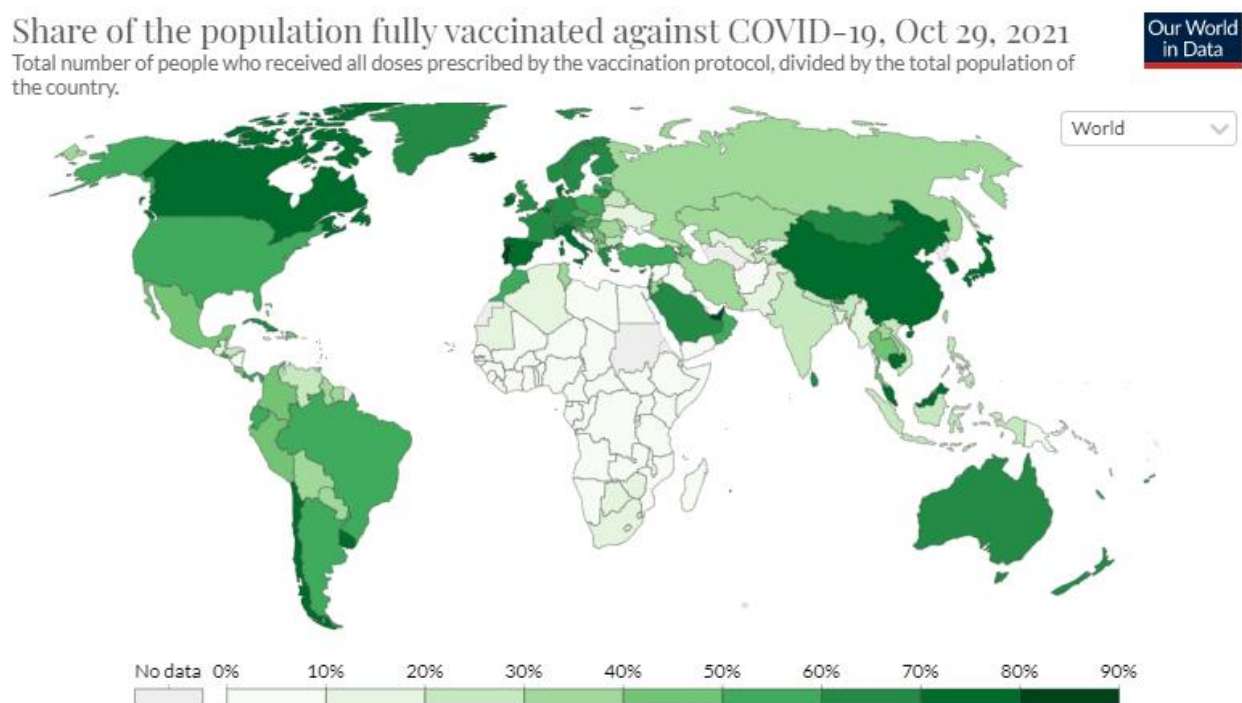


Figure 1.4: Share of the population fully vaccinated against COVID-19 as of 29 October 2021. The number of people who have received all doses was divided by the total population of the country. Figure taken from www.ourworldindata.org.

There are many different platforms that can be used to develop vaccines. These differ based on their development mechanisms, as well as their advantages and disadvantages. Vaccine platforms that have been developed include the contemporary whole vaccines consisting of live attenuated and inactivated vaccines. Subunit vaccines and viral vectored platforms have also been used extensively. The next-generation nucleic acid vaccines which include DNA and RNA vaccines, are additional platforms that can be used. In terms of vaccine platforms, this pandemic is revolutionary with the first licensing of a mRNA vaccine, which gives evidence of the many years that vaccines have been developed, and thus could be explored and exploited to develop an effective vaccine against COVID-19. As of December 2021, two years after the first cases were reported, there was one FDA approved vaccine, (Pfizer BioNTech's COMINARTY), three FDA authorized vaccines (Pfizer BioNTech, Moderna and Janssen) [148] and more approved for emergency use (Table 1). Several vaccine candidates based on these technologies are discussed in this review.

1.8.1 Live-attenuated vaccines

A contemporary and well-established vaccine platform, live attenuated vaccines are a form of vaccines that uses a weakened version of a slowly replicating live virus, and therefore does not cause disease. With the ability to induce a strong immune response with activation of both B and T cells, live attenuated vaccines have been used to develop some COVID-19 vaccines. The scalability of live attenuated vaccines is advantageous, particularly in a pandemic. Codagenix Inc developed COVI-VAC, a live attenuated vaccine made by recoding a segment of the SARS-CoV-2 spike glycoprotein to introduce 283 mutations and deleting the furin cleavage site. This vaccine is in Phase I of clinical trials, and is the only live-attenuated vaccine to be at this stage of development [149]. It showed production of high titres of neutralization antibodies in inoculated hamsters. Syrian golden hamsters inoculated intranasally with a single dose of COVI-VAC exhibited higher spike antibody titres and milder lung pathology than a group inoculated with wildtype virus. When challenged with wildtype SARS-CoV-2, the hamsters had low lung pathology and viral titres, and undetectable levels in the brains. The ability of live attenuated vaccines to target all components of the host's immune system thus producing broad immune responses was demonstrated by this vaccine candidate [141]. Live attenuated vaccines are also advantageous in the ability to induce strong and lasting immunity from a single dose, as evidenced by sCPD9, a genetically recoded live attenuated vaccine [150].

Table 1: Different vaccines approved against COVID-19. Table taken from The New York Times, 10 November 2021.

Leading vaccines

Developer	How It Works	Phase	Status
 Pfizer-BioNTech	mRNA	2 3	Approved in U.S., other countries. Emergency use in E.U., other countries.
 Moderna	mRNA	3	Approved in Switzerland. Emergency use in U.S., E.U., other countries.
 Gamaleya	Ad26, Ad5	3	Emergency use in Russia, other countries.
 Oxford-AstraZeneca	ChAdOx1	2 3	Approved in Brazil. Emergency use in U.K., E.U., other countries.
 CanSino	Ad5	3	Approved in China. Emergency use in other countries.
 Johnson & Johnson	Ad26	3	Emergency use in U.S., E.U., other countries.
 Vector Institute	Protein	3	Approved in Turkmenistan. Early use in Russia.
 Novavax	Protein	3	
 Sinopharm	Inactivated	3	Approved in China, U.A.E., Bahrain. Emergency use in other countries.
 Sinovac	Inactivated	3	Approved in China. Emergency use in other countries.
 Sinopharm-Wuhan	Inactivated	3	Approved in China. Limited use in U.A.E.
 Bharat Biotech	Inactivated	3	Emergency use in India, other countries.

Live attenuated vaccines are quick to develop and can therefore be mass produced and distributed for use in a rapidly growing pandemic. Although safe, immunogenic, and easy to produce, live attenuated vaccines run the risk of reversion to virulent forms of the virus. This was previously demonstrated in the oral polio virus vaccine which has, in rare cases, caused paralytic poliomyelitis in vaccinated people [151].

Netea and colleagues discovered that the Tuberculosis *Mycobacterium bovis* Bacillus Calmette-Guerin (BCG) BCG vaccine not only triggers memory cells specifically for the *Mycobacterium bovis* pathogen, but also triggers prolonged innate immunity against any infection that the immune system encounters [152]. Therefore, it was suggested as a possible effective vaccine candidate

against COVID-19. “Trained immunity” occurs when monocytes are epigenetically reprogrammed in the long term such that they are able to be responsive to new stimuli which they have not previously encountered [153], [154]. This was further confirmed in controlled randomized trials where vaccinated children in Guinea-Bissau were seen to be up to 50% protected against mortality from respiratory diseases, sepsis and fever after vaccination with BCG at neonatal stage [155], [156]. Effects of the BCG vaccine on respiratory tract infections were additionally investigated in adults, in Indonesia, and this too showed a significant reduction in acute upper respiratory tract infections after vaccination with BCG [157]. Considering the COVID-19 pandemic, several studies were assumed in a bid to exploit this function of BCG, for use against the SARS-CoV-2 virus, a respiratory tract virus. Clinical trials in several countries such as Australia (NCT04327206), and The Netherlands (NCT04328441) are investigating the effect of a BCG vaccination on the absence of healthcare workers from work due to COVID-19, as well as the incidence of COVID-19 as measured by a positive SARS-CoV-2 test, fever or at least one clinical symptom.

Although promising, there are several reasons why the use of BCG as a vaccine against COVID-19 could be a mistake. These include the inability to disprove confounding factors contributing to the reduced mortality after immunization with BCG [158], the global scarcity of the BCG vaccine, and the uncertainty on whether childhood immunity would still hold years later.

1.8.2 Inactivated vaccines

Inactivated vaccines have been approved as vaccines against SARS-CoV-2 (Table 1). Also a well-established platform which is additionally suitable for immunocompromised people, inactivated vaccines have been broadly used. These vaccines are relatively stable and simple to manufacture. The drawbacks of inactivated vaccines are limited immunogenicity and, in most cases, the requirement of an adjuvant to elicit a stronger immune response. It also requires the handling of vast amounts of live virus in development which is done in the expensive and not widely accessible BSL-3 facilities, presents a safety risk, and has limited capacity for large scale production [159].

Sinovac, a Chinese company, developed the Coronavac vaccine by inactivating strains of SARS-CoV-2 with a chemical called β -propiolactone [160]. Treatment with β -propiolactone causes the spike to transition from the preferred prefusion conformation which is a target for neutralizing

antibodies to the post-fusion conformation [140]. BALB/c mice, Wister rats and rhesus macaques were immunized with 3 or 6 µg of vaccine to assess the immunogenicity of the vaccine. Immunoglobulin antibody titres to the spike and receptor binding domains were produced and were higher in the higher dose groups, with no observation of disease after immunization. The neutralizing antibodies produced neutralized ten variants of SARS-CoV-2 indicating the ability of the vaccine to neutralize a broad range of variants of SARS-CoV-2. Efficacy trials in Turkey showed that the vaccine provided 83.5% protection in adults aged 18 – 59 years [161]. A study assessing the effectiveness of two inactivated SARS-CoV-2 vaccines, SARS-CoV-2 WIVO4 and HB02, showed that there was reduced risk of symptomatic COVID-19 disease after inoculation with either of the two vaccines [141]. This provided further support for the ability of this platform to be used for SARS-CoV-2 vaccine design.

1.8.3 Subunit vaccines

Subunit vaccines use one or more antigens, usually with the assistance of adjuvants, to produce immune responses. There are three types of subunit vaccines: protein subunits, polysaccharide subunits, and conjugate subunits. Of these, protein subunits have been used for COVID-19 vaccines. Subunit vaccines are safe and easily tolerated by the host's immune system because they only use a purified portion of the whole virus to trigger an immune response. They are also well-established and suitable for immunocompromised individuals, but it can take some time to find the suitable and best antigen combination. Because these vaccines use only a fragment of the pathogen, they induce a weaker immune response as compared to other vaccine platforms, and thus will usually require an adjuvant to strengthen the response. The ease of production of subunit virus-like particles (VLPs) has resulted in two vaccines being approved for use in the COVID-19 pandemic.

1.8.3.1 Virus-like particles (VLPs)

Virus-like particles (VLPs) are natural or synthetic nanostructures containing key structures of a virus without the genome. Because they do not contain infectious genetic material [162], these sub-unit vaccines are safe for use. VLPs self-assemble based on one or more protein, and are antigenic without causing disease [163]. Their size of 25-100nm and their structure allows them

to induce both humoral and cellular immune responses [164]. They are also versatile and flexible in design, which is ideal particularly when dealing with a rapidly evolving virus such as SARS-CoV-2 where different mutations arise and pose a threat to vaccine efficacy. Additionally, these vaccines are relatively easy to store which makes them accessible to countries with cold-chain challenges. Novavax, a pharmaceutical company, has developed a subunit vaccine (NVX-CoV2373) targeting the full-length SARS-CoV-2 S protein in the stable prefusion conformation [165]. When paired with the saponin-based Matrix-M adjuvant [166] this vaccine was shown to induce high titres of anti S IgG which was shown to neutralize the SARS-CoV-2 virus, blocked binding to the hACE2 receptor, and protected against SARS-CoV-2 challenge, without evidence of disease caused by the vaccine. Furthermore, NVX-CoV2373 was shown to induce T and B cells and had 95.6% efficacy against the original virus.

The receptor binding domain (RBD) was used in a study using the SpyTag/SpyCatcher technology where RBD displaying VLPs were developed [167]. The RBD was fused with the SpyTag peptide and subsequently conjugated to SpyCatcher003-mi3 VLP. This technology allows for high yields and results in a thermostable platform that can be freeze-dried for ease of transportation and distribution. Stability and resilience of the VLPs was tested and confirmed to be suitable for cold-chain logistics. The RBD-SpyVLPs were shown to induce superior neutralizing antibodies to that of convalescent individuals' sera. The neutralizing antibodies induced were additionally able to block the entry of SARS-CoV-2 into mice expressing ACE2 receptors thus confirming the immunogenicity of this candidate vaccine [141].

Medicago have developed a plant-based VLP, CoVLP, based on the stabilized full-length spike glycoprotein ectodomain fused to the transmembrane and cytoplasmic domains from influenza haemagglutinin. The vaccine was produced in *Nicotiana benthamiana* and the chimeric antigen was demonstrated to bud as a VLP [168]. Immunogenicity assessments revealed that when paired with the adjuvants ASO3 or CpG1018, the vaccine induced high titres of neutralizing antibodies and long-lasting T-cell responses. A phase 2/3 study of this vaccine showed that 35% of adults grouped as “healthy”, “older” and “those with comorbidities” seroconverted after the first dose of the vaccine paired with adjuvants, and this figure rose to 98% after a second dose which was administered 21 days later [169].

1.8.4 Nucleic acid-based vaccines

Nucleic acid-based vaccines can either be DNA based or RNA based and use selected coding sequences of a pathogen to induce an immune response against the expressed protein. Nucleic acid-based vaccines have shown promise thus resulting in the first ever emergency approved nucleic acid-based vaccines being used against COVID-19.

1.8.4.1 DNA-based vaccines

DNA-based vaccines are advantageous for comparatively low production cost, stability and the ability to be stored long-term [170] which is vital in a pandemic for distribution and storage purposes. A synthetic DNA vaccine targeting the SARS-CoV-2 spike protein was generated by Inovio Pharmaceuticals [102]. INO-4800 was developed from an already existing vaccine (INO-4700) against MERS-CoV which was in clinical trials, as well as SARS-CoV and Zika virus vaccine candidates. The spike was cloned into an expression vector before being evaluated for expression of RNA and the protein in vitro. This was quick and simple, particularly because of previous vaccine candidates that were leveraged thus allowing for a plug and play process. Immunogenicity assessments showed that the vaccine elicited both cellular and humoral responses as evidenced by the SARS-CoV-2 RBD antigen binding of IgG, neutralization of SARS-CoV-2 strains, blockage of ACE2 receptor binding, and high T-cell responses against the SARS-CoV-2, SARS-CoV and MERS-CoV S proteins. The production of T cell response is particularly important because neutralizing antibodies have been shown to decline rapidly [171]. The major drawback with DNA vaccines is their generally low immunogenicity.

1.8.4.2 RNA-based vaccines

RNA vaccines elicit strong humoral and cellular adaptive immune responses [172]. RNA encoding a specific antigen is used to develop these vaccines, which is quicker and easier than making a VLP, attenuating or inactivating a virus. Two large companies Moderna and Pfizer demonstrated this by having their mRNA-based vaccines ready and approved for emergency use less than a year after the first COVID-19 cases were reported. This is in comparison to a previous record of four years held by a mumps vaccine made in the 1960s [173]. The flexibility in vaccine design is an additional advantage of mRNA vaccines. SARS-CoV-2 messenger RNA (mRNA) vaccines are delivered in lipid-based nanoparticles that do not carry a charge. This allows for easy delivery of the genetic material into the host cell [174]. Once inside the cell the RNA is translated into proteins,

which triggers an immune response. The protein undergoes natural post-translational processing and packaging by the host cell [175]. Although highly advantageous, mRNA vaccines require cold storage which is a requirement that is difficult to meet for developing countries. They also require two dose regimens to elicit a strong and lasting immune response, a factor which may prove challenging in low-income countries where access to vaccines is sparse. The need for two doses presents an additional challenge in production to meet the global demand [176]. This being a newly approved technology for human use has induced fears and hesitancy, resulting in a low vaccination rate.

Moderna and Pfizer-BioNTech companies both produced mRNA vaccines against the SARS-CoV-2 S protein which are currently being administered globally [177], [178]. Pfizer-BioNTech's vaccine has become the first vaccine to be FDA licensed, allowing for a name change to "Cominarty" [148]. The BNT162b2 (Cominarty) vaccine had 94% efficacy in adults aged above 65 years when tested 14 days after administration [179]. An efficacy assessment in children aged 12 – 18 years old showed 93% effectiveness against hospitalization, which led to authorization for the use of this vaccine in children [180]. The mRNA-1273 vaccine was 94.1% efficacious against SARS-CoV-2 in adults aged 18 years or older [177] whilst a study assessing the persistence of antibodies after vaccination with this vaccine showed high titres in adults at six months post vaccination [181]. Both vaccines were safe as evidenced by the low severe adverse reactions to immunization. The rapid development speed coupled with the scalability and low cost of production (dependent on the amount of RNA in each vaccine dose) made these vaccine candidates leaders in the COVID-19 vaccine race.

1.8.5 Viral-vectored vaccines

Viral vectors use weakened viruses to introduce a specific gene or antigen into the cell. These are often genetically engineered to render them unable to replicate and unable to cause disease, which makes them safe to use as vectors. Live viral vector vaccines elicit strong immunogenicity and vigorous cytotoxic T lymphocyte (CTL) responses as they induce an immune response resembling a natural infection. The advantages of viral vector vaccines include the ability to target specific cells, the induction of humoral and cellular immune responses, and highly efficient gene transduction [182]. Frontrunners Astra-Zeneca/Oxford and Johnson and Johnson have both

developed adenoviral vectored vaccines targeting the SARS-CoV-2 spike protein and both are currently being administered globally. Other viral-vectored vaccine platforms that have been explored are the measles virus and poxviruses.

1.8.5.1 Adenovirus-vectored vaccines

Adenovirus-vectored vaccines have been widely used and explored for many infectious diseases. This platform induces a strong immune response and is largely scalable. Chimpanzee adenoviruses (ChAd) are an alternative to Ad5 because they have the ability to circumvent the pre-existing immunity challenge as only about 1% of people have antibodies against it [183] whilst Ad26 is a rarer virus in the human population and thus also serves as an alternative to Ad5 vectors [184]. The chimpanzee adenoviral vectored vaccine, ChAdOx1-S nCoV-19, produced by Astra-Zeneca and the University of Oxford has been shown to induce a strong and long-lasting immune response in both healthy adults and immunocompromised people [185], [186]. Owing to the ease of mass production, this vaccine was widely distributed to low-income countries such as South Africa where further trials were conducted. Four trials in the UK, Brazil and South Africa additionally showed the safety and 70% efficacy of the vaccine. Efficacy of 90% was seen in individuals who received one low dose followed by a standard dose whilst it was 62.1% in those who received two standard doses. These trials also showed that the vaccine can be stored at 2-8°C which made it ideal for distribution [185].

Using the same technology, Johnson and Johnson developed a human adenovirus type 26 based vaccine, Ad26.COV2-S which requires only a single dose to induce an immune response. The results of a randomized, double blind, placebo-controlled study showed that the Ad26.COV2-S vaccine protects against symptomatic COVID-19, asymptomatic SARS-CoV-2 infection and COVID-19 disease at low doses, whilst being safe as any adverse reactions were transient [186]. This being a single dose vaccine proved to be beneficial in global distribution, especially in countries where gaining access to two-dose regimen vaccines has been challenging. Whilst both the Astra-Zeneca and Johnson and Johnson vaccines were beneficial for global use, these vaccines were linked with rare blood clotting [187], [188] which halted their use in some countries. Two adenoviruses, Ad26 and Ad5 were used to deliver the full-length spike glycoprotein 21 days apart and were shown to be safe, immunogenic and offered 91.6% efficacy [189], [190]. However, because of the pre-existing immunity to adenovirus 5, countries like South Africa, with a high HIV

prevalence, have rejected the use of Sputnik V [191]. This was largely based on the premature termination of the STEP and PHAMBILI HIV-1 vaccine trials due to vaccine enhanced susceptibility to acquisition of HIV-1 caused by the adenovirus vector [192], [193]. This highlights a major drawback and risk of adenoviral-vectored vaccines which is the possibility of pre-developed anti adenovirus immunity. This is common in the human population and could result in increased enhanced immune responses [194], [195].

1.8.5.2 Poxvirus-vectored vaccines

Poxviruses, such as avipoxviruses like canarypoxvirus [196], and Modified Vaccinia Virus Ankara (MVA) have also been used as viral vectors for recombinant vaccines. Pox virus vaccines were first used in the 19th century by Benjamin Jesty and Edward Jenner when they used cow poxvirus as a vaccine for protection against smallpox. Cowpox virus (CPXV) and horsepox (HSPV) virus were used interchangeably until refinements were made in virus growth and vaccines were prepared from a single stock with little chance of recombination. Vaccinia virus (VACV) has become the prototype poxvirus and is being extensively used in vaccine development. The discovery of the ability to insert genes into the genomes of poxviruses allowed these viruses to be used as vaccine vectors [197].

There are several reasons why poxviruses are attractive for vaccine development. 1) Poxviruses replicate independently in the cytoplasm which takes away the risk of genomic integration into the host genome. 2) With a genome of up to 370 kbp [198], large and multiple foreign DNA sequences can be inserted into the poxvirus genome [199] without compromising the growth of the virus, thus allowing for expression of multiple antigens which could even be from different sources. 3) Freeze-dried poxvirus vaccines are thermostable, low-cost and easy to manufacture and administer. 4) Poxviruses can induce both antibody and cytotoxic T cell responses against heterologous antigens with long lasting immunity [200].

The DNA of poxviruses is not infectious. However, homologous DNA recombination occurs naturally and frequently within poxvirus infected cells (approximately 0.1%) and this feature has been exploited in the genetic engineering of recombinant poxvirus. Because of the ability to

genetically engineer recombinant poxviruses, a wide range of poxvirus vectors have been made, such as attenuated viruses NYVAC and ALVAC [199], [201], [202]. When assessed in a phase I clinical trial, antibody responses to the Japanese encephalitis virus in NYVAC vaccinated volunteers were stronger than responses induced by the ALVAC vaccine [203]. Another use of poxviruses is the pairing of modified recombinant poxvirus with adjuvants like IL-2 to enhance immunogenicity of recombinant vaccines [204], [205]. A study demonstrated that the use of IL-5 as an adjuvant induced longer lasting immune response as compared to IL-2 when paired with HIV gp160 recombinant vaccinia viruses [204].

1.8.5.3 MVA as a vaccine vector

Modified Vaccinia Ankara (MVA) has been safely used for human and animal vaccines and has been approved for human use [206]. This is a highly attenuated vaccinia virus that does not grow in human and most mammalian cells, besides BHK21 cells, but when introduced via infection prompts expression of early genes [207].

Mayr and colleagues produced MVA after 572 passages of chorioallantoic vaccinia virus (CVA) in primary chicken embryo fibroblasts (CEF) cells [208], which they were making in an effort to create a host restricted vaccine to eradicate smallpox virus at the University of Munich, Germany. After immunizing 120 000 individuals against smallpox using MVA, and having subjects show no adverse reactions as compared to other VACVs, interest in MVA arose. It was later discovered that 15% of the CVA genome was lost during the 572 passages resulting in mutations and deletions in six large genomic regions. The deleted genes are responsible for poxviral immune evasion and host interaction [209] thus MVA shows limited replication in some mammalian cell lines such as baby hamster kidney (BHK cells) [210].

MVA is advantageous for use because of its inability to complete its replication cycle in humans thus limiting the spread of MVA to human cells after vaccination, making it a safe option for immunocompromised individuals and neonates [211], [212], [213]. The large MVA genome also allows for insertions of large and multiple transgenes [214] which makes MVA useful as a vector. Another advantage of MVA is the lack of immunomodulatory genes in the genome, which leads to fast immune responses, making MVA an efficient adjuvant [215]. Previous research additionally showed that MVA can induce lasting cellular and humoral immune responses [216], [217], making it an ideal viral vector for vaccine development, an advantage that is particularly important with

highly infectious diseases such as COVID-19. Furthermore, MVA is low-cost because high titres of vaccine stock can be made in chick embryo fibroblasts and BHK cells thus large-scale manufacturing is possible [182].

A prime boost vaccine against the Ebola virus comprising a first dose with the adenovirus type 26 based vaccine, Ad26.ZEBOV, and a second dose with the MVA based vaccine, MVA-BN-Filo which encodes spike glycoproteins from Ebola, Sudan, Marburg viruses and the nucleoprotein from the Tai Forest virus has been approved for use. This vaccine clearly demonstrates that viral vectored vaccines allow for the insertion of multiple genes which promote a broad immune response. The immune response from this combination of vaccines has been shown to persist for a year, a first of its kind for Ebola vaccines [218]. Immunogenicity studies where participants were either given the prime boost Ebola vaccine or a placebo showed a significant antibody response, as well as at least 86% exhibiting an Ebola specific T-cell response in the prime-boost group. The T-cell response was stronger when the priming dose was the Ad26.ZEBOV vaccine followed by the booster MVA-BN-Filo vaccine, as compared to the opposite order [219].

A MVA vaccine, targeting the SARS-CoV-2 S protein has been developed. This consisted of a prime boost of either MVA/MVA, or DNA/MVA in mice. Results from these immunizations revealed the strong and robust induction of CD4⁺ and CD8⁺ T-cells, with the DNA/MVA regimen being stronger. High titres of IgG antibodies were induced by both vaccine regimens. Additionally, serum from vaccinated mice was shown to offer 100% protection against a lethal dose of SARS-CoV-2. The virus was further blocked from replicating in the lungs after immunization of humanized mice with two doses of MVA-CoV2-S [220]. These results demonstrated the strength and efficacy of an immune response from MVA based vaccines.

MVA would be a suitable vector platform for COVID-19 vaccines because of its safety, high immunogenicity, and the versatility that allows for multigene insertion.

1.8.5.4 The construction of recombinant MVA

As much as MVA is extremely advantageous and the poxvirus of choice for many, the process of constructing a recombinant MVA is lengthy hence the constant need for optimization strategies [221].

Figure 1.5 shows how a recombinant poxvirus is isolated. The first step when engineering a recombinant poxvirus is to produce a transfer plasmid containing the gene of interest, driven by a specific poxvirus promoter. The expression cassette in a transfer plasmid is flanked by 300-1000 bp of double stranded DNA homologous to the sequence in the parent virus DNA which allows for specific integration of the foreign gene/s into the poxvirus genome. A poxvirus promoter is required to drive transgene expression. Promoters can be chosen to fine-tune the strength and timing of transgene expression. Promoters are divided into three classes: early, intermediate, and late. Early promoters function before DNA replication whilst the intermediate and late promoters drive expression after DNA replication [222]. In order to ease the isolation of a recombinant poxvirus a fluorescent marker gene is often included with the foreign gene of interest. Markers assist in identifying recombinant viruses. In this project, eGFP is the marker used to identify the recombinant MVA virus. The flanking regions in the transfer vector guide homologous recombination between the transfer vector and the poxvirus DNA so generating the recombinant poxvirus.

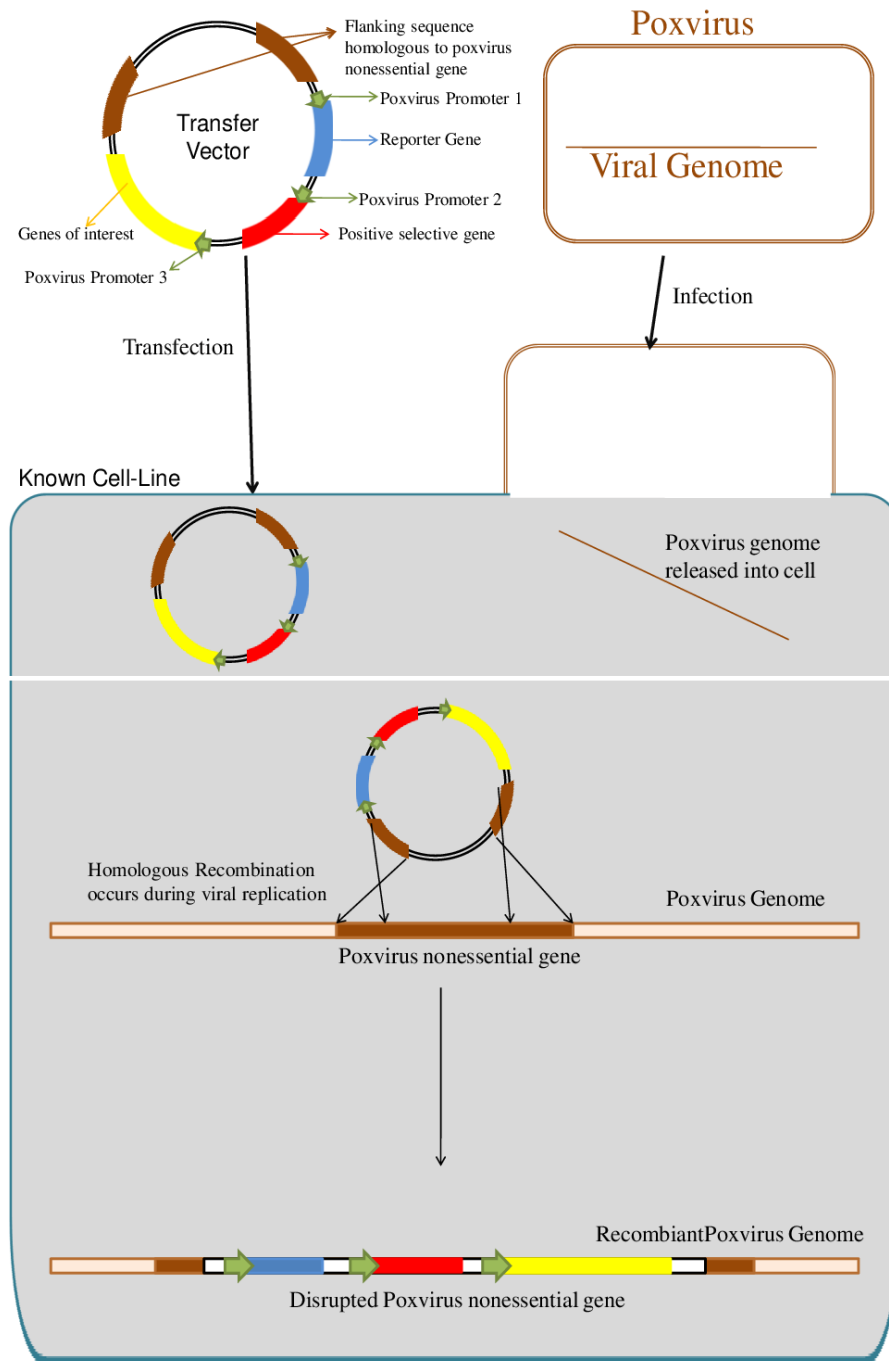


Figure 1.5: Generation of recombinant poxviruses by homologous recombination. A schematic representation of the poxvirus is shown on the top right; and a transfer vector (plasmid) is depicted on the top left. Viral DNA sequences adjacent to the genomic insertion site (flanking sequences) are cloned in the plasmid and target genes are inserted between these sequences and placed under transcriptional control of poxvirus-specific promoters. Recombinant viruses are generated by infection and simultaneous transfection of cells with vector plasmid DNA, resulting in recombination between homologous DNA sequences of plasmid and virus. The large box represents a poxvirus-infected, transfected cell, with a schematic of the viral genome and a plasmid designed for insertion of foreign DNA. Promoters, reporter genes, and host range selection genes are shown. In this project, the I8R and G1L locus was used as an insertion site. Figure taken from Hu (2010) [223].

1.8.6 Project rationale

At the start of the global pandemic, the numbers of infections and deaths were increasing exponentially, at a time when there was no vaccine available against COVID-19. There was a need to explore and evaluate which vector platform would be ideal for a COVID-19 vaccine. Our group explored the immunogenicity of an engineered version of the SARS-CoV-2 spike protein, based on the wildtype Wuhan virus across different platforms. The mediatory role played by the spike protein in allowing the virus to enter a host makes it the primary target of most vaccines against this coronavirus. Research on the SARS-CoV and MERS-CoV viruses of close relation to the SARS-CoV-2 virus have shown the immune response elicited by the spike protein, both humoral [224] and cell-mediated [225] to be protective, indicating that the spike is an ideal target to use for vaccine development. The broad aim of the study is to use the same gene insert to allow for direct comparison of different vaccination platforms: proteins (made in plants and eucaryotic cells), DNA, and poxviruses. This particular project entails the use of the poxvirus MVA as a live viral vector platform. MVA was selected due to the ease of genomic manipulation which allows for insertion of foreign genes to elicit immunogenicity against the gene of interest. This is a platform that our group has studied extensively for HIV-1 vaccines, which allowed us to leverage off of previous studies for application to this project.

This project had two aims. The first aim was to generate a recombinant MVA vaccine candidate encoding the modified soluble spike glycoprotein, S Δ TM from SARS-CoV-2. This involved cloning the engineered spike glycoprotein gene into an MVA transfer vector which had the necessary elements to produce a recombinant, generating the recombinant by infection of BHK21 cells with wild type MVA and transfection with the transfer vector, purification of the recombinant through a series of passages in RK13 cells, confirmation of the integration of the gene cassette into MVA, and finally, confirmation of expression of the antigen of interest, the SARS-CoV-2 spike glycoprotein. The second aim of this project was to test the immunogenicity of the recombinant MVA vaccine in mice to assess its suitability for future developments.

CHAPTER 2

MATERIALS AND METHODS

2.1. Cloning

2.1.1 Restriction enzyme digestion

Restriction enzyme digests were conducted using Fast Digest enzymes in a total volume of 20 µl. Each reaction contained 1 µl Fast Digest Enzyme (Thermofisher, South Africa), up to 1 µg plasmid DNA, 2 µl of 10x Fast Digest Green Buffer and dH₂O to make up the remaining volume. The reactions were incubated at 37 °C for 60 minutes to allow for optimum enzyme activity. In some cases, negative controls were included where the restriction enzyme was omitted.

2.1.2 Separation of DNA fragments by agarose gel electrophoresis

DNA fragments were subjected to agarose gel electrophoresis to separate fragments according to their molecular weight. An aliquot of 2 µl 10X Fast Digest buffer green was added to the sample prior to electrophoresis. Agarose gels were made up to 1% using SeaKem LE agarose (Lonza, South Africa) and 1 x TBE, which was supplemented with 0.5 µg/mL ethidium bromide (Merck, South Africa) to enable visualization of the DNA under UV light. The DNA was resolved on an agarose gel, suspended in 1 X TBE buffer, for 45 minutes at 100 V. A 10 µl aliquot of GeneRuler 1 kb DNA ladder (Thermo Fisher Scientific, South Africa) was resolved alongside the experimental samples to allow for estimation of the DNA band sizes. Agarose gels were viewed on the Gel Doc Go system (Bio-Rad, South Africa) and the images were captured using Image Lab 6.1 Software (Bio-Rad, South Africa).

2.1.3 Excision and recovery of DNA fragments from agarose gels

The bands of interest were excised from the gel with a scalpel using the Accuris SmartBlue Blue Light Transilluminator (Accuris Instruments, USA) to avoid DNA damage which could occur when using UV light for DNA visualisation. The DNA was then purified using the QIAquick Gel Extraction Kit (Qiagen, South Africa) according to the manufacturer's instructions. The gel slices were transferred to Eppendorf tubes and then weighed. Three volumes of Buffer QG were added to the agarose gel (volume:weight) before incubation at 50 °C for 10 minutes, to completely

dissolve the agarose. The tubes were vortexed every 2-3 minutes to mix the contents. The colour of the mixture was confirmed to be yellow before proceeding as this indicative of the pH of the solution. The ideal pH for DNA binding to the column matrix is $\text{pH} \leq 7.5$, which is signalled by the Buffer QG turning yellow at $\text{pH} \leq 7.5$. One gel volume of isopropanol was added to the sample which was then applied to the Qiaquick spin column. The column containing the sample was centrifuged at $\geq 10\,000 \times g$ for 1 minute to pass the solution containing DNA over the column matrix. The flow through was discarded and 0.75ml Buffer PE was added to the column. The sample was centrifuged again, for 1 minute, and the flow through was discarded. This was followed by an additional 1 minute centrifuge spin to remove any residual wash buffer. An aliquot of 30 μl pre-warmed Buffer EB was added to the centre of the Qiaquick membrane to elute the DNA, which was bound to the column matrix. The column was incubated with the buffer for 5 minutes before repeating the centrifugation step to elute the DNA. DNA was either used immediately or stored at $-20\text{ }^{\circ}\text{C}$.

2.1.4 Ligation of DNA fragments

The digestion of plasmid DNA yielded sticky ends, that were compatible between the plasmid backbone and the insert. Ligations were conducted using the Quick LigationTM Kit (New England BioLabs, South Africa). A 3:1 molar ratio of insert to backbone was used in a reaction volume of 20 μl . This comprised of 1 μl Quick Ligase (New England BioLabs, South Africa), 10 μl 2X Quick Ligase Reaction Buffer, insert, and vector backbone DNA. Controls were included where there was either 1) no insert DNA to assess self-ligation, 2) no insert DNA and no ligase to determine the prevalence of residual undigested DNA. Reactions were incubated at room temperature for 30 minutes.

2.1.5 Transformation of *Escherichia coli*

Escherichia coli (*E.coli*) DH10 chemically competent cells (Inqaba Biotechnology, South Africa) were transformed with an aliquot of 0.1 - 1 ng of plasmid, or 1.5 μl ligation reaction. After thawing the cells on ice, 15 μl of competent *E.coli* cells were added to an Eppendorf tube containing the plasmid DNA. The contents were gently mixed by pipette aspiration. The tubes were incubated on ice for 20 minutes and then heat shocked at $42\text{ }^{\circ}\text{C}$ for 30 seconds. The samples were then returned

to ice for a further 2 minutes. An aliquot of 400 µl of SOC Outgrowth Medium (New England BioLabs, USA) was added to the cells and the samples were incubated at 37 °C on a shaker, for 60 minutes, to allow for expression of the antibiotic selection genes. Aliquots of 25 µl, 50 µl and 150 µl of each sample were plated onto LB agar plates (Appendix A), supplemented with 25 µg/ml Chloramphenicol (Sigma-Aldrich, South Africa) for pSSPEX vectors and 100 µg/ml Carbenicillin for pMEX plasmid DNA. *E.coli* transformants containing the plasmids were grown in broth culture, with appropriate selection, and then stored as 25% glycerol stocks at -80 °C.

2.1.6 Isolation of DNA

Plasmid DNA for transfections was isolated from the *E.coli* cells using the QIAGEN® Plasmid *Plus* Maxi Kit. Small scale plasmid DNA isolation was done for screening putative clones using alkaline lysis.

2.1.6.1 Large scale isolation of plasmid DNA

Plasmid DNA was isolated using the QIAGEN® Plasmid *Plus* Maxi Kit (Qiagen, USA) in accordance with the protocol described for high yield DNA. Recombinant *E. coli* cells were propagated in 100 ml liquid media for large scale plasmid isolations. The bacterial culture was harvested by centrifuging at 6000 x g for 15 minutes at 4 °C and the pellet was resuspended in 8 ml Buffer P1. An aliquot of 8 ml Buffer P2 was added, and the tube was gently mixed until the contents became viscous. The sample was incubated at room temperature for 3 minutes to enable cell lysis to occur. Subsequently, 8 ml of Buffer S3 was added to neutralize the lysate and the solution was mixed by inverting 4-6 times. The lysate was then transferred to the QIAfilter cartridge and incubated at room temperature for 10 minutes to allow the cell debris to settle. While incubating the lysate, the QIAGEN Plasmid *Plus* spin column was placed into the QIAvac 24 Plus and Tube Extenders inserted. The plunger was gently inserted into the QIAfilter cartridge, and the lysate was filtered into a new tube enabling the removal of undesired cell debris. An aliquot of 5 ml binding buffer (BB) was added to the cleared lysate and inverted 4-6 times to mix. The addition of buffer BB promotes the binding of DNA to the spin column to maximise recovery of the recombinant plasmid. The lysate was transferred to the QIAGEN Plasmid *Plus* spin column, with a tube extender attached on the QIAvac 24 Plus. Approximately -300 mbar vacuum was applied until the liquid had been drawn through the column. The bound DNA was sequentially washed

with 0.7 ml endotoxin removal buffer (ETR) and 0.7 ml wash buffer (PE) which were drawn through the tube by application of a vacuum. The QIAGEN Plasmid *Plus* Maxi spin column was placed into a 2 ml collection tube and centrifuged at 10 000 x g for 1 minute to remove any residual wash buffer. The QIAGEN Plasmid *Plus* spin column was then placed into a clean 2 ml tube and 400 µl prewarmed elution buffer (EB) was added to the centre of the QIAGEN Plasmid *Plus* spin column. The column was incubated for 5 minutes and then centrifuged for 1 minute to elute the DNA. Plasmid DNA was stored at 4 °C.

2.1.6.2 Small scale isolation of plasmid DNA

Alkaline lysis was used to extract DNA from cells. A 12-well plate was simultaneously seeded with 0.1×10^6 RK13 cells and infected with recombinant virus. The infection was incubated at 37° C under standard cell culture incubator conditions for 72 hours. After 72 hours, the media was discarded and the cells were resuspended in 250 µl solution P1 (Appendix A) which contains glucose to maintain osmotic pressure outside the cells, Tris for maintenance of pH and EDTA to protect the DNA from degrading. An aliquot of 250 µl solution P2 (Appendix A) was added to lyse the cells by pipetting the mixture over the cells several times. The mixture was transferred to a 1.5 ml Eppendorf tube and 350 µl solution P3 (Appendix A) was added to neutralize the samples. The tube was gently inverted 4-6 times to ensure complete neutralization resulting in the formation of white precipitation of DNA and protein. The samples were spun at max RPM for 10 minutes on a benchtop centrifuge to separate the DNA and proteins; and 750 µl of clear supernatant was pipetted out into new 1.5 ml Eppendorf tubes. The DNA at this point was in the supernatant. An aliquot of 750 µl ice cold isopropanol was added to precipitate the DNA out of solution, and the samples were stored at -20° C for 1 hour. Samples were subsequently centrifuged on a benchtop centrifuge at maximum speed for 20 minutes. The isopropanol was carefully discarded so as not to disturb the pellet containing the DNA. To remove any residual salt, an aliquot of 70% ethanol was added, and the samples centrifuged for 1 minute at max speed. The ethanol was also carefully removed, and the pellet left to air dry for 5 minutes before being resuspended in 50 µl RO water.

2.2. Cell culture

2.2.1 Cell lines

Baby hamster kidney cells (BHK21) and rabbit kidney cells (RK13) were obtained from the American Type Culture Collection (ATCC). BHK21 cells were used for infection and transfection to generate recombinant Modified Vaccinia Ankara (MVA) virus. RK13 cells were used to select for recombinant MVA expressing the K1L host range gene.

2.2.2 Revival and expansion of frozen cells

A vial of cells was thawed and diluted in 9 ml pre-warmed 10% complete Dulbecco's Modified Eagle Medium [10% heat inactivated fetal calf serum (Gibco, South Africa) and 1% Penicillin-Streptomycin antibiotic) (Gibco, South Africa)] to dilute out the DMSO present in the freezing media. The cells were then transferred into a T25 cell culture flask (Thermo Fisher Scientific, South Africa) and grown at 37 °C with 70% relative humidity and 5% CO₂. Once the cells formed a confluent monolayer, the cells were expanded in T75 flasks (Thermo Fisher Scientific, South Africa).

2.2.3 Growth and maintenance of cell lines

Cells were maintained in T75 flasks, in a volume of 15 ml complete media (cDMEM) (Appendix A). Flasks were subcultured every 2-3 days at approximately 70-90% confluence. Spent media was removed from the flask, and the cells were washed with 10ml of phosphate buffer saline (PBS) pH 7.4 (Gibco, South Africa). A 3 ml aliquot of 1 X 0.25% trypsin-EDTA (Gibco, South Africa) was distributed over the cells and the flask was incubated for 2-5 minutes at 37 °C for the cells to lift. The flask was then agitated to dislodge the cells and the volume was adjusted to 10 ml with cDMEM to inactivate the trypsin. New flasks were seeded with 2-3 x 10⁶ cells and incubated as before.

2.2.4 Determination of cell count and cell viability

Viable cells were counted by staining with Trypan Blue (Lonza Bioscience, South Africa). When confluent, cells were lifted as described in Section 2.2.3. An aliquot of 50 µl of the cell suspension was mixed with an equal amount of Trypan Blue (0.4%) (Lonza Bioscience, South Africa) in an Eppendorf tube resulting in a 1:2 dilution. An aliquot of 10 µl was then pipetted onto a Neubauer chamber slide (Marienfield Superior, Germany), beneath a cover slip, and viewed using an optical microscope (Olympus CH20 10X). Four squares were counted and used to approximate the number of cells present in accordance with the following equation:

$$\text{Total cells/ml} = (\text{sum of cells in 4 squares}/4) * 10^4 * 2 \text{ (dilution factor)}$$

2.2.5 Preparation of frozen cell lines for long-term storage in liquid nitrogen

Cell lines were stored in liquid nitrogen for long term preservation. Cells were grown, trypsinized and counted as described in Sections 2.2.3 and 2.2.4 respectively, before being centrifuged at 180 x g for 5 minutes to collect the cells in a pellet. Pelleted cells were resuspended in freezing medium (Appendix A) to get to a total concentration of 1.5×10^6 cells/ml. Aliquots of 1 ml were dispensed into cryovials which were then transferred to a Mr. Frosty (storage container submerged in isopropanol to prevent rapid drop in temperature) and stored at -80°C for 48 hours, before being transferred to liquid nitrogen.

2.3. Transfection of mammalian cells

2.3.1 Transfection of cells with plasmid DNA

A 6-well plate was seeded with 2 ml per well of 0.3×10^6 cells/ml and incubated overnight. Cells, in the logarithmic phase of growth and approximately 70% confluent, were transfected with a transfection mixture containing 4 µl X-tremeGENE™ 9 (Thermo Fisher Scientific, South Africa), 500 µl DMEM (Gibco) and 4 µg of plasmid DNA to achieve a transfection reagent ratio of 1:1. Control wells of untransfected cells were prepared. The transfection mixture was incubated for 30 minutes at room temperature to allow DNA-transfection reagent complexes to form and 1 ml was added to the wells in a dropwise manner. The plate was incubated and monitored daily using under

a Zeiss Axio Vert.A1 fluorescent microscope where plasmids encoded a fluorescent reporter gene or under a light microscope to assess cell morphology where a reporter gene was absent.

2.4. Generation of MVA-SARS2-SΔTM

Recombinant MVA was constructed by first generating a transfer vector, pMVA-FNK1, which is diagrammatically depicted in Figure 2.1. The cloning strategy employed is shown in Figure 3.6 of the Results section together with a description of why specific genes were chosen. pMVA-FNK1 was designed with the mH5 vaccinia virus promoter driving expression of the antigen of interest, SARS-CoV-2-SΔTM, the early/late p7.5 promoter driving expression of eGFP as a fluorescent marker gene and the synthetic poxvirus promotor pSS driving expression of the K1L host range gene. These genes were placed between sequences homologous to the 3' ends of the convergent MVA open reading frames (ORFs) I8R on the left flank and G1L on the right flank (Figure 2.1). These promoters are regulated by pox polymerases and transcription of the genes is only possible in the presence of the polymerase.

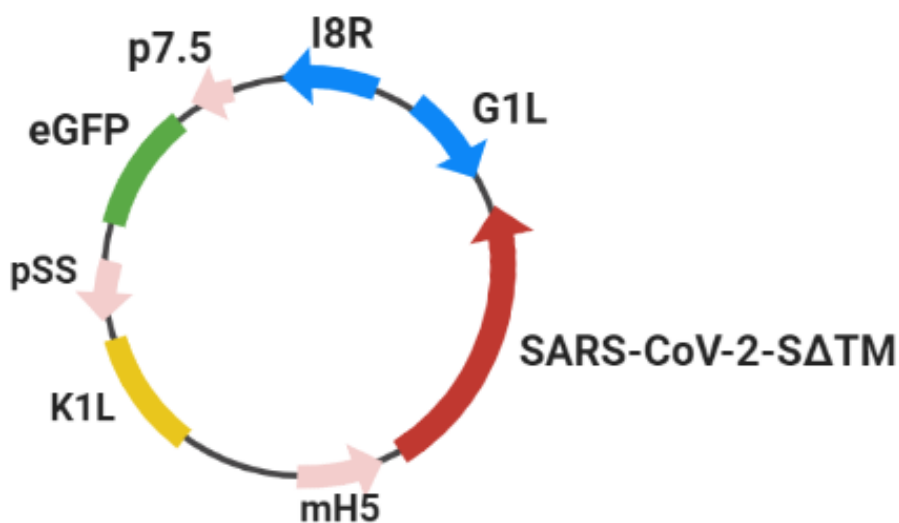
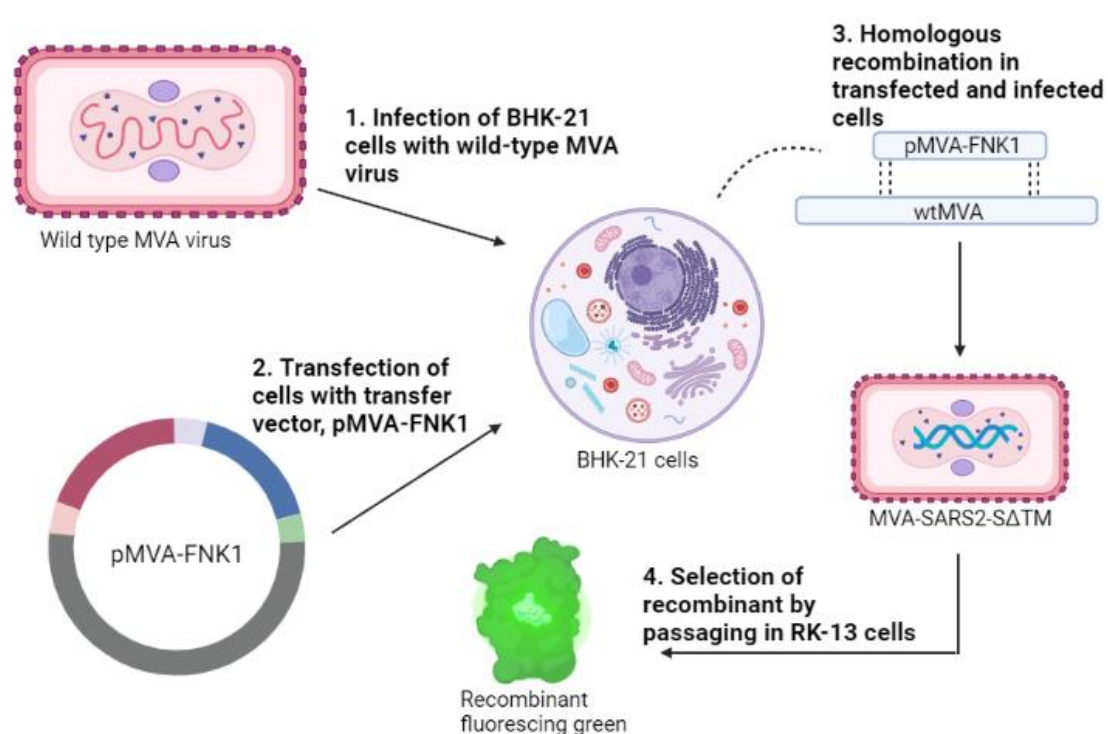


Figure 2.1: Diagram showing essential features of transfer vector pMVA-FNK1. I8R = MVA left flank, G1L = MVA right flank, promoter eGFP = green fluorescent protein, K1L = host range gene, p7.5 = early/late vaccinia virus promoter, pSS = synthetic vaccinia virus promoter, mH5 = vaccinia virus modified H5 promoter. This is not drawn to scale and the plasmid vector backbone is located between the 18R and G1L flanking sequences.

BHK21 cells, permissive for MVA, were infected with wild type MVA virus and transfected with pMVA-FNK1. Homologous recombination could take place between the identical regions of the

virus genome and the transfer vector, resulting in generation of recombinant MVA expressing the antigen of interest and the other genes located between the I8R and G1L flanking regions (Figure 2.2).

A)



B)



Figure 2.2: Schematic of experimental procedure to generate recombinant Modified Vaccinia Ankara (MVA) virus. A) BHK21 cells were infected with wild-type parent MVA virus and then transfected with a transfer vector (pMVA-FNK1) containing the modified SARS-CoV-2 spike glycoprotein gene (SΔTM). This was cloned between the MVA flanking sequences that corresponded to the ends of the open reading frames, I8R and G1L. The identical flanking regions between the transfer vector and wild-type MVA are able to recombine by a process of homologous recombination resulting in insertion of the foreign genes (K1L selection gene, eGFP marker gene, and SARS-CoV-2-SΔTM.) into the MVA genome thus generating recombinant MVA, MVA-SARS-2-SΔTM. Figure created using BioRender.com. B) A line diagram showing the integrated coding region. The K1L gene functions to positively select for the recombinant MVA in RK13 cells and subsequently allows for purification of the recombinant from the wildtype MVA virus which cannot grow in this cell line. Additionally, eGFP was used as a fluorescent marker to identify recombinant virus.

2.4.1 Infection of BHK21 cells with wild type MVA and transfection with pMVA-FNK1

A 6-well plate was seeded with 2 ml per well of BHK21 cells, at a concentration of 0.3×10^6 cells/ml and incubated overnight at 37 °C in a CO₂ incubator under standard growth conditions (Section 2.2.3). After 24 hours, cells were washed with 2 ml cDMEM before infection with 10 µl of MVA at a MOI of 0.5 and 1. Cells were incubated under standard growth conditions for 2 hours. After incubation, the media was removed and replaced with 2 ml fresh cDMEM.

Cells were then transfected as outlined in Section 2.3.1. Control wells of plasmid only, MVA only (MOI 0.5 and 1) and uninfected BHK21 cells were also prepared. The plate was incubated for 48 hours and viewed using a Zeiss Axio Vert.A1 fluorescent microscope to observe green fluorescence from eGFP expression. Green fluorescence is driven by the control of a poxvirus specific promoter p7.5. Therefore, only when a transfected cell is infected with a poxvirus (MVA), will there be eGFP expression.

2.4.2 Purification of recombinant MVA-SARS2-ΔTM

MVA-SARS2-ΔTM was passaged in RK13 cells to purify the recombinant virus from unwanted parental virus. Cells were freeze-thawed for three cycles to release the virus from the infected BHK21 cells. Freeze cycles were conducted at -80 °C for 30 minutes and thaw cycles were performed at 37 °C for 15 minutes. The cell lysate was mixed and transferred to a 2 ml cryovial tube to prepare for infection of RK13 cells. At passage 1, a concentration of 2×10^5 cells/ml was seeded into two 12-well tissue culture plates: one for MOI 1 and the other for MOI 0.5. Each well was infected with 10 – 200 µl of lysate. Plates were incubated for 3-5 days under standard growth conditions to allow for growth of the virus and the plates were observed daily using a Zeiss Axio Vert.A1 fluorescent microscope to monitor green fluorescence. This lysate was used for passage 2. For subsequent rounds of infections (passages 2 – 9), lysate of different volumes ranging from 20 µl to 200 µl were used to infect cells. At passages 3 and 4 the virus was serially diluted such that a single focus could be isolated in a single well of a 96-well plate. Each well containing a single focus was marked and those cell lysates were used for further passages in preparation for subsequent experiments. To expand the foci, subsequent passages were performed using 6-well and 12-well plates. PCR was performed using DNA isolated from cells in passage 7 to determine whether any residual parent MVA was present in the purified recombinant. PCR was performed

again using DNA derived from cells at passage 9, this time to confirm integration of the entire gene cassette into the MVA genome.

2.5. Validation and characterization of recombinant MVA-SARS2-SΔTM

After the recombinant was passaged several times in RK13 cells to purify the recombinant virus from the parental MVA, cell lysate from passages 7 and 9 was used to validate the integration of the whole gene cassette. The cell lysate was also used to confirm expression of SΔTM antigen by polymerase chain reaction (PCR) and Western blot (WB) analysis respectively.

2.5.1 Polymerase Chain Reaction (PCR)

The successful integration of the whole gene cassette flanked by I8R and G1L genes was confirmed using polymerase chain reaction (PCR). Genomic DNA was extracted using the QIAGEN® DNeasy Blood and Tissue Kit in accordance with the manufacturer's instructions for use in the PCR. Genomic DNA was isolated from infected eukaryotic cells to confirm, by PCR, that the recombinant MVA being passaged in RK13 cells, was indeed correct.

An aliquot of 20 µl Proteinase K was added before 200 µl Buffer AL was added and the cells were pipetted up and down. The mixture was incubated for 10 minutes at 56 °C to enable lysis to occur. An aliquot of 200 µl 96% ethanol was added before mixing thoroughly. The sample was pipetted into a DNeasy Mini spin column placed in a 2 ml collection tube and centrifuged at $\geq 6000 \times g$ for 1 minute to remove any residual contaminants. The flow through was discarded. An aliquot of 500 µl Buffer AW1 was added before the mixture was centrifuged for a further 1 minute at $\geq 6000 \times g$. An additional centrifugation for 3 minutes at $20\,000 \times g$ was performed to dry the DNeasy membrane and remove any residual ethanol. The DNeasy Mini spin column was placed in a 2 ml Eppendorf tube whilst an aliquot of 200 µl Buffer AE was added directly to the membrane to elute the DNA. This was incubated for 1 minute at room temperature and centrifuged for 1 minute at $\geq 6000 \times g$ for thorough elution.

A 12-well plate of RK13 cells was infected with increasing volumes of cell lysate from passage 7 as described in Section 2.4.2. The cells were incubated under standard growth conditions and monitored daily until the recombinant virus had spread, infecting as many cells as possible, without

the cells lifting. On day 4 the cells were harvested, and DNA was extracted using the aforementioned QIAGEN® DNeasy Blood and Tissue Kit. PCR was conducted as outlined in Table 2.1 using the primers reflected in Table 2.2. The reaction was performed using the KAPA 2G Robust HotStart ReadyMix kit in accordance with the protocol provided. PCR reactions were made up to a total of 50 µl, with 5 µl of DNA in each reaction. An aliquot of 25 µl KAPA2G Robust HotStart ReadyMix (Sigma-Aldrich, South Africa) containing dNTPs, a KAPA2G Robust DNA polymerase and a buffer, was added. The volume was adjusted with sterile dH₂O to make a total volume of 50 µl. The reactions were set up for 35 cycles, with cells only (no DNA) serving as a negative control; and parent MVA serving as a positive control. A 20 µl aliquot of the PCR products was resolved on a 0.6% agarose gel by electrophoresis as outlined in Section 2.1.2. The PCR binding sites and expected fragment sizes generated by the different primer pairs are depicted in Figure 2.3.

2.5.2 DNA sequencing

The PCR products were sequenced using the Sanger method by the Central Analytical Facilities at the University of Stellenbosch (South Africa). This was done to confirm the genetic integrity of the inserted gene, SΔTM using the sequencing primers outlined below in Table 2.3. PCR products were amplified using primers PEPV DRfwd and G1Lr2 and 100 ng/µl with 10 µl sample per primer pair was sent to be sequenced. Sequencing primers were designed to bind to the SΔTM gene insert. The sequence was analysed using CLC Genomics Workbench which enabled the sequence reads to be aligned to the reference sequence.

Table 2.3: Primers used to sequence MVA-SARS-2-SΔTM

Primer	Sequence
PEPV DRfwd	5'- GGGTGTAGCTAACAACCCTG - 3'
FNK1	5'- GTGAACCTGACTACCAGAAC – 3'
FNK2	5' - GGATGGAGAGCGAGTTTAGGG – 3'
FNK3	5' - GGATGGAGAGCGAGTTTAGGG – 3'
FNK4	5' - GGTGCTGAGCTTTGAGCTGC – 3'
FNK5	5' - CGCCTATACCATGTCCCTGG – 3'
FNK6	5' - GATGATCGCCCAGTACACATC – 3'
FNK7	5' - GCTATCACCTGATGAGCTTC – 3'
FNK8	5' - GCCTACGTGAGAAAGGATGG – 3'
FNK9	5' - TTT TCT GTC GAC GGG CCC G – 3'

Table 2.1: PCR cycling parameters for amplifying MVA-SARS2-SΔTM genomic DNA

Reaction Stage	Temperature (°C)	Duration
Initial denaturation	95	3 minutes
Denaturation	95	15 seconds
Annealing	53	15 seconds
Extension	72	6 minutes
Final extension	72	7 minutes

Table 2.2: Primers used for PCR validation of MVA-SARS2-SΔTM

Set	Primer	Primer sequence	Binding site
1 (Left end rMVA)	I8Rf2	5' - AGATGAACTTCAGGGTCAGC – 3'	Outside I8R flank
	eGFPprev	5' - GAAAAGGAAGAGTAGGAAGAG – 3'	eGFP gene
2 (Right end rMVA)	PEPVDRfwd	5' - GGGTGTAGCTAACAACCCTG – 3'	Outside K1L gene
	G1Lr2	5' - GGCCATGTGTAACACTAGAG – 3'	Outside G1L flank
3 (wtMVA)	I8Rf2	5' - GAAAAGGAAGAGTAGGAAGAG – 3'	Outside I8R flank
	G1Lf2	5' - GGCCATGTGTAACACTAGAG – 3'	Outside G1L flank

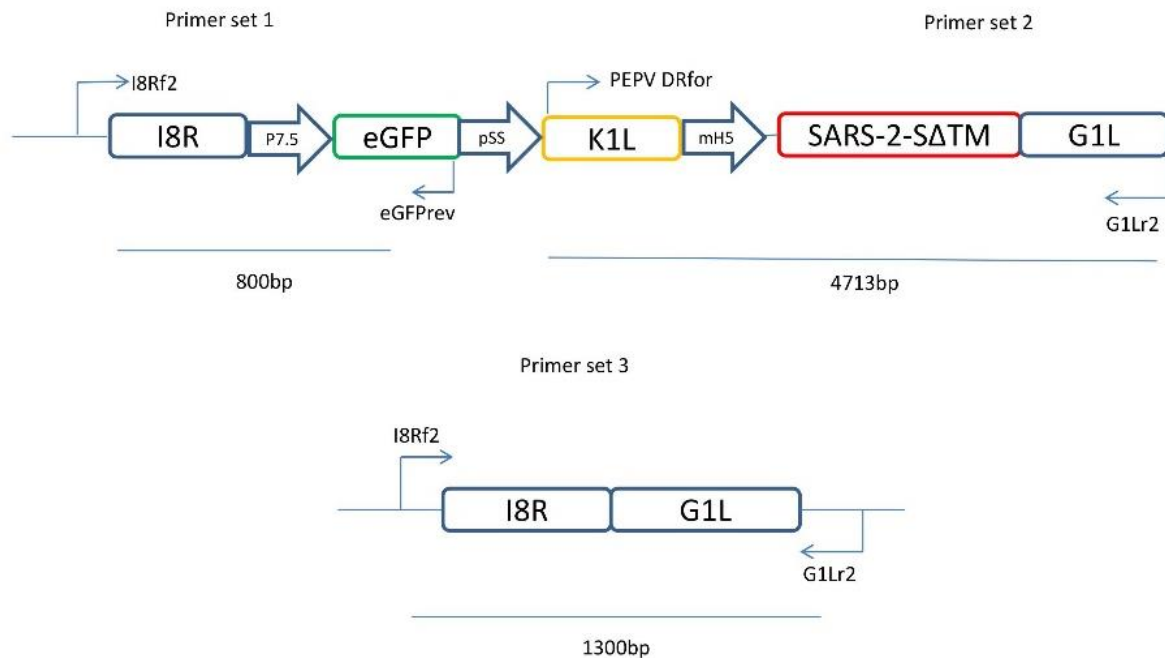


Figure 2.3: Schematic diagrams showing the binding sites of primer set 1 (I8Rf2 and eGFPprev) and primer set 2 (PEPV DRfwd and G1Lr2) in the recombinant virus (top) and primer set 3 for the wildtype parent MVA (bottom). PCR products are shown with expected product sizes as horizontal lines.

2.5.3 Western blot analysis

2.5.3.1 Preparation of media and cell lysate

Following transfection or infection, the spent growth media was removed and stored in Eppendorf tubes. The cells were rinsed with 500 µl 1XPBS. An aliquot of 200 µl Glo Lysis Buffer (Promega, South Africa) was dispensed over the cells before swirling the plate to ensure full coverage with the buffer. The cells were left at room temperature for 5 minutes to allow lysis to occur. The lysed cells were centrifuged for 10 minutes at 2500 x g to pellet insoluble host cell debris, and the supernatant was retained at –20 °C.

2.5.3.2 Sodium dodecyl sulphate - polyacrylamide gel electrophoresis (SDS PAGE)

Aliquots of 30 µl of media or lysate were mixed with 10 µl aliquots of 4X protein loading dye (Appendix A) and boiled at 95 °C for 5 minutes to denature the protein. Samples were loaded (15 – 20 µl) into the wells of 8% SDS page gels, alongside 10 µl Colour Protein Standard Broad Range (New England BioLabs, UK). The samples were resolved for 2 hours at 200 V in 1 X running buffer (Appendix A).

2.5.3.3 Immunoblotting of protein

The protein was transferred from the SDS gel to an Immuno-Blot PVDF membrane (Bio-Rad, USA) using the Bio-Rad Trans-Blot SD Semi-Dry Electrophoretic Transfer Cell apparatus (Bio-Rad, South Africa). A PVDF membrane (Bio-Rad, South Africa) was pre-soaked in methanol (Sigma-Aldrich, France) for 30 seconds to activate the membrane for protein binding. The membrane and extra thick blotting paper were both soaked in transfer buffer (Appendix A) for 5-10 minutes. The SDS-PAGE gel was placed on top of the activated PVDF membrane and the gel/membrane complex was sandwiched between the extra thick blotting paper. Transfer was conducted for 60 minutes at 25 V.

Following transfer, the membranes were blocked with 5% skim milk in PBS (Lonza, Belgium) for 2 hours at room temperature, whilst gently shaking. The blocked membranes were then incubated with a 1:5000 dilution of rabbit anti-SARS-CoV-2 Spike Glycoprotein S1 antibody (ab281311) (Abcam, South Africa) overnight. Membranes were washed with PBST wash buffer (Appendix A)

four times for 15 minutes, each with gentle agitation. The buffer was discarded, and the secondary goat anti-rabbit IgG (whole molecule) alkaline phosphate antibody (A3687) (Sigma-Aldrich, USA) (diluted 1:5000) was added to the membrane. The membrane was incubated at room temperature for 2 hours whilst shaking. The PBST washing steps were repeated as before, and the Western blot was developed for up to 1 hour using NBT/BCIP solution (KPL/SeraCare, USA). The reaction was terminated by washing the membrane in RO water.

2.5.4 Immunostaining of protein

A 12-well plate was seeded with 5×10^5 cells/ml in each well and then infected with MVA-SARS2-SΔTM. The infected cells were incubated for 72 hours. The spent media was removed and the cells were fixed with 500 µl 4% paraformaldehyde (Appendix A) with gentle agitation for 10 minutes. A 500 µl aliquot of 100% methanol (Sigma-Aldrich, France) was added and the plate was agitated for 1 minute. Cells were washed with PBS to remove excess methanol, before being blocked with 2% BSA (Appendix A) for 1 hour at room temperature. An aliquot of 500 µl SARS-CoV-2 spike rabbit primary antibody (1:500) was added to each well and the plate was incubated at room temperature for 90 minutes. Cells were again washed with PBS before an aliquot of 500 µl donkey anti-rabbit Cy3 conjugated secondary antibody (1:500) was added to each well and the incubation was repeated as before. The cells were washed twice with PBS, viewed using a Zeiss Axio Vert.A1 fluorescent microscope, and then imaged.

2.5. Growth of MVA-SARS2-SΔTM in BHK21 and RK13 cells

The growth of MVA-SARS2-SΔTM was evaluated in BHK21 and RK13 cells by daily observation of green fluorescence over a period of five days.

For this experiment 12-well plates were seeded with 5×10^5 cells/ml/well for each cell line and incubated under standard growth conditions overnight. Cells were infected with MVA-SARS2-SΔTM in triplicate at an MOI of 0.01. The virus was adsorbed for 2 hours before being removed from the wells. Cells were washed with 1 x PBS, to remove any unadsorbed virus, before 2 ml cDMEM was added to each well. Cells were visualized daily for green fluorescence, as an indication of viral growth.

2.6. Large scale production and titration of recombinant MVA-SARS2-SΔTM vaccine stocks

2.6.1 Growth of MVA-SARS2-SΔTM

In preparation for mice inoculations to test the immunogenicity of the vaccine, the recombinant MVA-SARS2-SΔTM was grown in RK13 cells. The cells were infected at an MOI of 0.001 in 8 x T175 flasks and incubated for three days. The flasks were freeze-thawed three times and the lysate was transferred to 50 ml falcon tubes. The lysate was centrifuged at 180 x g for 5 minutes and the supernatant was transferred to SS34 centrifuge tubes. The pellets were lysed with 2 ml 0.1M Tris-EDTA (pH 9) and centrifuged at 180 x g for a further 15 minutes. The supernatant was collected again and added to the SS34 tubes, which were then carefully underlayered with 1ml 36% sucrose (36% in PBS) to form a sucrose cushion. The supernatant was centrifuged at 15000 rpm using the Sorvall RC 5B Plus Refrigerated Centrifuge (Marshall Scientific, USA), at 4 °C for 1 hour. The pellet was resuspended in 0.5 ml PBS and stored in aliquots at -80 °C for further use. The MVA-SARS2-SΔTM stock was titrated in RK13 cells.

2.6.2 Titration of recombinant MVA-SARS2-SΔTM stocks

Titration was done to determine the total concentration of virus present in the vaccine stocks using the Median Tissue Culture Infectious Dose (TCID₅₀).

2.6.2.1 50% Tissue culture infective dose (TCID₅₀)

The TCID₅₀ method determines the endpoint of the highest dilution of virus which produces a cytopathic effect in 50% of infected cells. This method was first described by Reed and Muench [226] where the titre is expressed as logTCID₅₀/ml using the following formula:

$$h = \frac{(\% \text{ wells infected at dilution next above } 50\%) - 50\%}{$$

$$(\% \text{ wells infected at dilution next above } 50\%) - (\% \text{ wells infected at dilution next below } 50\%)}$$

The titre from this method is not comparable with the counting of foci method therefore a formula is needed to convert the TCID₅₀ titre to pfu [227]:

$$pfu = (TCID_{50} \text{ titre}) \times (0.69)$$

A confluent monolayer of RK13 cells was trypsinized and counted as described in 2.2.3 and 2.2.4. Cells were diluted in cDMEM to a final concentration of 4 x 10⁵/ml and 100 µl aliquots were

dispensed into each well of a 96-well plate. The virus, MVA-SARS2-SΔTM was serially diluted tenfold (Figure 2.4) using DMEM (no FCS). A total of 100 µl virus was pipetted into each well using 8 wells per dilution. One column was left uninfected as a negative control (cells only). The plates were incubated under standard growth conditions and checked for fluorescence daily, for 3 days.

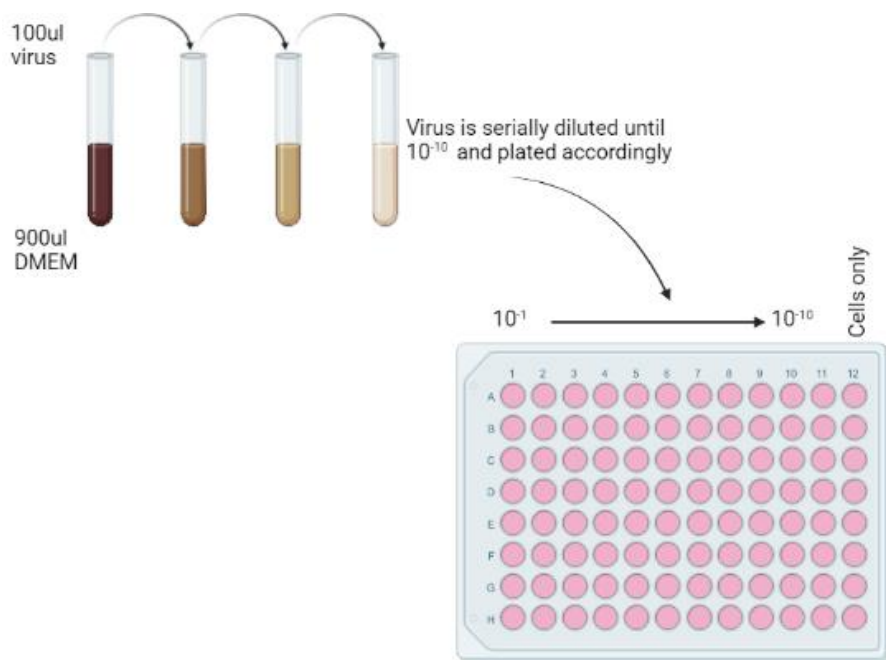


Figure 2.4. Virus titration. 10-fold serial dilutions of MVA-SARS-2-SΔTM were made and plated accordingly on a 96-well tissue culture plate containing 4×10^5 RK13 cells/ml. 100 µl virus was added into a falcon tube labelled “1” containing 900 µl DMEM (no FCS). The tube was vortexed and then 100 µl of the solution was added into the next tube labelled “2”, containing 900 µl DMEM. This process was repeated until the last dilution required (10^{-10}) and 100 µl of each dilution was dispensed into the wells accordingly. A row of cells was left uninfected to serve as a negative control. Plates were incubated and observed for cytopathic effect after 72 hours. Figure created using BioRender.com.

2.7. Immunologic evaluation of MVA-SARS-2-SΔTM in BALB/c mice

2.7.1 Mice vaccination regimen



Figure 2.5: Vaccination schedule used on BALB/c mice showing bleeds and inoculations. Balb/c mice were acclimatized from days -7 to 0, and pre-bled before intramuscular inoculation with 3×10^5 pfu on days 0, 21 and 42. Bleeds were taken on days 14, 35 and 56.

The immunogenicity of MVA-SARS-2-SΔTM was assessed in BALB/c mice. A group of five mice were inoculated intramuscularly with 3×10^5 pfu of the recombinant MVA-SARS-2-SΔTM, according to an experimental schedule (Table 2.4) that was approved by the University of Cape Town Animal Ethics Committee (reference UCTAEC020_024). Prior to the inoculations mice were anaesthetised with a mixture of ketamine/xylazine which was done with a peritoneal injection. Three inoculations were done at three week intervals with 50 µl MVA-SARS-2-SΔTM resuspended in 100 µl 1 X PBS (Gibco, South Africa). The vaccine was prepared by pipetting up and down to mix before being vortexed gently for 30 seconds. Mice were injected with 50 µl by needle in the right tibialis muscle.

2.7.1.1 Binding antibody Enzyme-Linked Immunosorbent Assay (ELISA)

The serum binding antibody ELISA was used to quantify SARS-CoV-2 spike glycoprotein antibody titres as previously described by Margolin *et al.*, [228].

A 96-well Maxisorb™ microtitre plate (NunC) was coated overnight at 4 °C with 10 ng/well of recombinant SARS-CoV-2 Spike Protein (S-ECD) (aa14-1213) His Tag Recombinant Protein (Invitrogen, USA).

The plate was washed three times with 1 X PBS (Gibco, South Africa) to remove any unbound antigen.

The plate was blocked with 5% blocking buffer (Appendix A) for 1 hour at room temperature to decrease non-specific binding. Five PBST (PBS containing 0.1% Tween (Roche, USA) washes followed, with tapping on paper towel to remove any residual buffer.

Pooled mice sera were diluted 1:10 and thereafter serially diluted 1:4 before plating in duplicate and incubation overnight. The plate was washed with PBST five times.

A goat polyclonal anti- mouse IgG HRP conjugate antibody (1:10 000) (ab97023) (Abcam, USA), was added and incubated at room temperature for 1 hour before repeating the five PBST washes.

The plate was developed by adding 100 µl/well of TMB ELISA Substrate (Abcam, USA) and incubating in the dark for 10 minutes to allow the signal to develop. The reaction was stopped by

adding 100 µl/well of 1N H₂SO₄. Readings were done on the SoftMax Pro software at 450nm and the data were calculated as previously described [229] before being plotted using GraphPad Prism.

2. 7.1.2 IFN γ - Enzyme-Linked Immune Absorbent Spot (ELISpot)

The ELISpot assay was used to measure the frequency of splenocytes secreting IFN γ as a measure of the induction of cellular immunity by the vaccine. A PVDF membrane 96-well plate was coated with mouse IFN γ SARS-CoV-2 capture antibody (1:200) and incubated at 4 °C overnight in a fridge.

The capture antibody was discarded, and wells were washed with 200 µl 1 x R10 ([1 ml Penicillin-Streptomycin antibiotic (Gibco, South Africa); 200mM L-Glutamine (Gibco, South Africa); 10 ml CTL-Test Medium (ImmunoSpot, Germany); 50mM 2-Mercapto-ethanol (Sigma-Aldrich, USA)]. Aliquots of 200 µl R10 were dispensed into each well and the plate was incubated for 2 hours at room temperature.

Spleens from the five vaccinated mice were harvested on day 56 and pooled. A cell suspension of splenocytes was prepared and centrifuged at 2500 rpm for 10 minutes on the Jouan CR422 Centrifuge (Thermo Scientific, USA). The supernatant was decanted, and the pellet was resuspended in 50 ml 1 X Rosewell Park Memorial Institute Medium 1640 with Glutamax (Gibco, South Africa) The solution was centrifuged again at 2500 rpm for 10 minutes, before resuspension in 1 X RPMI and removal of fibrin clots. After an additional round of centrifugation, the pellet was resuspended with 1 ml home-made Red Blood Cell Lysis buffer (Appendix A) to lyse red blood cells. The solution was mixed by inverting 5-6 times and then the samples was incubated for 1 -2 minutes at room temperature before being topped up with 1 X RPMI (Sigma-Aldrich, USA) to a final volume of 40 ml. The lysed cells were centrifuged at 1350 rpm for 7 minutes. The pellet was resuspended in 2 ml R10 growth media (Gibco, South Africa) and inverted several times for adequate mixing. The cells were counted as described in Section 2.2.4 and adjusted to a concentration of 5×10^6 / ml.

Peptides corresponding to the SARS-CoV-2 spike protein were synthesized (GenScript Biotech, USA), diluted in R10 medium to 0.2 µg/well, and used to stimulate splenocytes. The peptide pool included 316 peptides for the Severe Acute Respiratory Syndrome-related Coronavirus Virus 2

spike glycoprotein (cat# RP30020) (158 peptides for subunit A and 158 for subunit B) (Appendix C). A stimulant, ConA (Sigma-Aldrich, USA), was added at a concentration of 0.1 µg/well as a positive control to confirm the assay's functionality. An irrelevant peptide (0.2 µg/well) was included as a negative control. Peptides or stimulants were added in triplicate for each mouse. Additional controls of blank wells with R10 medium and no cells were added to determine background responses.

The blocking solution was discarded from each well and 100 µl peptides or stimulants were added, with ConA being added last. Splenocytes were added and plates were incubated at 37 °C, 5% CO₂ for 22-24 hours.

Cells were removed and wells were washed 3 times with 200 µl H₂O before being washed three times with PBST wash buffer (Appendix A). Excess buffer was removed by blotting the plate on a paper towel. Biotinylated detection antibody was added (1:250), and the plate was incubated for 2 hours at room temperature in the dark. The detection antibody was discarded, and wells were washed 3 times with PBST. The enzyme Streptavidin-HRP (0.5 mg/ml) (BD Biosciences, South Africa) was added prior to a 1-hour incubation at room temperature in the dark.

The Streptavidin solution was discarded, and plates were washed 3 times with PBST followed by three subsequent washes with 1 X PBS. The NovaRED Substrate (Vector Laboratories, USA) was added as per manufacturer's instructions. The reaction was allowed to continue for 10 minutes and thereafter was stopped by washing of the plate 5-6 times in running cold water. The plate was stored in the dark at room temperature overnight to facilitate before being viewed and spots counted with an automatic ELISPOT reader (CTL Technologies, Cleveland, Ohio) and the Immunospot Version 3.2 software. Spot numbers were averaged for the triplicate reactions per mouse.

The number of spots was averaged for each reaction from triplicate wells. The standard deviation and mean were calculated, adjusted and expressed as 1×10^6 spots forming units (sfu)/ml. The mean number of spots and standard deviation of the irrelevant peptide group were used as background responses. Positive responses to the SARS-CoV-2 spike A and B peptides were those greater than the mean plus two standard deviations. Background responses were subtracted from the positive SARS-CoV-2 spike A and B responses, and these were expressed as net sfu/ 10^6 splenocytes.

2. 7.1.3 Neutralization assays

Neutralizing antibody titres were assessed using a SARS-CoV-2 pseudovirus as previously described [228] for which I fully acknowledge Dr. Georgia Schafer (Full member, Institute of Infectious Diseases and Molecular Medicine) who conducted the assay. Briefly, the pseudoviruses were generated by co-transfection of HEK-293TT cells with an HIV plasmid pNL4-3.Luc.R-E- (aidsreagent #3418) and pcDNA3.3-SARS-CoV-2-spike Δ 18 prepared by Rogers *et al.*, [135]. Supernatants were harvested at 3 days post transfection and incubated with serially diluted sera (five-fold) heat-inactivated mouse plasma for 60 minutes at 37° C. HEK-293T cells expressing the ACE2 receptor were then transfected with the psuedovirus mixture and lysed at 3 days post infection using the Cell Culture Lysis Reagent (Promega). Luciferase activity was assessed using the GloMax[®] Explorer Multimode Microplate Reader (Promega) [230] and the Luciferase assay system (Promega). The half-maximal inhibitory dilution (ID₅₀) was calculated and plotted using a non-linear regression on GraphPad Prism [231].

CHAPTER 3:

RESULTS

This study entailed the construction and testing of a recombinant MVA encoding a modified form of the SARS-CoV-2 spike protein as a candidate. The spike antigen sequence was previously designed by Dr. Emmanuel Margolin to enable direct comparisons across different vector and subunit platforms, in a larger study beyond the scope of this thesis, as described in Section 1.8.6. For this project, the transfer vector, pMVA-FNK1, was constructed to generate the recombinant MVA expressing the SARS-CoV-2-SΔTM. BHK21 cells were infected with wildtype MVA and transfected with the transfer vector to enable homologous recombination to occur which resulted in the targeted integration of the antigen sequence into the viral genome. The recombinant virus, MVA-SARS2-SΔTM was purified from wildtype parental virus in RK13 cells through a series of passages, validated and characterized, before being grown to high titres and tested for immunogenicity in a mouse immunogenicity model.

The SARS-CoV-2 antigen used in this study was designed by Dr. Emmanuel Margolin (Post-doctoral scientist, Viral Vaccine Research Group, University of Cape Town) (Figure 3.1) as part of a larger initiative to compare different vector based and subunit platforms for SARS-CoV-2 vaccine development. As reported by Margolin *et al.*, the gene was optimized for expression in mammalian cells and the sequence was modified to improve processing, secretion and trimerization [228]. The native signal peptide was replaced with the Tissue Plasminogen Activator (TPA) leader sequence to improve secretion of the protein. The native RRAR furin cleavage site was replaced with a hexa-arginine sequence (RRRRRR) that was reported to improve the processing of HIV gp140 trimers in mammalian cells [232]. The coding sequence was prematurely terminated to remove the transmembrane and cytomembrane domains, resulting in a soluble antigen. A C-terminal trimerization motif (GCN4 Fibrin labelled FD) was added, followed by a his-tag (HHHHHH); both these sequences were preceded by upstream flexible linker sequences (GSGSGS and GSGGSGGSG respectively) [228].

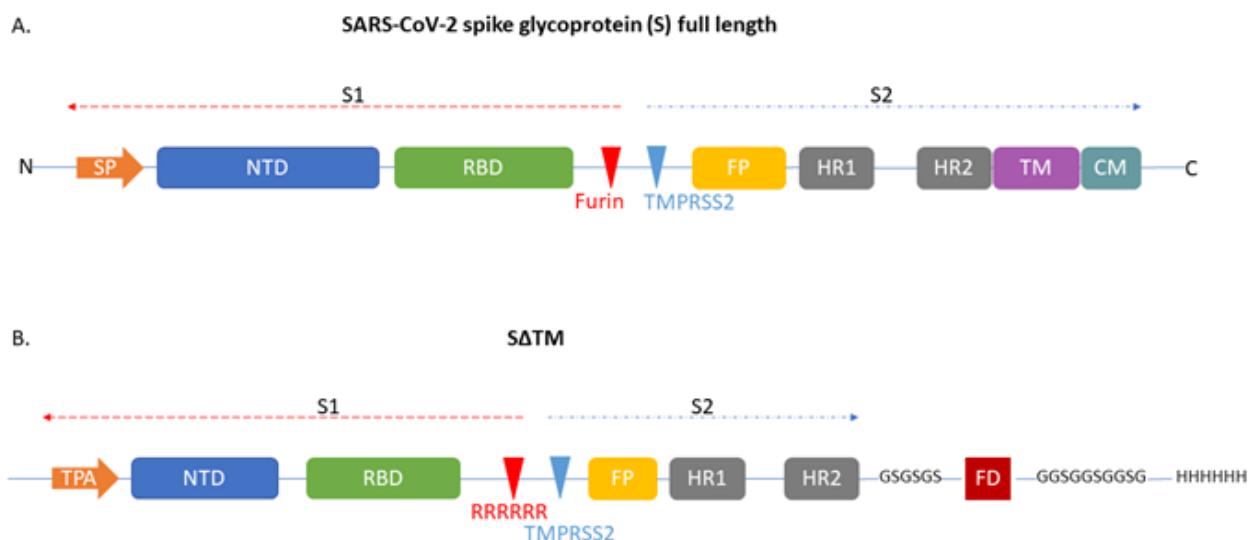


Figure 3.1: Design of the soluble SARS-CoV-2 spike antigen, SΔTM. The synthetic SΔTM gene sequence (B) was derived from the full-length SARS-CoV-2 S protein (A). The native signal peptide (SP) was replaced with the Tissue Plasminogen Activator leader sequence (TPA). The RRAR furin cleavage site was replaced with a hexa-arginine motif (RRRRRR). The protein was prematurely truncated to remove the transmembrane (TM) and cytoplasmic (CM) domains. A C-terminal trimerization motif (FD) and his-tag (HHHHHH) were added following flexible linker sequences (GSGSGS and GSGSGSGSGS, respectively). NTD = N-terminal domain; RBD = Receptor-binding domain; TMPRSS2 = Transmembrane protease, serine 2 recognition sequence; FP = Fusion peptide, HR1 = Heptad repeat 1; HR2 = Heptad repeat 2.

3.1 Construction of a mammalian expression plasmid encoding SARS-CoV-2 SΔTM

A mammalian expression plasmid expressing the SARS-CoV-2 SΔTM protein was constructed for use as a control for spike antigen expression. The expression plasmid was based on the pTHpCapR expression vector which was previously developed in our group [233]. The plasmid was obtained from a previous study where Dr. Tsungai Jongwe had cloned the HIV subtype C mosaic Gag antigen into the plasmid as a DNA vaccine [234]. The resulting plasmid was named pTJDNA4 whereas the plasmid backbone is referred to as pTHpCapR. Although the plasmid was originally developed as a DNA vaccine vector [233] it has also been used as a protein expression plasmid for HIV envelope antigen [235] and other recombinant viral glycoproteins that are structurally comparable to the SARS-CoV-2 spike protein [229].

The plasmid contains the gene of interest under the transcriptional control of the human cytomegalovirus (CMV) promoter which is downstream of a porcine circovirus enhancer element. The enhancer element has been shown to increase immunogenicity of plasmid vaccines as well as decreasing the dose necessary to induce immunity against prototype vaccine antigens [233].

To derive the plasmid in this project, the synthetic SARS-CoV-2-SΔTM gene, commercially synthesized by GenScript and provided in the pUC57 plasmid backbone, was cloned into the *HindIII* and *EcoRI* sites of the expression vector in place of the Gag insert (Figure 3.2).

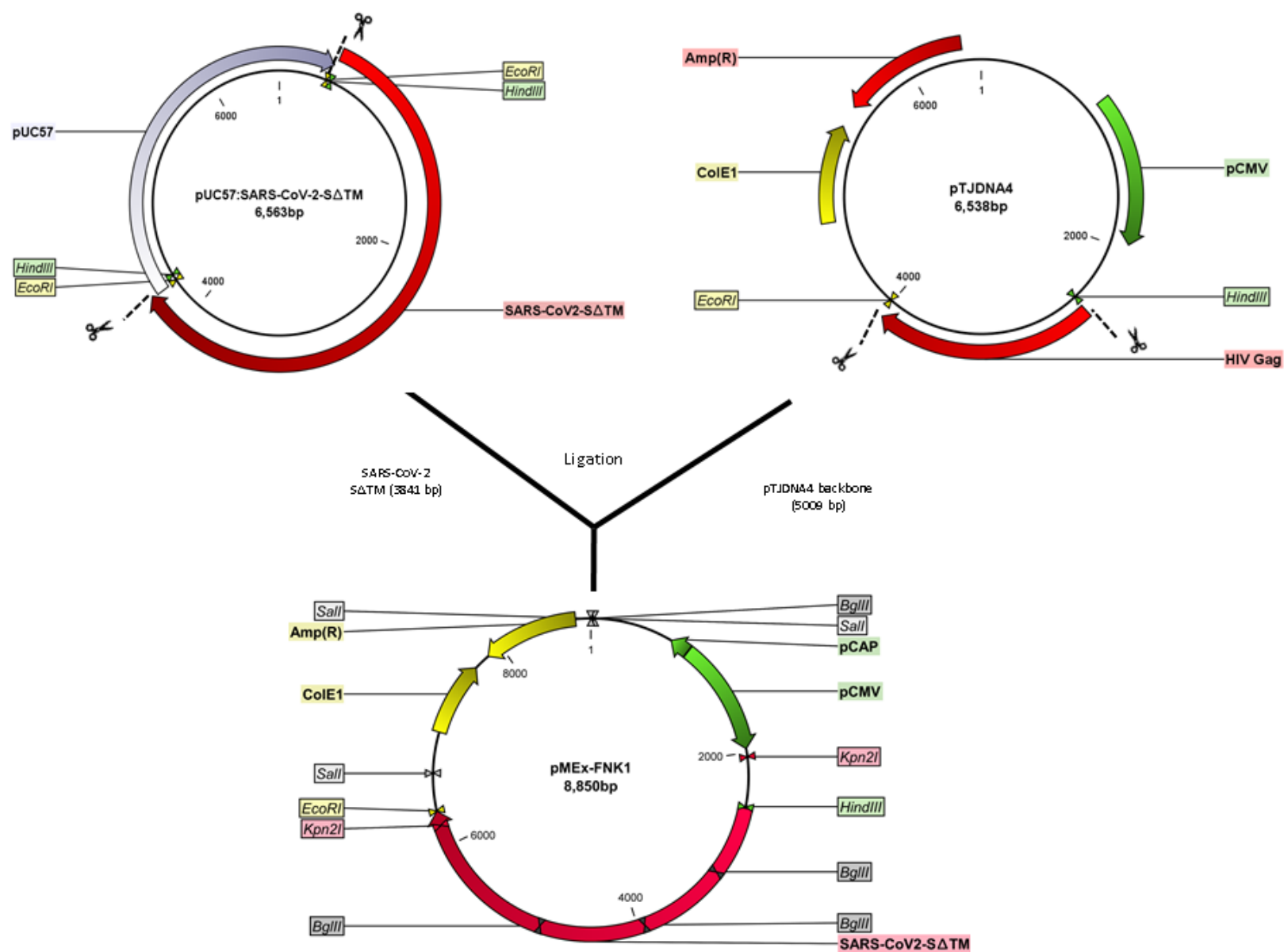


Figure 3.2: Cloning strategy used for the construction of pMEx-FNK1 mammalian expression plasmid encoding SARS-CoV-2 SΔTM. The pUC57:SARS-CoV-2-SΔTM plasmid and pTJDNA4 plasmids were both digested with *EcoRI* and *HindIII* to release the SΔTM gene and vector backbone respectively. The gene was ligated into the vector backbone, thereby replacing the Gag insert, to form the plasmid pMEx-FNK1. (ColE1 Origin = *E.coli* origin of replication, Amp^R = Ampicillin resistance gene, pCMV = human CMV promoter, pCAP = porcine circovirus type 1 enhancer element).

pUC57:SARS-CoV-2-SΔTM was digested with *EcoRI* and *HindIII* enzymes to release the SΔTM gene. pTJDNA4 was simultaneously digested with the same enzymes to release the vector backbone. Restriction products were resolved on a 0.8% agarose gel and fragments corresponding to the pTJDNA4 backbone (4.5 kb) and the SARS-CoV-2-SΔTM gene (3.8 kb) were recovered from the gel (Figure 3.3). The purified gene coding sequence was ligated into the pTJDNA4 vector backbone, thus forming the expression vector pMEx-FNK1. *E.coli* cells were transformed with the ligation products. Putative clones were grown in LB media and subjected to a DNA isolation. The resulting DNA samples were screened with restriction endonucleases *EcoRI* and *HindIII* to identify clones containing the SΔTM gene (Figure 3.3). Lanes 1, 3, 4, 5 and 6 show fragments of the correct size confirming the successful generation of pMEx-FNK1.

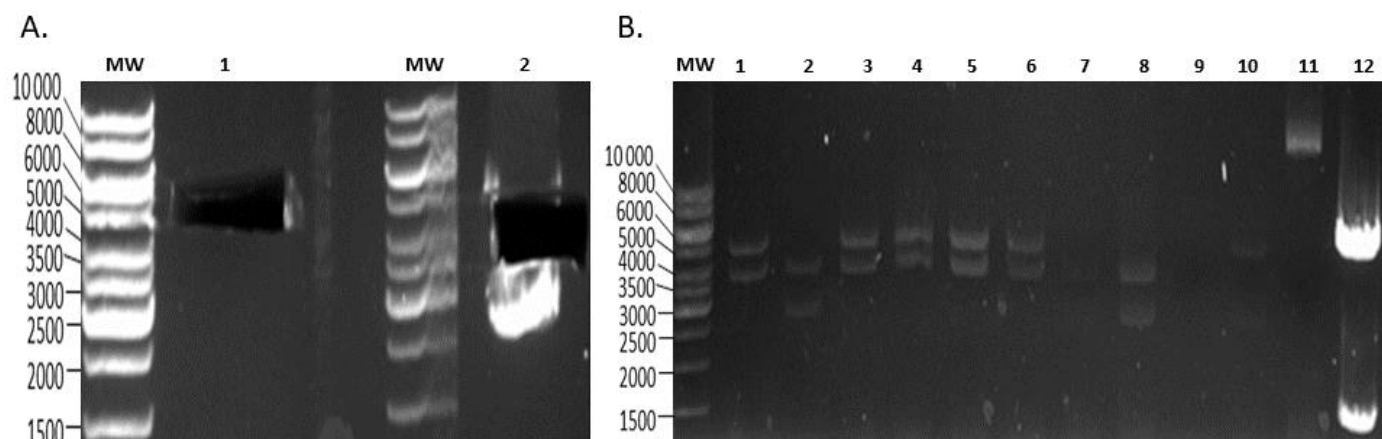


Figure 3.3: Construction of pMEx-FNK1 by subcloning the SARS-CoV-2-SΔTM gene sequence from pUC57 into the pTHpCapR backbone derived from pTJDNA4. A) Recovery of pTHpCapR backbone and SARS-CoV-2-SΔTM gene following restriction digest of pUC57: SARS-CoV-2-SΔTM and pTJDNA4 with *EcoRI* and *HindIII*. 1) pTJDNA4 backbone (4.5 kb); 2) SARS-CoV-2-SΔTM (3.8 kb). B) Screening of putative pMEx-FNK1 clones by restriction digest with *EcoRI* and *HindIII*. Samples were subjected to electrophoresis on a 0.8% agarose gel alongside a 5μl aliquot of GeneRuler 1kb DNA Ladder (MW). 1 – 10) Putative clones; 11) Uncut plasmid DNA from putative clone pMEx-FNK1; 12) pTJDNA4.

Clone 5 was propagated in liquid media and plasmid DNA was isolated for expression work. The integrity of the clone was further verified using restriction enzyme mapping with a series of enzymes as indicated in Table 3.1 and Figure 3.4. In the gel electrophoresis of this, the band of size 28 bp for the *SalI* enzyme was too small to visualize whilst the 958 and 811 bp bands for the *BglII* enzymes were run off the gel. Sequencing was conducted across the cloning junction using

primers pMEx-F (5'-GCAGTGTAGTCTGAGCAG-3') and pMEx-R (5'-CAAACAACAGATGGCTGG-3') which bound to regions of the vector flanking the insert.

Table 3.1: Restriction endonucleases used to confirm the integrity of pMEx-FNK1

Restriction Enzyme (s)	Expected products (bp)
<i>EcoRI</i> and <i>HindIII</i>	5004, 3846
<i>Sall</i>	6636, 2186, 28
<i>Kpn2I</i>	4691, 4159
<i>BglII</i>	3963, 3118, 958, 811,

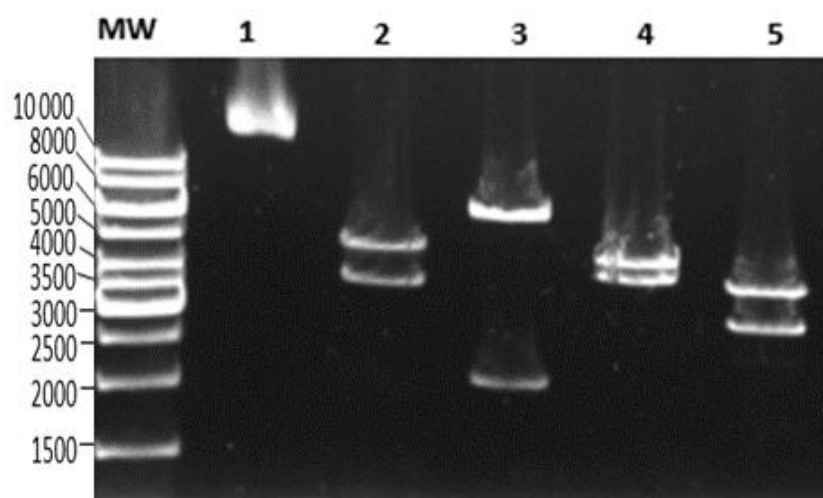


Figure 3.4: Agarose gel electrophoresis of restriction fragments derived from the cloned plasmid, pMEx-FNK1. 1) No endonuclease control; 2) *EcoRI* and *HindIII*; 3) *Sall*; 4) *Kpn2I*; 5) *BglII*. Samples were analysed by 0.8% agarose gel electrophoresis alongside an aliquot of 5 µl GeneRuler 1 kb DNA Ladder (MW)

3.1.1 Validation of SARS-CoV-2 SΔTM expression from the pMEx-FNK1 expression vector

Human Embryonic Kidney (HEK293) cells were transfected with pMEx-FNK1 to transiently express the antigen. As the plasmid does not contain a reporter gene, the transfection efficiency could not be directly determined, thus a positive control for transfection efficiency was included where HEK293 cells were transfected with peGFP, a plasmid expressing eGFP [236].

At 72 hours post infection, there was visible green fluorescence of cells (Figure 3.5) in the positive control sample, which suggested that the transfection was successful. Cell lysate and media were therefore harvested for Western blotting which is described in Section 2.5.3.

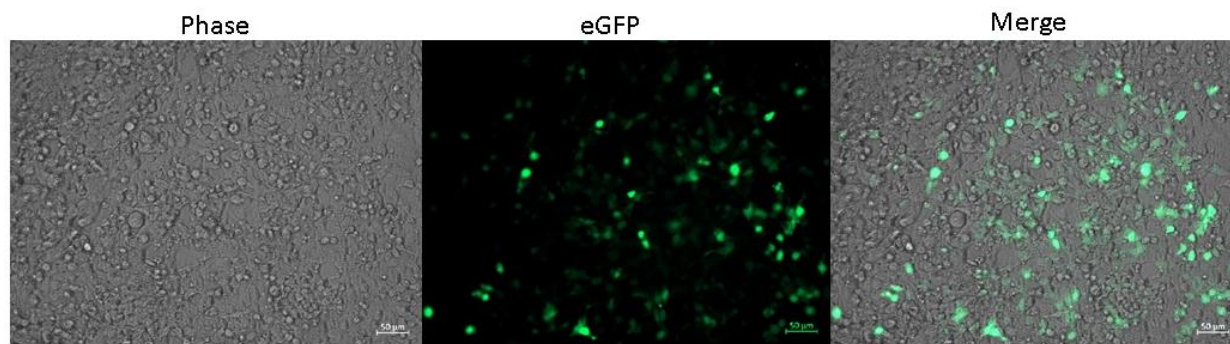


Figure 3.5: Visualization of eGFP expression as a surrogate marker for successful transfection of HEK293 cells. Cells were transfected with a transfection mixture comprising of peGFP plasmid and X-tremeGENE™ transfection mixture. The plate was incubated for 72 hours and viewed under a Zeiss Axio Vert.A1 fluorescent microscope over time to monitor eGFP expression as confirmation that the transfection was successful.

The construction of pMEx-FNK1 which expressed the SARS-CoV-2-SΔTM antigen enables the plasmid to be used as a positive control for expression of the immunogen from the recombinant MVA-SARS2-SΔTM vaccine. Western blot analysis showed that, despite the presence of a furin cleavage site, only a single product was observed of ~180 kDa which is the expected size of the uncleaved SΔTM protein. There were two non-specific bands in between 55 and 72 kDa in the negative cells only control, but nothing at ~180 kDa indicating that the protein of ~180 kDa in the test lane was SΔTM. The protein was present in both the media and lysate, with the media having a stronger signal. This confirms that the protein is being secreted as was intended by the inclusion of the TPA leader sequence. Lane 3 yielded a double band which could be as a result of two differentially modified products of the protein. Given that the signal yielded by the western blot is highly specific it is plausible that this could be the result of two differentially modified products

of the protein. Whilst not impossible, it is unlikely to represent two distinct glycoforms due to the high number of potential N-linked glycosylation sites which would more likely yield a more diffuse smear if there was high levels of heterogeneity.

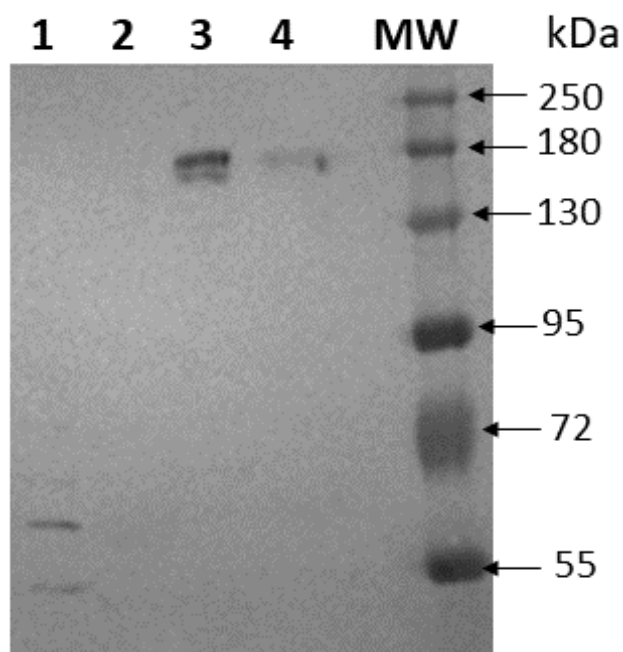


Figure 3.6: Western blot analysis to confirm expression of SARS-CoV-2-SΔTM in transfected HEK293 cells. **1**, media from untransfected HEK293 cells; **2**, lysate from untransfected HEK293 cells; **3**, media from HEK293 cells transfected with pMEx-FNK1; **4**, lysate from HEK293 cells transfected with pMEx-FNK1; **MW**, Ladder. The cell lysate and media were harvested after 72 hours. Samples were run alongside a 10µl aliquot of Colour Prestained Protein Standard, Broad Range.

3.2 Making of recombinant MVA

Generation of recombinant MVA requires the homologous recombination of wildtype MVA genomic DNA with a transfer vector containing homologous regions to allow for the integration of foreign genes into the MVA genome. In this study, a well characterized transfer vector was used to generate the recombinant virus which incorporated several elements to aid in the process [235]. In addition to the antigen of interest, the foreign gene cassette included a gene (K1L) to allow for positive selection of the recombinant as well as a fluorescent marker gene (eGFP) to enable the visualization of the recombinant.

3.2.1 Construction of transfer vector pMVA-FNK1

The backbone of the transfer vector encoding SARS-CoV-2-S Δ TM was derived from pSSPEx: Du151 Gly V2.1, as described by Van Diepen and colleagues [235]. The pSSPEx: Du151 Gly V2.1 plasmid was developed in a separate study and instead of the CAP256 had the Du151 gene. pSSPEx: Du151 Gly V2.1 contains the gene for a synthetic Du151 HIV-1 Envelope gp150 gene under the control of the mH5 promoter, a K1L selection gene under the control of the vaccinia virus pSS promoter and an eGFP gene under control of the vaccinia virus p7.5 promoter. These genes are placed between sequences homologous to the 3' ends of the convergent MVA ORFs I8R and G1L. K1L is a host range gene from vaccinia virus which allows for viral growth in the rabbit kidney cell line, RK13. RK13 cells, are non-permissive to the wildtype MVA virus, but permissive to the virus expressing K1L. This means that only the recombinant MVA can grow in RK13 cells thereby enabling its purification from unwanted parental virus. Additionally, eGFP is present as a fluorescent marker to enable the growth of the recombinant virus to be monitored. In order to insert the SARS-CoV-2 S Δ TM gene into MVA, the HIV Env gene in the pSSPEx Du151 Gly V2.1 plasmid vector backbone was replaced with the SARS-CoV-2 S Δ TM gene. Figure 3.7 shows the cloning strategy employed to modify the transfer vector. The resulting plasmid has the SARS-CoV-2 S Δ TM gene downstream of the vaccinia virus mH5 promoter.

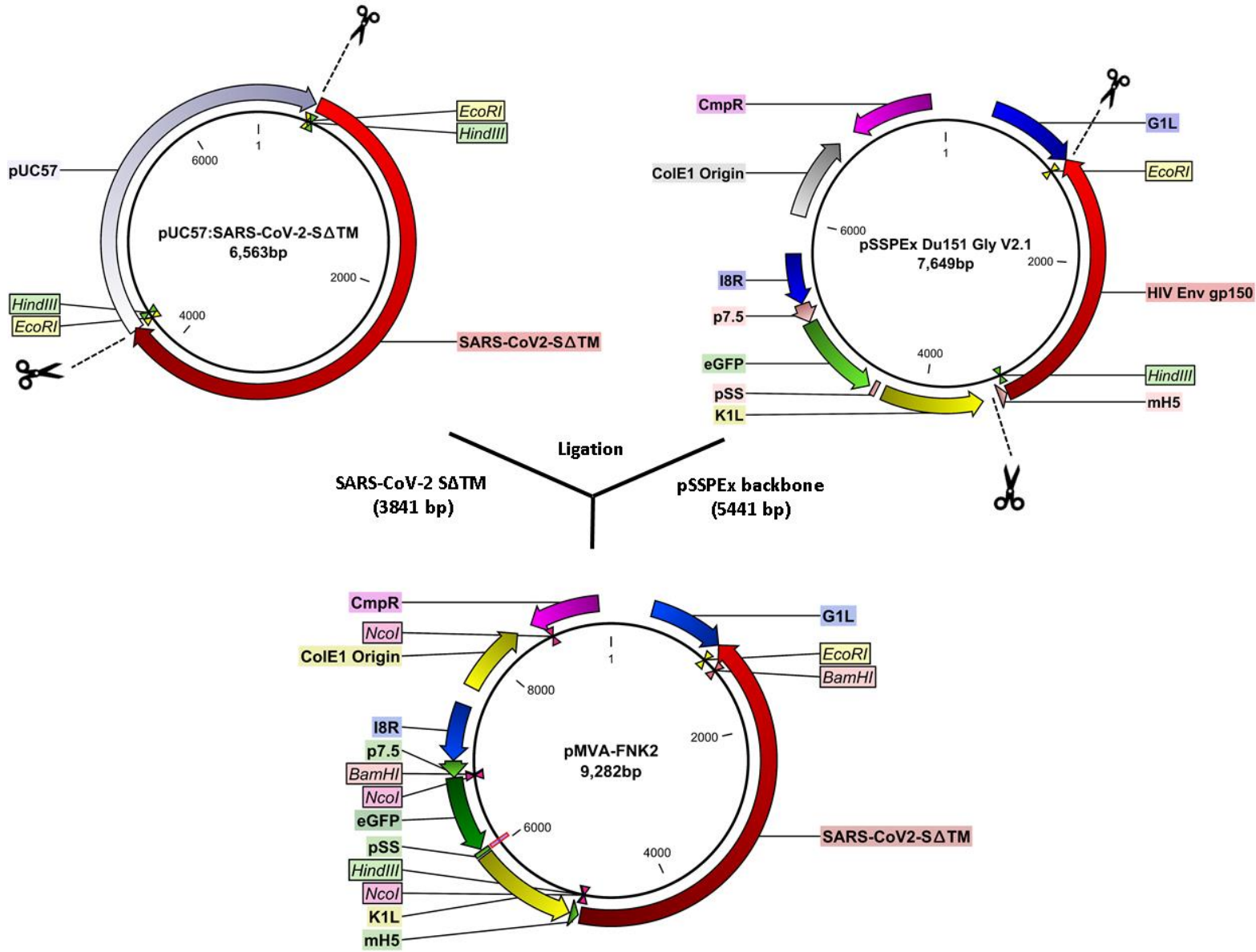


Figure 3.7: Overview of the cloning strategy used for the construction of transfer vector pMVA-FNK1. The pUC57:SARS-CoV-2-SΔTM and pSSPEX Du151 Gly V2.1 plasmids were both digested with *EcoRI* and *HindIII* to release the SΔTM gene and pSSPEX vector backbone respectively. The gene was then ligated into the vector backbone to form the plasmid pMVA-FNK2. (ColE1 Origin = *E.coli* origin of replication, *CmpR* = Chloramphenicol resistance gene, *G1L* = MVA right flank, *I8R* = MVA left flank; *eGFP* = green fluorescent protein, *K1L* = host range gene, *p7.5* = early/late vaccinia virus promoter, *pSS* = synthetic vaccinia virus promoter, *mH5* = vaccinia virus modified H5 promoter).

Restriction enzymes *EcoRI* and *HindIII* were used to digest the pSSPEX Du151 Gly V2.1 plasmid thus freeing the backbone. This in turn enabled the insertion of the SARS-CoV-2-SΔTM gene, which was excised from pUC57 using the same enzymes, *EcoRI* and *HindIII*. This approach generated complementary sticky between the vector backbone and the antigen gene sequence. As described in Section 2.1.3, restriction products were resolved on a 0.8% agarose gel and fragments corresponding to the pSSPEX Du151 Gly V2.1 backbone (5.5 kb) and the SARS-CoV-2-SΔTM gene (3.8 kb) were recovered from the gel (Figure 3.8A). The gene was ligated into the pSSPEX Du151 Gly V2.1 in place of the HIV *Env* gene, thus forming the transfer vector pMVA-FNK1. *E.coli* cells were transformed with the ligated products. Putative clones were then grown in LB media and subjected to small-scale DNA isolation. The resulting DNA samples were screened with restriction endonucleases *EcoRI* and *HindIII* to identify clones of the desired recombinant plasmid and the resulting restriction fragments were resolved by agarose gel electrophoresis (Figure 3.8B). The desired clones were expected to yield products of sizes 3.8 kb and 5.5 kb which correspond to the SΔTM insert and vector backbone, respectively.

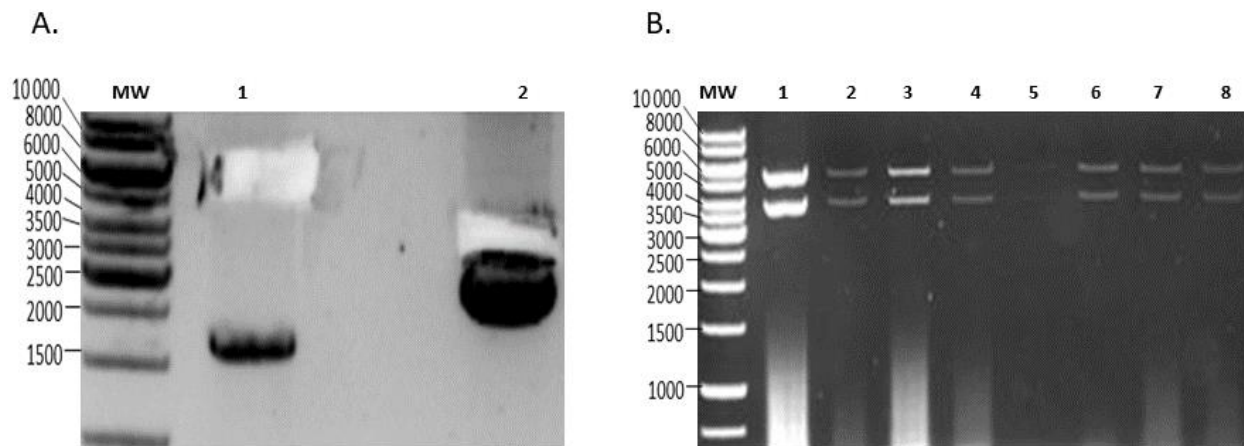


Figure 3.8: Construction of transfer vector pMVA-FNK1 containing the SARS-CoV-2 SΔTM gene sequence and screening for putative clones. A) Recovery of the pSSPEX backbone and SARS-CoV-2-SΔTM gene following restriction digestion and resolution of the resulting DNA products by agarose gel electrophoresis. 1) pSSPEX Du151 Gly V2.1 backbone; 2) pUC57:SARS-CoV-2-SΔTM. Both plasmids were digested with restriction endonucleases *EcoRI* and *HindIII* to free the pSSPEX vector backbone and the SΔTM gene respectively. B) Screening of putative pMVA-FNK1 clones. DNA was digested with restriction endonucleases *EcoRI* and *HindIII*. Samples were subjected to electrophoresis on a 0.8% agarose gel at 100 V for 45minutes alongside a 5μl aliquot of GeneRuler 1 kb DNA Ladder (MW).

One of the putative clones (lane 3) was grown up in 100 ml LB media and the culture was subjected to a large-scale DNA isolation to recover plasmid DNA for transfections. The integrity of the plasmid was also verified by restriction enzyme mapping as shown in Table 3.2 and Figure 3.9. The clone was mapped using restriction endonucleases. This yielded fragments of the expected sizes indicating that the correct eluted fragment was cloned. *EcoRI* and *HindIII* (Figure 3.8, lane 2) produced 3 fragments of 3842, 5441 and 9282 bp instead of 2 fragments of 3842 and 5441 bp. This could be explained by partial digestion of the full linear fragment.

BamHI and *NcoI* produced fragments of the anticipated size, indicating that the plasmid was indeed correct.

Table 3.2: Restriction endonucleases used to confirm the integrity of the plasmid pMVA-FNK1.

Restriction enzyme (s)	Expected products (bp)
<i>EcoRI</i> - <i>HindIII</i>	3842 bp, 5441 bp
<i>BamHI</i>	3731 bp, 5552 bp
<i>NcoI</i>	1808 bp, 1838 bp, 5577 bp

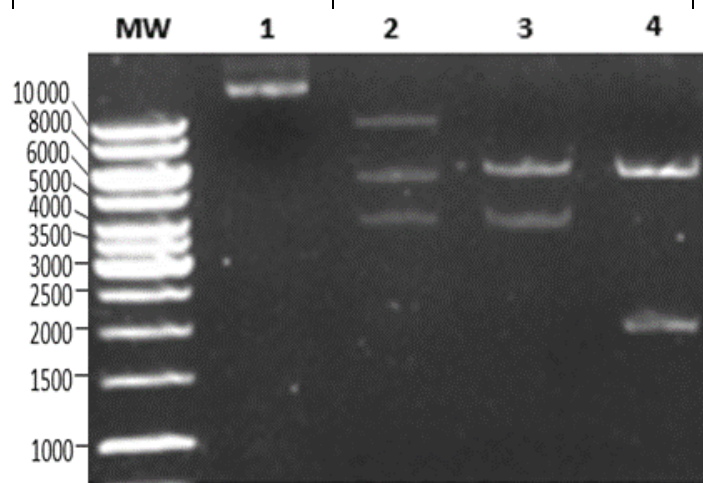


Figure 3.9: Confirmation of the integrity of the plasmid, pMVA-FNK1. The plasmid was

digested with restriction endonucleases. 1) No endonuclease control; 2) *EcoRI* and *HindIII*; 3) *BamHI*; 4) *NcoI*. Samples were analysed by 0.8% agarose gel electrophoresis alongside an aliquot of 5 µl GeneRuler 1 kb DNA Ladder (MW)

3.2.2 Generation of recombinant MVA by homologous recombination

To construct the recombinant MVA, MVA-SARS2-SΔTM, a monolayer of BHK21 cells was infected with wild type MVA at a multiplicity of infection (MOI) of 0.5 and 1. The infected monolayer was then transfected 2 hours later with pMVA-FNK1, as described in Section 2.4.1. The cells were viewed after 72 hours to assess efficiency of the experiment which was assessed based on morphology, density and fluorescence (Figure 3.10).

The morphology and density of the cells in each well differed. Cells in the uninfected control (D) were elongated and thin, typical of healthy BHK21 cells, whilst all the other wells had cells which were rounded, due to infection with virus which caused the observed cytopathic effect (Figure 3.10). Viruses cannot reproduce on their own, and require the host cell machinery to do so. In permissive cells such as BHK-21s which were used in this project, MVA typically causes inhibition of host cell protein synthesis, induction of cell damage, and eventually apoptosis [237], [238]. This results in the cytopathic effect of cells appearing rounded.

The density of cells in the infected and transfected wells was considerably lower than that of the controls, which provided more evidence of the impact of virus on the infected cells. As expected, green fluorescence was observed in the experimental wells with both wild type MVA and pMVA-FNK1 plasmid. This was due to the poxvirus specific promoter, p7.5 driving eGFP expression when cells contained both the virus and the transfer vector. This fluorescence could be due to either transient expression of eGFP in MVA-infected cells, or to eGFP being expressed from a recombinant MVA that was generated by homologous recombination. The higher MOI of 1 in row B showed more fluorescence than the MOI of 0.5 in row A, probably due to a higher number of cells being both infected and transfected, which is required for there to be expression of eGFP under control of the p7.5 poxvirus promoter. The BHK21 cells transfected with pMVA-FNK1 only in row C did not show fluorescence due to the lack of MVA present which is required for expression from the poxvirus promoter. However, the cells did exhibit some cytopathic effect which was likely due to the toxic effect of the transfection reagent on the cells. There is a “tug-of-war” between avoiding toxicity from the inherently toxic transfection reagent, and ensuring efficient transfection. This means a certain level of cytopathic effect will be visible when transfection reagent is used. In row D, the BHK21 cells were not infected with wildtype MVA, nor transfected with pMVA-FNK1 thus there was no expression of eGFP. These cells looked

healthy. Cells in rows E and F were round and lifted due to the infection with wild-type MVA virus. These did not fluoresce because of the absence of the transfer vector that encodes eGFP.

Cells from A and B were used for isolation of the recombinant MVA, which is summarized in Figure 3.11. The presence of the K1L gene in the recombinant allowed for growth of the recombinant only, in RK13 cells as described in Section 3.2.1.

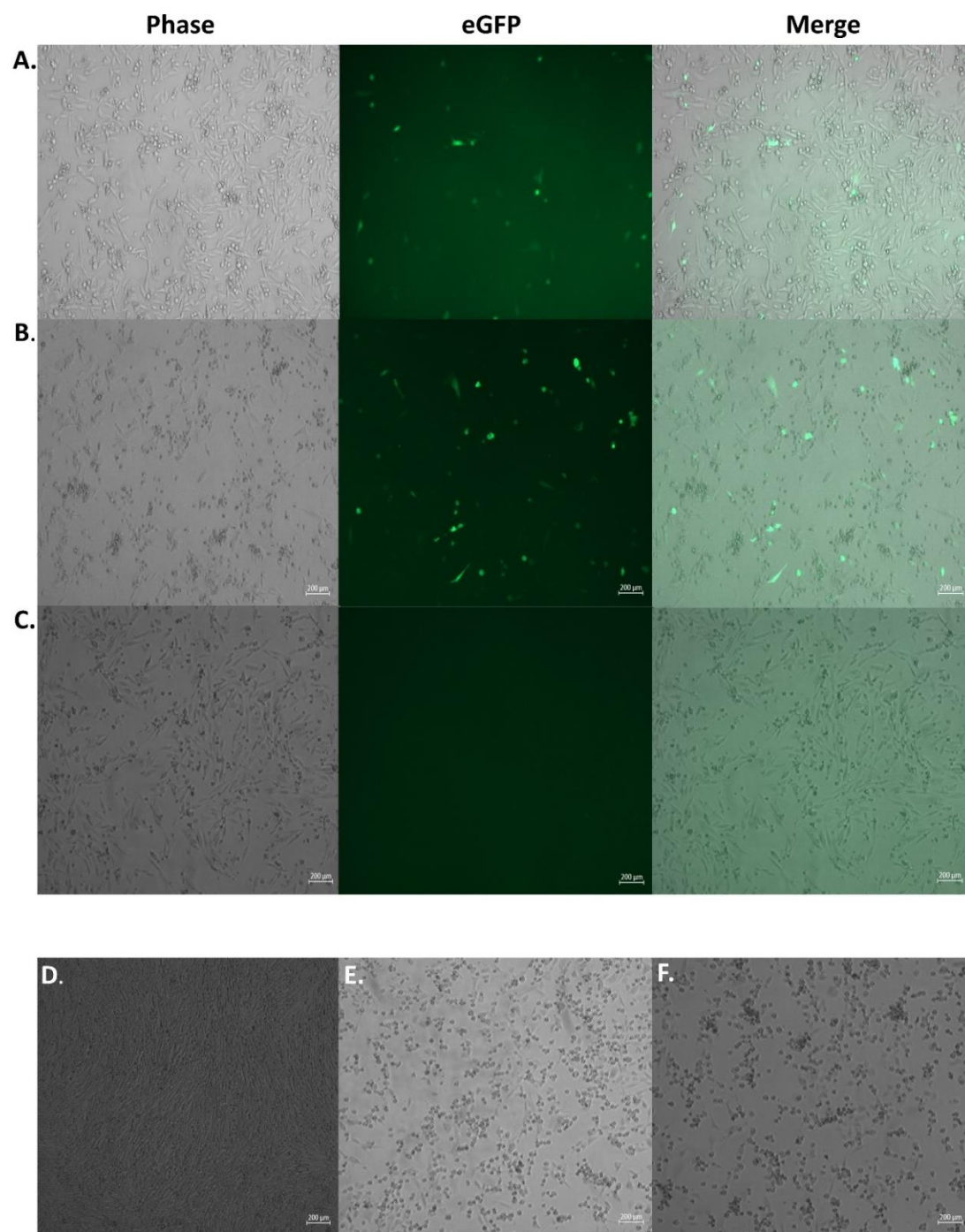


Figure 3.10: Expression of eGFP in BHK21 cells infected with wildtype MVA and transfected with pMVA-FNK2 plasmid encoding the Δ TM protein. A) BHK cells infected with MVA at an MOI of 0.5 and transfected with pMVA-FNK1. B) BHK cells infected with MVA at an MOI of 1 and transfected with pMVA-FNK1. C) Transfection control comprising BHK cells transfected with pMVA-FNK2 only. D) Cells only control comprising uninfected and untransfected cells. E) Infection control comprising BHK cells infected with MVA only at an MOI of 0.5. F) BHK cells infected with MVA only at an MOI of 1. Cells were visualized under phase contrast using transmitted light to visualize cell morphology (left panel), fluorescent light to show eGFP expression (central panel), and a merge of the two (right panel). Images are shown at 3 days post infection. D-F showed no green fluorescence

ad therefore only images viewed under transmitted light are shown (Zeiss Axio Vert.A1 fluorescent microscope at 5X magnification). Scale bars represent 200 μm .

Recombinant MVA was purified in RK13 cells through a series of passages where cells infected with putative recombinant were lysed by freeze/thawing and the lysate was used to infect fresh RK13 cells. Cell lysate from passages 1-3 containing multiple foci was used to infect cells, whilst cell lysate from wells with a single fluorescing focus was used at passage 4 and carried forward to passage 5 to purify the recombinant. Cells were infected with cell lysate from passage 5, and passaging continued until passage 9 where the recombinant was completely purified from parent MVA (Figure 3.11). PCR was performed at passages 7 and 9 to confirm that the recombinant was isolated from the parent MVA, whilst western blot analysis was conducted at passage 9 to confirm that the spike protein was being expressed.

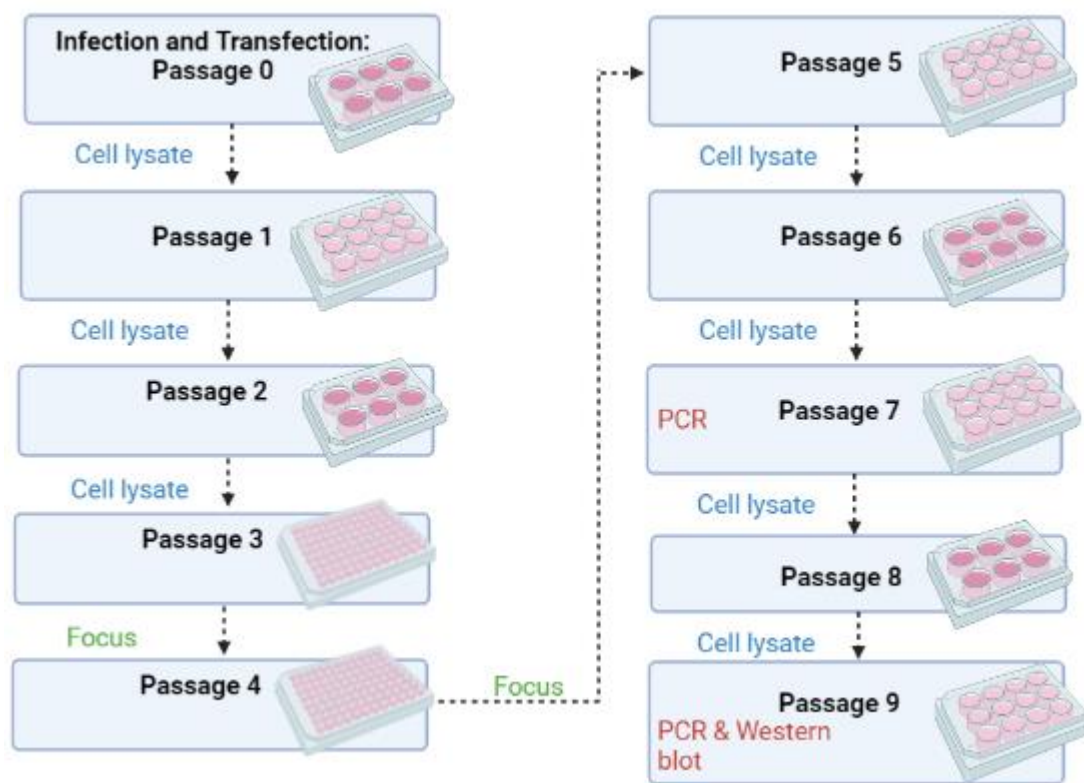


Figure 3.11: Flow diagram outlining the process used to generate and purify recombinant MVA-SARS2- ΔTM in mammalian cells. BHK21 cells were infected with parent MVA and transfected with pMVA-FNK1 to generate the recombinant MVA, MVA-SARS2- ΔTM . Crude cell lysate containing putative recombinant virus was used to infect RK13 cells to purify the desired recombinant from undesired parent virus. Cell lysate was used from passages 1-3 whilst lysate from wells with a single fluorescing focus was used to purify the recombinant in passages 3 and 4. Purification in RK13 cells continued using cell lysate in passages 5-9. The diagram outlines the tissue culture plates used for each passage, and whether cell lysate from wells with multiple green foci or lysate from a single focus was used for the next infection. Stages at which PCR and Western Blot were performed are also shown. Figure was created using BioRender.com.

Many fluorescent cells were visible after the first passage in RK13 cells, (P1); at this stage the fluorescence could still be due to transient expression of eGFP in MVA-infected cells. By P2 the fluorescence was clearly present in clumps of cells as opposed to individual cells, suggesting a recombinant virus had been generated and was spreading from a single infected cell (Figure 3.12). At P3 the virus continued to spread forming more clumps of fluorescent cells. Vaccinia viruses produce a vaccinia virus growth factor (VGF) which stimulates proliferation of cells thus facilitating the spread of infection, which causes clustering of cells as virus particles spread to neighbouring cells [239], [240]. The clumping could also be due to cell apoptosis as a response to, and a way to control the spread of viral infection. At passage 4, RK13 cells were seeded into a 96-well tissue culture plate to isolate single foci. The cell lysate from P3 was serially diluted in order to completely dilute out the parental virus that could still be present yet undetectable, and to isolate a single fluorescent focus. A single fluorescing focus was visible at P4, and this was used going forward. The non-fluorescing foci were no longer visible in most wells by P4, which suggested that residual parental MVA had largely been eliminated. The wells without any non-fluorescing foci were used. By passage 7 the green fluorescence had spread and there was no indication that parent MVA was present. At passage 9 only fluorescing foci were present, which suggested the successful purification of recombinant MVA.

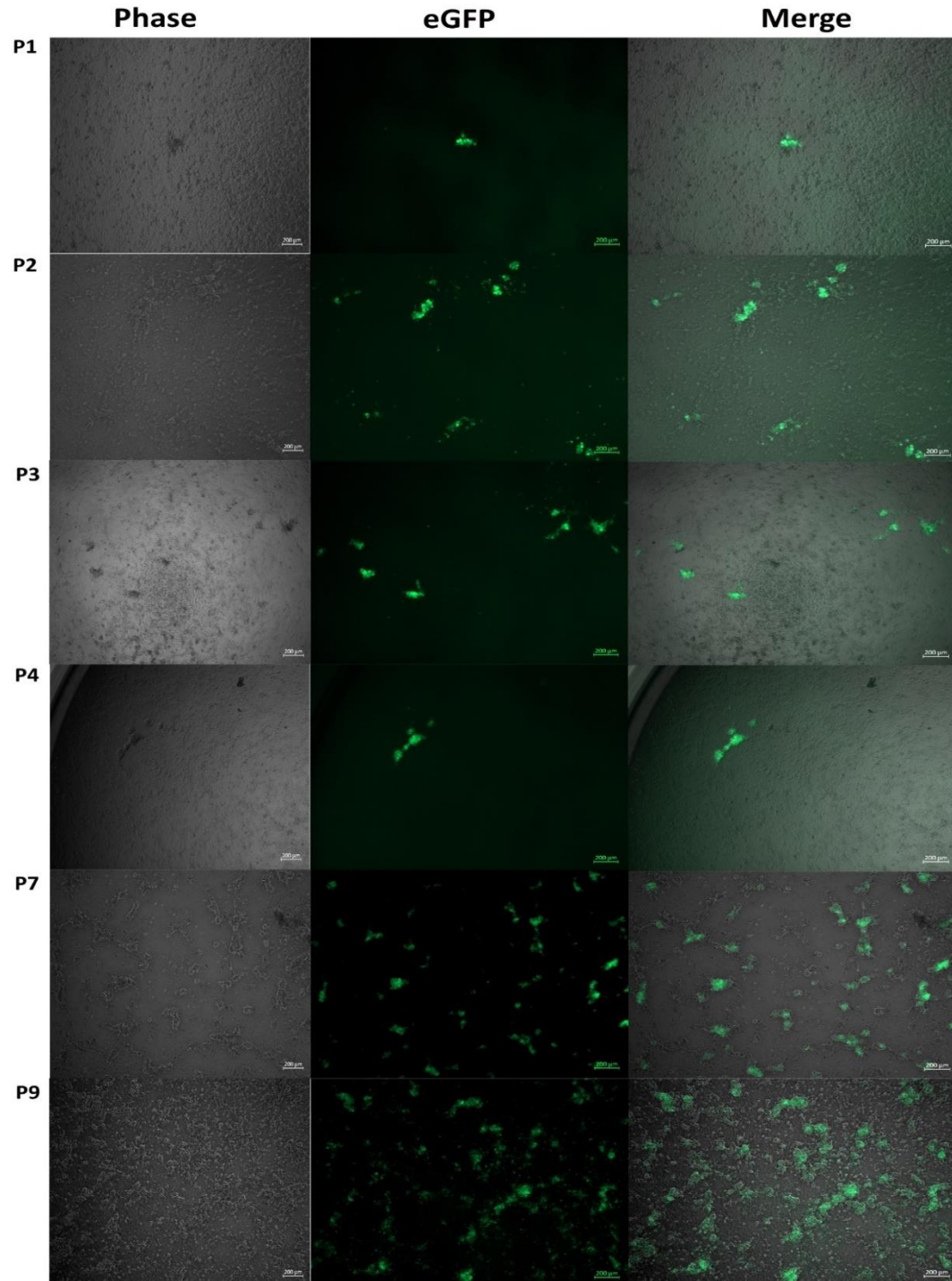


Figure 3.12: Enrichment of MVA-SARS-2-SΔTM by serial passage in RK13 cells. P1, P2, P3, P4, P7 and P9 refer to passages 1, 2, 3, 4, 7 and 9 respectively. eGFP is expressed from the recombinant virus. Cells were visualized under UV light at 3 days post infection. At each passage images were taken using transmitted light, fluorescent light and merged light. (Zeiss Axio Vert.A1 fluorescent microscope at 5X magnification. Scale bars represent 200 µm).

3.3 Validation and characterization of recombinant MVA-SARS-2-SΔTM by PCR and Sequencing

To confirm that the recombinant was correct with respect to the site of insertion of the foreign gene cassette and retention of the gene of interest, the region of recombination was amplified by PCR and sequenced, as described in Section 2.5.2.

3.3.1 PCR to confirm integrated gene cassette in the recombinant virus

A series of PCR reactions were used to verify the successful integration of the gene cassette into the MVA genome and to confirm that no residual parent virus was present. Using primer sets 1 (I8Rf2 and eGFPrev), and 2 (PEPV DRfor and G1Lr2), the left and right ends, respectively, of the gene cassette were amplified (Figure 3.12 (I)). Expected fragments of 800 bp and 4713 bp were generated for primer sets 1 (left end) and 2 (right end), respectively (Figures 3.13 II A and Figure 3.13 II B respectively). Primer set 3, which covers the entire gene sequence, was not able to amplify the large fragment of 9283 bp for the recombinant. However, the PCR successfully amplified a product of 1300 bp for the parent MVA. No fragment of 1300 bp was amplified from the putative recombinant. This indicated that there was no residual parent virus present in the purified recombinant.

3.3.2 Sequencing to confirm the genetic integrity of the DNA in the recombinant virus

The genetic integrity of the recombinant MVA-SARS2-SΔTM was confirmed by Sanger sequencing of the PCR products described in Section 3.3.1. Specifically, the 4713bp PCR product shown in Figure 3.13 II B was sequenced. The resulting sequence was analysed on Qiagen CLC Sequence Viewer by comparing the sequence runs of the PCR product to the known sequence of the SΔTM antigen. The genetic integrity of MVA-SARS2-SΔTM was successfully confirmed. The sequence of the SARS-CoV2-SΔTM can be found in Appendix B.

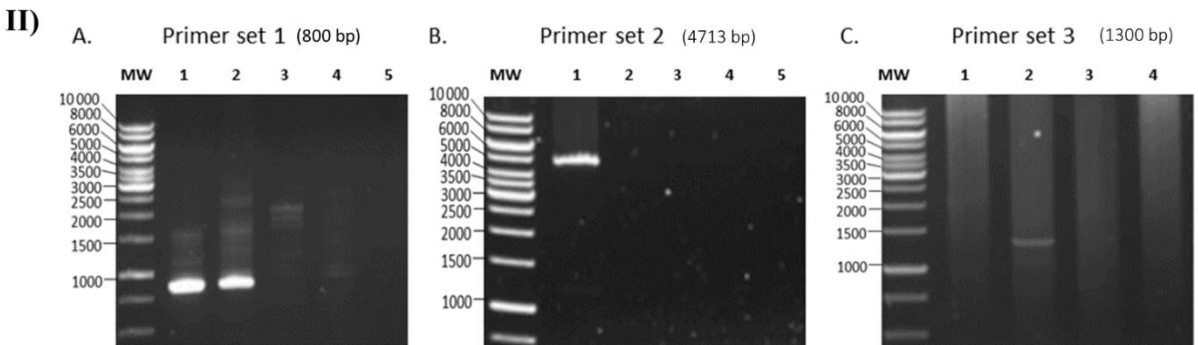
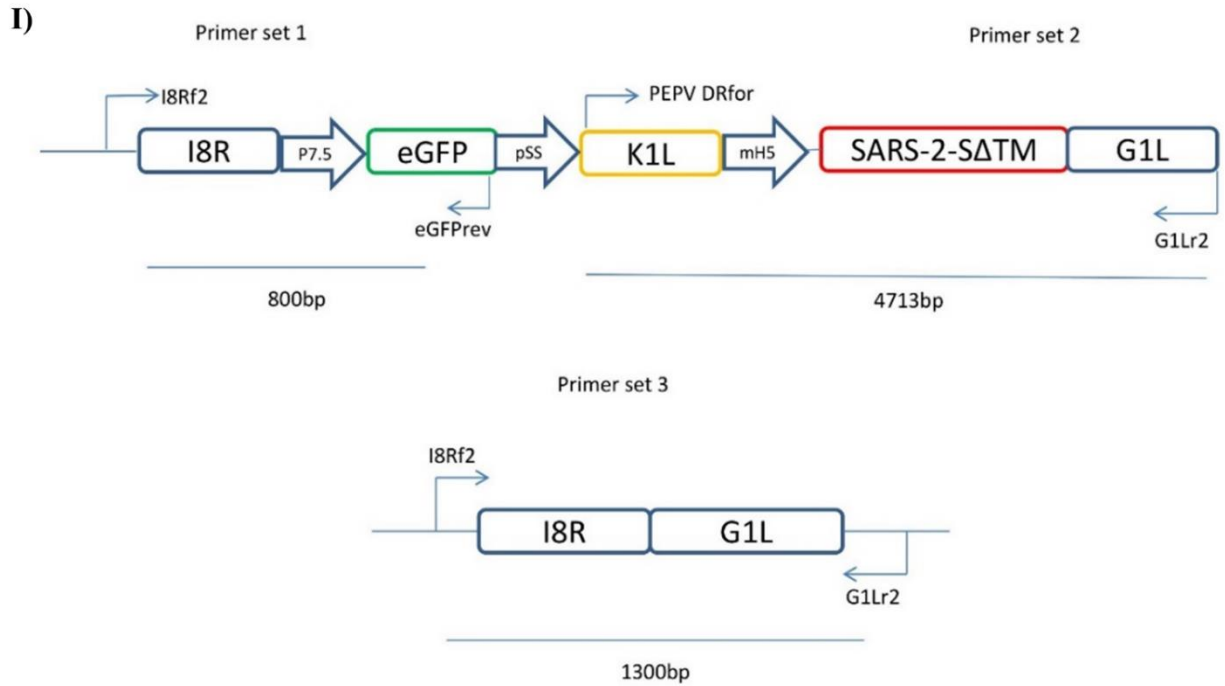


Figure 3.13: PCR amplification to confirm integration of the desired gene cassette into MVA. I) Schematic diagrams showing the binding sites of primer sets 1 (eGFPprev and I8Rf2 – 800 bp), 2 (PEPVDRfwd and G1Lr2 – 4713 bp) and 3 (I8Rf2 and G1Lr2 – 1300 bp); in the recombinant virus (top) and parent MVA (bottom). PCR products are shown with expected product sizes as horizontal lines. II) Agarose gels showing PCR products amplified by A) primer set 1: 1) MVA-SARS2-SΔTM 10 μl; 2) MVA-SARS2-SΔTM 15 μl; 3) Wild-type MVA; 4) Uninfected cells; 5) Water (no DNA template); B) primer set 2: 1) MVA-SARS2-SΔTM; 2) Wild-type MVA 3) Uninfected cells; 4) Water (no DNA template); 5) Empty lane; and C) primer set 3: 1) MVA-SARS2-SΔTM; 2) Parent MVA 3) Uninfected cells; 4) Water (no DNA template). The DNA samples were subjected to electrophoresis on 0.8%, 0.6% and 0.8% agarose gels respectively, alongside 5 ul aliquot of GeneRuler 1 kb DNA Ladder (MW).

3.4 Confirmation of expression of SARS-CoV2-SΔTM by Western blot analysis and immunofluorescence

Following confirmation of the genetic integrity of the SΔTM gene, the next step was to confirm that the recombinant virus expressed the antigen. Two techniques were used to assess the antigen of interest, SΔTM. Western blot analysis shows the protein size after separation by polyacrylamide agarose gel electrophoresis (PAGE) under denaturing conditions, whereas immunofluorescence shows protein expression *in vitro*.

3.4.1 Western blot analysis to confirm expression of SΔTM from the recombinant virus

Expression of SΔTM in cells infected with the recombinant virus was confirmed by western blotting of media and lysate. The immunoblot showed a product of ~180 kDa corresponding to the SΔTM protein (Figure 3.14) in both the media (lane 7) and lysate (lane 8), with a stronger signal visible in the lysate. The same band was observed for cells transfected with the positive control vector pMEx-SARS-2-SΔTM (lane 9). The cells only control yielded no bands confirming that the observed signal was specific to the protein of interest.

The faint band (lane 7) could be explained by a more dilute product being present in the media as opposed to the more concentrated product in the lysate. When extracting protein, there was only 200 µl lysate as compared to 2 ml media. The presence of the protein in the media strongly suggests that the protein was being secreted from the infected cells as would be expected following the addition of the TPA leader sequence. The successful detection of a band at the expected size confirmed that the protein was being expressed from the recombinant MVA-SARS2-SΔTM.

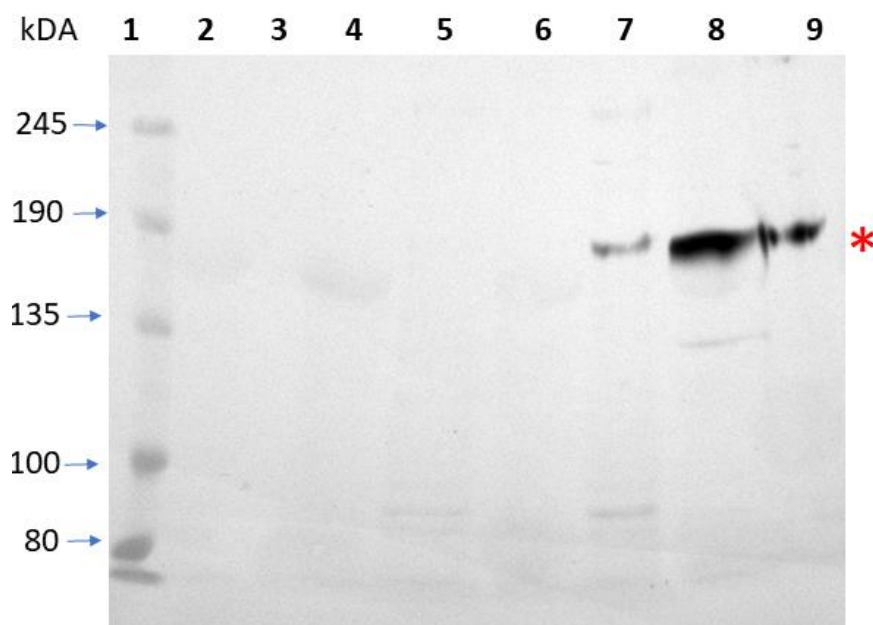


Figure 3.14: Validation of SARS-CoV-2-S Δ TM expression in infected RK13 cells. Western blot analysis on media and cell lysate to confirm expression of S Δ TM in RK-13 cells infected with passage 9 stock of MVA-SARS-2S Δ TM. **1**, Molecular weight marker; **2**, Cells only media; **3**, Cells only lysate; **4**, Empty lane; **5**, Media from cells infected with 20 μ l from P9; **6**, Lysate from cells infected with 20 μ l from P9; **7**, Media from cells infected with 50 μ l from P9; **8**, Lysate from cells infected with 50 μ l from P9; **9**, pMEx: SARS2-S Δ TM lysate as positive control. The cell lysate and media were harvested after 72 hours. Samples were run alongside a 10 μ l aliquot of Color Protein Standard Broad Range. The red asterisk indicates the product of interest.

3.4.2 Immunostaining to detect and confirm expression of S Δ TM from the recombinant virus

Immunofluorescence was performed to visualize the SARS-CoV-2 S Δ TM protein. This would serve as additional confirmation of expression and could give an indication of the cellular distribution of the product. HEK293 cells were infected with MVA-SARS2-S Δ TM and fixed as described in Section 2.5.4, before observation under fluorescent light whereby red Cy3 fluorescence was visible (Figure 3.15). The antibody has a red fluorophore therefore the red signal was visualized to signify expression. The presence of red fluorescence in the first and second rows of Figure 3.15 served as confirmation of S Δ TM protein expression in cells infected with MVA-SARS2-S Δ TM. The protein appeared to have a diffuse distribution in the cells. In row 3, the cells only control yielded no fluorescence, as did the experimental control of secondary antibody (with no primary antibody) in the last row. This confirmed that the SARS-CoV-2-S Δ TM protein is expressed by the recombinant MVA.

3.4.3 Growth of MVA-SARS2-SΔTM in BHK21 and RK13 cells

Growth of recombinant MVA-SARS2-SΔTM was assessed in the two permissive cell lines, BHK21 and RK13 cells. This was conducted to identify a suitable cell line to expand the recombinant for immunogenicity studies. The assessment was made qualitatively based on fluorescence levels only. The two cell lines were infected with MVA-SARS2-SΔTM at MOI 0.01 and observed daily for green fluorescence over a period of five days (Figure 3.16).

On day 1, the virus was growing and spreading well. By day 2, there was significant growth in both cell lines. On day 3, the cells were still attached although most of them had rounded (Figure 3.16). This suggested that the optimum harvest time point for the recombinant is three days post infection. On day 4, cells lifted and exhibit a cytopathic effect. Both BHK21 and RK13 cells proved to be favourable for the growth of the recombinant MVA-SARS2-SΔTM, although the fluorescence differed in the two cell lines. The growth of the recombinant in BHK21 cells was more diffuse whilst in RK13 cells it was more focussed. It was assumed that the level of fluorescence was indicative of the amount of viral growth. Titrations should have been performed to further assess the growth quantitatively but could not be done due to time constraints. Based on the fluorescence levels, it was decided to grow the virus in RK13 cells.

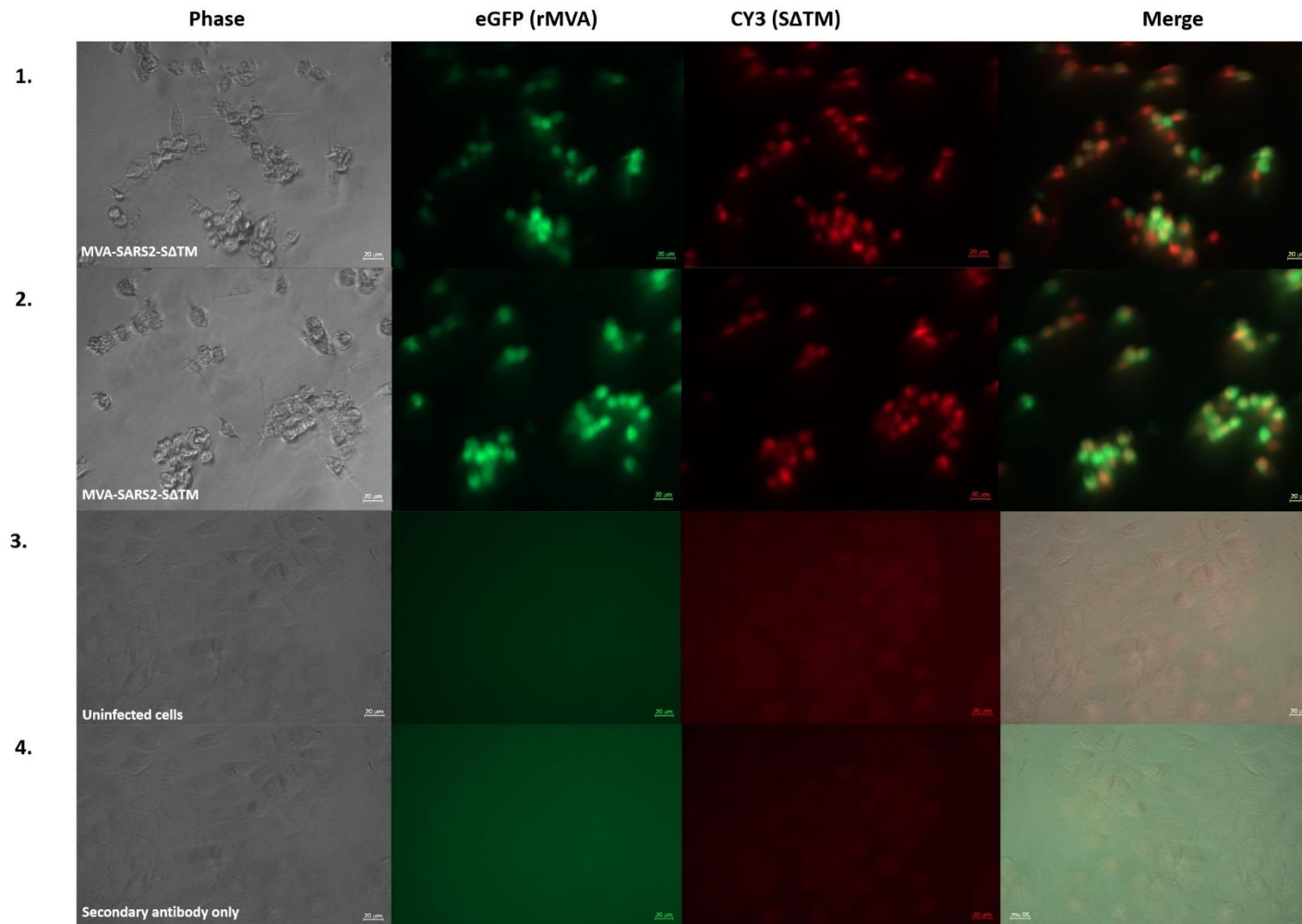


Figure 3.15: Immunofluorescence to detect expression of antigen SΔTM expression following infection of HEK293 cells with recombinant MVA-SARS2-SΔTM. In the experimental samples (rows 1 and 2) cells were infected with MVA-SARS2-SΔTM and fixed with 4% paraformaldehyde and 100% methanol before being blocked with 2% BSA. Primary rabbit anti-SARS-CoV-2 Spike Glycoprotein S1 antibody was added, followed by secondary donkey-anti-rabbit-Cy3 antibody (red). Both primary and secondary antibodies were used in the first and second rows (MVA-SARS2-SΔTM). Row 3 (uninfected cells) did not have any virus, whilst the row 4 shows uninfected cells treated with secondary antibody only (Zeiss Axio Vert.A1 fluorescent microscope at 40X magnification).

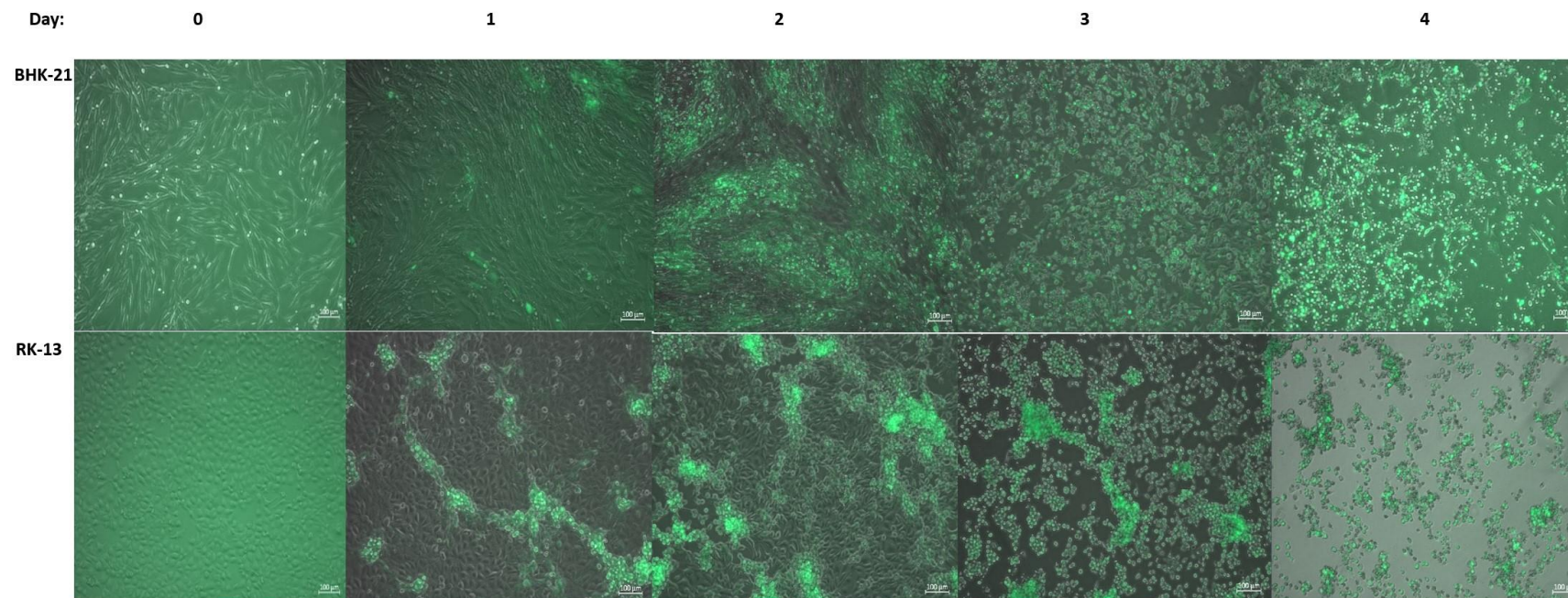


Figure 3.16: Observation of MVA-SARS2-SΔTM growth in BHK21 and RK13 cells by visualization of eGFP expression at different time points post infection. Cells were infected with MVA-SARS-2-SΔTM. Cells were infected at MOI 0.01 and observed over a series of 5 days to observe fluorescence as an indication of viral growth. (Zeiss Axio Vert.A1 fluorescent microscope at 40X magnification).

3.4.4 Scale up of MVA-SARS2-SΔTM

MVA-SARS2-SΔTM was grown in RK-13 cells as described in Section 2.6.1 and titrated as described in Section 2.6.2. Figure 3.17 shows the results of the titration. The titre was determined as follows:

$$h = \frac{(\% \text{ wells infected at dilution next above } 50\%) - 50\%}{(\% \text{ wells infected at dilution next above } 50\%) - (\% \text{ wells infected at dilution next below } 50\%)}$$

$$h = \frac{100-50}{100-12.5}$$

$$h = 0.57$$

$$\text{Titre} = 1 \times 10^{5.57} \text{ TCID}_{50}/100\mu\text{l}$$

$$\text{Titre} = 1 \times 10^{6.57} \text{ TCID}_{50}/\text{ml}$$

$$= 0.69 \times 1 \times 10^{6.57} = \mathbf{3.9 \times 10^6 \text{ ffu/ml}}$$

Day 4	
Well	% wells infected
-1	100
-2	100
-3	100
-4	100
-5	100
-6	12.5
-7	0
-8	0
-9	0

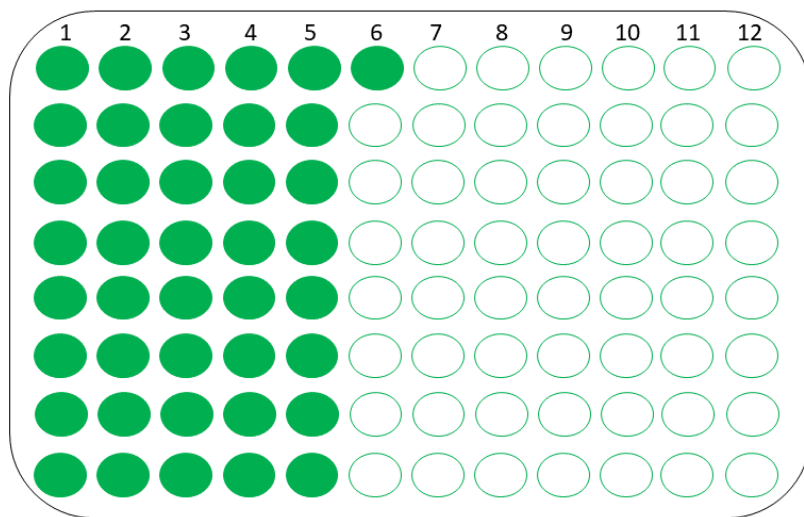


Figure 3.17: Titration of MVA-SARS-2-SΔTM. A 96 well plate of RK-13 cells was infected with serially diluted MVA-SARS-2-SΔTM. Green fluorescing foci were observed (Zeiss Axio Vert.A1 fluorescent microscope at 5X magnification) daily and counted after 96 hours. Calculations were made using the Reed and Muench method [226].

A titre of 3.9×10^6 ffu/ml was obtained in a total volume of 2 ml.

3.5 Immunogenicity of MVA-SARS-2-SATM in mice

3.5.1 Quantification of serum binding antibodies in immunized mice

Pooled mice sera were used to quantify the levels of binding antibodies induced by vaccination. The sera were pooled due to a low volume recovered from individual mice (~50 µl per bleed). Binding antibodies were quantified by ELISA using commercially bought SARS-CoV-2 spike glycoprotein (aa14-1213) that was produced in *E.coli* (Invitrogen, USA). The serum was tested at four time points namely before inoculation (pre-bleed) and after each immunization (Figure 3.18). Antibodies increased after the first immunization followed by a very slight decrease after the second. This unexpected negligible decrease could be explained by inadequate mixing of the vaccine after freeze-thawing in preparation for inoculation. Although this was a deviation from other MVA-based SARS-CoV-2 vaccines which showed increased antibody levels from the first to the second immunization [241], [242], it was similar to Margolin *et al.*, who found a plateau in binding antibodies after the second immunization [228]. Generally, it is expected that antibody titres will increase with each dose, until a certain threshold level is reached. This was followed by a larger increase in antibodies after the third immunization.

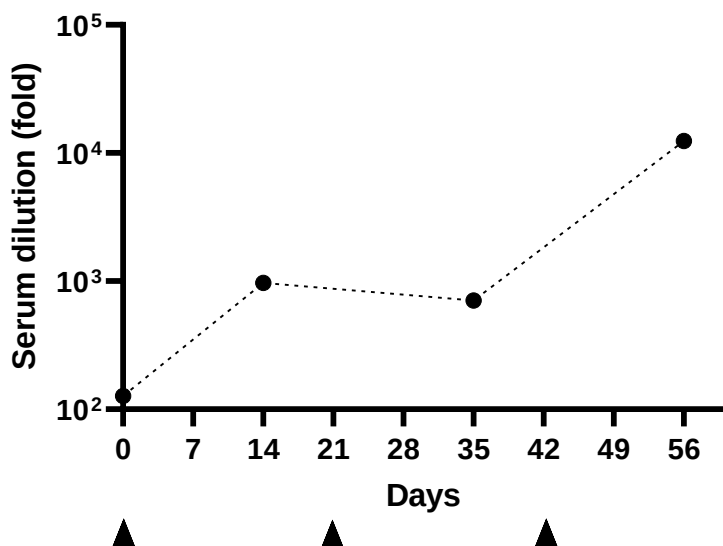


Figure 3.18: Quantification of serum binding antibodies by ELISA over the course of the experiment from day 0 to day 56. Mice were vaccinated with 50 µl of 3×10^5 pfu on day 0, 21 and 42 (represented by arrows). Mice sera were pooled before adding the samples to the ELISA plate therefore standard error bars are not shown.

3.5.2 Determination of the frequency of IFN- γ secreting splenocytes following immunization

An ELISPOT assay was used to determine the frequency of IFN- γ secreting splenocytes, as described in Section 2.7.1.2. Responses from peptides of two pools (A and B) spanning across the full length of the spike glycoprotein were recorded. The peptide pool included 316 peptides, 15mers with 11 aa overlap, for the SARS-CoV-2 spike glycoprotein (cat# RP30020) (158 peptides for pool A and 158 for pool B). The mean for the spike peptide A pool was 1398.1 SFU/ 10^6 with a range from 294 SFU/ 10^6 to 1330 SFU/ 10^6 splenocytes. The mean for the spike peptide B pool was 188.9 SFU/ 10^6 with the most responsive mouse exhibiting 191.3 SFU/ 10^6 splenocytes (Figure 3.19). A negative control of an irrelevant peptide yielded negligible responses (mean 89.4 SFU/ 10^6 splenocytes) whilst the positive control, Con A, had a high magnitude of responses, as expected (mean 2610.3 SFU/ 10^6 splenocytes).

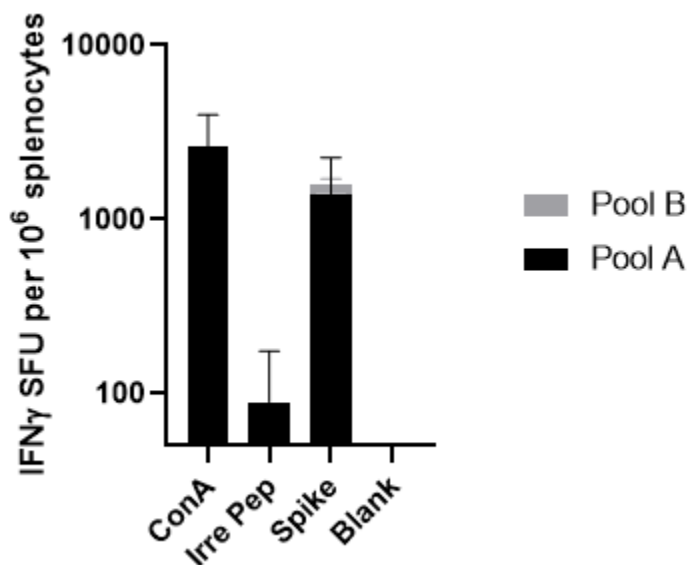


Figure 3.19: IFN- γ ELISPOT analysis of SARS-CoV-2 S T-cell responses in mouse splenocytes. Mice were vaccinated with 50 μ l of 3×10^5 pfu on day 0, 21 and 42 and spleens were harvested on day 56. Splenocytes were pooled from five mice and incubated in the absence of peptide (R10 medium only), stimulated with an irrelevant peptide or stimulated with SARS-CoV-2 S1 and S2 peptides. Bars represent the mean number of spots of triplicate reactions for 10^6 splenocytes. Positive responses were considered to be those greater than 3-sigma (average of blank + 3x standard deviation of the blank).

3.5.3 Quantification of serum neutralizing antibodies

Serum neutralization antibodies were investigated in an assay where the serum from the vaccinated mice was tested to determine if it could block infection with a SARS-CoV-2 pseudo virus. The assay was conducted by Dr. Georgia Schafer (Full member, Institute of Infectious Diseases and Molecular Medicine, University of Cape Town) where the pseudovirus was made based on the HIV backbone in HEK293TT cells, and thereafter the virus was pseudotyped with the pcDNA3.3-SARS-CoV-2-spike $\Delta 18$. Infectivity was assessed by measuring luciferase activity, which is produced by cells as a response to infection with the virus. Thereafter, this was quantified by calculating the half-maximal inhibitory dilution (ID_{50}). The results are given in Table 3.3. The day 56 serum did not give a higher titre than the pre-bleed serum and so the result for neutralization is negative. It is unexpected that despite the induction of binding antibodies, no neutralizing antibodies were detected.

Table 3.3: Mean ID_{50} values in pooled sera of mice immunized with MVA-SARS2-S Δ TM.

Day post immunization	Average ID_{50}
0 (pre-bleed)	30,4
14	5,1
35	7,2
56	30,4

CHAPTER 4:

DISCUSSION

Given the ongoing COVID-19 pandemic fueled by the continued evolution of the SARS-CoV-2 genome, second generation vaccines are needed which elicit improved immunity and can be manufactured at scale for resource-limited regions. Despite the availability of a number of different vaccines, very little consideration has been given to developing countries resulting in inequitable vaccine distribution. This has exacerbated the already dire economic, social and health implications of the pandemic. In view of this, our group has been exploring several vaccine platforms that are amenable to the African context where cost, manufacturing capacity and logistics for storage and distribution are a challenge [243]. These include poxvirus vector-based platforms, DNA vaccines, and subunit vaccines that are produced in plants [228].

In this study, we constructed a recombinant MVA vaccine expressing a soluble SARS-CoV-2 spike ectodomain that was engineered to promote secretion from infected cells following vaccination. MVA is a highly immunogenic platform which has been shown to induce both humoral and cellular responses [244]. Given the backdrop of the African continent where challenges span from manufacturing to distribution of vaccines, a low-cost production vaccine platform such as MVA is ideal. Furthermore, these vaccines are thermostable, easy to manufacture and easy to administer. Additionally, MVA has a large and stable genome for insertion of a large antigen such as the SARS-CoV-2 spike glycoprotein, a strategy which has been used by several others [241], [242]. MVA replicates in the cytoplasm which minimizes integration into the host cell genome and renders MVA vaccines safe for human use. Considering the context of Africa's vaccine manufacturing challenges, we leveraged off previously successfully constructed MVA vaccines [235], [245] to construct a SARS-CoV-2 vaccine expressing the SΔTM protein. The present study aimed to develop, characterize, and assess immunogenicity of the vaccine using in vitro cell culture experiments and a mouse model of immunogenicity.

4.1 SΔTM antigen design

The SΔTM gene was previously designed based on the spike glycoprotein, which is the sole target of neutralizing antibodies, and is also targeted by cellular responses in infection. The larger aim of

the project was to compare vaccine platforms, hence the use of the same antigen for construction of the different vaccine candidates for this comparison. A soluble antigen is better for producing recombinant proteins; therefore, the matched protein was used in this study. Unique to the SARS-CoV-2 virus is the furin cleavage site in the spike glycoprotein which has been documented to have increased infectivity [246]. In our design, the furin cleavage site was replaced with a hexa-arginine sequence (RRRRRR). This strategy was previously employed by Binley and colleagues [232], and was reported to improve the processing of HIV gp140 trimers in mammalian cells. However, when the project was initiated, the impact of having the hexa-arginine sequence on improved immunogenicity was unclear. The antigen in our study was further modified by deleting the transmembrane and cytoplasmic domains, a strategy that has also been used by Jia *et al.*, [247] to compare SARS-CoV-2 vaccines expressing various versions of the spike protein, and a combination of the membrane and nucleocapsid proteins. Results from this present study showed that the SΔTM version had the highest expression levels, due to the deletion of the transmembrane domain. An additional modification employed for our design was the replacement of the native leader sequence with the tissue plasminogen activator signal sequence, which has previously been shown to improve secretion of the desired protein, and was demonstrated to improve immunogenicity of a MVA based tuberculosis vaccine [248].

A mammalian expression plasmid expressing the matched SARS-CoV-2 SΔTM protein was used as a positive control in this study. The plasmid was based on the pTHpCapR expression vector which was previously designed and used in our group [233].

4.2 Construction of a transfer vector pMVA-FNK2

A transfer vector, pMVA-FNK1 was designed to generate the recombinant for this study. A site of insertion in the MVA genome was selected in between the convergent I8R and G1L open reading frames, which were shown by Moss *et al.*, [249] to be useful for expression without any loss of genes during recombination, resulting in a stable recombinant. This insertion site has also been previously used successfully in our group to generate HIV-1 vaccines [235], [250] and has been proven to be stable and allowing for insertion of transgenes [251]. In this study, the SARS-CoV-2-SΔTM gene was inserted under the transcriptional control of the strong modified early/late vaccinia virus promotor, mH5 [252]. A reporter gene, eGFP was included under the control of the

p7.5 promoter [253] and a selection gene, K1L was included, driven by the strong early/late synthetic pSS promoter [254]. Poxvirus promoters are important in that they uniquely drive poxviral transcription, due to the specific recognition of the promoters by the poxviral transcriptional machinery. The poxvirus RNA polymerase only recognizes uniquely poxvirus promoters during transcription, which is controlled by early, intermediate, and late genes. Thus, the inclusion of the poxvirus p.75 promoter to drive expression of the reporter green fluorescence gene, eGFP, allowed for visualization of the generated recombinant MVA-SARS2-SΔTM, as all three foreign genes (eGFP, K1L and SARS-CoV-2-SΔTM) were inserted between the I8R and G1L flanking sequences. In this study, the SΔTM gene was cloned into the MVA pSSPEX backbone, putative clones were confirmed by restriction enzyme digestion, and plasmid DNA was purified for use in transfections.

4.3 Generation of recombinant MVA-SARS2-SΔTM

BHK21 cells were infected with wildtype MVA and transfected with pMVA-FNK1 to generate the recombinant. Lysates were passaged in RK13 cells for several rounds until lysate from fluorescent foci isolated in a single well of a 96-well plate were expanded. Expanding green, fluorescent foci signified the growth of the recombinant. The host range K1L gene discriminated against parental MVA in RK13 cells, thus making it easy to purify the recombinant. Although the K1L gene provided stringent selection as described in Section 3.2.1, and the eGFP gene made it easy to identify fluorescent cells, there was still a possibility that non-fluorescent parental virus would still be present in the background, although in small quantities.

Additionally, in the serial passages to purify recombinant MVA from the parental MVA, Wyatt *et al.*, [255] have previously demonstrated that genomic loss or rearrangement is a possible occurrence due to spontaneous mutations. Thus, the recombinant in our project was characterized by PCR to rule this out, and to confirm that there was no residual parent MVA. A pair of primers that were designed to amplify the left end of the gene cassette (primer set 1), and a pair for the right end (primer set 2) successfully amplified products of the expected sizes of 800 bp and 4713 bp respectively. A third primer pair was designed to detect the wildtype MVA sequence, spanning the I8R - G1L region. Interestingly, these primers were not able to successfully amplify the large DNA insert in the recombinant, MVA-SARS2-SΔTM. The large size of the recombinant (9282

bp) could have contributed to this as it is generally difficult to amplify large fragments [256]. The use of “long fragment polymerases” designed to specifically amplify fragments larger than 6 kbp could have yielded a positive result. The GC-rich sequence of the recombinant (Appendix B) could also have contributed to the inability to amplify the whole foreign gene cassette in the recombinant. The difficulty in amplifying templates that have a high percentage of GC runs is a well-documented challenge and, as such, there are many protocols that have been developed to circumvent this [257], [258], [259], [260]. In this project, neither the use of Taq polymerase nor dimethyl sulfoxide (DMSO) was successful. Due to time constraints further protocols were not explored.

4.4 Confirmation of expression of protein SΔTM

Expression of the protein SΔTM by the recombinant was confirmed by Western blot analysis and immunofluorescence. The expected size of the SARS-CoV-2 SΔTM was 180 kDa. This was seen in the western blot analysis and corresponded to the exact same size as that of the matched SARS-CoV-2 SΔTM protein which was used as a positive control. This plasmid (described in Section 3.1) was previously constructed to express HIV-1 genes but, in this case, was modified to serve as a control to validate the expression of SARS-CoV-2-SΔTM. The immunofluorescence showed expression of the protein which could be visualized by red fluorescence around the cells.

MVA is known to replicate in BHK21 cells and very rarely in any other mammalian cells [210] and as such this cell line is largely the choice for propagation of MVA. RK13 cells on the other hand, were discovered to be permissive to the growth of MVA in the presence of the K1L gene [261]. With this logic, the growth of MVA-SARS2-SΔTM in this project was assessed in the two cell lines, BHK21 and RK13s to determine which cell line would produce a higher yield for bulking up of the virus. Based on fluorescence spread over a series of four days, we observed that harvesting of the virus in both cell lines was optimal on day 2. By day 3, the cells had rounded and were beginning to lift. This was important to note when bulking the vaccine up for further use in immunogenicity studies. The growth appeared to be similar in the two cell lines with respect to timing, however the manner of viral spread differed. In RK13 cells the growth appeared more diffuse whilst the growth was more localized in BHK21 cells. Titrations were necessary to

accurately quantify the growth of virus, but these were not done due to time constraints. It was decided to grow the virus in RK13 cells, for further use in immunogenicity studies.

4.5 Immunogenicity assessments in mice

The evaluation of antibodies has been widely used as a measure of immunity to SARS-CoV-2. As discussed in Section 1.7, the ability of a vaccine to induce a neutralizing antibody response correlates with a certain level of immune protection. Khoury *et al.*, assessed neutralizing antibodies in vaccinated and convalescent individuals to confirm that the presence of neutralizing antibodies afforded a level of protection, although the study additionally uncovered that this protection was likely to wane over time [134]. Consistent with this, Earle *et al.*, found a strong positive correlation between the production of both neutralizing and binding antibodies and vaccine efficacy [138]. Together, these studies indicated that antibodies protected against SARS-CoV-2, and therefore are a necessary endpoint to consider in vaccine design. Assays to assess production of antibodies are non-standardized, with different groups employing different methods when testing their vaccine's immunogenicity. This presents a challenge when reporting and comparing data and results from different studies. The enzyme-linked immunoassay (ELISA) is a test which produces quantitative data that can be used to infer the immunity of a vaccine, and therefore this was used in our study. We immunized mice, to measure the humoral response by quantification of serum binding antibodies. Antibodies from pooled mice serum, after three inoculations with the MVA-SARS2-SΔTM vaccine, bound to the antigen as days post-inoculation increased. We observed that there was a slight decrease after the second immunization which could have been due to inadequate mixing of the vaccine after freeze-thawing in preparation for inoculation. Elicited antibodies were highest on day 56 which was the final bleed after the third inoculation. These results were somewhat consistent with Margolin *et al.*, where the same antigen elicited robust antibodies from the onset of immunization, with a plateau after the second inoculation in a plant produced vaccine [228]. Similarly to our study, Yong *et al.*, constructed a DNA based vaccine expressing the soluble spike with a deletion of the transmembrane domain which elicited high titres of binding antibodies in mice and non-human primates [262] as compared to a full length version of the spike.

Disappointingly, extremely low neutralizing activity was seen in the mice. The serum from the final bleed after the last inoculation on day 56 did not give a higher titre than the serum from the

pre-bleed, thus there was no neutralization. It was, however, interesting to note that the same antigen was previously used by Margolin *et al.*, for plant production of the spike glycoprotein, which successfully yielded high titres of neutralizing antibodies, and provided confirmation that the truncated spike protein has the ability to induce neutralizing antibodies [228]. A possible explanation for the inability of MVA-SARS2-SΔTM to induce neutralizing antibodies in this study could be the low expression of the spike protein that was seen in the western blot results, where the analysis yielded a faint band of the protein. This has been previously reported in DNA based vaccines where codon optimization has been employed to increase protein expression and subsequently immunogenicity of the vaccine [263], [264]. Additionally, the vaccine titre that was used in this study to inoculate the mice was comparatively low, at 10^5 . A dose dependent relationship was demonstrated by Meseda *et al.*, [241] where increasing titres of IgG were observed with an increase in dose from 10^5 to 10^7 . Similarly, Sundaram *et al.*, showed that the higher doses of vaccines (either DNA vaccines or chemically inactivated vaccines with adjuvants) elicited higher titres of neutralizing antibodies [265]. These data collectively suggest that a stronger neutralization ability could have been induced if the vaccine titre was higher.

Cellular responses to SARS-CoV-2 vaccines have also been studied. T-cell responses to SARS-CoV-2 can be protective, and in severe disease have been shown to be destructive with the induction of inflammation and subsequently lung damage [263], [264], [266]. The measure of a good vaccine includes the ability to induce a protective cellular response without it becoming toxic. This can be measured by an ELISPOT assay which in this study showed that MVA-SARS2-SΔTM induced a high frequency of IFN- γ producing cells in Balb/c mice. Our findings were similar to those of Sundaram *et al.*, which demonstrated the ability of a whole-virus psoralen inactivated SARS-CoV-2 vaccine combined with the adjuvant Advax-2 to induce high levels of IFN- γ producing cells [265]. Interestingly in this study, there was a Th1 bias where the vaccine coupled with adjuvant Advax-2 produced stronger T-cell responses as opposed to antibody responses. When paired with the adjuvant alum, the vaccine induced a stronger Th2 response. Both conditions were dose dependent. In our study, MVA-SARS2-SΔTM followed a pattern similar to the vaccine coupled with Advax-2 whereby there was a Th1 bias which could likely be explained by the low dose of the MVA based vaccine used for inoculations. The response to the S1 peptide pool (A pool) was greater than that of the S2 peptide pool (B pool). This could likely be due to the length of the S2 which was shorter in our SΔTM gene. A similar response pattern was seen in the

AZD1222/Covishield vaccine where there was a greater response rate to the S1 peptide pool as compared to the S2 pool [267]. In contrast, a study assessing T-cell responses in convalescent individuals and vaccinated individuals showed a higher magnitude of response to the S2 peptide pool in both groups [268].

In summary, we successfully constructed and characterized a recombinant MVA vaccine expressing a soluble SATM gene. We have shown that this vaccine induced both cellular and humoral immunity, although neutralizing antibodies were negligible. This work constitutes a starting point towards the development of an MVA-vectored SARS-CoV-2 vaccine and further development will be required to improve immunogenicity of the vaccine. Additionally, work in our group is ongoing to determine how this platform compares to other vector-based modalities encoding the matched antigen.

4.6 Future work

Although significant progress in structure-based vaccine design has been made, improvement in antigen design would have a significant effect in improving the immunogenicity of this vaccine. Given that the spike is highly metastable and often requires modifications, the addition of prolines has been demonstrated to be one mechanism that can be employed to achieve stability [269]. The study showed that adding prolines to stabilize the spike protein in its prefusion conformation was largely advantageous for inducing stronger immune responses. This strategy has been employed by several others, and has recently been modified by Schaub and colleagues to include six prolines instead of two [270]. The authors demonstrated that the large and heavily glycosylated spike protein was difficult to stabilize in its prefusion conformation, and this was remedied by the addition of four prolines to form the HexaPro design [270].

Overall improvement of the MVA-SARS2-SATM and other vaccines is necessary to accommodate the evolving SARS-CoV-2 virus. It is possible that the inclusion of other important antigens such as the nucleocapsid protein could result in a more immunogenic vaccine. Coexpression with the highly expressed nucleocapsid, or generation of nucleocapsid-only expressing vaccines is worth exploring [271], [247], [272]. Due to the fact that it is relatively more stable as compared to the spike and plays a crucial role in viral replication, the protein is a target for cytotoxic T-cells [273]. This has been demonstrated by several others and is being shown as a viable target [274], [275],

[272]. Studying T-cells and their role in mitigating severe disease has proven challenging [276] yet it is crucial to understand this for design of next generation vaccines. Given the frequently mutating virus and high levels of breakthrough infections, T-cell immunity has been shown to be important because T-cells have the ability to recognize parts of SARS-CoV-2 that are not frequently mutating such as the nucleocapsid [277]. Additionally, it has been demonstrated that severe and fatal COVID-19 disease is characterized by lymphopenia and high levels of the biomarker CXCL10, which were shown to be strongly positively correlated with T-cell death when compared in ICU and non-ICU patients [278]. These findings together elucidate the role of T-cells which is critical as the virus continues to evade host immunity. Further investigations could be done to determine the effect of including a shorter length of our designed antigen, with either the S1 only or S2 only subunits. Kim *et al.*, 2021 demonstrated that the S1 subunit alone given at high dose produced high titres of neutralizing antibodies. However, when compared to a low-dose of a full-length spike vaccine, the S1 subunit produced lower neutralizing antibodies [279]. It is fundamental for vaccines to be “updated” through employment of strategies in order to continue offering protection to humans. Considering the continuing COVID-19 pandemic, vaccine development is an ongoing process. The mutation of the SARS-CoV-2 virus presents an additional challenge which shifts vaccine development to focusing on the effect of already approved and administered vaccines on emerging variants. Booster shots are in discussion with some countries already administering these for several vaccines [280], [281], [282]. The timing of the booster shots and questions of whether to get the same shot or a different vaccine as a booster are all under consideration with the aim of eliciting as safe, strong, broad and long-lasting immune responses as possible.

Combining vaccines expressing different immunogenic antigens has been shown to induce both T-cells and antibodies [235]. A possible application of this would be the combination of the MVA-SARS2-SΔTM vaccine with another vaccine, such as priming with the S protein and boosting with another antigen e.g. the RBD. This approach has been explored whereby it was shown that priming with the S protein and boosting with either the S or the RBD was highly immunogenic. On the other hand, this study observed that priming with the RBD and boosting with the S or the RBD was not immunogenic [283].

Once further work has been done to improve immunogenicity of the MVA-SARS2-SΔTM vaccine, evaluations on dose safety would be necessary to determine the toxicity of the vaccine. This would involve varying doses to conclude on adverse effects and what the “right” dose is. Hamster challenges will be set up where hamsters will be vaccinated and subsequently challenged with variants of the SARS-CoV-2 virus. Following this, hamsters will be assessed for histopathological changes, viral load and weight changes to evaluate the vaccine’s ability to provide protection against severe infection.

APPENDICES

APPENDIX A: REAGENT FORMULATIONS

Luria Bertani agar (100ml)

Tryptone	1 g
Yeast extract	0.5 g
NaCl	0.5 g
Agar	1.5 g
Water	Adjust to 100 ml

10% (v/v) Complete Dulbecco's Modified Eagle Medium (cDMEM)

Dulbecco's Modified Eagle Medium	450 ml
10% Heat inactivated Fetal Calf Serum	50 ml
1% Penicillin-Streptomycin antibiotic	5 ml

Freezing medium

	Concentration (v/v)
Heat inactivated Fetal Calf Serum	80%
Dimethyl Sulfoxide (DMSO)	10%
DMEM	10%

4X protein loading buffer

100% Glycerol	4 ml
1.5M Tris/HCl (p.H 6.8)	1.6 ml
SDS	0.8 g
Bromophenol blue	4 mg
β -mercaptoethanol	0.5 ml
ddH ₂ O	3.9 ml

10 X SDS page running buffer

SDS	10 g
Tris	30.3 g
Glycine	29 g
ddH ₂ O	adjust to 1000 ml

10 X transfer buffer

SDS	3.7 g
Tris	58 g
Glycine	144.1 g
ddH ₂ O	adjust to 1000 ml

PBST

10 X PBS	100 ml
10% Tween 20	10 ml
ddH ₂ O	Adjust to 1000 ml

Block buffer

1 X PBS	100 ml
Instant skim milk power	5 g
10% Tween 20	1 ml

4% (v/v) stacking gel (5 ml)

40% Acryl-bisacrylamide mix	0.5 ml
1.5 M Tri (pH 6.8)	0.63 ml
10% SDS	50 µl
10% APS	50 µl
TEMED	5 µl
ddH ₂ O	3.8 ml

8% (v/v) resolving gel (10 ml)

40% Acryl-bisacrylamide mix	2 ml
1.5 M Tri (pH 8.8)	2.5 ml
10% SDS	0.1 ml
10% APS	0.1 ml
TEMED	6 µl
ddH ₂ O	5.3 ml

2% Bovine Serum Albumin (BSA)

BSA	2 g
1 X PBS	100 ml

4 % Paraformaldehyde

Paraformaldehyde powder	40 g
1 X PBS	800 ml

Red Blood Cell Lysis buffer

NH ₄ CL	8.34 g
EDTA	0.037 g
NHCO ₃	1 g
ddH ₂ O	1000 ml

Resuspension Solution P1

Tris-HCL (1 M, pH 8.0)	2.5 ml
20% (w/v) glucose	0.91 g of glucose in 4.55 ml of ddH ₂ O
EDTA (0.5 M, pH 8.0)	2 ml
ddH ₂ O	0.95 ml
RNase (200 µg/ml)	400 µl

Lysis buffer P2

Sodium hydroxide (200mM)	8 g in 900 ml of ddH ₂ O
1% (v/v) SDS	100 ml of 10% SDS in 1 L of ddH ₂ O

Neutralization buffer P3 (100 ml)

Potassium acetate (3.0 M, pH 4.8)	4.41 g
Glacial acetate	7.5 ml
ddH ₂ O	adjust to 15 ml

G1L-SARS-CoV-2 SΔTM-GGTGGACCGGTAAGCTTCT-mH5- CCGGGTTATAG-K1L-
CGTGGCGGTCGACTCTAA-pSS- GAGCTCAGAAAAA-eGFP- GGT- p7.5- ATCGA-I8R

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TCTGCAGGGCGGGCTCCTGCGCCGAAGGTCCATCCGCTTGTGATGGTTCCTGCCAGCA
GGGCAGATGTGTACTGGGCGATCATCTCATCTGTCAGCAGTGGAGGCAGCACGGTC
AGGCCATTAACTTCTGGGCACAGATCAGGTCGCGGGCTGCGATGTCGCCCAGGCA
ATCGCCATACTGCTTGATGAAGCCGGCATCGGCCAGGGTCACCTTGTTGAACAGCAG
GTCCTCGATAAAGCTCCTCTTAGAAGGCTTGGAAGGATCGGGCAGGATCTGGCTGA
AGTTGAAGCCGCCAAAGTCCTTGATGGGTGGGGTCTTGTAAGATCTGCTTCACCTGGG
CGAACACCTCCTGTGTGTTCTTATCCTGCTCCACTGCGATGCCTGTCAGGGCCCTATT
CAGCTGGGTACAAAAGGAGCCGTACTGCAGCAGCAGGTTAGAGCACTCGGTGGAAT
CGCCACAGATATACATTGTGCAGTCCACGGATGTCTTGGTTCATGCTCACGGGCAGGA
TCTCTGTGGTCACGCTGATTGTGAAGTTGGTTGGGATGGCGATGCTATTGTTAGAGT
AGGCCACGCTGTTCTCGGCGCCCAAGGACATGGTATAGGCGATGATAGACTGGGAT
GCCACGCTTCTCCGGCGCCTTCTCCGTGGAGAGTTTGTCTGGGTCTGGTAGGAGGCA
CAGATGCCGGCGCCGATTGGGATGTCGCACTCATAAGAATTGTTACGTGCTCTGCT
CCGATCAGGCATCCGGCGCGTGTCTGGAACACGTTGGAGCCGGTGCTGTACACCCTC
CATGTTGGGGTCAGCTGATCGGCGTGGATGGCCACAGGCACCTCGGTACAATTCACG
TCCTGATACAGCACGGCCACCTGGTTGGATGTATTGGTGCCGGGTGTGATCACAGAC
ACGCCGCCGAAGGAGCAGGGTGTGATATCCAGGATCTCCAGGGTCTGGGGGTCCCG
CACGGCGTCTGTGGTATCTGCGATGTCCCGGCCGAAGTCTGAAATGGCAGGAAGTT
CTTGTTGCTCTCGGTCAGCACGCCTGTGCCGGTCAGGCCGTTGAAGTTGAAGTTCAC
GCACTTGTTCTTCACCAGATTGGTGGAATTCTTAGGTCCGCACACTGTTGCTGGTGCG
TGCAGCAGCTCAAAGCTCAGCACCACTCTGTAAGGCTGATAGCCACGCCGTTG
GTGGGCTGGAAGCCGTAAGACTGCAGTGGGAAATAACAGTTAAAGCCCTCCACGCC
ATTGCAAGGGGTAGAGCCGGCCTGGTAGATCTCTGTGGAGATGTCCCGCTCGAAGG
GCTTCAGATTGGACTTTCTAAACAGCCGGTACAGATAATTGTAGTTGCCGCCCACTT
TGCTATCCAGATTGTTAGAGTTCCAGGCGATCACGCAGCCGGTGAATCGTCTGGCA
GCTTATAATTGTAGTCGGCGATCTTGCCCTGTCTGTCCAGGTGCGATCTGGCGCACCTC
GTCGCCCCTGATCACGAAAGAATCGGCGTACACGTTGGTAAAGCACAGGTCATTCA
GCTTTGTGGGGGACACGCCATAGCACTTAAAGGTGCTGAAAGAGGCGGAGTTGTAC
AGCACGCTATAGTCGGCCACGCAGTTAGAGATGCGCTTCCTATTCAGGCGTACACG
CTGGCGAATCTGGTGGCGTTGAACACCTCGCCAAAGGGGCACAGGTTTGTGATATTT
GGAAATCTCACGATGGACTCGGTTGGCTGCACCCGGAAATTGCTTGTCTGATAGATG
CCCTTCTCCACGGTAAAGCTCTTCAGTGTACACTTTGTCTCAGACAGGGGGTCCAGT
GCGCAATCCACTGCGTCTGTGATGGTGCCATTCTCGTTGTACTTCAGCAGGAAGGTC
CGAGGCTGCAGATAGCCACATAGTAGGCTGCTGCTCCTGCGGTCCATCCGCTAGAG
GAGTCGCCTGGTGTGAGGTAGGAGCGGTGCAGGGCCAGCAGTGTCTGAAACCTGGT
GATGTTGATGCCGATTGGCAGATCCACCAGAGGCTCCAGGGCGCTGAAGCCCTGAG
GCAGGTCCCGCACCAAGGTTGATGGGGGTGTGCTTAGAGTAGATCTTGAAGTAGCCAT
CGATATTCTTAAACACGAACTCCCGCAGGTTCTTGAATTTGCCCTGCTTGCCCTCCA
GGTCCATCAGGAAAGGCTGGCTCACGTACTCAAATGTGCAATTGTTGGCGCTAGAAT
ACACCCTAACTCGCTCTCCATCCAAGACTTATTGTTCTTGTGATAGTACACGCCCA
GGAAGGGATCATTACAAAAGTGAAGTGCACACCTTGATGACCACGTTGGTGGCA
TTGTTACGATCAGCAGGGACTGTGTCTTGCTGTCCAGTGTGGTGCCAAAGATCCAG
CCGCGGATGATGTTAGACTTCTCGGTGGAGGCGAAGTACACGCCATCGTTAAAGGG
CAGCACTGGATTGTGCAACCTCTTTGTGCCATTGGTGCCGGACACGTGGATGGCGTG
GAACCAGGTCACGTTAGAAAAGAATGGCAGAAACAGATCCTGTGTGGAGTGCAGCA
CGGAGCTGCGAAACACCTTGTGAGGATAGTACACGCCCTTGTGAAGCTATTGGTAT

AGGCAGGGGGCAGCTGAGTTCTGGTAGTCAGGTTACGCGGGCTCACAAACACGGCT
CCGCACAGCAGCAGCACGCAGCACAGTCCTCTTTTCATAGCATCCATGGTGGACCGG
TAAGCTTCTAGATATTTATGATTATTTCTCGCTTTCAATTTAACACAACCCTCAAGAA
CCTTTGTATTTATTTTCAATTTTCCCGGTTATAGGATATTGATTCTTCTAATGAAA
ATCTTTAGAATGAATAGAGATATGTTAATAACAGGGTTGTTAGCTACACCCATTTTT
GTAGCTAGTTGTTTACAATCGGGCCCGTCGACAGAAAAATTAGTTCTTCTTTACACA
GTTTACGTACATAAGTCTTAGTTCTTTGTTCTTAGAGATGATCTCATCCAACCTTGTTG
TTCTTTACGATATCCAAATCCTTTGTGTAAGAATCAGACTTGATTCTACATGTTTTT
CGATGATCATCTTCGCGATTTCCGCATCATCCAATAGTACGTTTTCTAGGTTTCGCAGA
GTAGATGTTGATATCGTACTTAAACAAGGCTTGTAGCATTTTCGATATCCTTGTTATCA
ATCGCTAGCTTGATATCCGGGATAAACAACAACTGTTGTTTGTGTTTGTAGATGTC
ATGTAATCTAGTAGTAGGATCATCATATCTACATGTCCGTTCTTGATTGTGATATGGA
TACAAGATAGTAGGATCGCTAGATCAAATGTAGACGGGATTTTCAGATAGAAAGTAA
GATACGATAGATACATCGTTTAGCATTACGGCATGGTAAAAAGATGTTTTCCATCCT
GTCTTTCCGTAAACATTAGTCTCCAATTTTTCTTAACAAATAGCTTTACTGTTTGCA
TGTTTCCAGAATCTACCGCGTAGTACAACGCTGTGTTTCCCTTATCATCAAATTGACT
GTCGTCTAAACCAGAAAAACAACAGGATTTTCACAATCTTAGTGTCTCTAGTGTAGC
AGCTTGGTGCAGAGGGAACCTCGTTTTCCAATAAGTTCTTTAGAGCACCTGCATTGAG
TAGAGTGCAGACCAATCGTACATTGTTGTCTGCAATAGCGTAGTAAGTACTGAGTG
ACCGTGTACGTCAGCTTTGAAAGTGTCTTAGAACTTAAGAAAGACTTCAATTGTTT
ACTTTTCCATGTGTTGATTCTACTCAAATCCATCGTGGCGGTGCGACTCTAATATTTAT
ATTCCAAAAAATAAAATTTCAATTTGAGCTCAGAAAAATTACTTGTACAG
CTCGTCCATGCCGAGAGTGATCCCGGCGGCGGTACGAACTCCAGCAGGACCATGT
GATCGCGCTTCTCGTTGGGGTCTTTGCTCAGGGCGGACTGGGTGCTCAGGTAGTGGT
TGTCGGGCAGCAGCACGGGGCCGTCGCCGATGGGGGTGTTCTGCTGGTAGTGGTCG
GCGAGCTGCACGCTGCCGTCTCGATGTTGTGGCGGATCTTGAAGTTCACCTTGATG
CCGTTCTTCTGCTTGTGCGCCATGATATAGACGTTGTGGCTGTTGTAGTTGTACTCCA
GCTTGTGCCCCAGGATGTTGCCGTCTCCTTGAAGTCGATGCCCTTCAGCTCGATGC
GGTTCACCAGGGTGTGCGCCCTCGAACTTCACCTCGGCGCGGGTCTTGTAGTTGCCGT
CGTCCTTGAAGAAGATGGTGCCTCCTGGACGTAGCCTTCGGGCATGGCGGACTTGA
AGAAGTCGTGCTGCTTCATGTGGTCGGGGTAGCGGCTGAAGCACTGCACGCCGTAG
GTCAGGGTGGTTCACGAGGGTGGGCCAGGGCACGGGCAGCTTGCCGGTGGTGCAGAT
GAACTTCAGGGTCAGCTTGCCGTAGGTGGCATCGCCCTCGCCCTCGCCGGACACGCT
GAACTTGTGGCCGTTTACGTGCGCGTCCAGCTCGACCAGGATGGGCACCACCCCGGT
GAACAGCTCCTCGCCCTTGCTCACCATGGTCATTCGACGGGATCCGTCAGTGTCTTT
ATGATTCTACTTCCTTACCGTGCAATAAATTAGAATATATTTTCTACTTTTACGAGAA
ATTAATTATTGTATTTATTATTTATGGGTGAAAACTTACTATAAAAAGCGGGTGGG
TTTGGAATTAGATCGATTTAGTTATTATCTACAGGAACAAATATAGTATCTGAAATC
ATATTCATATATCCCGTTAGAGGTCTATGATAATATATAGTAGCGTTTGTTCGGTTAT
AGACACCGAATAATATTTTACAAAAGTGTATATACGTATCATCATCTTTATGTTTAA
AATTTAAAATCTTAATTCGTAAATTTAGAGATAAAATGGCTTCTTGTACAATACTTGT
TAATTCCTCCCGTCTCTCAAATATCCAACCTCCTCAGCGAGAATAGGACTTAGTAC
ATAAAGTTTAGCATACTCTATCATCTTCATATAATAATTGGATAATAATTTATTCCAT
GTTTCTGTACTAATATCGAACGAGTCTATATATTCCTTTGTACGCCATAGAATATCCA
AATTTGTAGGGATTATAAACAATCTTCGGGGAGTGTTAGATTAACTTATTAGCGT
ACAATATATAATTATGTAGAAATTCTGAATCTATTTCGCTGTATAGACTTCATATAAG

ACAGATCATAGAAATATACGTATGTCCCAGGGGTACCGTTAATTAACCTGGCCTCATG
GGCCTTCGCATCACTGCCCCGCTTTCCAGTCGGGAAACCTGTCGTGCCAGCTGCATTA
ACATGGTCATAGCTGTTTCCTTGCGTATTGGGCGCTCTCCGCTTCCTCGCTCACTGAC
TCGCTGCGCTCGGTTCGTTTCGGGTAAAGCCTGGGGTGCCTAATGAGCAAAAGGCCAG
CAAAAGGCCAGGAACCGTAAAAAGGCCGCGTTGCTGGCGTTTTTCCATAGGCTCCG
CCCCCTGACGAGCATCACAAAAATCGACGCTCAAGTCAGAGGTGGCGAAACCCGA
CAGGACTATAAAGATAACCAGGCGTTTCCCCCTGGAAGCTCCCTCGTGCGCTCTCCTG
TTCCGACCCTGCCGCTTACCGGATACCTGTCCGCTTTCTCCCTTCGGGAAGCGTGGC
GCTTTCTCATAGCTCACGCTGTAGGTATCTCAGTTCGGTGTAGGTCGTTTCGCTCCAAG
CTGGGCTGTGTGCACGAACCCCCCGTTCAGCCCGACCGCTGCGCCTTATCCGGTAAC
TATCGTCTTGAGTCCAACCCGGTAAGACACGACTTATCGCCACTGGCAGCAGCCACT
GGTAACAGGATTAGCAGAGCGAGGTATGTAGGCGGTGCTACAGAGTTCTTGAAGTG
GTGGCCTAACTACGGCTACACTAGAAGAACAGTATTTGGTATCTGCGCTCTGCTGAA
GCCAGTTACCTTCGGAAAAAGAGTTGGTAGCTCTTGATCCGGCAAACAAACCACCG
CTGGTAGCGGTGGTTTTTTTTGTTTGCAAGCAGCAGATTACGCGCAGAAAAAAAGGAT
CTCAAGAAGATCCTTTGATCTTTTCTACGGGGTCTGACGCTCAGTGGAACGAAAAC
CACGTTAAGGGATTTTGGTCATGAGATTATCAAAAAGGATCTTCACCTAGATCCTTT
TAAATTAATAAATGAAGTTTTAAATCAATCTAAAGTATATATGAGTAACTTGGTCTG
ACAGTTATTACGCACCGCCCTGCCATTCATCACAACTGCTGCAGTTCGTTTCAGCA
TACGGCCACATGAAAGCCATCACACACCGCATGATGCACCTGAATGGCCAGCGGC
ATCAGCACTTTATCGCCCTGGGTATAATATTTGCCCATGGTAAACACCGGCGCAAAA
AAGTTATCCATGTTCGCCACGTTTCAGATCGAAGCTGGTAAAGCTCACCCACGGATTC
GCGCTCACAAAGAACATGTTTTTCGATGAAGCCTTTCGGAAAATAGGCCAGGTTTTCG
CCATAGCACGCCACATCCTGGCTATAAATATGCAGGAACCTGGCGAAAATCATCATG
ATATTCGCTCCACAGGCTGCTAAAGGTTTCGGTCTGTTTCATGAAACACGGTATAGCA
CGGATGCACGCTATCCCAAATCACCAGTTCGCCATCTTTCATGGCCATACGAAATTC
CGGATGCGCGTTCATCAGACGGGCCAGAATATGAATAAACGCCGGGTAGAATTTGT
GTTTGTTTTTTTTTACGGTTTTTCAGAAACGCGGTGATATCCAGCTGCACGGTCTGGTT
ATAGGTGCACTGCGCCACGCTCTGAAACGCTTCAAAATGTTCTTTACGATGCCACTG
GCTAATATCCACGGTGGTATAGCCGGTGATTTTTTTTTTCCATACTCTTCCTTTTTCAAT
ATTATTGAAGCATTTATCAGGGTTATTGTCTCATGAGCGGATACATATTTGAATGTAT
TTAGAAAAATAAACAAATAGGGGTTCGCGCACATTTCCCCGAAAAGTGCCAC

APPENDIX C:

Table 2.5 GenScript SARS-CoV-2-r spike peptide sequence

MFVFLVLLPLVSSQCVNLTTRTQLPPAYTNSFTRGVYYPDKVFRSSVLHSTQDLFLPFFS
NVTWFHAIHVSGTNGTKRFDNPVLPFNDGVYFASTEKSNIIRGWIFGTTLDSKTQSLIV
NNATNVVIKVFCEQFCNDPFLGVYYHKNNKSWMESEFRVYSSANNCTFEYVSQPFLMDLE
GKQGNFKNLREFVFKNIDGYFKIYSKHTPINLVRDLPQGFSALEPLVDLPIGINITRFQT
LLALHRSYLT PGDSSSGWTAGAAAYYVGYLQPRTELLKYNENGTITDAVDCALDPLSETK
CTLKSFTVEKGIYQTSNFRVQPTESIVRFPNITNLCPFGEVFNATRFASVYAWNRRKRISN
CVADYSVLVNSASFSTFKCYGVSPTKLNDLCFTNVYADSFVIRGDEVQRQIAPGQTGKIAD
YNYKL PDDFTGCVIAWNSNNLDSKVGGNYNLYRLFRKSNLKPFERDISTEIQAGSTPC
NGVEGFNCYFPLQSYGFQPTNGVGYQPYRVVLSFELLHAPATVCGPKKSTNLVKNKCVN
FNFNGLTGTGVLTESNKKFLPFQQFGRDIADTTDAVRDPQTLEILDITPCSFGGVSVITP
GTNTSNQVAVLYQDVNCTEVPVAIHADQLTPTWRVYSTGSNVFQTRAGCLIGAEHVNNSY
ECDIPIGAGICASYQTQTSNPRRARSVASQSIIAYTMSLGAENSVAYSNNNSIAIPTNFTI
SVTTEILPVSMTKTSVDCTMYICGDSTECSNLLLQYGSFCTQLNRALTGIAVEQDKNTQE
VFAQVKQIYKTPPIKDFGGFNFSQILPDPSKPSKRSFIEDLLFNKVTLADAGFIKQYGDC
LGDIAARDLICAQKFNGLTVLPPLLTDEMIAQYTSALLAGTITSGWTFGAGAALQIPFAM
QMAYRFNGIGVTQNVLYENQKLIANQFNSAIGKIQDSLSTASALGKLQDVVNQNAQALN
TLVKQLSSNFGAISSVLNDILSRDKVEAEVQIDRLITGRLQSLQTYVTQQLIRAAEIRA
SANLAATKMSECVLGQSKRVDFCGKGYHLMSFPQSAPHGVVFLHVTYVPAQEKNFTTAPA
ICHDGKAHFPREGVFVSNGTHWFVTQRNFYEPQIITDNTFVSGNCDVVIGIVNNTVYDP
LQPELDSFKEELDKYFKNHTSPDVLGDISGINASVVNIQKEIDRLNEVAKNLNESLIDL
QELGKYEQYIKWPWYIWLGFIAGLIAIVMVTIMLCCMTSCCCLKGCCSCGSCCKFDEDD
SEPVLKGVKLHYT

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