UNIVERSITY of the
WESTERN CAPE

TRANSIENT TRANSGENE EXPRESSION OF HUMAN CORONAVIRUS NL63 ORF3 PROTEIN IN A BACULOVIRUS SYSTEM

A Masters Research Thesis

by

Kerwin Liedeman

Submitted in fulfilment of the requirements for the degree of

Magister Scientiae

Department of Medical Bioscience

Faculty of Natural Sciences

University of the Western Cape

Approved by Research Advisor: Professor Burtram Fielding

18 December 2020

DECLARATION

I declare that this thesis, "**Transient Transgene Expression of Human Coronavirus NL63 ORF3 Protein in a Baculovirus System**" is my own work, that it has not been submitted for any degree or examination in any other university, and that all the sources I have used or quoted have been indicated and acknowledged by complete references. Submitted to the University of the Western Cape for the degree of *Magister Scientiae (M.Sc.) Medical Biosciences*

Kerwin William Liedeman: _____

Date Signed: 18 December 2020

ACKNOWLEDGEMENTS

I would like to extend a few words of gratitude to the following individuals, groups and organisations who have all made an invaluable impact on me and my academic journey:

- My supervisor, Professor B.C. Fielding, who accepted me in his lab and whose tutelage and mentorship throughout my postgraduate career has been instrumental to my future in the science field.

- The Poliomyelitis Research Foundation and the National Research Foundation, for funding my studies throughout my postgraduate career.

- To my fellow colleagues in the Molecular Biology and Virology Lab, Bianca Gordon, Palesa Makoti and Jihaan Adonis, for their time, patience, and extended coffee breaks.

- A heartfelt thank you to Lisa Benjamin, for the belief you showed in me and countless messages of encouragement when I needed them the most.

- My godparents, Gustav and Ursula Louw, my brother, Ciaran Liedeman, and finally, my parents, Lilian Liedeman and Leon Liedeman, to whom all my work is dedicated to.

ABSTRACT

Insect-derived baculoviruses have been used extensively as a safe and versatile research model for transgenic protein expression. Preclinical studies have revealed the promising potential of Baculoviruses as a delivery vector for a variety of therapeutic applications, including vaccination, tissue engineering and cancer treatments. Coronaviruses are enveloped viruses containing linear, non-segmented ribonucleic acid. Human coronavirus NL63 was first discovered in the Netherlands in January 2004, where a 7-month-old girl presented with an acute respiratory tract infection that was later established to predominantly infect infants, the elderly and immunocompromised individuals. In addition to the known non-structural and structural proteins of coronaviruses, an accessory protein known as open reading frame 3 which is conserved in the *Coronaviridae* family has not been extensively researched. Open reading frame 3 encodes a putative membrane-bound protein. This study cloned the open reading frame 3 viral gene of 741 base pairs into the baculovirus expression construct via competent bacterial cell lines. Open reading frame 3-Baculovirus particles were generated in *Spodoptera frugiperda* insect cells. Recombinant cells containing the viral protein gene were used to infect healthy *Spodoptera frugiperda 9* cells at varying ratios of multiplicity of infection over a fixed time-course. The open reading frame 3 viral protein was not detected by quantification methods at a molecular weight of 26 kilo Dalton, due to polyclonal antibody degradation.

TABLE OF CONTENTS

LIST OF ABBREVIATIONS

µg	Microgram
µL	Microlitre
ACE-2	Angiotensin converting enzyme 2
***Ac*MNPV**	*Autographa californica* multicapsid nucleopolyedrovirus
ARTI	Acute respiratory tract infection
BEVS	Baculovirus expression vector system
bp	Base pairs
BSA	Bovine serum albumin
cDNA	Complementary DNA
CoV	Coronavirus
COVID-19	Coronavirus Disease 2019
CPE	Cytopathic effect
DNA	Deoxyribonucleic acid
dNTP	Deoxy nucleoside triphosphate
dsDNA	Double-stranded DNA
E. coli	*Escherichia coli*
ER	Endoplasmic reticulum
ERGIC	Endoplasmic reticulum-Golgi intermediate compartment
EtBr	Ethidium bromide
FBS	Foetal bovine serum
GV	Granulovirus
HCoV	Human coronavirus
HRP	Horseradish Peroxidase
IPC	Infection prevention and control
kB	Kilobases
kDa	Kilo Dalton
LRTI	Lower respiratory tract infection
MDRO	Multidrug resistant organism
MERS	Middle East Respiratory Syndrome
MERS-CoV	MERS coronavirus
mL	Millilitre
mM	Millimolar
MOI	Multiplicity of infection

mRNA	Messenger RNA
nCoV	Novel coronavirus
nm	Nanometer
NPV	Nucleopolyhedrovirus
ORF	Open reading frame
PBS	Phosphate buffered saline
PCR	Polymerase chain reaction
PFU	Plaque forming units
PVDF	Polyvinylidene fluoride
RIPA	Radioimmunoprecipitation assay
RNA	Ribonucleic acid
R_t	Transmission rate
RT-PCR	Reverse transcription polymerase chain reaction
SARS	Severe acute respiratory syndrome
SARS-CoV	SARS coronavirus
SDS-PAGE	Sodium dodecyl sulfate polyacrylamide gel electrophoresis
SF9	*Spodoptera frugiperda*
sg mRNA	Subgenomic messenger RNA
S.O.C.	Super optimal broth with catabolic repressor
ssRNA	Single stranded RNA
TMPRR2	Transmembrane protease serine 2
TNF-∝	Tumour necrosis factor-∝
TRS	Transcription-regulating sequence
URTI	Upper respiratory tract infection
VLP	Virus-like particle
WHO	World Health Organization

LIST OF FIGURES

LIST OF TABLES

PRESENTATIONS AND PUBLICATIONS

South Africa

Conference Poster Presentations from this Masters Thesis

1. **Liedeman, K.** and Fielding, B. *Transient Transgene Expression of Human Coronavirus NL63 ORF3 in a Baculovirus System*. Virology Africa 2020, Cape Town, South Africa (February 2020).

International

Conference Poster Presentations from Masters Research Internship Exchange

1. **Liedeman, K.**, Cardwell, L. and Daniels, M. *Gene Knockout of POSH using the CRISPR-Cas9 System in Human and Murine cell lines*. Summer Research Exchange (MU-iCATS), University of Missouri School of Medicine. Columbia, Missouri, United States of America (July 2018).

CHAPTER 1

INTRODUCTION

Human Coronaviruses

Classification and Origins

Viral Classification

Coronaviruses are members of the viral family *Coronaviridae* of the order *Nidovirales*, which are enveloped viruses containing linear, non-segmented RNA. According to Baltimore viral classification, coronaviruses are graded as Group IV due to them containing positive-sense single-stranded RNA (+) (ssRNA). Contemporary nomenclature groups coronaviruses into four distinct genera: *Alphacoronavirus, Betacoronavirus, Gammacoronavirus*, and *Deltacoronavirus.*

The *Alphacoronaviruses* (formerly coronavirus group I) and the *Betacoronaviruses* (formerly coronavirus group II) are known as the Human coronaviruses (HCoV). Furthermore, the *Deltacoronaviruses* and *Gammacoronaviruses* are coronaviruses of zoonotic origin.

Origins of HCoVs

The genomes of coronaviruses have not been extensively researched, as discovery of the viruses date back to the 1960s when they were first associated with URTIs in children (Kahn & McIntosh, 2005). At the time, only 2 human coronaviruses had been discovered - HCoV-229E and HCoV-OC43 until November 2002, when a farmer in China's Guangdong province was confirmed to have the novel case of Severe Acute Respiratory Syndrome (SARS), caused by the etiological agent SARS-CoV-1 (Zhong et

al., 2003). Subsequently, due to the severity of the SARS outbreak, which killed 774 of 8096 (4.1%) infected people, the World Health Organisation (WHO) scientific fraternity initiated a sense of urgency to research coronaviruses in depth (Goh et al., 2004). Two years later, due to reactive stance on SARS and proactive pursuits to other novel Human coronaviruses, a new virus HCoV-NL63 was isolated from a 7-month-old girl in the Netherlands whose symptoms included coryza, conjunctivitis, fever and bronchiolitis (Van Der Hoek, Pyrc, & Berkhout, 2006).

HCoV symptoms are classified as acute respiratory tract infections (ARTIs) which include predominantly upper respiratory tract infections (URTIs), lower respiratory tract infections (LRTIs) and pneumonia-like infections (Sloots et al., 2008). HCoV-NL63 infects predominantly young children, the elderly and immunocompromised individuals, which suggests that the virus is opportunistic and infects individuals with underdeveloped or inadequate immune systems (Burger, Dempers, & de Beer, 2014). In 2005, the next novel coronavirus discovery—HCoV-HKU1—was isolated from a 71-year-old man in Hong Kong who displayed symptoms pathognomonic of respiratory disease (Woo et al., 2005). Slowly, over time, with scientific studies of CoVs becoming more prevalent, almost a decade later in 2012, a 60-year-old man was diagnosed with Middle East Respiratory Syndrome (MERS) which was contracted in the Kingdom of Saudi Arabia (Azhar et al., 2016). Currently, at the time of writing, a 7th HCoV was isolated in the Wuhan region of Hubei province in China in late 2019 and was subsequently named SARS-CoV-2; the etiological agent of the coronavirus disease 2019 (COVID-19) pandemic (Lu et al., 2020).

Coronaviridae—Structure and Genome

Structural Similarity

In a period of almost 2 decades, from and including, the discovery of SARS-CoV-1 in 2002 up until SARS-CoV-2 in 2019 5 of the 7 total human infecting coronaviruses were discovered, as researchers were actively looking at infections which were previously being diagnosed as general respiratory tract

infections. Researchers have since applied *in silico* and clinical research techniques, particularly on structural proteins: spike (S), envelope (E), membrane (M) and nucleocapsid (N) (Figure 1.1) and also non-structural/accessory proteins in the human coronavirus genomes to conclude that human coronaviruses are phylogenetically similar (Graham, Donaldson, & Baric, 2013; Shi et al., 2015).

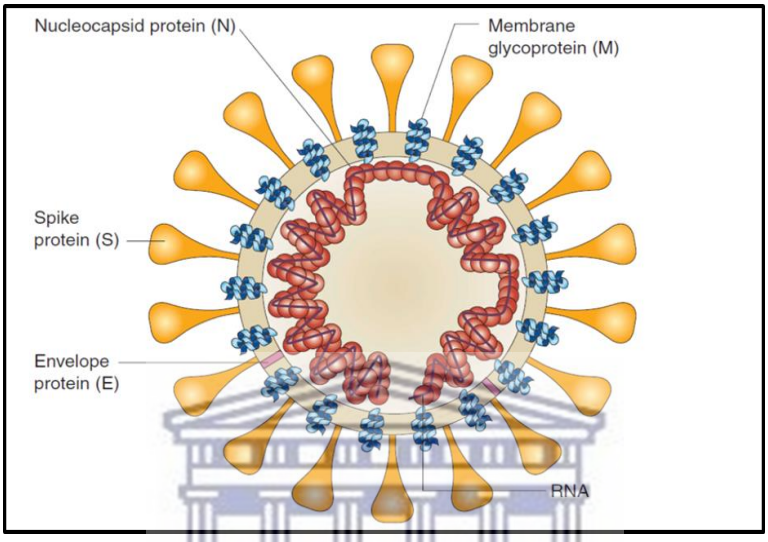

Figure 1.1: Structural proteins of HCoV-NL63

Schematic diagram of the coronavirus structure - Membrane (M), envelope (E), spike (S) and nucleocapsid (N). The viral surface proteins (spike, envelope, and membrane) are embedded in a lipid bilayer envelope derived from the host cell. The single-stranded positive-sense viral RNA is associated with the nucleocapsid protein. *Source*: (Peiris, Guan, & Yuen, 2004)

HCoV Genome Mutations

The coronavirus genome displays high frequencies of mutation with approximately 1 mutation occurring with every genome replication cycle (Moya, Holmes, & González-Candelas, 2004). Categorically RNA viruses display a high rate of recombination, which subsequently contributes to their overall genetic diversity (Pérez-Losada et al., 2015). These factors are what permit RNA-viruses to adapt relatively quickly to changes in their environment and ensuring virus propagation. Recombination is thought to be controlled by a "copy choice mechanism" whereby the RNA polymerase enzyme switches to a different copy template during RNA synthesis: -1 frameshift slippage

as the RNA polymerase encounters the pseudoknot during translation (Simon-Loriere & Holmes, 2011). RNA viral genomes are able to gain and lose coding domains which is a major contributing factor as to why there will always be an emergence of new viral strains, each with their own inherent novel traits, who have the ability to establish infection in a vast range of hosts (Van Boheemen et al., 2012). Genome sizes of coronaviruses are known to be the largest of RNA-viruses; thus denoting the fact that there are many sites within a coronavirus genome where mutational and recombinant events can occur (Woo et al., 2009).

HCoVs as Agents of Emerging Infectious Diseases

Coronaviruses in the 21st Century

HCoV-NL63 infections

HCoV-NL63 is not a cause for concern as there has not been a significant mortality rate in confirmed cases. Although changing the current paradigm to a more clinical approach where HCoVs are screened for regularly, and not only when a patient displays ARTIs with origins that cannot be explained would help advance the field of HCoVs significantly. To affirm the need for proper Human coronavirus screening, a team of researchers analysed nasopharyngeal swabs from 3677 children under the age of 3 from 6 countries across Western Europe over a period of 2 years. The samples were taken at outpatient points by paediatricians and inpatient points where children were hospitalised. The results indicated that 69 children tested positive for HCoV-NL63 infection (3.9%), with 38 of the 69 positive cases having visited the outpatient clinics and 31 were already hospitalised (Van Der Hoek et al., 2010). HCoV-NL63 was detected at a frequency of less than 2% in South African patients hospitalized with severe acute respiratory illness (Subramoney et al., 2018).

Further studies concluded that 75% of children infected with HCoV-NL63 were under the age of 6 months and required hospitalisation, indicating that the younger age group were more susceptible to

severe infections (Esper et al., 2005; Fouchier et al., 2004). Furthermore; a study in the Netherlands containing cross-sectional samples established that 75% of children in the age group 2.5 years were HCoV-NL63 seropositive (Dijkman et al., 2008). This indicates that a significant proportion of children are becoming infected and primary health care workers need a method to screen for HCoV-NL63, provided the infant displays ARTIs of unknown origins.

In a study done in Cape Town, South Africa 1055 respiratory samples were taken from children aged 13 days to 5 years at the Red Cross War Memorial Children's Hospital during the period 2003 to 2004, with the subsequent data published in 2008. The study concluded that 9 (0.8%) infants were infected with either HCoV-229E or HCoV-NL63—uncertainty due to limitations in specificity of samples tested—and 6 of the 9 infected children were not co-infected with a secondary pathogen (Smuts, 2008). This study indicates that Human coronaviruses are a cause for serious concern in South Africa and not restricted to Europe and Asia. Furthermore, the Red Cross War Memorial Children's Hospital study elucidated that the children who were infected with coronaviruses were infected equally in winter and summer, but information is still limited to describe seasonality of HCoVs (Subramoney et al., 2018).

Similar Infection Pathways in HCoVs

Angiotensin Converting Enzyme 2 Receptor Binding for Entry into the Host Cell

A pivotal point in human coronavirus research came to light when researchers established that the spike protein of SARS-CoV-1 and the spike protein of HCoV-NL63 use the same receptor angiotensin converting enzyme 2 (ACE-2) for entry into the host cell (Hofmann et al., 2005). The spike protein forms projections on the surface of the virion which is fundamental in host tropism and mediates membrane fusion for infection of the host cell. A more recent research study definitively established that SARS-CoV-1, SARS-CoV-2 and HCoV-NL63 all share ACE-2 as the natural receptor; although the SARS-CoV-2 ACE-2 complex contains a higher number of contacts, larger interface area, and decreased

interface residue fluctuations relative to the SARS-CoV–1 ACE-2 complex, and thus have a higher binding affinity and infection rate (Brielle, Schneidman-Duhovny, & Linial, 2020). SARS-CoV-2 spike protein binds to the catalytic site of ACE-2 to gain access to the host cell cytosol (Figure 1.2).

The spike protein undergoes proteolytic cleavage via tumour necrosis factor-α (TNF)-∝ and type II transmembrane protease serine 2 (TMPRR2), which allows for clathrin-dependent endocytosis of SARS-CoV-2 ACE-2 complex in to the host cell (Brojakowska et al., 2020).

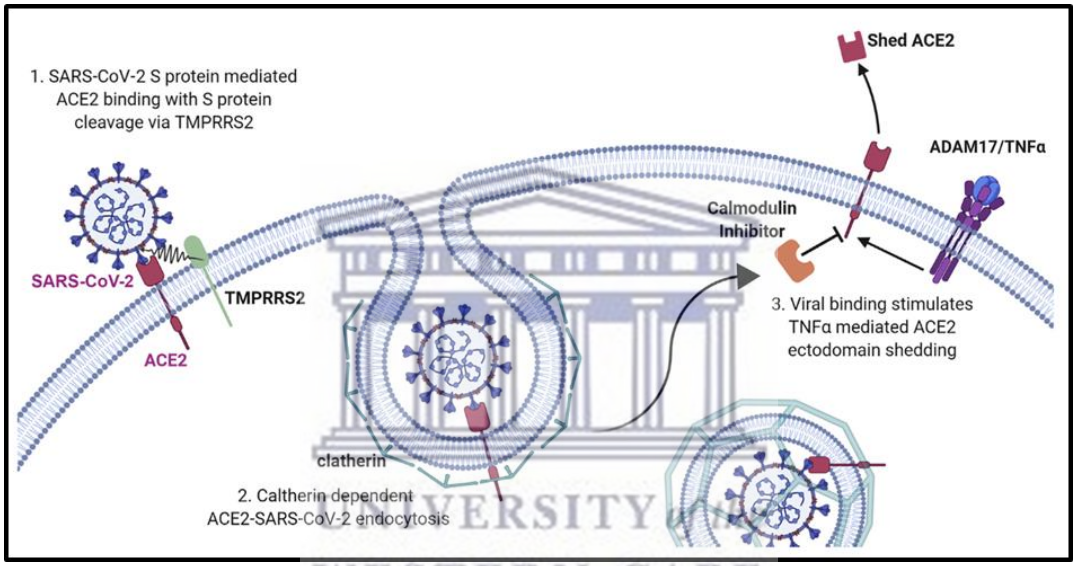

Figure 1.2: Mechanism of entry for SARS-CoV-2 via ACE-2

Spike proteins on the surface of the coronavirus bind to human ACE-2 receptors on the surface of the target host cell. The TMPRSS2 binds to and cleaves the ACE-2 receptor. In the process, the spike protein is activated. The cleaved ACE-2 and activated spike protein facilitate viral entry. TMPRSS2 expression increases cellular uptake of the coronavirus (Brojakowska et al., 2020).

CHAPTER 2

LITERATURE REVIEW

HCoV-NL63

Viral Genome and Replication

Viral Genome

HCoV-NL63 genome is 27553 base pairs in size (GenBank: AY567487.2) which is capped at 5'-end and polyadenylated at 3'-end (Abdul-Rasool & Fielding, 2010). The genome order of HCoV-NL63 is graphically shown in Figure 2.1 (Pyrc, Berkhout, & Van Der Hoek, 2007). Replicase genes account for the first two thirds of HCoV-NL63 genome which codes the polyprotein replicase translated from 1a and 1b (Woo et al., 2010).

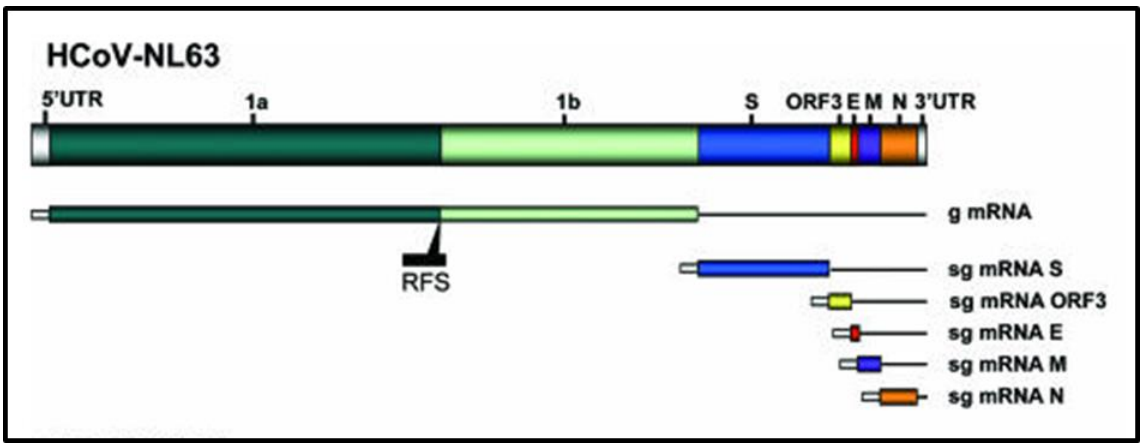

Figure 2.1: Schematic organization of the HCoV-NL63 genome

Corresponding subgenomic (sg) mRNAs generated during the discontinuous transcription process are shown (Pyrc, Berkhout, & Van Der Hoek, 2007).

Six mRNAs are produced in the infected cell membrane replication centres comprising of the full genomic mRNA as well as a nested set of 5 subgenomic mRNAs (Abdul-Rasool & Fielding, 2010). These mRNAs encode the structural and non-structural/accessory proteins (Brockway et al., 2003; Pyrc, Berkhout, & Van Der Hoek, 2007). The genes encoding for the viral proteins are found at the 3'-end of the genome; S: 180-190 kDa, ORF3: 26kDa, M: 26 kDa, E: 9 kDa and N: 50-60 kDa. Furthermore, number of non-structural/ accessory genes are unique to specific CoVs, are interspersed among the viral structural genes and are therefore termed group-specific genes. The functionality of these specific accessory genes is deemed dispensable *in vitro*, but are hypothesised to be essential in the host (Mcbride & Fielding, 2012).

Virus Entry into Host Cell

HCoV-NL63 has been shown to be amenable to *in vitro* culture on LLC-MK2 and Vero-B4 cell lines, and display CPE and clear viral replication. SARS-CoV-1 can also be cultured with these cell lines, however, HCoV-229E, which is most closely related to HCoV-NL63, cannot be expressed in these cell lines (Schildgen et al., 2006). A later study then concluded that the ACE-2 binding site—spike protein of HCoV-NL63—has a lower affinity interaction with ACE-2 than SARS-CoV-1, although the residues required for contact are similar; thus, reaffirming the statement that human coronaviruses are similar and also inherently different due to them being intrinsically polymorphic (Mathewson et al., 2008).

The spike protein of HCoV-NL63 must therefore be in the correct conformation to bind to the ACE-2 receptor. The ACE-2 receptor is found throughout the human body, but more densely populated in the ciliated epithelium of the LRT. This explains why HCoV-NL63, SARS-CoV-1 and SARS-CoV-2 cause infection of the respiratory system. SARS-CoV-1 and SARS-CoV-2 cause severe respiratory symptoms upon infection of the host; where in contrast the effects of HCoV-NL63 are far milder considering they both employ the ACE-2 binding mechanism. This effect in virulence can be attributed to HCoV-NL63 having a reduced binding affinity to ACE-2 and the inherent immune response of the infected

individual (Mathewson et al., 2008).

Virus Replication *In Vitro*

Coronaviruses are classified as Group IV viruses based on their method of replication (Figure 2.2).

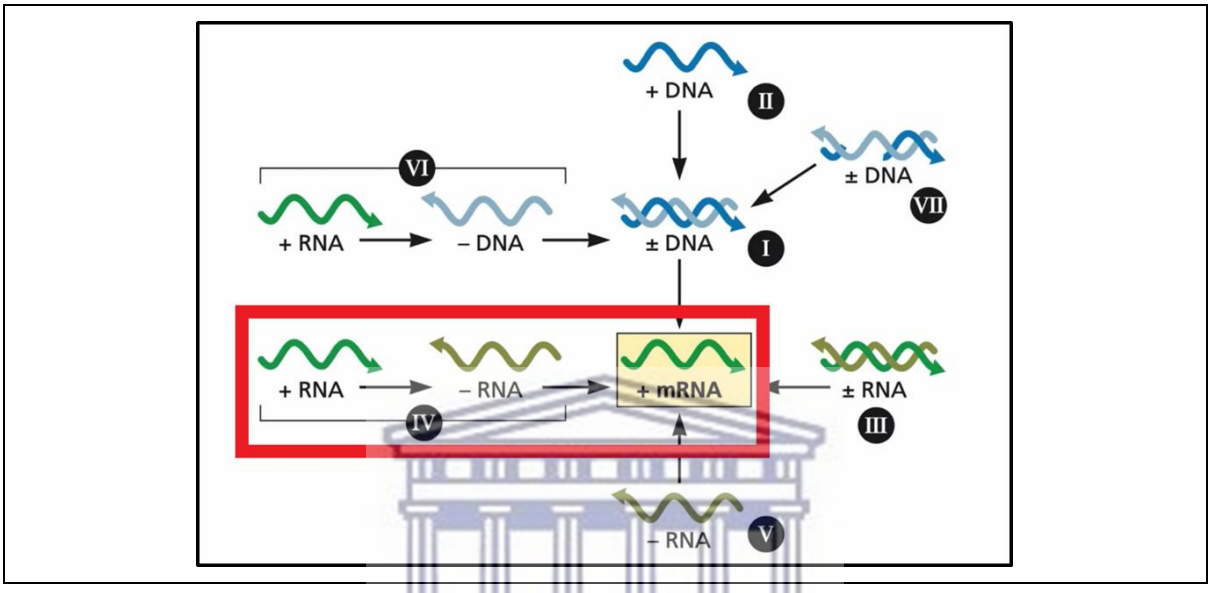

Figure 2.2: Coronavirus genome replication according to the Baltimore classification system

The Baltimore classification allows relationships among various viral genomes and the pathway to mRNA to be determined. All viruses must direct the synthesis of mRNA that is decoded by the host's translational machinery. Highlighted in red is the method of translation followed by coronaviruses, thus establishing them as Group IV viruses according to the Baltimore classification system (Flint et al., 2015)

Firstly, translating its genomic (+) ssRNA to (-) ssRNA, followed by translating the (-) ssRNA to mRNA for discontinuous translation of the genomic/subgenomic RNA (Flint et al., 2015). Virulence of HCoV-NL63 may evolve through recombination with other viruses due to the nature of RNA viruses' ability to readily recombine consequently creating detrimental effects for the host in the case of co-infection (Pyrc, Berkhout, & Van Der Hoek, 2007). Infection of a host cell by a coronavirus is known to exhibit CPE in *in vitro* studies which include rounding of cells, syncytium, detachment of cells from culture vessels, membrane rupturing of infected cell and apoptotic cell mediated death via caspase activity (Schildgen et al., 2006).

Open Reading Frame 3

ORF3 Gene Sequence

In addition to the known structural proteins, all coronaviruses have one or more non-structural/accessory proteins whose functions have not been fully elucidated. HCoV-NL63 ORF3 is a highly conserved accessory protein among HCoVs. The accessory gene is present at nucleotide position 24541–25219 of the genome and was identified between the spike (S) and envelope (E) genes. Additionally, the ORF3 gene has a unique nucleotide composition which appears as a uracil-rich and adenine-poor region within the genome, indicating a recent gene transfer event from another viral or cellular origin (Pyrc, Berkhout, & Van Der Hoek, 2007).

Furthermore, the subgenomic ORF3 mRNA is characterised as being polycistronic due to each ORF being translated into a polypeptide which is the subunit of a more complex protein. The ORF3 gene of HCoV-NL63 plays a role in infectivity and pathogenesis (Figure 1.2).

This ORF is the result of a -1 ribosomal frameshift that is triggered by a pseudoknot (Figure 2.3) (Giedroc, Theimer, & Nixon, 2000; Herald & Siddell, 1993; Pyrc et al., 2004). This secondary structure in the mRNA causes ribosomes translating from 5'- to 3'-end to pause at the position of the slippery sequence upstream. Consequently, this slowing or pausing of the ribosome during translation increases the frequency at which frameshifting occurs, thus enhancing the relative amounts of the proteins encoded by the downstream reading frames (Cann, 2016). This discontinuous method of transcription during synthesis of the (-) sg mRNA is governed by a transcription-regulating sequence (TRS) motif that contains the core sequence 5'-ACGAAC-3', which is highly conserved in coronaviruses (Yount et al., 2006).

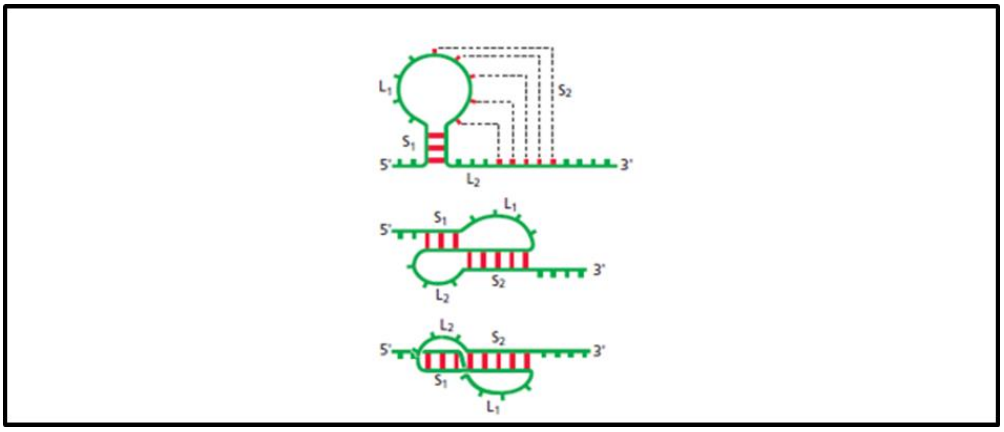

Figure 2.3: Schematic of a pseudoknot

Top - Stem 1 (S_1) is formed by base pairing in the stem-loop structure, and stem 2 (S_2) is formed by base pairing of nucleotides in the loop with nucleotides outside the loop. **Middle** - A different view of the formation of stems S_1 and S_2. **Bottom** - Coaxial stacking of S_1 and S_2 resulting in a quasicontinuous double helix causing the pseudoknot (Flint et al., 2015).

ORF3 Protein Amino Acid Sequence

HCoV-NL63 ORF3 encodes a putative membrane-bound accessory glycoprotein found in both group IA and IB coronaviruses (Fielding & Suliman, 2009). The ORF3 protein is expressed from a distinct sg mRNA encoding a putative 225 amino acid sequence resulting in a protein of about 25.6 kDa in size with 3 transmembrane domains – positions listed in (Figure 2.4) (Pyrc et al., 2004). Furthermore, three potential N-glycosylation sites occur at amino acid positions 16, 119 and 126, of which only the first is used because the sites at positions 119 and 126 are located within the transmembrane domains (Pyrc et al., 2004).

Moreover, there is subcellular localization of the ORF3 protein along the secretory pathway ERGIC (endoplasmic reticulum, Golgi, plasma membrane). Also, there is colocalization of HCoV-NL63 ORF3 protein with other structural proteins in the ERGIC, and finally the inclusion of the ORF3 protein in virions which give support to the viral assembly and budding processes (Müller et al., 2010).

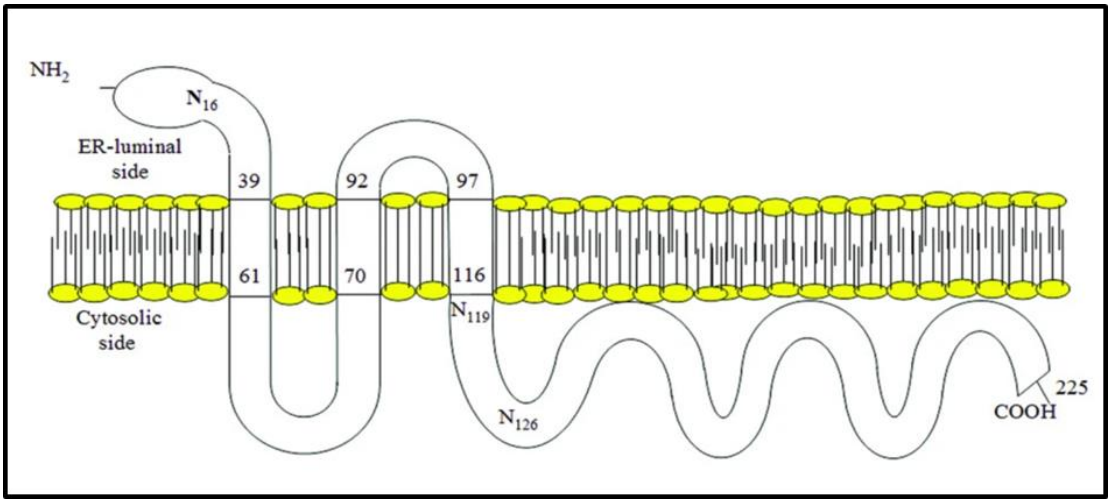

Figure 2.4: Transmembrane characteristics of HCoV-NL63 ORF3

Image displays ORF3 topology and glycosylation. N-linked glycosylation sites are indicated by an "N" at the respective localizations with an index number identifying the amino acid position (Pyrc et al., 2004).

In silico computational modelling comparing HCoV-NL63 ORF3 amino acid sequences to homologues from selected coronavirus isolates established that NL63 ORF3 is most similar to the *Betacoronavirus* HCoV229E ORF4 (43% identity; 62% similarity). Additionally, the study showed BtCoV, a coronavirus of animal origin affecting bats, having an ORF3 homologue to NL63 ORF3 (34% identity and 60% similarity) substantiating the relationship between this human and bat coronavirus having a similar zoonotic origin (Fielding & Suliman, 2009).

A range of further hypotheses indicate the HCoV-NL63 ORF3 expressed protein has functional similarities to the non-structural/accessory proteins of SARS-CoV-1 and possibly SARS-CoV-2 which include involvement in antigen decoy functions (Huang et al., 2006), interference with the regulation of expression of NFκB-dependent cytokines (Kanzawa et al., 2006; Narayanan, Huang, & Makino, 2008), modulation of spike protein endocytosis (Tan et al., 2004) and down-regulation of the expression of the spike protein on the cell surface (Tan, Lim, & Hong, 2006). This research further validates the rationale for researching NL63 ORF3 as similarities exist within the *Coronaviridae* family on a genetic and possible clinical level.

Heterologous Protein Expression

Protein Expression Systems

Overview

Many factors play a role in selecting a suitable recombinant protein expression system. These factors include preserving the biological and functional actions of the protein of interest after expression, and post-translational modifications of the protein. To date there are plenty of expression systems which have been engineered to generate recombinant viral DNA, proteins and peptides. The most widely used expression systems in both academia and industry comprise of engineered *Escherichia coli*, insect cell expression using the Baculovirus Expression Vector System (BEVS), yeasts and transient expression of recombinant proteins in mammalian cells (Assenberg et al., 2013). The aforementioned systems have been thoroughly characterised in academic literature for the production of heterologous proteins.

Bacterial expression systems are widely used due the shortened timeframe as opposed to other protein expression systems; although they are also known for their safety, low cost and convenience. A shortfall of the system is misfolding of proteins which causes considerable stress and toxicity towards the bacterial host cell (Gasser et al., 2008). This is due to the fact that foreign DNA hosts such as *E. coli,* the most used prokaryote and well-studied bacterial strain, do not have the necessary cell machinery to execute post-translational modifications and processing in the manner that higher order eukaryotes are capable of *E. coli* has been reported to have the following caveats: incapable of facilitating the formation of disulphide bonds, the cells lack the secretion mechanisms for optimal and efficient release of the recombinant protein in the culture medium which ultimately leaves researchers with low protein yield (Rosano & Ceccarelli, 2014). Furthermore, bacterial cell protein expression systems are predominantly used in academia and industry for simple uncomplex and large-

scale protein expression (Yokoyama, 2003). Eukaryotic organism-based systems, on the other hand, are more widely used for recombinant protein expression. The eukaryote cell-based recombinant systems machinery allows for correct folding of the proteins; as well as post-translational modifications which include phosphorylation and glycosylation. The subsequent recombinant proteins produced are biologically functional and can be implemented in downstream applications (Kost, Condreay, & Jarvis, 2005). These scientific applications include the use of recombinant proteins as suitable candidates in vaccine development and as antigens for detection and screening of antibodies in whole blood (Ren et al., 2004).

Published data on potential vaccines are predominantly recombinant viral protein/antigen-based, for example highly purified virus-like particles (VLPs) which are comprised of a viral structural protein expressed by recombinant Baculovirus techniques. In the midst of the ongoing SARS-CoV-2 pandemic, a recombinant SARS-CoV-2 spike protein using the baculovirus-silkworm system are used in ongoing vaccine development strategies (Fujita et al., 2020). Furthermore; the Baculovirus system is being used in the case of pandemic preparedness in development of a cross-protective influenza H5N1 vaccine (Prabakaran & Kwang, 2014) and a vaccine against avian infectious bronchitis virus (IBV) by co-expressing S1 and N Proteins using a similar baculovirus system (Yuan et al., 2018).

The aforementioned reasons are indicative as to why eukaryotic expression systems such as insect, mammalian and yeast are favoured by researchers as opposed to bacterial expression systems for expression of recombinant proteins and peptides. The implementation of these recombinant expression models in research laboratories and industry, where high output production of proteins is required, allow for several downstream applications. These include but are not limited to vaccine production or generation of purified antigens for diagnostics (Ren et al., 2004; van Oers, Pijlman, & Vlak, 2015).

Bac-to-Bac® Baculovirus Expression System

The Family Baculoviridae

The viral family comprises large viruses with circular double-stranded DNA genomes ranging from 80–180 kB. The virions are enveloped, contain rod-shaped nucleocapsids and are embedded in distinctive occlusion bodies (Harrison et al., 2018). Baculovirus-infected insect and mammalian cells facilitate the production of glycosylated membrane (Altmann, 1999).

Baculovirus Expression Vector System (BEVS)

In order to study the HCoV-NL63 ORF3 accessory viral protein, a substantial yield of the recombinant protein is required. Notably, BEVS is the system of choice for large-scale production of recombinant antigens which are functionally and biologically active (Mirzaei et al., 2014). The system allows for researchers to produce bioactive proteins from inexpensive culture medium instead of using expensive extraction techniques (Yin et al., 2007).

BEVS for Heterologous Protein Expression

The rod-shaped baculoviruses are known to exclusively infect a small range of insect species, thus making them safe to utilize in low biosafety environments, owing to their low-risk biosafety profile (Airenne et al., 2013). *Baculoviridae* are divided in to 2 genera: Nucleopolyhedrovirus (NPV) and Granulovirus (GV), based on taxonomy and morphology of their occlusion bodies (Rohrmann, 2019). Besides, research indicates that NPV expresses two genes which are not of value in driving viral DNA synthesis, although these genes contain promoter regions which are harnessed for the purposes of driving heterologous protein expression in insect cells (van Oers & Vlak, 2007). The nonessential genes in question encode the proteins p10 and polyhedron which constitute the viral occlusion bodies and are present in fibrillar structures. These proteins are shown in research to be expressed in high levels during the late phase of viral infection when exploited (van Oers, 2006).

Autographa californica Multicapsid Nucleopolyedrovirus (*Ac*MNPV)

*Ac*MNPV is a member of the Baculovirus family that researchers have studied and used extensively in production of recombinant antigens for proteins using insect cell lines (Abdel-Moneim, Giesow, & Keil, 2014). The *Ac*MNPV Baculovirus contains a genome of significant size (135 kB) and remains the preferred choice for large-scale recombinant protein expression needs (Carstens, 2009).

Recombinant *Ac*MNPVs have shown to enter mammalian cells and release their genomic DNA, however, the recombinant virus cannot replicate in the mammalian cells, thus being indicative that BEVS have a high level of biosecurity as they are non-pathogenic to humans (Fabre et al., 2020).

*Ac*MNPV are engineered to facilitate expression in various insect cell lines. These cell lines are isolated from lepidopteran species (fall army worms), with common cell lines derived from *Spodoptera frugiperda* (SF9 and SF21 cells) (van Oers & Vlak, 2007). The Baculoviruses double-stranded DNA genomes are able to accommodate insertions of large genes; the transgene capability of *Ac*MNPV is extending beyond 100 kB (Naik et al., 2018). The *Bac-to-Bac® Baculovirus Expression System* developed by Luckow *et al*. (1993) makes use of a Baculovirus-containing transfer vector containing; polyhedron/p10 promoter for transcription initiation, a gentamicin resistance gene and 2 Transposon (Tn7) elements which allow site directed transposition of the bacterial expression plasmid into the Baculovirus DNA/Bacmid.

Aims and Objectives

The significance of human coronaviruses as the aetiological agents causing mild to serious upper and lower respiratory tract infections within the human population has been well established and documented. In the last decade, a considerable amount of resources has been dedicated to disease control measures involving coronaviruses, in addition to a sizeable increase in peer-reviewed research

to better understand their periodic devastating impacts; both clinically and epidemiologically. Published research has also indicated that the viral genetic structure and function aid in the aforementioned efforts of understanding coronaviruses.

The HCoV-NL63 ORF3 gene encodes a membrane-bound accessory protein which is highly conserved within the *Coronaviridae* family. Therefore, the elucidation of the intra-viral protein-protein interactions of ORF3 could provide insight into virion assembly within infected cells during viral transcription, translation and replication.

The primary aim of this research project was to clone the HCoV-NL63 ORF3 gene into a Baculovirus insect cell system for gene of interest protein expression. The recombinant HCoV-NL63 ORF3 protein generated will be suitable reagents for further molecular and protein interaction studies.

CHAPTER 3

MATERIALS AND METHODS

Materials

Laboratory Equipment, Consumables, Reagents and Kits Used

Equipment

Table 3.1 shows the list of technical equipment used in this study.

Table 3.1: List of technical equipment used

Type of Equipment	Model	Supplier
Autoclave	HL-340	Lab and Scientific Equipment Co., CPT
Biosafety fume hood	1200	Labscheme Allchem
Centrifuge (4˚C)	L500 tabletop low speed centrifuge	LGM International Inc., USA
Fridge (4˚C and -20˚C)	Express cool No Frost	LG, South Africa
Freezer (-80˚C)	Evosafe series VF720-86	Snijders Scientific, Holland
Gel electrophoresis casting tray	Hoefer™ HE 33 Mini Submarine Unit	Labnet International, Inc. NJ, USA
Gel electrophoresis tank	Hoefer™ Submarine Gel SUB13 Midi Horizontal	Labnet International, Inc. NJ, USA
Haemocytometer	Neubauer	Superior Marienfeld, Germany
Heating block	AccuBlock Digital Dry Bath	Labnet International, Inc. NJ, USA
Ice Maker	AF 80	Scotsman, South Africa
Incubator	Series 2000	Scientific, South Africa

Incubator (Tissue Culture)	ESCO CelCulture CO_2 Incubator	Scientific, South Africa
Laminar flow	SCR-2A1	ESCO, Singapore
Magnetic stirrer	MSH B	Laboratory Equipment, Germany
Microcentrifuge	M-24 A	Boeco, Germany
Microscope (Tissue culture)	XDS-1B	Lasec, South Africa
Microwave	Concave reflex system	Defy, South Africa
SDS-PAGE Tank	Mini-Protean Tetra System	Bio-Rad
Rocker	S 2025-220	Labnet International, New Jersey
Pipettes	Discovery Pro Starter 4 pack	HTL Lab Solutions, Poland
Power Pack	PowerPac HC	Bio-Rad, Singapore
Power Supply	Power Station 200	Labnet International, Inc. NJ, USA
Power Supply	Power Station 300 PLUS	Labnet International, Inc. NJ, USA
Shaking incubator	J1000	Snijders Scientific. Holland
Thermocycler	Multi-gene II	Labnet International, Inc. USA
UV Trans illuminator	DigiDoc-It Darkroom	UVP, Inc. Bio Imaging Systems, USA.
Vortex Mixer	Vortex-Genie 2	Lasec, South Africa
Waterbath	Memmert	Lasec, South Africa
Weighing scale	AQT – 1500	Adam Equipment, South Africa

Consumables

Table 3.2 shows the list of consumables used in this study.

Table 3.2: List of consumables used

Type	Supplier
96-well Microplate	Lasec, South Africa
Erlenmeyer flask	Duran, Germany
Eppendorf microcentrifuge tubes	ExtraGene, USA.
Gloves (nitrile)	Super Care, Thailand

Parafilm	Pechiney, USA
Polypropylene tubes	SPL Life Sciences, Korea
Pipette tips	SPL Life Sciences, Korea
Schott bottles	Duran, Germany
Serological pipettes (non-pyrogenic)	SPL Life Sciences, Korea
Sterile syringe filters	Biosmart, South Africa
Syringes	Dura Surge
Tissue culture flasks	SPL Life Sciences, Korea

Reagents

Table 3.3 shows the list of reagents used in this study.

Table 3.3: List of reagents used

Chemical reagent	Supplier
100% Ethanol	Saarchem, South Africa
SeaKem LE Agarose (Lonza)	Whitehead Scientific (Pty) Ltd, USA
Cellfectin™ II Reagent	Invitrogen, South Africa
DNA Markers (1kb and 100bp)	Promega, South Africa
Ethidium bromide	Promega, South Africa
Grace's Insect Medium, supplemented	Gibco, Life Technologies
Grace's Insect Medium (2X), supplemented	Gibco, Life Technologies
Grace's Insect Medium, unsupplemented	Gibco, Life Technologies
Heat-inactivated Foetal Bovine Serum	Gibco, Life Technologies
Loading dye (6x)	Promega, South Africa
Phosphate Buffered Saline (PBS)	Lonza, BioWhittaker, USA.
Pre-stained protein markers	Thermo-Fisher, South Africa
Sodium Chloride (NaCl)	Merck, Germany
Tris (hydroxymethyl)-aminomethane	Merck, Germany
Trypan Blue Stain	Lonza. BioWhittaker, USA
TMB Membrane Peroxidase Substrate	KPL, USA

Laboratory Kits

Table 3.4 shows the list of laboratory kits used in this study.

Table 3.4: List of laboratory kits used

Kit	Supplier
NucleoBond® Xtra Midi	Separations, South Africa
Pierce™ BCA Protein Assay Kit	Invitrogen, South Africa
SDS Gel Preparation Kit	Invitrogen, South Africa
Wizard® Plus SV Minipreps DNA Purification System	Promega, South Africa
Wizard® SV Gel and PCR Clean-Up System	Promega, South Africa

Methodology

Overview Outlining HCoV-NL63 Expression in a Baculovirus

Preliminary Cloning of HCoV-NL63 ORF3 into pGEM®-T Easy Vector

Firstly, the gene of interest, HCoV-NL63 ORF3, will undergo preliminary cloning into pGEM®-T Easy Vector (*Promega*) as it allows for a "plug-and-play" system for gene insertion into the vector via single nucleotide T-overhangs. Secondly, the BEVS outlined in this study requires the gene of interest DNA to be at a starting concentration of >0.5 µg/µL, which will be obtained via the *Machery-Nagel NucleoBond Xtra Midi kit*.

A RT-PCR of the HCoV-NL63 ORF3 gene from the parent vector pCAGGS will be amplified via full-length HCoV-NL63 ORF3 forward and reverse primers. These primers will be designed to incorporate the restriction sites *Bam*H I and *Xho*I, at the gene of interest 5'- and 3'-ends, respectively. HCoV-NL63 ORF3 amplicons will be excised from the PCR agarose gel and purified. The purified gene fragment will undergo ligation into the pGEM®-T Easy Vector via T-overhangs in the vector MCS, creating a pGEM-

ORF3 construct for ligation and transformation into *Promega JM109 High Efficiency Competent Cells. JM109 E. coli* cells contain an ampicillin resistance gene and a *LacZ* gene; with the former being activated upon gene insertion and the latter repressed due to the aforementioned gene insertion in the vector to allow for blue/white colony screening (Figure 3.1). The pGEM-ORF3 construct will be purified and will undergo a double restriction endonuclease digest as a confirmatory checkpoint of correct gene insertion. Furthermore, due to the DNA binding limitations of the columns within the miniprep kit (0.1–0.2 µg/µL); glycerol stocks of *JM109 E. coli* pGEM-ORF3 cells will be grown up as large broths and purified *Machery-Nagel NucleoBond Xtra Midi kit* to obtain >0.5 µg/µL of DNA.

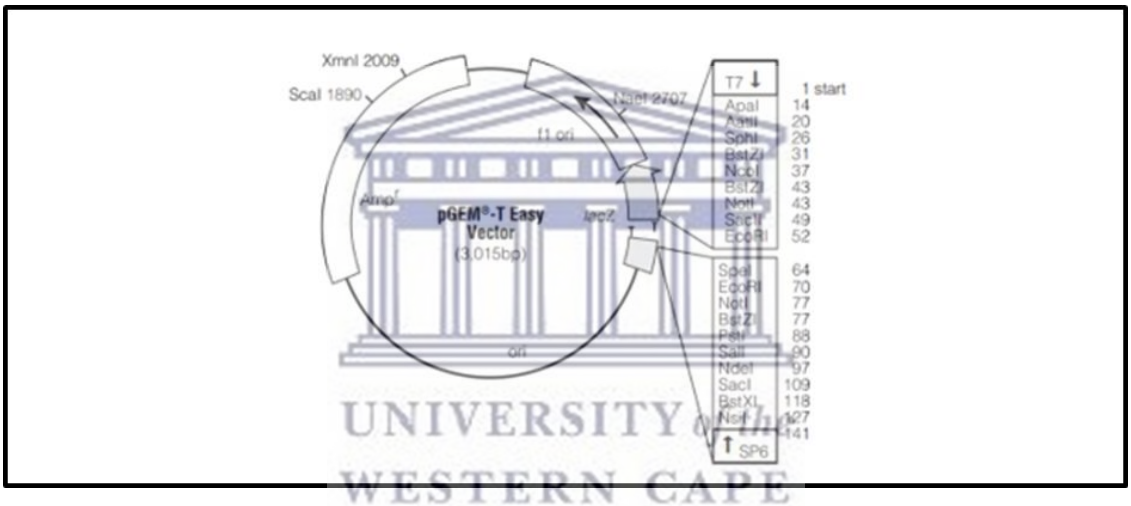

Figure 3.1: pGEM®-T Easy Vector map and sequence reference points

The map illustrates the different elements of pGEM®-T Easy Vector; containing the T7 and SP6 promoters to initiate transcription (with MCS located in between), lacZ gene for hydrolysis of X-Gal for blue/white colony selection, f1 origin and Ampicillin resistance gene (Promega).

Expression of HCoV-NL63 ORF3 with the *Bac-to-Bac® Baculovirus Expression System*

The subsequent high concentration purified DNA will be ligated into the pFastBac™1 transfer vector at restriction sites *Bam*H I and *Xho*I (Figure 3.2). The pFastBac™1 transfer vector contains both ampicillin and gentamicin resistance genes which only facilitate growth of *E. coli* cells with successfully ligated DNA products. The pFastBac-ORF3 construct will then be transformed into *MAX Efficiency™*

DH5∝E. coli competent cells for colony selection via PCR prior to generation of larger cultures.

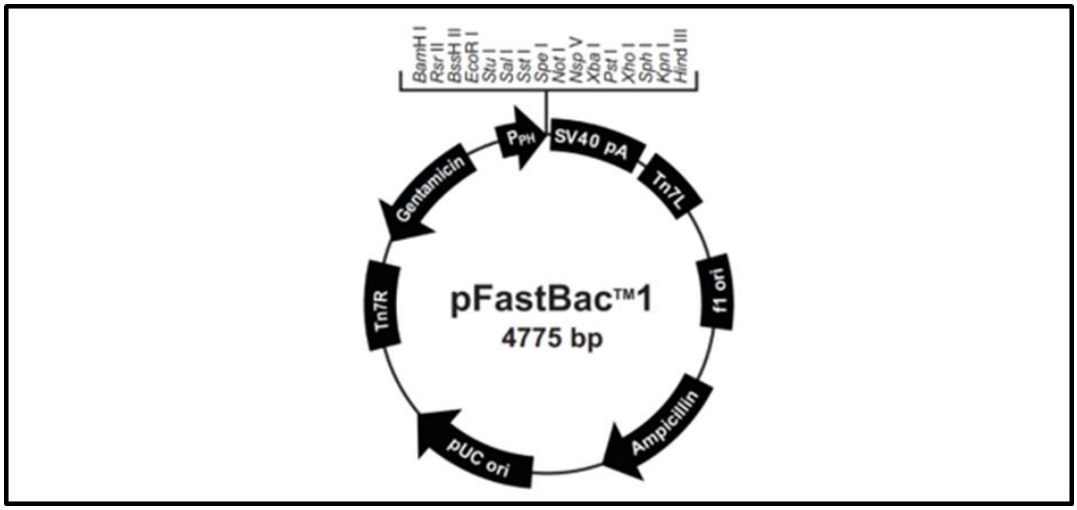

Figure 3.2: pFastBac™1 vector map and features

The map illustrates the different elements of pFastBac™1. The bacterial plasmid contains the polyhedron promoter (P_{PH}), multiple cloning site (MCS) that permits restriction enzyme-mediated ligation of HCoV-NL63 ORF3; the SV40 polyadenylation signal, Tn7L and Tn7R elements, f1 origin, Gentamicin-and Ampicillin-resistant genes and a pUC origin that allows for high-copy replication and maintenance of the gene of interest in the E. coli cell. The ORF3 gene will be cloned within the MCS of pFastBac plasmid (Thermo Fisher Scientific).

Miniprep pFastBac-ORF3 DNA will then be transformed into *MAX Efficiency™ DH10Bac Competent Cells* which will facilitate the transposition of pFastBac-ORF3 plasmid into the Bacmid to generate the Bacmid-ORF3 construct. Transposition into the Bacmid DNA adds kanamycin- and tetracycline-resistance genes which will only facilitate *E. coli* cell growth of the transposed DNA of interest. *DH10Bac E. coli* will undergo blue/white colony selection for generation of larger broths for plasmid DNA purification once more with the *Machery-Nagel NucleoBond Xtra Midi kit,* as SF9 cell transfection requires a DNA concentration of 0.5 µg/µL as a minimum guideline. The midiprep Bacmid-ORF3 construct will undergo a M13 primer and gene specific primer RT-PCR to confirm that the gene of interest is present in the Bacmid and in the correct orientation. The flow diagram in Figure 3.3 highlights the methodology of Baculovirus DNA generation.

Generation of ORF3-Baculovirus for HCoV-NL63 ORF3 Gene Expression

After RT-PCR confirmation of the transposition of the gene of interest into the Bacmid the purified Bacmid-ORF3 DNA will then be used to transfect SF9 insect cells with the *Cellfectin® II reagent*. This will result in the generation and production of an HCoV-NL63 ORF3 Baculovirus stocks from SF9 cell supernatant. Figure 3.4 highlights the workflow of SF9 cell infection with Baculovirus DNA.

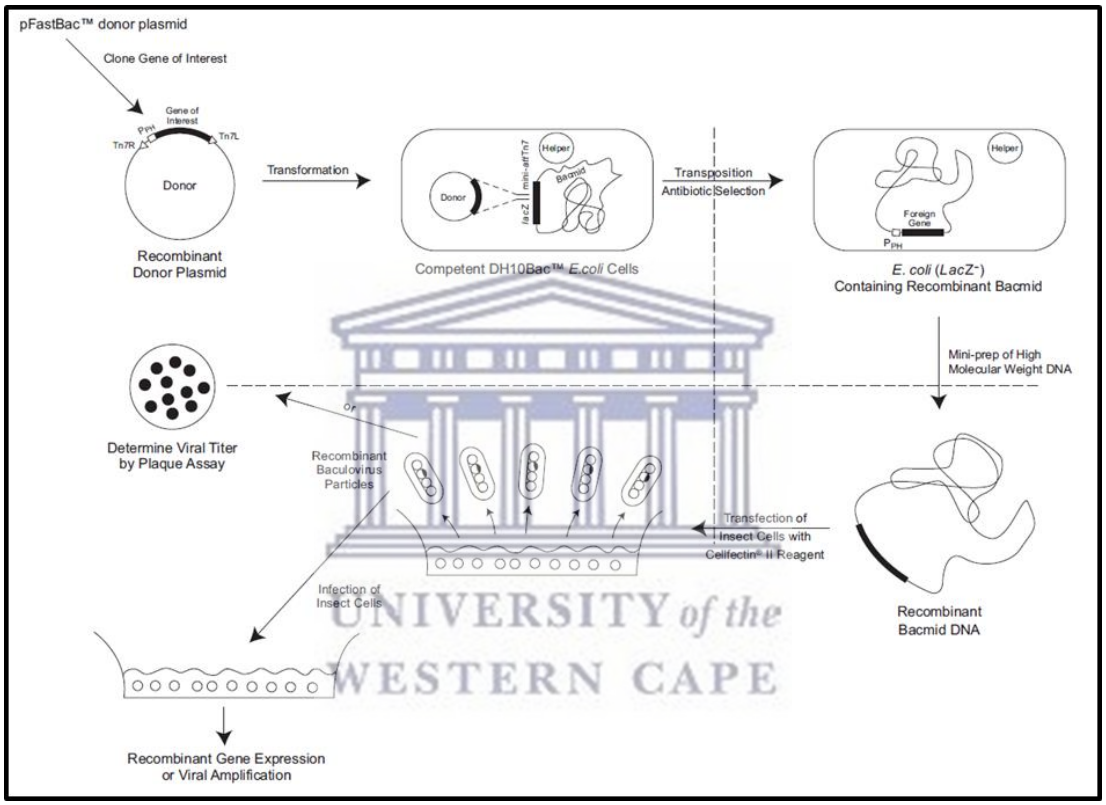

Figure 3.3: Diagram of the Bac-to-Bac® System

The figure depicts the generation of recombinant baculovirus and the expression of the gene of interest (Thermo Fisher Scientific).

The transfection Baculovirus produced is labelled P0 stock and is used as a method of screening. As a fixed Bacmid-DNA lipid complex concentration is used over a time course and amplification is dependent on cell CPE. This preliminary P0 stock will then be used to infect a fresh batch of SF9 cells to amplify the Baculovirus to generate a P1 stock which will be quantified by a viral plaque assay. Subsequently if the P1 stock viral titre is above $1x10^6$ pfu/mL it can be used for protein expression

studies via Western blot. If the ORF3-Baculovirus DNA titre is below the aforementioned baseline figure the P1 stock will be amplified to a P2 stock and a viral plaque assay will be conducted to establish the P2 stock viral titre.

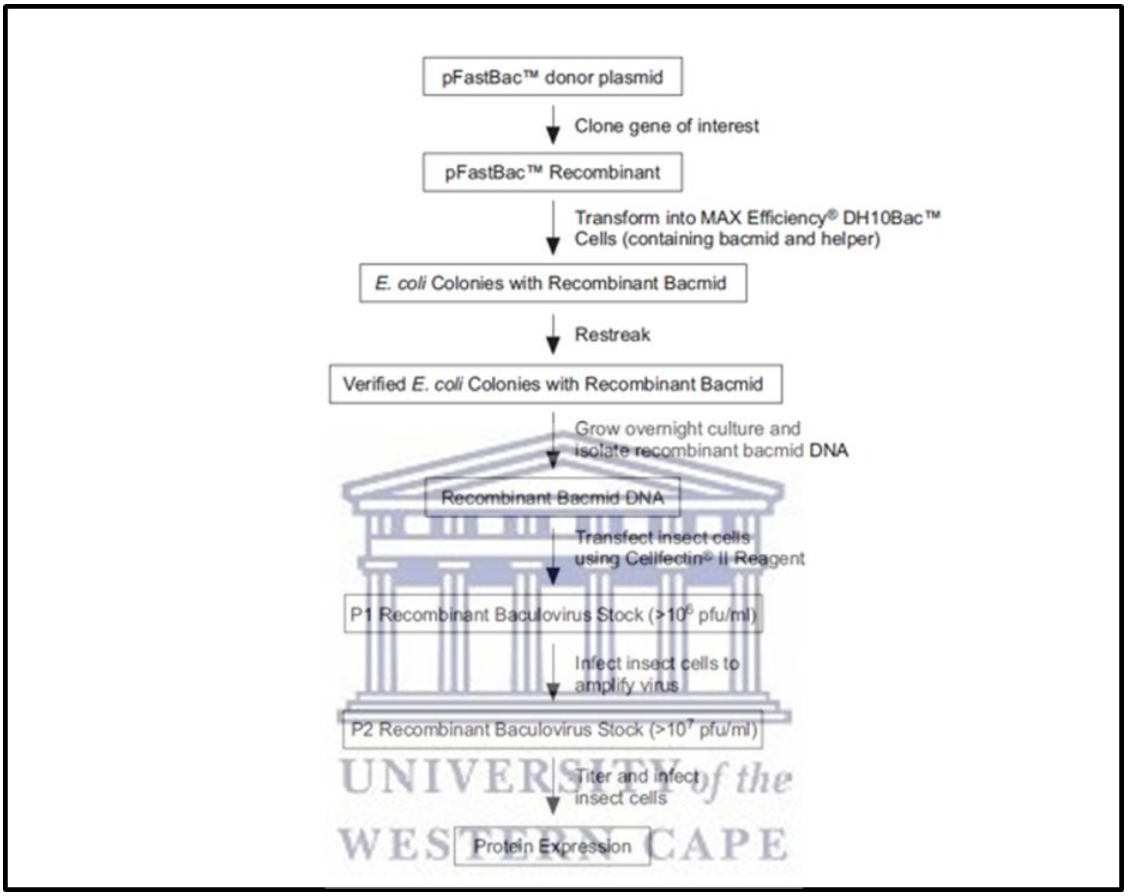

Figure 3.4: Bac-to-Bac® Baculovirus Expression System workflow

Flow diagram depicting the necessary checkpoints for expressing HCoV-NL63 ORF3 protein (Thermo Fisher Scientific).

HCoV-NL63 ORF3 Protein Expression Studies

Baculovirus plaque expression in SF9 cell lysates will then allow for HCoV-NL63 ORF3 protein expression studies at varying multiplicity of infection (ΔMOI) over a fixed time course. Protein expression lysates will be quantified via SDS-PAGE and Western blot, thus indicating the relative levels at which HCoV-NL63 ORF3 protein expressed.

Molecular Cloning of HCoV-NL63 ORF3 Gene

Sample Collection and Preparation, and Plasmid-DNA Constructs

Reference samples of full-length recombinant construct of HCoV-NL63 ORF3 in parent vector pCAGGS were provided by Professor B.C. Fielding's Molecular Virology Laboratory (Medical Biosciences Department) at the University of the Western Cape.

Generating Recombinant pGEM-ORF3

RT-PCR to Amplify HCoV-NL63 ORF3 Gene

A polymerase chain reaction (PCR) assay generated the HCoV-NL63 ORF3 gene from the reference pCAGGS-ORF3 plasmid-DNA constructs, along with an HCoV-NL63 ORF3 positive control to validate the presence of the gene of interest within the constructs at the size of ≈720 bp. PCR primers to generate the full-length HCoV-NL63 ORF3 gene were synthesised by Inqaba Biotechnical Industries (Ltd) Pty, South Africa; HCoV-NL63 ORF3 full-length forward and HCoV-NL63 full-length reverse (Table 3.5). Primers incorporated restriction sites *Bam*H I (full-length forward) and *Xho*I (full-length reverse) for downstream cloning into the *Bac-to-Bac™ Baculovirus Expression System*. The *GoTaq® Flexi DNA Polymerase* kit was used for this PCR application (Table 3.6).

Table 3.5: Full-length primers used for RT-PCR amplification

Gene of Interest	Primer	Primer sequence
HCoV-NL63 ORF3	Forward	5'-GCGGATCCGCCACCATGATGCCTTTTGGTGGCC-3'
HCoV-NL63 ORF3	Reverse	5'-CCGCTCGAGATTAATCGAAGGAACATC-3'

PCR cycling parameters are listed in Table 3.7. The PCR products were loaded on a 1% (w/v) agarose gel containing EtBr, along with 1 kB and 100 bp DNA Ladders. The agarose gel was run at 80 Volts for

80 minutes with variable amperage. Agarose gel bands were visualised with a UV transilluminator which illuminated bands at the expected size of ≈720 bp.

PCR Clean Up

Amplified genes of the correct size of ≈720 bp were excised from the agarose gel and placed in 2 mL Eppendorf tubes. The PCR gel clean-up was completed using the *Wizard® SV Gel and PCR clean-up System* according to manufacturer's guidelines.

Table 3.6: HCoV-NL63 ORF3 RT-PCR mix

Reagent	Volume
DNA (0.01 µg/µL)	2 µL
dNTP mix (10 mM)	0.5 µL
Buffer (5x)	5 µL
$MgCl_2$ (25 mM)	1.5 µL
Forward Primer (100 mM)	1 µL
Reverse Primer (100 mM)	1 µL
Nuclease free H_2O	13.5 µL
***Taq* polymerase (5 units/µL)**	0.5 µL
Total	25 µL

Table 3.7: T-PCR cycling parameters of HCoV-NL63 ORF3 gene

Stage	Temperature	Time	Number of Cycles
Initial denaturation	93°C	3 minutes	1 cycle
Denaturation	94°C	45 seconds	30 cycles
Annealing	54°C	45 seconds	
Extension	72°C	5 minutes	
Final extension	72°C	7 minutes	1 cycle

Ligation of ORF3 into pGEM®-T Easy Vector

High concentration plasmid DNA was required to for cloning into the *Bac-to-Bac™ Baculovirus Expression System* which was achieved by initial cloning of the ORF3 into the pGEM®-T Easy Vector (*Promega*) using TA-overhang ligation method, following the manufacturers guidelines in the *pGEM®-T and pGEM®-T Easy Vector Systems* manual. A ligation reaction was set up with the purified PCR amplified HCoV-NL63 ORF3 gene, along with ligation positive and negative controls (Table 3.8). Ligation was conducted at 4°C overnight (14-18 hours).

Table 3.8: HCoV-NL63 ORF3 ligation into pGEM vector

Reagent	ORF3 Experimental 1	Ligation Positive control	Ligation Negative control
pGEM®-T Easy Vector	1 µL	1 µL	1 µL
2x Rapid Ligation Buffer	2 µL	2 µL	2 µL
ORF3 PCR Product	8 µL	-	-
Control Insert DNA	-	2 µL	-
T4 DNA Ligase	1 µL	1 µL	1 µL
Nuclease Free Water	8 µL	14 µL	16 µL
Total	**20 µL**	**20 µL**	**20 µL**

Transformation of pGEM-ORF3 Into JM109 *E. coli* Cells

Post-overnight incubation the ligation product pGEM-ORF3 was transferred in to sterile 2-mL tube. Thawed *JM109 High Efficiency Competent Cells* were added and gently mixed before being incubated at 4°C for 20 minutes. Transformant tubes were heat-shocked in a dry bath at 42°C for 45 seconds before being returned to ice for 2 minutes. Luria-Bertani (LB) broth was added to each tube and incubated for 90 minutes at 37°C with shaking (150 rpm).

Each transformation culture was plated in duplicate on LB media (Ampicillin, IPTG and X-Gal) agar plates and incubated overnight (16–24 hours) at 37°C for blue/white colony selection. The following day plates were transferred to 4°C for 1 hour to facilitate blue colour development of JM109 colonies.

JM109 *E. coli* Blue/White Colony Selection for Colony PCR and LB Media Starter Cultures

The colonies appear as white if a gene has been successfully ligated into the pGEM plasmid, and blue if there is no insert ligated into the pGEM plasmid. Well isolated colonies were picked with sterile pipette tips (6 white and 2 blue colonies) which were each added to respective PCR reaction mixes momentarily. The pipette tip for each colony picked was then ejected into 10 mL LB Broth (0.1% Ampicillin) and placed in a shaking incubator at 37°C for 16 hours to generate starter cultures.

To validate the successful ligation and transformation of ORF3 into the pGEM plasmid a colony PCR was run according to the parameters listed in Table 3.7. The PCR product was loaded on a 1% agarose gel containing EtBr, along with 1 kB and 100 bp DNA ladder. The agarose gel was run at 80 Volts for 80 minutes with variable amperage. Agarose gel bands were visualised with a UV transilluminator which illuminated bands at the expected size of ≈720 bp when viewed.

Mini-Prep pGEM-ORF3 Cultures

The colony PCR indicated successful ligation and transformation of the HCoV-NL63 ORF3 gene into the pGEM vector. Subsequently, glycerol stocks of the cultures were made by combining 900 µL culture in the ratio 3:1 with sterile 50% (v/v) aqueous glycerol and vortexed thoroughly before being stored at -80°C. The remainder of the culture was used for a mini-prep to isolate pGEM-ORF3 plasmid DNA. Miniprep was conducted with the *Wizard® Plus SV Minipreps DNA Purification System* according to manufacturer guidelines. Miniprep plasmid DNA was then quantified with a nanodrop to determine DNA concentration of 0.099 µg/µL. Miniprep DNA was stored at -20°C.

Restriction Endonuclease Double Digest Confirming Successful ORF3 Insertion Into pGEM Vector

Miniprep DNA of pGEM-ORF3 was digested with restriction enzymes *Bam*H I and *Xho*I *(Promega)* and Buffer H *(Promega)* (Table 3.9). pGEM-ORF3 constructs were digested in a thermocycler for 1 hour at 37°C, restriction enzymes inactivated for 15 minutes at 65°C and held at 4°C. The double digest product was diluted with Blue/Orange Loading dye 6x and was run on a 1% (w/v) agarose gel containing EtBr at 80 Volts for 80 minutes with variable amperage. Agarose gel bands were visualised with a UV transilluminator which illuminated bands of the ORF3 gene insert at the expected size of ≈720 bp, and pGEM vector at 3 kB.

Table 3.9: Double restriction enzyme digest pGEM-ORF3

Reaction Tube	pGEM-ORF3 Experimental 1	pGEM-ORF3 Experimental 2	pXJ40-N-FLAG Positive control
Nuclease-free water	36.5 µL	35.5 µL	41.5 µL
Buffer H	5 µL	5 µL	5 µL
BSA	0.5 µL	0.5 µL	0.5 µL
DNA	6 µL	7 µL	1 µL
***Bam*H I**	1 µL	1 µL	1 µL
***Xho*I**	1 µL	1 µL	1 µL
Total	**50 µL**	**50 µL**	**50 µL**

pGEM-ORF3 Glycerol Stock Starter Cultures for Midiprep

100 µL of the pGEM-ORF3 glycerol stock was inoculated into 10 mL LB broth (0.1% Ampicillin) and incubated shaking for 8 hours at 37°C to generate starter cultures for larger broths. After incubation 4 mL starter culture was inoculated in to 400 mL 37°C LB broth (0.1% Ampicillin) and incubated shaking for 14 hours at 37°C to generate larger broths.

pGEM-ORF3 Midiprep

To generate high DNA concentration transfection grade plasmid DNA pGEM-ORF3 400 mL LB broth cultures were purified using the *NucleoBond Xtra Midi kit* according to the manufacturer guidelines. Purified pGEM-ORF3 DNA was quantified using a nanodrop and yielded a DNA concentration of 0.75 µg/µL. Midiprep samples were stored at 4°C.

PCR to Confirm ORF3 Gene in High Concentration pGEM-ORF3 Midiprep Sample

A PCR was run with high the concentration DNA (0.75 µg/µL); pGEM-ORF3 midiprep constructs, along with an ORF3 positive control to validate the presence of the gene of interest within the constructs at the size of ≈720 bp. Full-length ORF3 primers were used with the *GoTaq® Flexi DNA Polymerase* kit and 1 ul plasmid DNA as template to confirmed presence of the inserted gene segment by PCR based on protocol in Table 3.10. PCR cycling parameters are as listed in Table 3.7. The PCR product was loaded on a 1% (w/v) agarose gel containing EtBr, along with 1 kB and 100 bp DNA Ladders. The agarose gel was run at 80 Volts for 80 minutes with variable amperage. Agarose gel bands were visualised with a UV transilluminator which illuminated bands at the expected size of ≈720 bp.

Table 3.10: PCR to confirm ORF3 in high concentration pGEM-ORF3 midiprep

Reagent	Volume
DNA (0.075 µg/µL)	1 µL
dNTP mix (10 mM)	0.5 µL
Buffer (5x)	5 µL
$MgCl_2$ (25 mM)	1.5 µL
Forward Primer (100 mM)	1 µL
Reverse Primer (100 mM)	1 µL
Nuclease free H_2O	14.5 µL
***Taq* polymerase (5 units/µL)**	0.5 µL
Total	25 µL

Generation of Recombinant pFastBac™1-ORF3

Restriction Endonuclease Double Digest Generating High Concentration ORF3 Gene

Midiprep DNA of pGEM-ORF3 and vector pFastBac™1 were digested in the required ratios with restriction enzymes *Bam*H I and *Xho*I *(Promega)* and Buffer B *(Promega)* (Table 3.11). ORF3 constructs were digested in a thermocycler for 2 hours at 37°C, restriction enzymes inactivated for 20 minutes at 65°C and held at 4°C.

The double digest was diluted with Blue/Orange loading dye and was run on a 1% (w/v) agarose gel containing EtBr, along with 1 kB and 100 bp DNA ladders. The agarose gel was run at 80 Volts for 80 minutes with variable amperage.

Agarose gel bands were visualised with a UV transilluminator which illuminated bands of the ORF3 gene insert at the expected size of ≈720 bp and pGEM vector at 3 kB, along with *Bam*H I and *Xho*I double digested circular pFastBac™1 vector with a band of 4.7 kB. Experimental digest product bands were excised from the agarose gel and purified using *Wizard® SV Gel and PCR clean-up System* according to the manufacturer's guidelines.

Table 3.11: Double restriction enzyme digest pGEM-ORF3 for Bac-to-Bac sub-cloning

Reaction Tube	pGEM-ORF3 Experimental	pFastBac™1 vector	pXJ40-N-FLAG Positive control
Nuclease Free Water	32.5 µL	30.5 µL	41.5 µL
Buffer H	5 µL	5 µL	5 µL
BSA	0.5 µL	0.5 µL	0.5 µL
DNA	8 µL	10 µL	1 µL
***Bam*H I**	2 µL	2 µL	1 µL
***Xho*I**	2 µL	2 µL	1 µL
Total	**50 µL**	**50 µL**	**50 µL**

Ligation of ORF3 into pFastBac™1 Vector

Sub-cloning the viral gene DNA into pFastBac™1 Vector; the ORF3 gene was ligated into the vector using sticky end cloning with restriction sites *Bam*H I and *Xho*I (Table 3.12). Ligation was conducted at 4°C overnight (14–18 hours).

Table 3.12: HCoV-NL63 ORF3 ligation into pFastBac™1 vector

Reagent	ORF3 Experimental 1	Ligation Positive control	Ligation Negative control
pFastBac™1 Vector 0.5 µg/µL (open circular)	2 µL	-	-
2x Rapid Ligation Buffer	8 µL	8 µL	8 µL
pGEM-ORF3 DNA 0.75 µg/µL	9 µL	-	-
pFastBac™1 Vector 0.5 µg/µL (circular)	-	2 µL	2 µL
Control insert DNA	-	2 µL	-
T4 DNA Ligase	1 µL	1 µL	1 µL
Nuclease-Free Water	-	7 µL	9 µL
Total	20 µL	20 µL	20 µL

Transforming pFastBac™1-ORF3 Into DH5∝ *E. coli* Competent Cells

Post-overnight incubation the ligation product pFastBac-ORF3 was transferred into sterile 2-mL tubes. Thawed *MAX Efficiency™ DH5∝ E. coli competent cells* were added and gently mixed before being incubated at 4°C for 20 minutes. Transformant tubes were heat-shocked in a dry bath at 42°C for 45 seconds before being returned to ice for 2 minutes. Pre-warmed 37°C LB broth (900 µL) was added to each tube and incubated for 90 minutes at 37°C with shaking (150 rpm). Each transformation culture was pelleted by centrifugation at 3000 rpm for 10 minutes at room temperature. Excess LB broth was removed from tubes leaving ≈200 µL for resuspension and plating. Transformants were spread plated on LB agar plates (0.1% Ampicillin). Plates were incubated overnight (16–24 hours) at 37°C.

DH5∝ *E. coli* Colony Picking for Colony PCR and LB Media Starter Cultures

To validate the successful ligation and transformation of ORF3 into pFastBac™1 a colony PCR was run. DH5∝ *E. coli* competent cells do not allow for blue/white colony selection therefore a colony PCR must be undertaken as a confirmation step. Four well isolated colonies were picked with sterile pipette tips which were each added to PCR reaction mixes momentarily. The pipette tip for each colony picked was then ejected into 10 mL LB Broth (0.1% Ampicillin) and placed in a shaking incubator at 37°C for 16 hours to generate starter cultures. The colony PCR, gel electrophoresis and amplicon visualisation was done as already described.

Mini-Prep pFastBac™1-ORF3 Cultures

The colony PCR indicated successful ligation and transformation of the viral gene ORF3 into the pFastBac™1 vector. Subsequently, glycerol stocks of the cultures were made by combining culture with 50% (v/v) aqueous glycerol in the ratio 3:1 and vortexed thoroughly before being stored at -80°C. The remainder of the culture was used for a mini-prep to isolate pFastBac-ORF3 plasmid DNA. Mini-prep was conducted with the *Wizard® Plus SV Minipreps DNA Purification System* according to manufacturer guidelines. Mini-prep plasmid DNA was then quantified with a nanodrop to determine DNA concentration of 0.132 µg/µL, where a minimum plasmid DNA concentration required for transposition into DH10Bac Competent Cells is 0.1 µg/µL. Mini-prep DNA was stored at -20°C.

Restriction Endonuclease Double Digest Confirming Successful ORF3 Insertion Into pFastBac™1

Miniprep DNA of pFastBac-ORF3 was digested in the required ratios with restriction enzymes *Bam*H I and *Xho*I and Buffer B (Table 3.13). ORF3 constructs were digested in a thermocycler for 1 hour at 37°C, restriction enzymes inactivated for 15 minutes at 65°C and held at 4°C. Double digest products was diluted with 6x Loading dye and run on a 1% (w/v) agarose gel containing EtBr at 80 Volts for 80 minutes with variable amperage. Agarose gel bands were visualised with a UV transilluminator which

displayed bands of the ORF3 gene insert at the expected size of ≈720 bp, and pFastBac™1 vector at 4.7 kB.

Table 3.13: Double restriction enzyme digest pFastBac™1-ORF3 for confirmation of gene insertion

Reaction Tube	pFastBac™1-ORF3 Experimental 1	pFastBac™1-ORF3 Experimental 2	pFastBac™1-ORF3 Experimental 3	pXJ40-N-FLAG Positive control
Nuclease Free Water	39 µL	39 µL	38.5 µL	41.5 µL
Buffer B	5 µL	5 µL	5 µL	5 µL
BSA	0.5 µL	0.5 µL	0.5 µL	0.5 µL
DNA	3.5 µL	3.5 µL	4 µL	1 µL
***Bam*H I**	1 µL	1 µL	1 µL	1 µL
***Xho*I**	1 µL	1 µL	1 µL	1 µL
Total	**50 µL**	**50 µL**	**50 µL**	**50 µL**

Sequencing HCoV-NL63 ORF3 Gene Within pFastBac™1 Vector

Samples of pFastBac-ORF3 miniprep were sequenced with pFastBac™1 plasmid specific forward and reverse primers to ensure no base-pair mutations occurred. DNA sequencing using Sanger method were conducted by Inqaba Biotechnical Industries (Ltd) Pty, South Africa. Results were viewed with MEGA X: Molecular Evolutionary Genetics Analysis version 10.1.8 (Kumar et al., 2018) and extracted sequences were aligned using the multiple sequence alignment in ClustalX2 version 2.1 (Larkin et al., 2007). Files were subsequently extracted to GeneDoc version 2.7 (Nicholas & Nicholas, 1997) for viewing (Appendix 1).

Generating Recombinant Bacmid-ORF3 DNA

Transforming pFastBac™1-ORF3 Into DH10Bac E. *coli* Competent Cells

The ORF3 gene was confirmed to have been successfully cloned into pFastBac™1. Purified mini-prep pFastBac-ORF3 DNA was subsequently transformed into *MAX Efficiency™ DH10Bac Competent Cells (Thermo Fisher)*. *DH10Bac competent cells* were thawed and sterile 2-mL polypropylene tubes were pre-chilled on ice for 20 minutes. pFastBac-ORF3 mini-prep DNA was diluted to a concentration of 1 ng/µL and 5 µL of diluted plasmid DNA was added to 100 µL of thawed *DH10Bac cells* in the pre-chilled tubes before being incubated on ice for 30 minutes. The cells were heat-shocked in a heating block for 45 seconds at 42°C without shaking and then returned to ice for 2 minutes. 900 µL of room temperature Super Optimal Broth with Catabolic repressor (S.O.C.) media was added to each tube of cells and placed in a 37°C shaking incubator for 4 hours at 225 rpm.

After incubation of the pFastBac-ORF3 transformants, 10-fold serial dilutions of the constructs were prepared with S.O.C. media ranging from 10^{-1} to 10^{-3}. S.O.C media plates were prepared containing 50 µg/mL kanamycin, 7 µg/mL gentamicin, 10 µg/mL tetracycline, 100 µg/mL X-gal, and 40 µg/mL IPTG. 100 µL of each serial dilution transformant was spread plated on S.O.C. agar plates, which was incubated at 37°C for 48 hours. Following incubation plates were transferred to 4°C for 1 hour to facilitate blue colour development. Once pFastBac-ORF3 was transformed into the bacterial cells the process of transposition into the Bacmid will occur and thus generating recombinant Bacmid-ORF3 constructs.

DH10Bac *E. coli* Blue/White Colony Selection for S.O.C. Media Starter Cultures

To validate the transformation of pFastBac-ORF3 and subsequent transposition of ORF3 into the Bacmid 10 white and 2 blue colonies were picked with sterile pipette tips. The pipette tip for each colony picked was then ejected into 10 mL S.O.C. media prepared containing 50 µg/mL kanamycin, 7

µg/mL gentamicin, 10 µg/mL tetracycline and placed in a shaking incubator at 37°C for 8 hours to generate starter cultures. Post incubation glycerol stocks of the cultures were made by combining culture with 50% (v/v) aqueous glycerol in the ratio 3:1 and vortexed thoroughly before being stored at -80°C.

Bacmid-ORF3 Larger Cultures for Midiprep

To generate high concentration transfection grade Bacmid-ORF3 DNA 4 starter cultures were inoculated 1:100 into 400 mL of pre-warmed S.O.C. media containing 50 µg/mL kanamycin, 7 µg/mL gentamicin, 10 µg/mL tetracycline. Bacmid-ORF3 inoculated S.O.C. media was incubated shaking for 14 hours at 37°C to generate larger broths. Following *Machery-Nagel NucleoBond Xtra Midi kit* instructions according to manufacturer guidelines 2 midiprep Bacmid-ORF3 samples were generated. Purified Bacmid-ORF3 DNA samples were quantified using a nanodrop and yielding a DNA concentration of 0.45 µg/µL and 0.49 µg/µL respectively. Midiprep samples were stored at 4°C.

PCR of Recombinant Bacmid-ORF3 DNA

Recombinant Bacmid DNA is greater than 135 kB in size, thus a restriction endonuclease digest is not feasible. PCR analysis of Bacmid-ORF3 midiprep DNA will verify the presence of the ORF3 gene of interest in the recombinant Bacmid. M13 primers (Table 3.14) that hybridize to sites flanking the mini-attTn7 site within the lacZα-complementation region was used to facilitate PCR analysis (Figure 3.5).

To confirm the transposition of ORF3 into the Bacmid in the correct orientation the PCR was designed to use combinations of HCoV-NL63 ORF3 gene specific primers (Table 3.7) and M13 primers so that the amplification of ORF3 occurs on both the areas flanking the mini-*att*Tn7 region and within the ORF3 gene of interest. In Table 3.15 the expected band sizes of the amplified regions were calculated to validate the ORF3 transposition and orientation within the Bacmid based on forward and reverse primer binding.

Table 3.14:M13 primers used for PCR amplification

***mini-attTn7* binding site**	**Primer**	**Primer sequence**
M13	Forward	5'-CCCAGTCACGACGTTGTAAAACG-3'
M13	Reverse	5'-AGCGGATAACAATTTCACACAGG-3'

Promega GoTaq® Flexi DNA Polymerase kit was optimised for this Bacmid-ORF3 amplification (Table 3.16). Both M13 and gene specific full-length primers were synthesised by Inqaba Biotechnical Industries (Ltd) Pty, South Africa and have a common annealing temperature of 54°C - PCR cycling parameters are listed in Table 3.17.

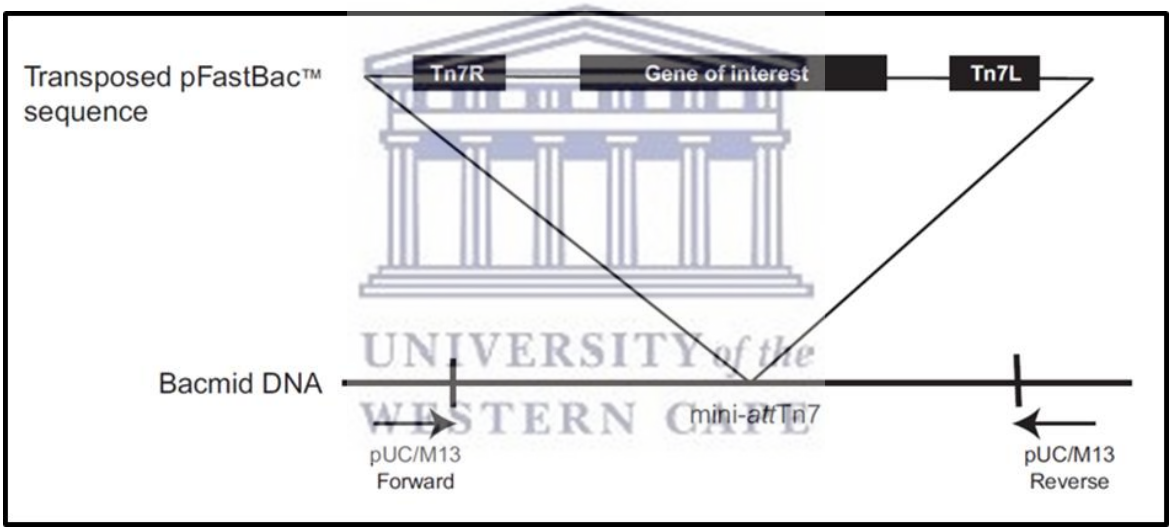

Figure 3.5: Schematic displaying flanking binding sites of M13 primers to mini-attTn7

The region spanning M13 forward primer binding site to mini-attTn7 length is 128 bp and region spanning M13 reverse primer binding site to mini-attTn7 length is 145 bp. Thus, indicating that if no gene is inserted into the Bacmid a band of ≈273 bp is visible on agarose gel electrophoresis (Thermo Fisher Scientific).

The PCR was product loaded on a 1% (w/v) agarose gel containing EtBr, along with 1 kB and 100 bp DNA ladders. The agarose gel was run at 80 Volts for 80 minutes with variable amperage. Agarose gel bands were visualised with a UV transilluminator which displayed bands at the expected sizes listed in Table 3.15.

Table 3.15:M13 and HCoV-NL63 full-length primer combinations to confirm ORF3 gene transposition and orientation in the Bacmid

Reaction Tube	Forward Primer	Reverse Primer	Expected size
1	M13 Forward	M13 Reverse	3041 bp
2	M13 Forward	HCoV-NL63 ORF3 Reverse	2390 bp
3	HCoV-NL63 ORF3 Forward	M13 Reverse	1320 bp
4	HCoV-NL63 ORF3 Forward	HCoV-NL63 ORF3 Reverse	741 bp

Table 3.16: Bacmid-ORF3 PCR mix

Reagent	Volume
DNA (0.01 µg/µL)	1 µL
dNTP mix (10 mM)	1 µL
Buffer (5x)	10 µL
$MgCl_2$ (25 mM)	3 µL
Forward Primer (100 mM)	1.25 µL
Reverse Primer (100 mM)	1.25 µL
Nuclease free H_2O	32 µL
***Taq* polymerase**	0.5 µL
Total	50 µL

Table 3.17:RT-PCR cycling parameters for Bacmid-ORF3

Stage	Temperature	Time	Number of Cycles
Initial- denaturation	93°C	3 minutes	1 cycle
Denaturation	94°C	45 seconds	
Annealing	54°C	45 seconds	35 cycles
Extension	72°C	5 minutes	
Final extension	72°C	7 minutes	1 cycle

Producing Recombinant HCoV-NL63 ORF3 Baculovirus

Growth and Maintenance of SF9 Insect Cells in Cell Culture

SF9 Insect Cell Culture

Spodoptera frugiperda insect cells were used as the host for baculovirus expression. Cultures of SF9 cells were grown in complete Supplemented Grace's Insect Medium*;* which was enhanced with 10% *FBS* and 1% *anti-mycotic*. Insect cells were maintained as adherent cells grown in a monolayer in 25-cm^2 and 75-cm^2 sterile tissue culture flasks in a sterile laminar flow hood. Cells were incubated at 27°C in a humidified incubator. SF9 cells were routinely sub-cultured at 2-3-day intervals.

SF9 Insect Cell Storage

Stocks of SF9 insect cells were routinely suspended in cryo-media (Grace's Insect media supplemented with 20% FBS and 10% DMSO) at low passage number and stored at -80°C until required. Insect cells were passaged 2 times minimum after removal from cryo-storage for downstream experiments and were propagated up until passage 6 maximum.

Transfection

Bacmid-ORF3 Midiprep DNA

Bacmid-ORF3 midiprep DNA was quantified with a nanodrop to establish a concentration of 0.45 µg/µL. The *Bac-to-Bac® Baculovirus Expression System* manual states a minimum 0.5 µg/µL Bacmid DNA is needed for transfection, however constructs are functionally different and dependent on the type of gene which is cloned into the Bacmid. The workflow for the process of generating ORF3 Baculovirus is outlined in Figure 3.3.

Seeding Cells for Transfection

SF9 insect cells were carefully monitored and determined to be in log phase. At 90% confluency,

passage 2 cells were detached and spun down at 3000 rpm for 3 minutes to yield a pellet. The SF9 cell pellet was resuspended in fresh complete media containing 10% FBS and 1% Anti-mycotic to create a cell suspension for cell count and seeding. A cell count was done on the SF9 cell suspension with an inverted light microscope and haemocytometer; 1:1 (v/v) dilution of cell suspension and trypan blue. The transfection protocol from the *Bac-to-Bac® Baculovirus Expression System* required a minimum of 95% total cell viability of which the criteria was met with the equation below.

$$Percentage\ SF9\ cell\ viability = \frac{\#\ of\ viable\ cells}{Total\ \#\ of\ cells} \times 100$$

SF9 insect cell suspension was subsequently seeded in a 6-well plate format with 2 mL per well at a density of $4x10^5$/mL and allowed to attach for 30 minutes. After incubation to allow cell attachment the media from the 6-well plate was removed and replaced with 15% plating media Supplemented Grace's Insect Medium (10% FBS, without anti-mycotic) and 85% Unsupplemented Grace's Insect Medium (without FBS and anti-mycotic).

Bacmid-ORF3 Transfection

Bacmid-ORF3 DNA-lipid complexes were prepared for transfection in 5 steps as follows.

1. Cellfectin® II reagent was vortexed and 8 µL was combined with 100 µL Unsupplemented Grace's Medium (without FBS and anti-mycotic) in sterile 2 mL tubes per transfection well required. Tubes vortexed briefly to mix.

2. A final concentration of ≈1 µg recombinant Bacmid-ORF3 DNA was required for transfection per well. Therefore 2 µL of Bacmid-ORF3 DNA (0.45 µg/µL) was diluted in 100 µL Unsupplemented Grace's Medium (without FBS and anti-mycotic) in sterile 2 mL tubes per transfection well required. Tubes vortexed briefly to mix.

3. The appropriate diluted Cellfectin® II tube contents *(Step 1)* were combined with the appropriate diluted DNA (Step2), thus generating Bacmid-ORF3 DNA lipid complex with a total volume ≈210 µL. Tubes were mixed gently and incubated for 20 minutes at room temperature

4. Post incubation the ≈210 µL DNA-lipid mixture (Step 3) was added dropwise onto the attached cells of the respective well to transfect. Cells were incubated at 27°C for 5 hours.

5. After the 5 hours the media containing transfection mixture was removed (Step 5). Replaced media in wells with 2 mL of fresh complete Supplemented Grace's Insect Medium (10% FBS, 1% anti-mycotic). Plates were incubated at 27°C.

Isolating P0 Viral stock

Transfection media was collected at 24; 48; 72 and 96 hours, taking note of CPE in relation to the SF9 cells-only and SF9 cells + Cellfectin® II background controls for reference. Once the transfected cells demonstrated signs of late stage infection the medium containing virus was collected from each well (≈2 mL) and transferred to sterile 15 mL tubes. Tubes were centrifuged at 500 × g for 5 minutes to remove cells and large debris. The clarified supernatant was transferred to fresh 15 mL tubes by filter sterilising with low protein binding 0.22-µm filters. This was the P0 viral stock of the ORF3 Baculovirus which is stored at 4°C protected from light.

Determination of Viral Titre of ORF3-Baculovirus Stock

Amplification of P0 Transfection Stock Generating P1 Viral Stock

To amplify the P0 viral stock to a P1 viral stock SF9 cells were infected with transfection P0 viral stock showing late stage infection (72 hours). A multiplicity of infection (MOI) was set at the lower limit of 0.05 MOI for better baculovirus quality and the viral titre was set at a baseline of $1x10^6$ pfu/mL, in accordance with the *Bac-to-Bac™ Baculovirus Expression System*. Fresh SF9 cells were seeded in 6-well plate format at $1x10^6$ cells/mL and incubated for 1 hour to attach. The equation below

determined that 100 µL of the 72-hour P0 ORF3-Baculovirus stock must be added dropwise to the fresh SF9 cells for amplification.

$$Inoculum\ required\ (mL) = \left(\frac{MOI\ (pfu/cell) \times \#\ of\ cells}{titer\ of\ viral\ stock\ (pfu/mL)} \right)$$

After 48 hours, significant CPE was noted in relation to the SF9 cells-only background control for reference. The P1 infection media was collected at 48 hours post-infection, duplicate wells were pooled (≈2 mL/well) and transferred to sterile 15 mL tubes. The tubes were centrifuged at 500 × g for 5 minutes to remove cells and large debris. The clarified supernatant was transferred to fresh 15 mL tubes by filter sterilising with low protein binding 0.22-µm filters. This was the P1 viral stock of the ORF3 Baculovirus which was stored at 4°C protected from light.

Viral Plaque Assay to Determine Titre of P1 Viral Stock

To quantify and determine how many plaque forming units the P1 viral stock of the ORF3 Baculovirus contained (viral titre) a plaque assay was carried out. Secondly the assay provides a high purity plaque purified virus for subsequent infections.

Working in a laminar flow Passage 3 SF9 cells were seeded in 6-well plate format at $5x10^5$ cells/mL and incubated for 1 hour to allow cell adherence. Concurrently serial dilutions (1:10) of the P1 viral stock were made with Supplemented Grace's Medium (without FBS and anti-mycotic) ranging from 10^{-1} to 10^{-12}. Post attachment incubation the growth media was removed from all wells. Wells were labelled in duplicate, which included cells-only control and those to be infected with the P1 viral stock ranging from lowest viral concentration 10^{-12} to the highest 10^{-5}. 1 mL of Supplemented Grace's Medium (without FBS and anti-mycotic) was added to each of the cells-only control wells and 1 mL of each respective P1 viral stock serial dilution (10^{-12} to 10^{-5}) was added to the appropriately labelled wells. Infected plates were incubated for 45 minutes.

During incubation plaquing media for agarose overlay to immobilise ORF3-Baculovirus plaques was made as follows:

1. In a 100 mL sterile glass autoclave bottle 20.8 mL 2x Grace's Insect Cell Culture Medium was combined with 4.2 mL FBS.

2. Then 12.5 mL sterile dH_2O and 12.5 mL 4% Agarose gel (40°C) was added to the mixture above (Step 1).

3. Plaquing media generated (Step 2) was stored in a 40°C water bath until infected cells completed the 45-minute incubation.

After incubation, working one plate at a time to avoid desiccation of the cell monolayer, the diluted infection media was removed from the 6-well plate. The Plaquing media was retrieved from the water bath and place in the laminar flow hood. 2 mL of the agarose overlay was dispensed into each well, taking care to avoid air bubble formation in the wells. The Plates were covered in foil to allow agarose overlay to solidify in the laminar flow for 1 hour at room temperature. The viral plaque assay was incubated at 27°C for 7-10 days with humidity. The viral plaques were present on day 5 post-infection.

MTT Stain to Quantify Viral Plaques

Non-formazan MTT stain was used as a SF9 cell viability stain to view and quantify ORF3-Baculovirus plaques on day 7 post-infection of plaque assay. The stain was made by combining 10% (w/v) MTT diluted with sterile 1x PBS and filter sterilised through a 0.22-µm filter before being stored in the dark at 4°C. Plaque assay 6-well plates were removed from incubator and transferred to laminar flow hood with the light off (MTT stain is light sensitive). 200 µL MTT stain was added to each well containing the agarose overlay.

Plates were returned to 27°C incubator for 4 hours to allow the stain to develop. Living SF9 cells

displayed a Blue/Black stain and the definitive clear areas in the cell monolayer were indicative of no viable SF9 cells, these were the ORF3-Baculovirus plaques. The number of plaques present in each well for the duplicate infection dilutions of ORF3-Baculovirus were recorded. Using the formula below the titre of the P1 viral stock was quantified and further amplification of the P1 viral stock was required, as protein expression studies via Western blot require a viral titre of $1x10^6$ pfu/mL.

$$Titre\ (pfu/mL) = Number\ of\ plaques \times dilution\ factor \times \left(\frac{1}{1\ mL\ inoculum/well}\right)$$

Picking Viral Plaques for Plaque Purification

After quantifying P1 stock infection plaques and recording findings, 5 viral plaques were picked from the dilution ratio wells 10^{-6} and 10^{-7} with sterile Pasteur pipettes. Each agarose plug containing a singular plaque was transferred to a sterile 2 mL tube containing 500 µL Supplemented Grace's Medium (without FBS and anti-mycotic). Viral plaques in media suspension were thoroughly mixed by vortexing.

Passage 4 SF9 cells were seeded in a 6-well plate format at a density of $5x10^5$ and allowed to incubate for 1 hour for cell attachment. Each of the 5 agarose plugs in suspension were used to infect the SF9 cells in a 6-well plate by adding 100 µL of the suspension dropwise per experimental well. Each plate contained a cells-only control and 5 wells of plaque infected SF9 cells. Plates were incubated at 27°C for 72 hours.

Plaque Purified Viral Stock Collection

After 72 hours incubation, SF9 supernatant media was collected, pooling duplicate wells (≈2 mL/well) for 5 cell supernatants collected and transferred to sterile 15 mL tubes. The tubes were centrifuged at 500 × g for 5 minutes to remove cells and large debris.

Amplification of Plaque Purified Viral Stock Generating Plaque Purified P2 Stock

Another round of amplification was deemed necessary as the calculated viral titre of the P1 viral stock used for the plaque assay was on the lower end of the threshold for protein expression studies via Western blot, a viral titre of $1x10^6$ pfu/mL was needed. To amplify the collected plaque purified supernatant from above - fresh SF9 cells were infected with the plaque purified stocks. The plaque purified stocks originating from the plaque assay wells with the most optimal dilution range for expected viral titre were selected (viral plaques #1 and #4).

Passage 3 SF9 cells were seeded in 6-well plate format at $1x10^6$ cells/mL and incubated for 1 hour to allow the cells to attach. The plaque purified stocks from viral plaques #1 and #4 were used to infect a 6-well plate of SF9 cells with a MOI 0.05 by adding 100 µL dropwise per well. Each plate contained a cells-only control and 5 wells of plaque infected SF9 cells. Plates were incubated at 27°C for 72 hours.

P2 Plaque Purified Viral Stock Collection

After 72 hours incubation, SF9 supernatant media was collected, pooling duplicate wells (≈2 mL/well) and transferred to sterile 15 mL tubes. The tubes were centrifuged at 500 × g for 5 minutes to remove cells and large debris. The clarified supernatant was transferred to fresh 15 mL tubes by filter sterilising with low protein binding 0.22-µm filters. This was the unquantified P2 viral stock of the ORF3-Baculovirus which is stored at 4°C protected from light.

Viral Plaque Assay to Determine Titre of P2 Viral Stock Post Amplification

A second and final plaque assay was carried out using the plaque purified P2 viral stock to quantify and determine how many plaque forming units were in the P2 viral stock. The viral stock plaque #1 from plaque purification amplification was used. Working in a laminar flow Passage 4 SF9 cells were seeded in 6-well plate format at $5x10^5$ cells/mL and incubated for 1 hour to allow cell adherence. Concurrently serial dilutions (1:10) of P2 viral stock (viral plaque #1) were made with Supplemented

Grace's Medium (without FBS and anti-mycotic) ranging from 10^{-1} to 10^{-14}. Post attachment incubation the growth media was removed from all wells. The wells were labelled in duplicate, which included cells-only controls and those to be infected with P2 viral stock ranging from lowest viral concentration 10^{-14} to the highest 10^{-4}. 1 mL of Supplemented Grace's Medium (without FBS and anti-mycotic) was added to each cells-only control well and 1 mL of each respective P2 viral stock serial dilution (10^{-14}-10^{-4}) was added to the appropriately labelled well. Infected plates were incubated for 45 minutes. During incubation plaquing media for agarose overlay to immobilise ORF3-Baculovirus plaques was completed as listed in the methodology above for P1 stock plaque assay.

MTT Stain to Quantify Viral Plaques

Plaque staining procedure for P2 stock quantification was completed as listed earlier in methodology.

HCoV-NL63 ORF3 Protein Expression in SF9 Insect Cells

ORF3-Baculovirus SF9 Cell ΔMOI Infection

P2 Viral Stock Infection at ΔMOI for Protein Expression

Passage 4 SF9 cells were seeded in 24-well plate format at $1.5x10^6$ cells/mL and incubated for 1 hour to attach. The quantified P2 viral stock was used to infect SF9 cells in a 24-well plate with a ΔMOI range (0.1; 1; 2.5; 5 and 10), where a MOI of 1 relates to 1:1 ratio of plaque forming units per SF9 cell. The formula below was used to calculate volume of inoculum required from the P2 viral stock to infect the SF9 cells at each desired MOI.

$$Inoculum\ required\ (mL) = \left(\frac{MOI\ (pfu/cell) \times \#\ of\ cells}{titer\ of\ viral\ stock\ (pfu/mL)}\right)$$

The plate contained a cells-only control and replicates of the infections at the ΔMOI range. Plates were incubated at 27°C for 72 hours.

Collecting ΔMOI Supernatants and Generating Cell Lysates for Protein Expression

After 72 hours incubation SF9 supernatant media was collected, pooling duplicate wells (≈0.3 mL/well) and transfer to sterile 2 mL tubes. The tubes were centrifuged at 500 × g for 5 minutes to remove cells and large debris. The clarified supernatant was transferred to sterile 2 mL tubes by filter sterilising with low protein binding 0.22-µm filters and stored at 4°C protected from light. Once supernatants were collected the remaining ΔMOI SF9 cells were lysed for protein expression studies. 100 µL of 4°C RIPA lysis buffer was added to each well and incubated in laminar flow hood for 10 minutes. Cell scrapers were used to further detach and lyse the SF9 cells. The lysed cells in buffer were then pooled according to cells-only control and each respective MOI (≈0.5 mL per set of replicates) and transferred to sterile 2 mL tubes. Lysate tubes were then incubated on ice for 20 minutes. After incubation on ice the lysates were spun down in a microcentrifuge for at 13000 rpm for 30 minutes at 17°C. Cells-only control and ΔMOI lysate supernatants (soluble fractions) were removed to new sterile tubes and cell pellets (insoluble fractions) remained. Lysate tubes were stored at -20°C. The ΔMOI supernatants and ΔMOI lysates (soluble and insoluble fractions) were collected for downstream SDS-PAGE and Western blot analysis.

ΔMOI Supernatants and Lysates Total Protein Quantification

Total protein harvested from the supernatants and lysates were required to be a known variable in the scope of ORF3-Baculovirus protein studies, as concentration of total protein loaded is standardised to 30 µg per Lane. Total protein contained in supernatant and lysate samples for downstream SDS-PAGE analysis was quantified with the *Pierce™ BCA Protein Assay Kit* according to the manufacturer's guidelines.

HCoV-NL63 ORF3 Protein Detection

Target Protein Linearization and Separation

SDS Gel Casting

SDS Gel Preparation Kit was used for SDS-PAGE applications. An 8% stacking gel and 12.5% separating gel were cast according to the manufacturer's guidelines in 10 Lane 1-mm thick casting trays. SDS-PAGE gels were cast the day prior to loading and running to allow gel to solidify completely. Gels were stored wrapped in cling film submerged in 1X SDS running buffer at 4°C.

SDS-PAGE

SDS gels were run in duplicate to allow for a Coomassie stain for total loaded protein detection and the second gel for wet transfer to PVDF membrane for Western blot protein detection of HCoV-NL63 ORF protein. Quantified lysate samples were thawed on ice for 30 minutes. Gels were loaded in SDS-PAGE tank and filled to the required level for 2 gels with 1X SDS running buffer. Experimental and control samples were diluted 1:1 with Laemmli sample buffer so that each sample was standardised to contain 30 µg total protein. Samples were then incubated at 100°C for 20 minutes in a heating block before being returned to ice prior to loading in the SDS gel. 10 µL of *Prestained PAGE Ruler* was loaded in at least 1 Lane in each gel and samples are loaded in each Lane until well is filled. SDS-PAGE tank was run on *PowerPac™ HC High-Current Power Supply* at 6 mA for 15 minutes, allowing leftover samples to be loaded in the 8% stacking gels respective Lanes where necessary. Once all Lanes had been stacked with 30 µg total protein and samples entered the 12.5% separating gel the power supply's current was lowered to 2 mA and run to completion (≈1 hour).

SDS-PAGE Coomassie Stain

The gel was removed from running tank once the sample had run through the entire polyacrylamide matrix. The gel was removed from the glass backing plates of the casting tray and the stacking portion

of the gel was trimmed and discarded. The gel was placed in a plastic container and was rinsed 3 times with dH_2O. Coomassie stain was added until gel was completely submerged and agitated on rocker for 1 hour. After the hour the Coomassie stain was discarded and the Coomassie de-stain was added to cover the gel before being returned to the rocker. After 15 minutes the de-stain liquid was discarded and replaced with fresh de-stain and the gel was returned to the rocker for overnight agitation. Following staining, the gel was rinsed 3 times with dH_2O and left covered in dH_2O for better viewing of proteins present.

HCoV-NL63 ORF3 Target Protein Detection

SDS-PAGE Total Protein Wet Transfer for Western Blot

The gel was removed from the running tank once the sample had run through the entire polyacrylamide matrix. The gel was removed from the glass backing plates of the casting tray and the stacking portion of the gel was trimmed and discarded. PVDF membrane (0.45 µm) was trimmed to size of gel (8.5 cm x 6 cm) and protective layers were removed. Membrane was activated by submersion in 100% methanol for 2 minutes. Once the membrane was calibrated it was transferred to 4°C 1x Transfer Buffer for priming for 10 minutes. The gel and PVDF membrane were loaded into *Mini Trans-Blot® Cell* cassette according to the manufacturer's guidelines and the tank was filled to the required level with 4° C 1x Transfer Buffer. Protein transfer from gel to membrane was run at 90 V for 90 minutes in a 4°C cold room. Once run had completed the membrane was removed from the tanks cassette and was ready for further experiments.

Ponceau Stain

After the gel proteins were successfully transferred to the PVDF membrane, the membrane was placed in a plastic container and Ponceau staining liquid was added. The membrane was agitated on a rocker for 5 minutes at room temperature. Once the membrane had taken up the Ponceau stain adequately the staining liquid was discarded. The stain was removed from the membrane by rinsing with sterile

dH_2O. Once relevant protein bands had been viewed the membrane was repurposed for Western blot protein detection.

Western Blot HCoV-NL63 ORF3 Protein Expression

To analyse the HCoV-NL63 ORF3 protein expressed by the *Bac-to-Bac™ Baculovirus Expression System* a protein specific primary antibody was required. This final aspect of the study validating the viral protein was part of an umbrella study being undertaken in our lab. No HCoV-NL63 ORF3 antibody is commercially available for purchase and a fellow PhD student within the lab had synthesised a set polyclonal HCoV-NL63 ORF3 antibodies in 2015 while on a research exchange at The Institute of Molecular and Cell Biology (IMCB), Singapore. The blood serum samples from a set of 7 immunised mice were shipped from Singapore to the University of the Western Cape in storage at -80°C since 2016. The PVDF membrane from the SDS-PAGE wet transfer was transferred to a sterile 50 mL tube and blocked on a tube roller to prevent non-specific protein binding with 5% (w/v) BSA in 1x PBS at room temperature for 2 hours. The membrane was then transferred to a new sterile 50 mL tube for primary antibody probing. Primary antibody ORF3 polyclonal (MAP + KLH conjugated) serum was diluted 1:1000 (v/v) in 2% (w/v) BSA in 1x PBS and incubated on a tube roller overnight at 4°C. Following primary antibody probing the membrane underwent 3x 15-minute wash cycles in wash buffer, 0.1% (v/v) Tween-20 in 1x PBS at room temperature.

The PVDF membrane was transferred to a new sterile 50 mL tube for secondary antibody probing. The secondary antibody m-IgGκ bp-HRP was a recombinant anti-mouse antibody suitable for binding to mouse IgGκ light chain immunoglobulins which is HRP conjugated. Secondary antibody was diluted 1:1000 (v/v) in 2% (w/v) BSA in 1x PBS and incubated on a tube roller for 1 hour at room temperature. Following secondary antibody probing the membrane underwent 3x 15-minute wash cycles in wash buffer, 0.1% (v/v) Tween-20 in 1x PBS at room temperature. The membrane was covered with 1 mL pre-warmed TMB membrane peroxidase substrate and incubated for 10 minutes in the absence of

light. After incubation a colorimetric change was observed, and where appropriate, protein bands were viewed and protein size in kDa quantified in relation to the pre-stained PAGE ruler.

CHAPTER 4

RESULTS AND DISCUSSION

Molecular Cloning of HCoV-NL63 ORF3 Gene

HCoV-NL63 ORF3 PCR Amplification from pCAGGS Parent Vector

RT-PCR to Amplify HCoV-NL63 ORF3 Gene

The full-length sequence of HCoV-NL63 ORF3 gene were determined using the NCBI database (https://www.ncbi.nlm.nih.gov/nuccore/) (GenBank: AY567487.2). The gene of interest HCoV-NL63 ORF3 was amplified from the parent vector construct pCAGGS-ORF3. The successful amplification of the gene of interest (ORF3) using full-length complementary primers is illustrated in Figure 4.1. The expected product sizes were ≈720 bp.

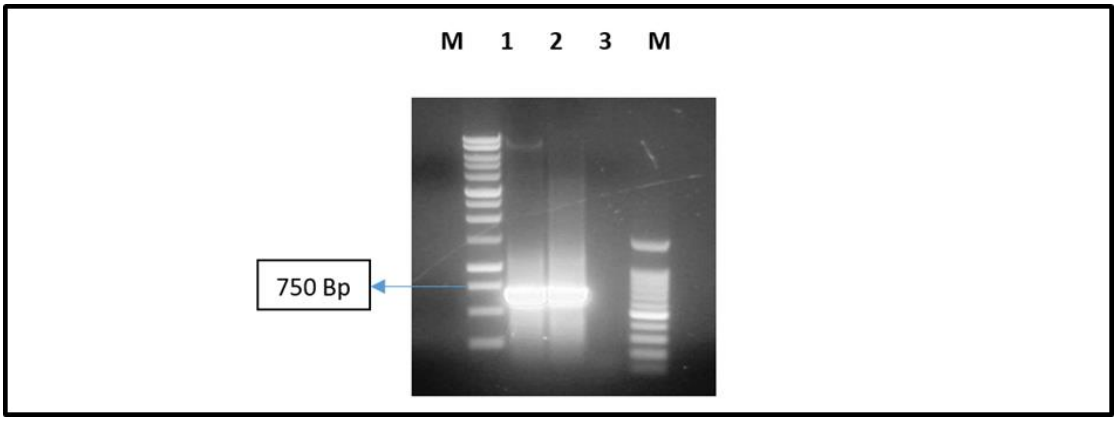

Figure 4.1: RT-PCR to amplify HCoV-NL63 ORF3 gene from pCAGGS-ORF3 reference sample using full-length ORF3 gene specific primers

Agarose gel figure labelled from left to right. Lane M: 1 kB DNA Marker; **Lane 1:** Amplification of HCoV-NL63 full-length ORF3 (≈720 bp); **Lane 2:** Positive (+) control.

The positive (+) control used was pXJ40-ORF3; **Lane 3:** Negative (-) control. The negative (-) control contained all PCR reagents except reference template (pCAGGS-ORF3); **Lane M** 100 bp marker.

Colony PCR of JM109 *E. coli* to Validate Ligation of pGEM-ORF3 Via Blue/White Colony Selection

Identification of the amplified gene by size chromatography is not completely accurate and further validation is required. The viral gene was subsequently ligated into the pGEM® T-easy vector for sequencing with M13 primers, which will also allow for identification of any base pair mutations. Following ligation into pGEM® T-easy vector, JM109 competent *E. coli* was transformed with the plasmid. The transformed cells were inoculated onto LB agar plates and incubated overnight at 37°C after which, single colonies were selected and inoculated into LB media and corresponding PCR mix. Successful amplification of pGEM-ORF3 using gene-specific primers that amplify full-length HCoV-NL 63 ORF3 is shown in Figure 4.2. Results were used to select cultures with correct gene constructs from the colonies that were picked, all white clones were positive for HCoV-NL63 ORF3. That is evident from the amplification of approximately 720 bp products. The samples were run on a 1% (w/v) agarose gel.

Restriction Enzyme Digest pGEM-ORF3 Miniprep Samples

Experimental pGEM-ORF3 miniprep samples 1 and 2 were quantified with a nanodrop which yielded concentrations of 99.15 ng/µL and 92.55 ng/µL respectively.

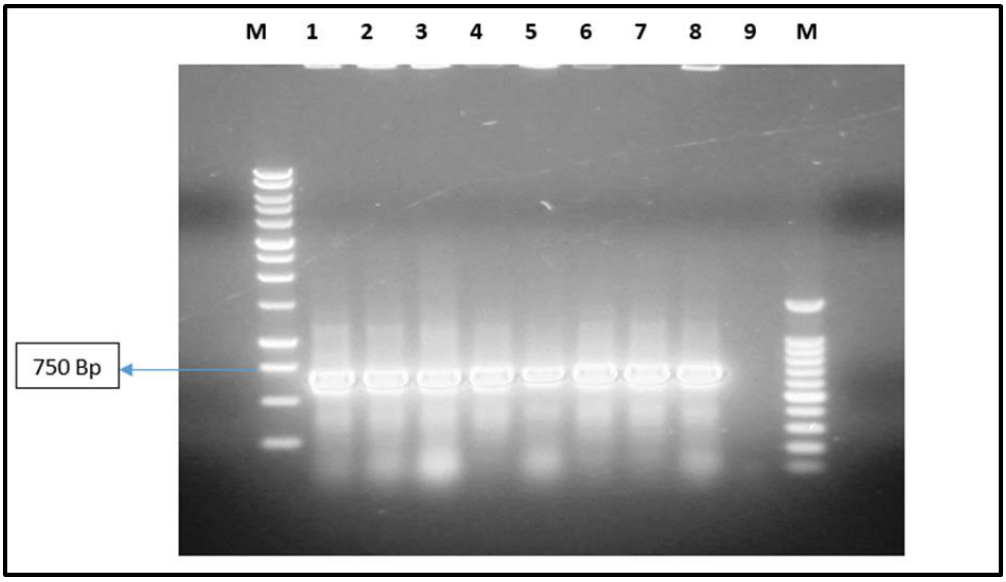

Figure 4.2: Colony PCR to confirm successful ligation of HCoV-NL63 into pGEM®-T Easy Vector

Agarose gel figure labelled from left to right. **Lane M:** 1 kB DNA Marker; **Lanes 1-6:** white colonies showing amplification of HCoV-NL63 full-length ORF3 (≈720 bp); **Lanes 7-8:** blue colonies showing amplification of HCoV-NL63 full-length ORF3 (≈720 bp); Lane 9: Negative (-) control. The negative (-) control contained all PCR reagents except DNA colony template; **Lane M:** 100 bp marker.

The results obtained after restriction enzyme digest with *Bam*H I and *Xho*I from miniprep samples of HCoV-NL63 ORF3 pGEM constructs are shown in Figure 4.3. A positive (+) control pXJ40-N-FLAG, with its own respective construct size, was used as *Bam*H I and *Xho*I were used as restriction sites during cloning. The agarose gel picture below displays several distinct fragments (bands) of interest for the 2 experimental samples loaded; the largest band which is the pGEM plasmid (≈3 kB), the middle band contains a partially digested pGEM vector (≈2 kB), and the lower band which is the ORF3 gene of interest (≈720 bp). Lanes 1 demonstrates successful restriction or release of the insert ORF3 (≈720 bp) from the sequencing vector pGEM. In Lane 2 there is no release of the gene of interest from pGEM, although it appears that either restriction enzyme *Bam*H I or *Xho*I has digested within the pGEM vector itself (the same band is apparent in Lane 1). Lane 3 positive (+) control shows pXJ40-N-FLAG, digested with restriction enzymes *Bam*H I and *Xho*I, with the pXJ40 vector (≈5 kB) and N-FLAG gene (≈1.2 kB). The results confirm successful ligation of HCoV-NL63 ORF3 into pGEM sequencing vector.

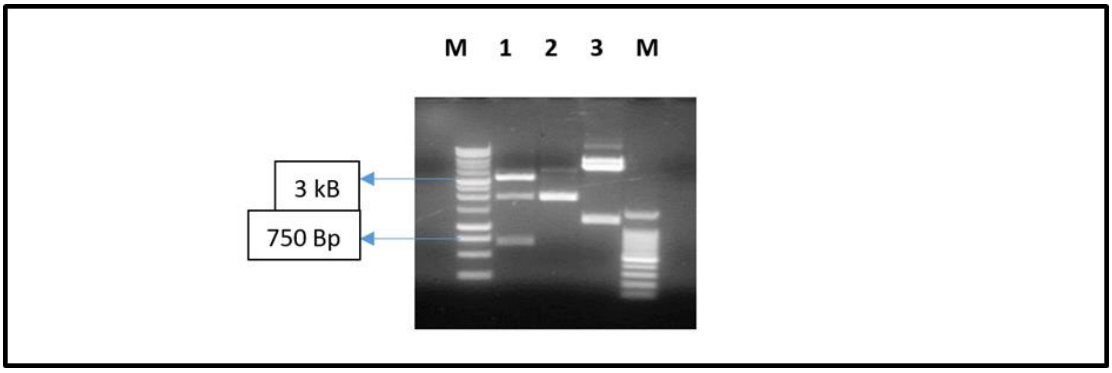

Figure 4.3: Restriction enzyme digest pGEM-ORF3 miniprep samples with restriction enzymes BamH I and XhoI

Agarose gel figure labelled from left to right. **Lane M:** 1 kB DNA Marker; **Lane 1:** digestion of pGEM-ORF3 miniprep sample 1 releasing gene of interest (≈720 bp) from pGEM vector (≈3 kB); **Lane 2:** digestion of pGEM-ORF3 miniprep sample 2 with no gene of interest released; **Lane 3:** Positive (+) control pXJ40-N-FLAG digestion showing pXJ40 vector (≈5 kB) and gene N-FLAG (≈1.2 kB); **Lane M:** 100 bp marker.

Restriction Enzyme Digest pGEM-ORF3 Midiprep Samples

pGEM-ORF3 glycerol stock transformants were grown up in LB media and a midiprep was conducted yielding plasmid DNA with a concentration of 0.75 µg/µL. The results obtained after restriction enzyme digest with *Bam*H I and *Xho*I from the high plasmid concentration experimental midiprep sample of HCoV-NL63 ORF3 pGEM construct is shown in Figure 4.4.

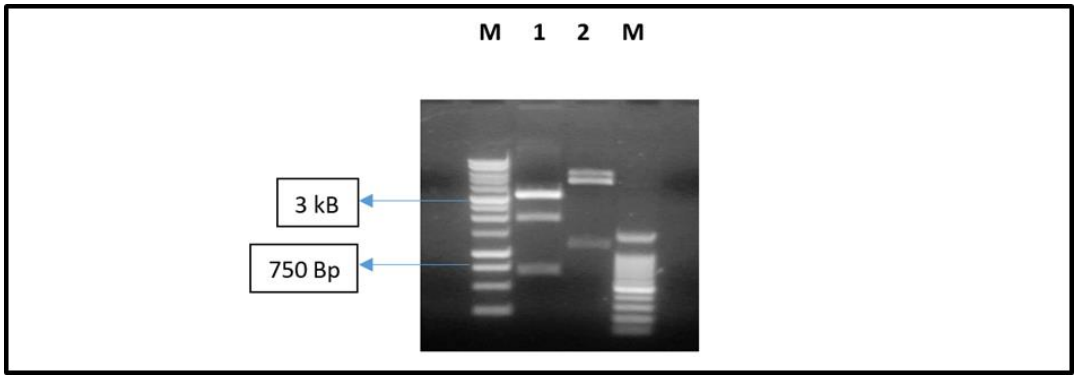

Figure 4.4: Restriction enzyme digest pGEM-ORF3 midiprep sample with restriction enzymes BamH I and XhoI

Agarose gel figure labelled from left to right. **Lane M:** 1 kB DNA Marker; **Lane 1:** digestion of pGEM-ORF3 midiprep releasing gene of interest (≈720 bp) from pGEM

vector (≈3 kB); **Lane 2:** Positive (+) control pXJ40-N-FLAG digestion showing pXJ40 vector (≈5 kB) and gene N-FLAG (≈1.2 kB); **Lane M**: 100 bp marker.

A positive (+) control pXJ40-N-FLAG, with its own respective construct size, was used as *Bam*H I and *Xho*I were used as restriction sites during cloning. The agarose gel image displays several distinct fragments (bands) of interest for the experimental sample loaded; the largest band which is the pGEM plasmid (≈3 kB), the middle band contains a partially digested pGEM vector (≈2 kB), and the lower band which is the ORF3 gene of interest (≈720 bp). Lanes 1 demonstrates successful restriction or release of the insert ORF3 (≈720 bp) from the sequencing vector pGEM. Lane 2 positive (+) control shows pXJ40-N-FLAG, digested with restriction enzymes *Bam*H I and *Xho*I, with the pXJ40 vector (≈5 kB) and N-FLAG gene (≈1.2 kB). below indicates where the digested ORF3 DNA is ligated into the MCS of the digested pFastBac™1 vector to create pFastBac-ORF3 plasmid construct.

PCR to Confirm HCoV-NL63 ORF3 Gene

The gene of interest (ORF3) was successfully amplified as illustrated in Figure 4.5. The expected product sizes were ≈700 bp.

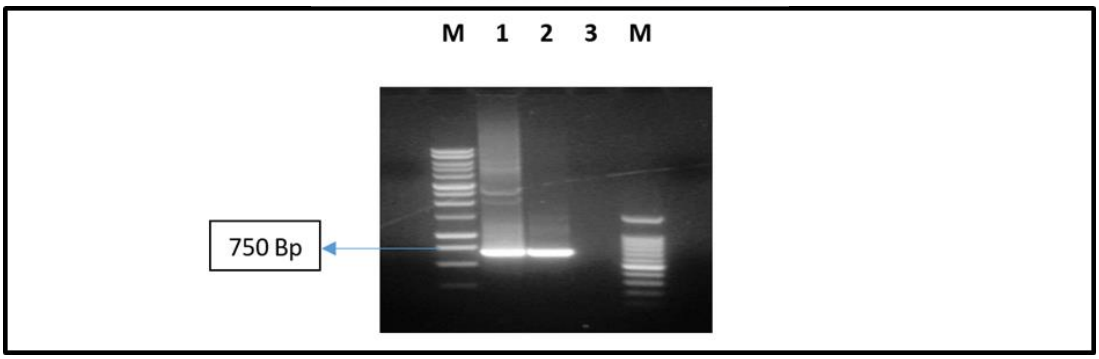

Figure 4.5: RT-PCR to confirm HCoV-NL63 ORF3 Gene from pGEM-ORF3 midiprep using full-length ORF3 gene specific primers

Agarose gel figure labelled from left to right. **Lane M:** 1 kB DNA Marker**; Lane 1:** Amplification of HCoV-NL63 full-length ORF3 (≈720 bp); **Lane 2:** Positive (+) control. The positive (+) control used was pXJ40-ORF3; **Lane 3:** Negative (-) control. The negative (-) control contained all PCR reagents except reference template (pCAGGS-ORF3); **Lane M**: 100 bp marker.

Generating Recombinant pFastBac™1-ORF3 Construct

Restriction Enzyme Digest pGEM-ORF3 Midiprep Sample and pFastBac™1 Empty Vector

A restriction enzyme digest was completed to: firstly, confirm correct ligation of the gene of interest into pGEM®-T Easy vector and, secondly, to digest pGEM-ORF3 DNA concentration of 6 µg and 5 µg empty circular pFastBac™1 vector with enzymes *Bam*H I and *Xho*I for entry into the *Bac-to-Bac™ Baculovirus Expression System* (Figure 4.6). Lane 1 contained the digested pGEM-ORF3 0.75 µg/µL midiprep sample which released the HCoV-NL63 ORF3 gene; Lane 2 contained linearized digested pFastBac™1 vector and Lane 3 the positive control pFastBac-N-FLAG (*Figure 4.6*:). Lanes 1 and 2 were excised and purified with the *Promega Wizard® SV Gel and PCR clean-up System* kit for downstream ligation and transformation. Primers that were used to amplify the full-length gene from the reference construct were designed to incorporate cut sites for *Bam*H I (N-terminus) and *Xho*I (C-terminus) into the Multiple Cloning Site (MCS) of pFastBac™1.

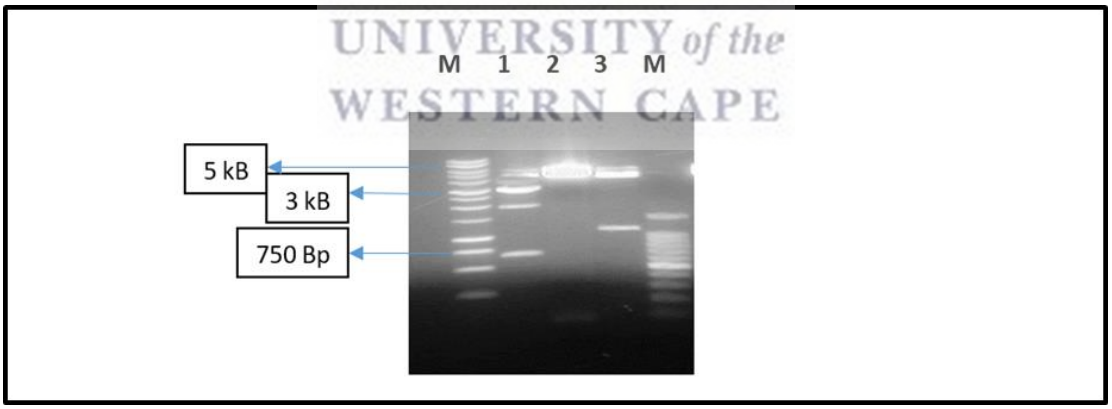

Figure 4.6: Restriction enzyme digest pGEM-ORF3 midiprep sample and pFastBac™1 empty vector

Agarose gel figure labelled from left to right. **Lane M:** 1 kB DNA Marker; **Lane 1:** digestion of pGEM-ORF3 midiprep releasing gene of interest (≈720 bp); **Lane 2:** linearized digested pFastBac™1 vector (≈4.7 kB); **Lane 3:** Positive (+) control pFastBac-N-FLAG digestion showing pFastBac™1 vector (≈4.7 kB) and gene N-FLAG (≈1.2 kB); **Lane M:** 100 bp marker.

Colony PCR of DH5∝ *E. coli* to Validate Ligation of pFastBac-ORF3

From the colonies that were picked, all clones in Lanes 1-6 were positive for HCoV-NL63 ORF3 Figure 4.7). All bands of interest (≈720 bp) confirmed successful pFastBac-ORF3 ligation and transformation. Although the band intensity of Lane 6 qualitatively indicates low HCoV-NL63 ORF3 DNA copy numbers in relation to Lanes 1-5.

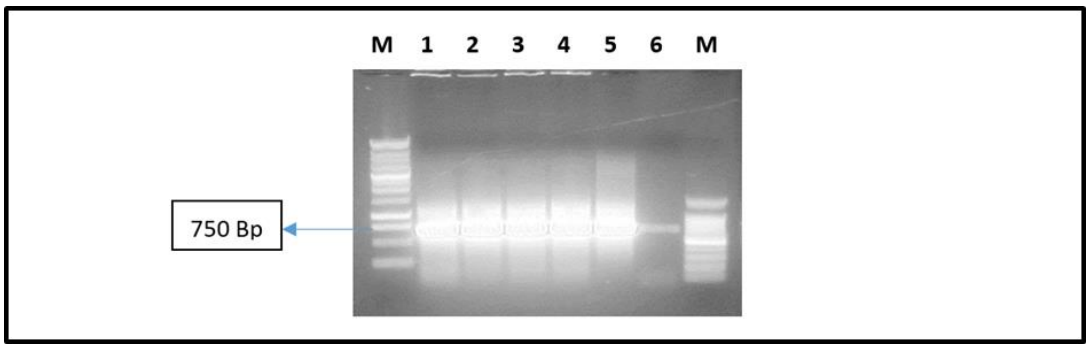

Figure 4.7: Colony PCR to confirm successful ligation and transformation of HCoV-NL63 into pFastBac™1 vector

Agarose gel figure labelled from left to right. **Lane M:** 1 kB DNA Marker; **Lanes 1-6:** DH5∝ E. coli colonies showing amplification of HCoV-NL63 full-length ORF3 (≈720 bp); **Lane M:** 100 bp marker.

Sequencing pFastBac-ORF3 miniprep

Sequencing results indicated a fragment of 741 bp and was entered into the NCBI blast database confirming 100% gene similarity to the GenBank: AY567487.2 reference sequence. Furthermore, the gene sequence results were viewed with MEGA X: Molecular Evolutionary Genetics Analysis (Kumar et al., 2018) and extracted sequences were aligned using the multiple sequence alignment in ClustalX (Larkin et al., 2007). Files extracted to GeneDoc (Nicholas & Nicholas, 1997) for viewing are shown in APPENDIX 1.

Transposition of pFastBac™1-ORF3 Generating Bacmid-ORF3 DNA

Transformation of pFastBac-ORF3 into DH10Bac *E. coli* Competent Cells and Bacmid Transposition

White and blue colony growth were present on serial dilution spread plates 10^{-1} and 10^{-2}, no growth occurred on 10^{-3}. White colony growth is the qualitative confirmation of successful HCoV-NL63 ORF3 transposition into the Bacmid.

DH10Bac *E. coli* Midiprep

A total of 4 well isolated white colonies were picked and cultures underwent a miniprep which yielded DNA concentrations of 0.45 µg/µL and 0.49 µg/µL, respectively. Based on nanodrop sample purity readings, the Bacmid-ORF3 of 0.45 µg/µL sample was deemed more acceptable for downstream experiments

PCR Confirming Recombinant Bacmid-ORF3 DNA

DNA isolated from white colonies (0.45 µg/µL midiprep sample) generated an amplicon size of 2.3 kB + the size of the insert of 741 bp, thus equalling a band of ≈3 kB as a positive result for successful transposition of Bacmid-ORF3 (Figure 4.8). Lane 1 shows amplification using M13 forward and reverse primers. The product size is 3041 bp, which was the calculated expected size of the amplicon. A combination of the primers was used to further confirm correct orientation and successful transposition of the pFastBac-ORF3 gene. This is demonstrated in Lanes 2 and 3; In Lane 2, M13 forward primer was used in combination with the ORF3-specific reverse primer. In Lane 3, M13 reverse primer was used in combination with ORF3-specific forward primer. Furthermore, the results show successful generation of recombinant Bacmid DNA that contains HCoV-NL 63 ORF genes. The manual states a band of 300 bp is indicative of no gene transposed into the Bacmid.

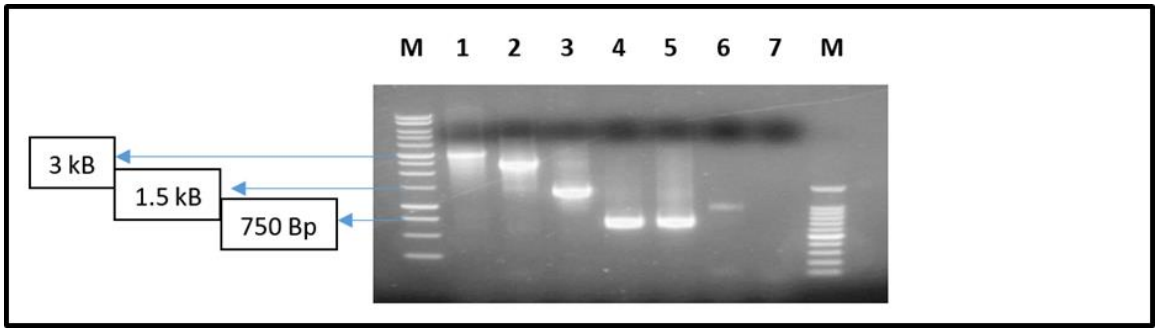

Figure 4.8: RT-PCR confirming recombinant Bacmid-ORF3 in midiprep sample

Agarose gel figure labelled from left to right. **Lane M:** 1 kB DNA Marker; **Lane 1:** shows amplification using M13 forward and reverse primers with a 3041 bp product; **Lane 2:** shows amplification using M13 forward primer with the ORF3-specific reverse primer with a 2390 bp product; **Lane 3:** shows amplification using ORF3-specific forward primer and M13 reverse primer with a 1320 bp product; **Lane 4:** shows amplification ORF3-specific forward and reverse primers with a 741 bp product; **Lane 5:** Positive (+) control. The positive (+) control used was pFastBac-ORF3 midiprep amplified with ORF3-specific forward and reverse primers with a 741 bp product; **Lane 6:** Positive (+) transposition control. The transposition control used was Bacmid-GUS midiprep amplified using M13 forward and reverse primers with a 4200 bp product; **Lane 7:** Negative (-) control containing all PCR reagents except reference template (Bacmid-ORF3); **Lane M:** 100 bp marker.

Producing Recombinant HCoV-NL63 ORF3 Baculovirus

Transfection Generating ORF3-Baculovirus

Production and Collection of P0 Transfection Viral Stock

Transfection with 1 µg Bacmid-ORF3 DNA displayed the most favourable results, cell supernatant was the P0 viral stock. Fresh SF9 cells were infected with the P0 transfection stock and P1 stock was collected at 48 hours post-infection.

Viral Plaque Assay

Quantification to Establish Viral Titre of P1 Stock

The P1 stock showed definite CPE to cells post-infection, although the recombinant virus required the titre to be determined for infection at ΔMOI. The initial viral plaque assay after MTT staining

determined the titre of the P1 ORF-Baculovirus stock was concentration of $6.5x10^6$ pfu/mL was too low. According to the *Bac-to-Bac™ Baculovirus Expression System* manual for protein expression studies a viral stock titre of minimum of $10x10^6$ pfu/mL is required.

Viral Plaque Purification and Amplification Generating P2 Plaque Purified Stock

Amplification and Generation of P2 Stock Viral Stock

P1 viral stock was amplified via plaque picking and infection. A total of 5 plaque purified P1 viral stocks were generated, with each originating from a single viral plaque to enhance ORF3-Baculovirus purity. The 5 viral plaque P1 stocks were ranked in levels of virulence based on CPE caused to infected cells. Plaque purified P1 stocks originating from viral plaque assay labelled as plaques 1-5 were amplified by independently infecting fresh SF9 cells over a 72-hour time course. Harvested P2 viral stocks were expected to have a far higher viral. The P2 viral stock originating from plaque 1 of the plaque purified P1 stock showed the higher infectivity compared to the plaque purified P1 stocks originating from viral plaques 2–5.

Final Viral Plaque Assay to Quantify Titre of P2 Viral Stock

Final Viral Plaque Assay to Establish Viral Titre of P2 Stock

SF9 insect cells were infected with the P2 viral stock ranging from lowest viral concentration 10^{-14} to the highest 10^{-4}. Viral plaques were present in experimental wells 3 days post-infection. On day 7 post-infection the cells-only control and viral infection experimental wells were stained for ORF3-Baculovirus plaques (Figure 4.9).

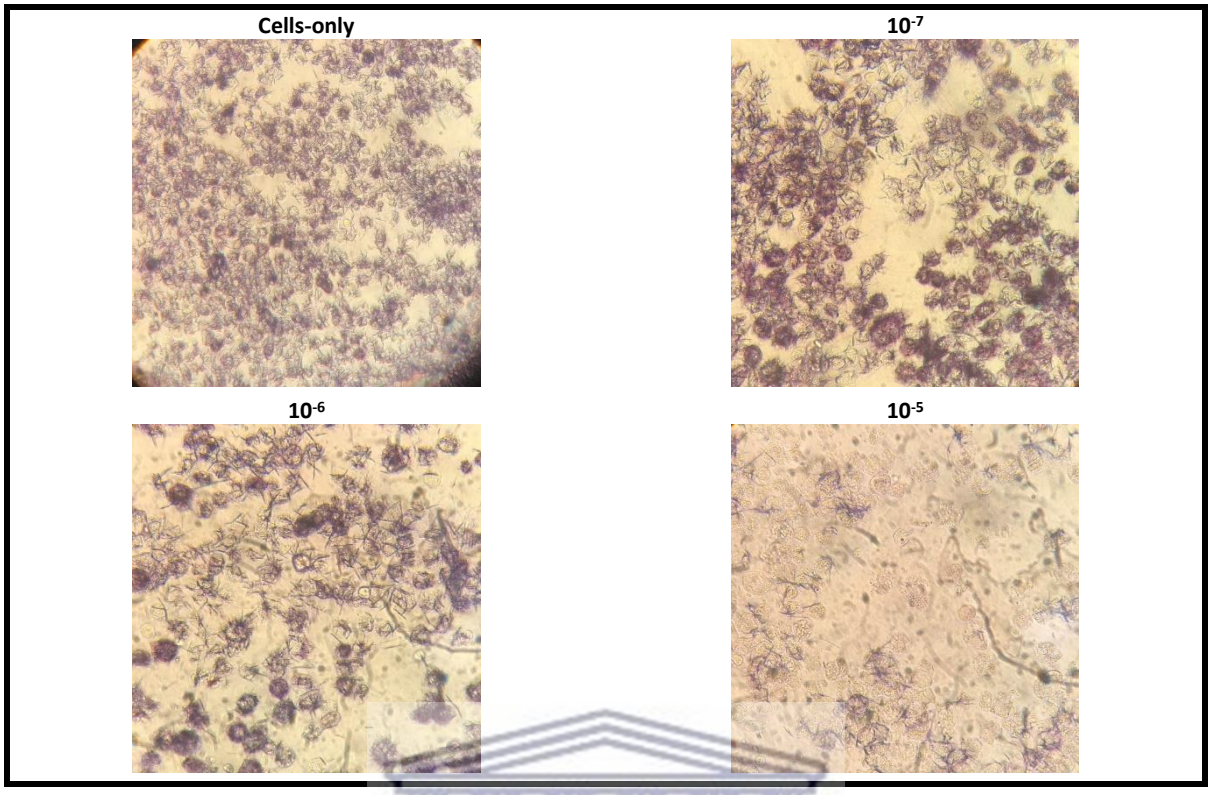

Figure 4.9: Final viral plaque assay quantifying P2 viral stock

MTT stained ORF3-Baculovirus plaques 7 days post-infection. Image displays cells-only control well and P2 viral stock infection for wells with viral infection concentrations 10^{-7} to 10^{-5} which was determined to be the optimal range for calculating the P2 stock titre. Cells viewed under inverted light microscope.

Living SF9 cells displayed a blue/black stain and the definitive clear areas in the cell monolayer were indicative of non-viable/infected SF9 cells, these were the ORF3-Baculovirus plaques.

Quantified P2 High Titre ORF3-Baculovirus Stock

The viral plaque assay after MTT staining determined that the titre of the viral plaque 1 P2 plaque purified ORF-Baculovirus stock was $15x10^6$ pfu/mL, as 15 plaques were counted in the dilution well and calculated according to the equation listed in the methodology. This plaque purified ORF3-Baculovirus P2 viral stock titre is well above the minimum threshold of $10x10^6$ pfu/mL, according to the *Bac-to-Bac™ Baculovirus Expression System* manual for protein expression studies.

ORF3-Baculovirus Protein Expression

ORF3-Baculovirus SF9 cell ΔMOI Assay for Varying HCoV-NL63 ORF3 Protein Expression Levels

SF9 Cell Infection

The known titre concentration plaque purified P2 viral stock originating from viral plaque 1 was used to infect SF9 cells at the ΔMOI range set of 0.1; 1; 2.5; 5 and 10 pfu/mL. After the fixed time course incubation of 72 hours, apart from MOI of 0.1 and 1, CPE was viewed in infected SF9 cells.

ORF3-Baculovirus Lysate Collection and Quantification

Infected Cell Lysate Collection

The SF9 ORF3-Baculovirus infected SF9 cell lysates were quantified via spectrophotometry at 562 nm and generated an R^2 value of 0.9885.

HCoV-NL63 ORF3 Target Protein Detection

SDS-PAGE ORF3-Baculovirus Lysates

Sample Loading

SF9 cell lysates generated from ΔMOI 72-hour infection containing Baculovirus expressing HCoV-NL63 ORF3 protein was denatured and loaded at a constant total protein concentration of 30 µg per Lane on SDS-PAGE.

Coomassie Stain SDS-PAGE ORF3-Baculovirus 72-hour ΔMOI Lysates

SDS-PAGE

SDS-PAGE gels containing ΔMOI lysates underwent Coomassie stain and de-stain. SDS gel (Figure 4.10) contains 30 µg total protein per Lane for all lysates loaded - cells-only and experimental ΔMOI lysates. The cells-only control is separated by an empty Lane and the ΔMOI range was loaded in ascending order (0.1; 1; 2.5; 5 and 10 pfu/mL). Only ~55 kDa band was visible in all sample lanes.

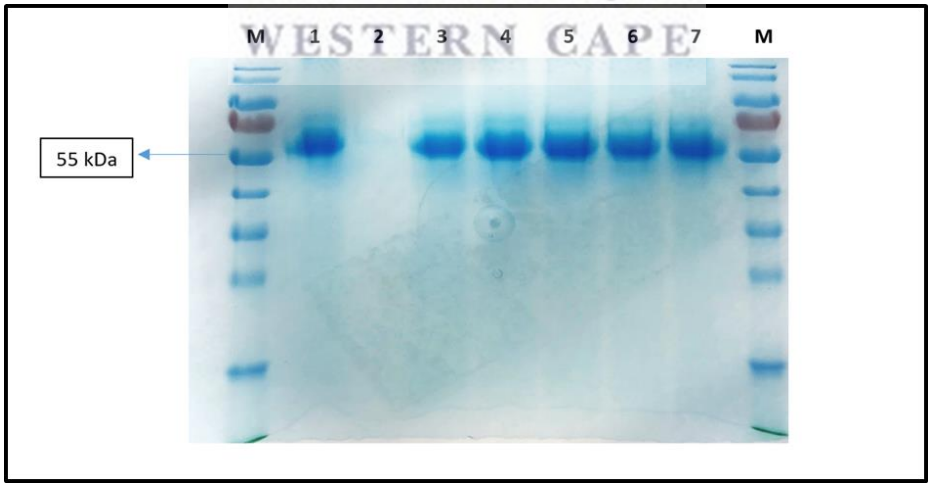

Figure 4.10: Coomassie stain SDS-PAGE ORF3-Baculovirus 72-hour ΔMOI lysates

SDS-PAGE labelled left to right. **Lane M**: Prestained PAGE ruler; **Lane 1:** Cells-only control; **Lane 2:** Empty; **Lane 3:** MOI 0,1 pfu/mL; **Lane 4:** MOI 1 pfu/mL; **Lane 5:** MOI 2,5 pfu/mL **Lane 6:** MOI 5 pfu/mL; **Lane 7:** MOI 10 pfu/mL.

Ponceau Stain ORF3-Baculovirus 72-Hour ΔMOI Lysates

Ponceau Stain

The SDS gel containing ΔMOI lysates underwent a wet transfer for total protein migration to a PVDF membrane, which was subsequently Ponceau stained. The PVDF membrane (Figure 4.11) contains 30 µg total protein for all lysates loaded - cells-only and experimental ΔMOI lysates. The cells-only control is separated by an empty Lane and the ΔMOI range was loaded in ascending order (0.1; 1; 2.5; 5 and 10 pfu/mL). Again, only a ~55 kDa band visible.

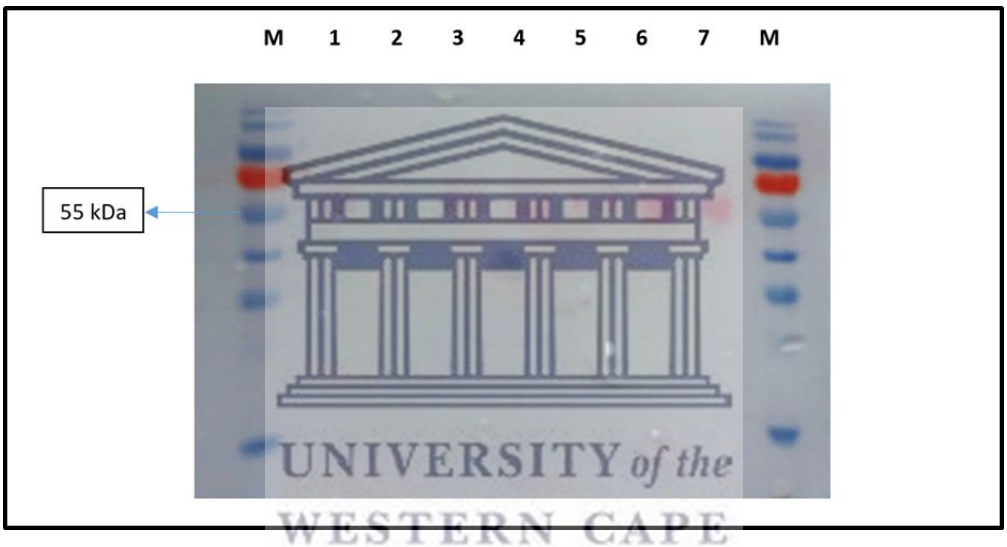

Figure 4.11: Ponceau stain ORF3-Baculovirus 72-hour ΔMOI lysates

Ponceau stained PVDF membrane labelled left to right. **Lane M:** Prestained PAGE ruler; **Lane 1:** Cells-only control; **Lane 2:** Empty; **Lane 3:** MOI 0,1 pfu/mL; **Lane 4:** MOI 1 pfu/mL; **Lane 5:** MOI 2,5 pfu/mL; **Lane 6:** MOI 5 pfu/mL; **Lane 7:** MOI 10 pfu/mL.

Western Blot ORF3-Baculovirus 72-Hour Infection with Plaque 1-5 P2 Viral Stocks

Western Blot

The membrane underwent Western blot as discussed in methodology, using lab synthesised polyclonal mice serum as primary antibody probing and secondary antibody probing with m-IgGκ bp-HRP recombinant commercial antibody. The PVDF membrane (Figure 4.12) contains 30 µg total protein loaded for all samples loaded; cells-only and experimental plaque purified P2 stocks. Now a

~25.6 kDa band was visible in lanes 2,3, 4, 5 and 6.

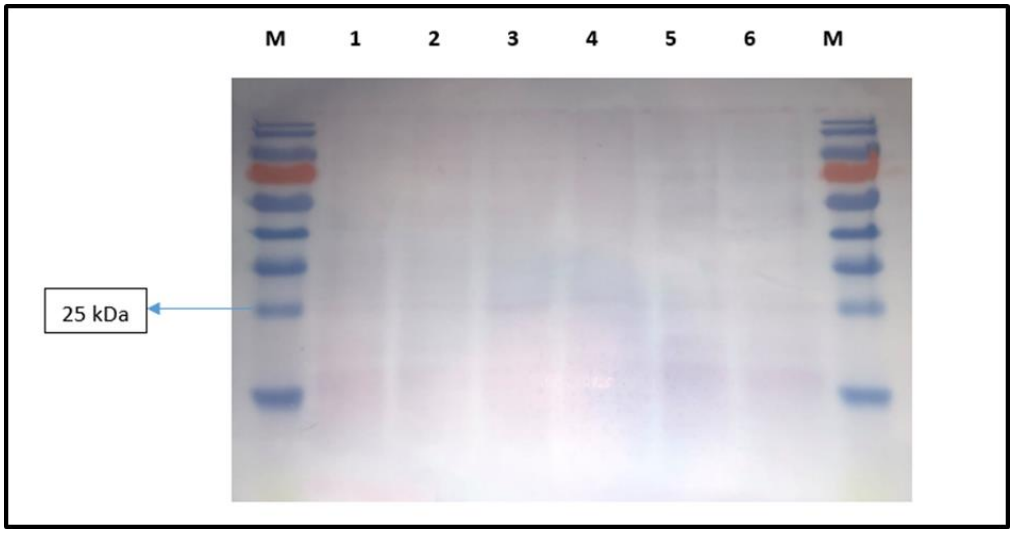

Figure 4.12: Western blot ORF3-Baculovirus 72-hour infection SF9 cell supernatant P2 stocks of viral plaques 1-5

Western blot using lab synthesised polyclonal mice serum as primary antibody probing and secondary antibody probing with m-IgGκ bp-HRP recombinant commercial antibody. Membrane labelled left to right. **Lane M:** Prestained PAGE ruler; **Lane 1:** Cells-only control; **Lane 2:** Viral plaque 1 P2 stock; **Lane 3:** Viral plaque 2 P2 stock; **Lane 4:** Viral plaque 3 P2 stock; **Lane 5:** Viral plaque 4 P2 stock; **Lane 6:** Viral plaque 5 P2 stock.

Conclusion

Viruses from the *Coronaviridae* family have some of the largest viral genomes and are characterised to contain (+) ssRNA which is a major contributing factor causing genetic recombination; as the "copy-choice" mechanism which permits the loss and gaining of coding domains (Figure 2.1) leads to the emergence of novel strains (Van Boheemen et al., 2012). The physical structure of coronaviruses, shown in Figure 1.1 display a great similarity by having the same structural proteins (spike, envelope, membrane and nucleocapsid); which have been of great value to researchers who have used various *in silico* and bioinformatics modelling techniques to draw inferences on lethality amongst HCoVs (Peiris, Guan, & Yuen, 2004). Similarly, the importance of the non-structural/accessory ORF proteins

are of great significance as they contain the TRS motif which aid in transcription regulation of viral structural proteins and the colocalization of structural proteins in the ERGIC (Müller et al., 2010; Yount et al., 2006).

This study aimed to clone and express the HCoV-NL63 ORF3 protein using the *Bac-to-Bac® Baculovirus Expression System;* as the use of recombinant proteins have been shown to be of importance in vaccine development and as antigens for detection and screening of antibodies (Ren et al., 2004).

The molecular cloning and subsequent expression of individual proteins assist to elucidate the functioning of the protein. By expressing the ORF3 protein in an expression vector, a greater understanding of the functional and molecular properties can be determined.

The recombinant protein produced with the Baculovirus system can be used for gene delivery of therapeutics which include; vaccine therapy, diagnostics, and medical research sectors involving public health (van Oers, Pijlman, & Vlak, 2015).

Promega restriction enzymes *BamH* I and *Xho* I along with *Promega* Buffer B, only having a 75% digestion efficiency, therefore agarose gels of digested ORF3 constructs display bands for the vector, the gene of interest and a third band of partially undigested vector.

The rationale of obtaining the purified HCoV-NL63 ORF3 proteins expressed with the *Bac-to-Bac® Baculovirus Expression System* was that conformational changes of the transmembrane glycoprotein could be further studied and possibly used in vaccine research (Pyrc et al., 2004). As the ORF3 protein boosts the relative amounts of the proteins encoded by the downstream reading frames (Cann, 2016) and the inclusion of the ORF3 protein in virions supports the viral assembly and budding processes of HCoV-NL63 (Müller et al., 2010).

However, the ΔMOI protein lysates harvested, which were denatured with Laemli's incubation buffer,

loaded at a fixed total protein concentration of 30 µg/µL on SDS-PAGE indicated a protein product of 57 kDa present in the control and experimental Lanes (Figure 4.10). Therefore, apart from the protein being present in the cells-only control lysates and experimental ΔMOI protein lysates the outcome does not align with published literature which states the size of HCoV-NL63 ORF3 is 25.6 kDa (Pyrc et al., 2004). The 55 kDa protein seen via SDS-PAGE and Coomassie stain is most likely a cellular protein with no relation to ORF3 and the experiment was repeated numerous times at multiple denaturation temperatures for validation (figures included in APPENDIX 2).

However after secondary antibody probing and incubation with a chemiluminescent substrate the Western blot in Figure 4.12 indicate proteins transferred at the expected size of approximately 25.6 kDa. Subsequent repeated attempts with ΔMOI protein lysates (figures included in APPENDIX 2), did not detect the target size protein with the listed antibodies.

In conclusion, these results are indicative that the gene of interest HCoV-NL63 ORF3 was successfully cloned into the *Bac-to-Bac® Baculovirus Expression System* which was the main objective of this Masters research project. The RT-PCR containing the Bacmid-ORF3 DNA which amplified the Bacmid vector at its M13 binding sites and gene-specific sites within the vector were a clear indication that HCoV-NL63 ORF3 was successfully cloned into this BEVS, based on the calculated amplicon size contained in the manufacturer's instructions. Furthermore, the Bacmid-ORF3 DNA was used to successfully transfect SF9 cells and subsequently generate ORF3-Baculovirus particles. Two successful viral plaque assays further confirmed the production of Baculoviral plaques which were quantified for HCoV-NL63 ORF3 expression studies. There is no commercially available HCoV-NL63 ORF3 antibody, further research is required to fully assess serological responses to the expressed ORF3 protein. Unfortunately, protein expression indicated weak binding of the primary antibody to proteins expressed.

Future Perspectives

Possible areas of future research include the synthesis of a new HCoV-NL63 ORF3 antibody for protein expression studies. The antibody produced could further be used for immunofluorescence microscopy assays to indicate the host cellular localisation of HCoV-NL63 ORF3 proteins. Finally, as the expression of the protein of interest falls under a greater umbrella project within the Molecular Biology and Virology Lab, the ORF3-Baculovirus could be used in transfection studies to gain a greater understanding of the ORF3 accessory protein role in viral assembly and packaging HCoV-NL63 within host cell lines.

REFERENCES

Abdel-Moneim, A., Giesow, K., & Keil, G. (2014). High-Level Protein Expression Following Single and Dual Gene Cloning of Infectious Bronchitis Virus N and S Genes Using Baculovirus Systems. *Viral immunology, 27*, 75.

Abdul-Rasool, S., & Fielding, B. (2010). Understanding Human Coronavirus HCoV-NL63. *The Open Virology Journal, 4*, 76.

Airenne, K., Hu, Y., Kost, T., Smith, R., Kotin, R., Ono, C., Matsuura, Y., Wang, S., & Ylä-Herttuala, S. (2013). Baculovirus: an insect-derived vector for diverse gene transfer applications. *Molecular therapy : the journal of the American Society of Gene Therapy, 21*, 739.

Altmann, F. (1999). Insect cells as hosts for the expression of recombinant glycoproteins. *Glycoconjugate Journal, 16*, 109.

Assenberg, R., Wan, P., Geisse, S., & Mayr, L. (2013). Advances in recombinant protein expression for use in pharmaceutical research. *Current Opinion in Structural Biology, 23*, 393.

Azhar, E., Lanini, S., Ippolito, G., & Zumla, A. (2016). The Middle East Respiratory Syndrome Coronavirus – A Continuing Risk to Global Health Security *Emerging and Re-emerging Viral Infections*: Springer International Publishing.

Brielle, E., Schneidman-Duhovny, D., & Linial, M. (2020). The SARS-CoV-2 Exerts a Distinctive Strategy for Interacting with the ACE2 Human Receptor. *Viruses, 12*, 497.

Brockway, S., Clay, C., Tao Lu, X., & Denison, M. (2003). Characterization of the Expression, Intracellular Localization, and Replication Complex Association of the Putative Mouse Hepatitis Virus RNA-Dependent RNA Polymerase. *Journal of Virology, 77*, 10515.

Brojakowska, A., Narula, J., Shimony, R., & Bander, J. (2020). Clinical Implications of SARS-CoV-2 Interaction With Renin Angiotensin System. *Journal of the American College of Cardiology, 75*, 3085.

Burger, M. C., Dempers, J. J., & de Beer, C. (2014). Profiling the approach to the investigation of viral infections in cases of sudden unexpected death in infancy in the Western Cape Province, South Africa. *Forensic Science International, 239*, 27.

Cann, A. (2016). Chapter 5 - Expression *Principles of Molecular Virology*: Academic Press.

Carstens, E. (2009). AcMNPV as a model for baculovirus DNA replication. *Virologica Sinica, 24*, 243.

Dijkman, R., Jebbink, M., El Idrissi, N., Pyrc, K., Muller, M., Kuijpers, T., Zaaijer, H., & Van Der Hoek, L. (2008). Human Coronavirus NL63 and 229E Seroconversion in Children. *Journal of Clinical Microbiology, 46*, 2368.

Esper, F., Weibel, C., Ferguson, D., Landry, M., & Kahn, J. (2005). Evidence of a Novel Human Coronavirus That Is Associated with Respiratory Tract Disease in Infants and Young Children. *The Journal of Infectious Diseases, 191*, 492.

Fabre, M., Arrías, P., Masson, T., Pidre, M., & Romanowski, V. (2020). Baculovirus-Derived Vectors for Immunization and Therapeutic Applications *Emerging and Reemerging Viral Pathogens*: Elsevier.

Fielding, B., & Suliman, T. (2009). Comparative analysis of human coronavirus-NL63 ORF3 protein homologues. *African Journal of Biotechnology, 8*, 3175.

Flint, S., Racaniello, V., Rall, G., Skalka, A., & Enquist, L. (2015). *Principles of Virology: Pathogenesis and Control*: Wiley.

Fouchier, R., Hartwig, N., Bestebroer, T., Niemeyer, B., De Jong, J., Simon, J., & Osterhaus, A. (2004). A Previously Undescribed Coronavirus Associated with Respiratory Disease in Humans. *Proceedings of the National Academy of Sciences, 101*, 6212.

Fujita, R., Hino, M., Ebihara, T., Nagasato, T., Masuda, A., Lee, J., Fujii, T., Mon, H., Kakino, K., Nagai, R., Tanaka, M., Tonooka, Y., Moriyama, T., & Kusakabe, T. (2020). Efficient production of recombinant SARS-CoV-2 spike protein using the baculovirus-silkworm system. *Biochemical and Biophysical Research Communications, 529*, 257.

Gasser, B., Saloheimo, M., Rinas, U., Dragosits, M., Rodríguez-Carmona, E., Baumann, K., Giuliani, M., Parrilli, E., Branduardi, P., Lang, C., Porro, D., Ferrer, P., Tutino, M., Mattanovich, D., & Villaverde, A. (2008). Protein folding and conformational stress in microbial cells producing recombinant proteins: a host comparative overview. *Microbial cell factories, 7*, 11.

Giedroc, D., Theimer, C., & Nixon, P. (2000). Structure, stability and function of RNA pseudoknots involved in stimulating ribosomal frameshifting. *Journal of Molecular Biology, 298*, 167.

Goh, P., Choi, Y., Shuo, S., Tan, Y., Fielding, B., Tan, T., Ooi, E., Lim, S., & Hong, W. (2004). Cellular Characterization of SARS Coronavirus Nucleocapsid. *EXCLI Journal, 3*, 91.

Graham, R., Donaldson, E., & Baric, R. (2013). A decade after SARS: strategies for controlling emerging coronaviruses. *Nature Reviews Microbiology, 11*, 836.

Harrison, R., Herniou, E., Jehle, J., Theilmann, D., Burand, J., Becnel, J., Krell, P., Van Oers, M., Mowery, J., & Bauchan, G. (2018). ICTV Virus Taxonomy Profile: Baculoviridae. *Journal of General Virology, 99*, 1185.

Herald, J., & Siddell, S. (1993). An 'elaborated' pseudoknot is required for high frequency frameshifting during translation of HCV 229E polymerase mRNA. *Nucleic Acids Research, 21*, 5838.

Hofmann, H., Pyrc, K., Van Der Hoek, L., Geier, M., Berkhout, B., & Pohlmann, S. (2005). Human coronavirus NL63 employs the severe acute respiratory syndrome coronavirus receptor for cellular entry. *PNAS, 102*, 7988.

Huang, C., Narayanan, K., Ito, N., Peters, C., & Makino, S. (2006). Severe Acute Respiratory Syndrome Coronavirus 3a Protein Is Released in Membranous Structures from 3a Protein-Expressing Cells and Infected Cells. *Journal of Virology, 80*, 210.

Kahn, J., & McIntosh, K. (2005). History and Recent Advances in Coronavirus Discovery. *The Pediatric Infectious Disease Journal, 24*, 223.

Kanzawa, N., Nishigaki, K., Hayashi, T., Ishii, Y., Furukawa, S., Niiro, A., Yasui, F., Kohara, M., Morita, K., Matsushima, K., Le, M., Masuda, T., & Kannagi, M. (2006). Augmentation of chemokine production by severe acute respiratory syndrome coronavirus 3a/X1 and 7a/X4 proteins through NF-κB activation. *FEBS Letters, 580*, 6807.

Kost, T., Condreay, J., & Jarvis, D. (2005). Baculovirus as versatile vectors for protein expression in insect and mammalian cells. *Nature Biotechnology, 23*, 567.

Kumar, S., Stecher, G., Li. M, Knyaz, C., & Tamura, K. (2018). MEGA X: Molecular Evolutionary Genetics Analysis across computing platforms (Version 10.1.8).

Larkin, M., Blackshields, G., Brown, N., Chenna, R., McGettigan, P., McWilliam, H., Valentin, F., Wallace, I., Wilm, A., Lopez, R., Thompson, J., Gibson, T., & Higgins, D. (2007). Clustal W and Clustal X version 2.1 (Version 2.1).

Lu, R., Zhao, X., Li, J., Niu, P., Yang, B., Wu, H., Wang, W., Song, H., Huang, B., Zhu, N., Bi, Y., Ma, X., Zhan, F., Wang, L., Hu, T., Zhou, H., Hu, Z., Zhou, W., Zhao, L., Chen, J., Meng, Y., Wang, J., Lin, Y., Yuan, J., Xie, Z., Ma, J., Liu, W., Wang, D., Xu, W., Holmes, E., Gao, G., Wu, G., Chen, W., Shi, W., & Tan, W. (2020). Genomic characterisation and epidemiology of 2019 novel coronavirus: implications for virus origins and receptor binding. *The Lancet, 395*, 565.

Mathewson, A., Bishop, A., Yao, Y., Kemp, F., Ren, J., Chen, H., Xu, X., Berkhout, B., Van Der Hoek, L., & Jones, I. (2008). Interaction of severe acute respiratory syndrome-coronavirus and NL63 coronavirus spike proteins with angiotensin converting enzyme-2. *Journal of General Virology, 89*, 2741.

Mcbride, R., & Fielding, B. (2012). The Role of Severe Acute Respiratory Syndrome (SARS)-Coronavirus Accessory Proteins in Virus Pathogenesis. *Viruses, 4*, 2902.

Mirzaei, N., Nategh, R., Soleimanjahi, H., Amirmozafari, N., Rezaei, F., & Azad, T. (2014). Construction of Recombinant Bacmid Containing M2e-Ctxb and Producing the Fusion Protein in Insect Cell Lines. *Iranian Red Crescent Medical Journal, 16*.

Moya, A., Holmes, E., & González-Candelas, F. (2004). The population genetics and evolutionary epidemiology of RNA viruses. *Nature Reviews Microbiology, 2*, 279.

Müller, M. A., van der Hoek, L., Voss, D., Bader, O., Lehmann, D., Schulz, A. R., Kallies, S., Suliman, T., Fielding, B. C., Drosten, C., & Niedrig, M. (2010). Human Coronavirus NL63 Open Reading Frame 3 encodes a virion-incorporated N-glycosylated membrane protein. *Virology Journal, 7*, 6.

Naik, N., Lo, Y., Wu, T., Lin, C., Kuo, S., & Chao, Y. (2018). Baculovirus as an efficient vector for gene delivery into mosquitoes. *Scientific Reports, 8*.

Narayanan, K., Huang, C., & Makino, S. (2008). SARS coronavirus accessory proteins. *Virus Research, 133*, 113.

Nicholas, K., & Nicholas, H. (1997). GeneDoc: a tool for editing and annotating multiple sequence alignments (Version 2.7.000).

Peiris, J., Guan, Y., & Yuen, K. (2004). Severe acute respiratory syndrome. *Nature Medicine, 10*, S88.

Pérez-Losada, M., Arenas, M., Galán, J., Palero, F., & González-Candelas, F. (2015). Recombination in viruses: Mechanisms, methods of study, and evolutionary consequences. *Infection, Genetics and Evolution, 30*, 296.

Prabakaran, M., & Kwang, J. (2014). Recombinant baculovirus displayed vaccine. *Bioengineered, 5*, 45.

Pyrc, K., Berkhout, B., & Van Der Hoek, L. (2007). The Novel Human Coronaviruses NL63 and HKU1. *Journal of Virology, 81*, 3051.

Pyrc, K., Jebbink, M., Berkhout, B., & Van Der Hoek, L. (2004). Genome structure and transcriptional regulation of human coronavirus NL63. *Virology Journal, 1*, 7.

Ren, A., Xie, Y., Kong, Y., Yang, G., Zhang, Y., Wang, Y., & Wu, X. (2004). Expression, Purification and Sublocalization of SARS-CoV Nucleocapsid Protein in Insect Cells. *Acta Biochimica et Biophysica Sinica, 36*, 754.

Rohrmann, G. (2019). Baculovirus Molecular Biology *Baculovirus Molecular Biology*: National Center for Biotechnology Information.

Rosano, G., & Ceccarelli, E. (2014). Recombinant protein expression in Escherichia coli: advances and challenges. *Frontiers in microbiology, 5*, 172.

Schildgen, O., Jebbink, M., De Vries, M., Pyrc, K., Dijkman, R., Simon, A., Müller, A., Kupfer, B., & Van Der Hoek, L. (2006). Identification of cell lines permissive for human coronavirus NL63. *Journal of Virological Methods, 138*, 207.

Shi, J., Zhang, J., Li, S., Sun, J., Teng, Y., Wu, M., Li, J., Li, Y., Hu, N., Wang, H., & Hu, Y. (2015). Epitope-Based Vaccine Target Screening against Highly Pathogenic MERS-CoV: An In Silico Approach Applied to Emerging Infectious Diseases. *PLoS ONE, 10*, e0144475.

Simon-Loriere, E., & Holmes, E. (2011). Why do RNA viruses recombine? *Nature Reviews Microbiology, 9*, 617.

Sloots, T., Whiley, D., Lambert, S., & Nissen, M. (2008). Emerging respiratory agents: New viruses for old diseases? *Journal of Clinical Virology, 42*, 233.

Smuts, H. (2008). Human coronavirus NL63 infections in infants hospitalised with acute respiratory tract infections in South Africa. *Influenza and Other Respiratory Viruses, 2*, 135.

Subramoney, K., Hellferscee, O., Pretorius, M., Tempia, S., Mcmorrow, M., Von Gottberg, A., Wolter, N., Variava, E., Dawood, H., Kahn, K., Walaza, S., Madhi, S., Cohen, C., Venter, M., & Treurnicht, F. (2018). Human bocavirus, coronavirus, and polyomavirus detected among patients hospitalised with severe acute respiratory illness in South Africa, 2012 to 2013. *Health Science Reports, 1*, e59.

Tan, Y. J., Lim, S., & Hong, W. (2006). Understanding the accessory viral proteins unique to the severe acute respiratory syndrome (SARS) coronavirus. *Antiviral Research, 72*, 78.

Tan, Y. J., Teng, E., Shen, S., Tan, T., Goh, P., Fielding, B., Ooi, E., Tan, H. C., Lim, S., & Hong, W. (2004). A Novel Severe Acute Respiratory Syndrome Coronavirus Protein, U274, Is Transported to the Cell Surface and Undergoes Endocytosis. *Journal of Virology, 78*, 6723.

Van Boheemen, S., De Graaf, M., Lauber, C., Bestebroer, T., Stalin Raj, V., Moh Zaki, A., Osterhaus, A., Haagmans, B., Gorbalenya, A., Snijder, E., & Fouchier, R. (2012). Genomic Characterization of a Newly Discovered Coronavirus Associated with Acute Respiratory Distress Syndrome in Humans. *mBio, 3*, e00473.

Van Der Hoek, L., Ihorst, G., Sure, K., Vabret, A., Dijkman, R., De Vries, M., Forster, J., Berkhout, B., & Uberla, K. (2010). Burden of disease due to human coronavirus NL63 infections and periodicity of infection. *Journal of Clinical Virology, 48*, 104.

Van Der Hoek, L., Pyrc, K., & Berkhout, B. (2006). Human coronavirus NL63, a new respiratory virus. *FEMS Microbiology Reviews, 30*, 760.

van Oers, M. (2006). Vaccines for viral and parasitic diseases produced with baculovirus vectors. *Advances in Virus Research, 68*, 193.

van Oers, M., Pijlman, G., & Vlak, J. (2015). Thirty years of baculovirus–insect cell protein expression: from dark horse to mainstream technology. *Journal of General Virology, 96*, 6.

van Oers, M., & Vlak, J. (2007). Baculovirus genomics. *Curr Drug Targets, 8*, 1051.

Woo, P., Huang, Y., Lau, S., & Yuen, K. (2010). Coronavirus Genomics and Bioinformatics Analysis. *Viruses, 2*, 1804.

Woo, P., Lau, S., Chu, C., Chan, K., Tsoi, H., Huang, Y., Wong, B., Poon, R., Cai, J., Luk, W., Poon, L., Wong, S., Guan, Y., Malik Peiris, J., & Yuen, K. (2005). Characterization and Complete Genome

Sequence of a Novel Coronavirus, Coronavirus HKU1, from Patients with Pneumonia. *Journal of Virology, 79*, 884.

Woo, P., Lau, S., Huang, Y., & Yuen, K. (2009). Coronavirus Diversity, Phylogeny and Interspecies Jumping. *Experimental Biology and Medicine, 234*, 1117.

Yin, J., Li, G., Ren, X., & Herrler, G. (2007). Select what you need: A comparative evaluation of the advantages and limitations of frequently used expression systems for foreign genes. *Journal of biotechnology, 127*, 335.

Yokoyama, S. (2003). Protein expression systems for structural genomics and proteomics. *Current Opinion in Chemical Biology, 7*, 39.

Yount, B., Roberts, R., Lindesmith, L., & Baric, R. (2006). Rewiring the Severe Acute Respiratory Syndrome Coronavirus (SARS-CoV) Transcription Circuit: Engineering a Recombination-Resistant Genome. *Proceedings of the National Academy of Sciences, 103*, 12546.

Yuan, Y., Zhang, Z., He, Y., Fan, W., Dong, Z., Zhang, L., Sun, X., Song, L., Wei, T., Mo, M., & Wei, P. (2018). Protection against Virulent Infectious Bronchitis Virus Challenge Conferred by a Recombinant Baculovirus Co-Expressing S1 and N Proteins. *Viruses, 10*, 347.

Zhong, N., Zheng, B., Li, Y., Poon, L., Xie, Z., Chan, K., Li, P., Tan, S., Chang, Q., Xie, J., Liu, X., Xu, J., Li, D., Yuen, K., Peiris, J., & Guan, Y. (2003). Epidemiology and cause of severe acute respiratory syndrome (SARS) in Guangdong, People's Republic of China, in February, 2003. *The Lancet, 362*, 1353.

APPENDIX 1

Hcov-Nl63 Orf3 Gene Sequence Alignment

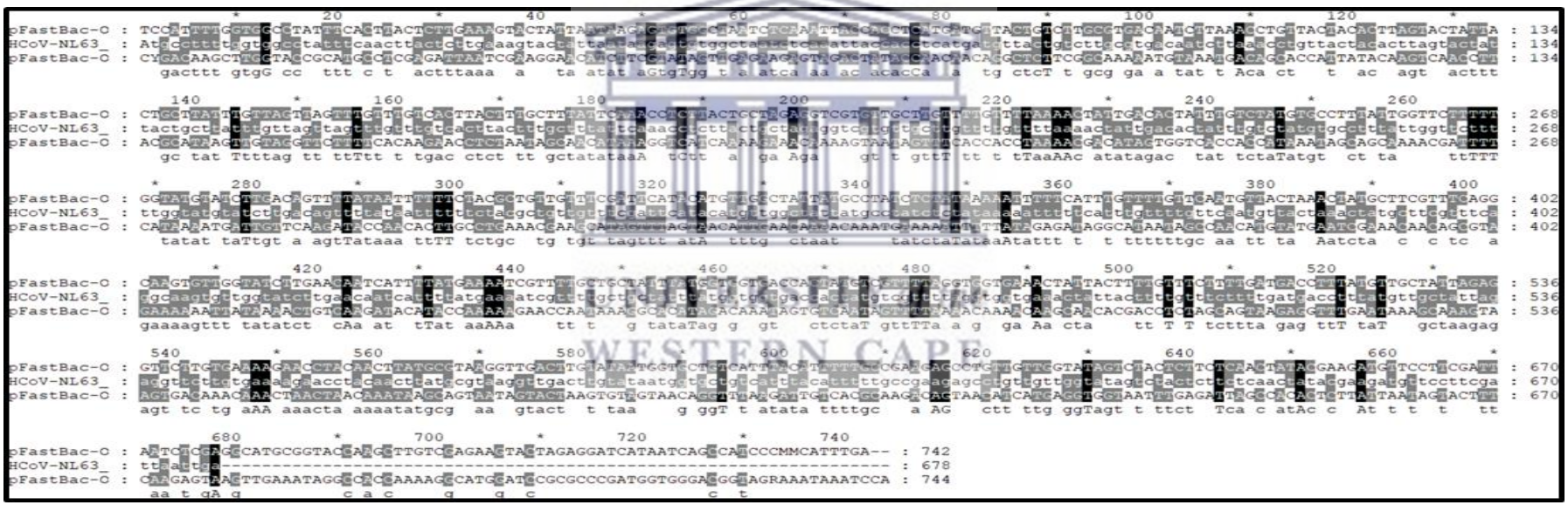

Image above depicts sequencing alignment result of pFastBac-ORF3 Forward (Top), HCoV-NL63 ORF3 NCBI (Middle) and pFastBac-ORF3 Reverse (Bottom).

APPENDIX 2

SDS-PAGE

Image above contain SDS PAGE repetitions where ORF3-Baculovirus protein lysates of 30 µg per Lane were linearized at room temperature, 40°C and 80°C respectively.

Western Blot

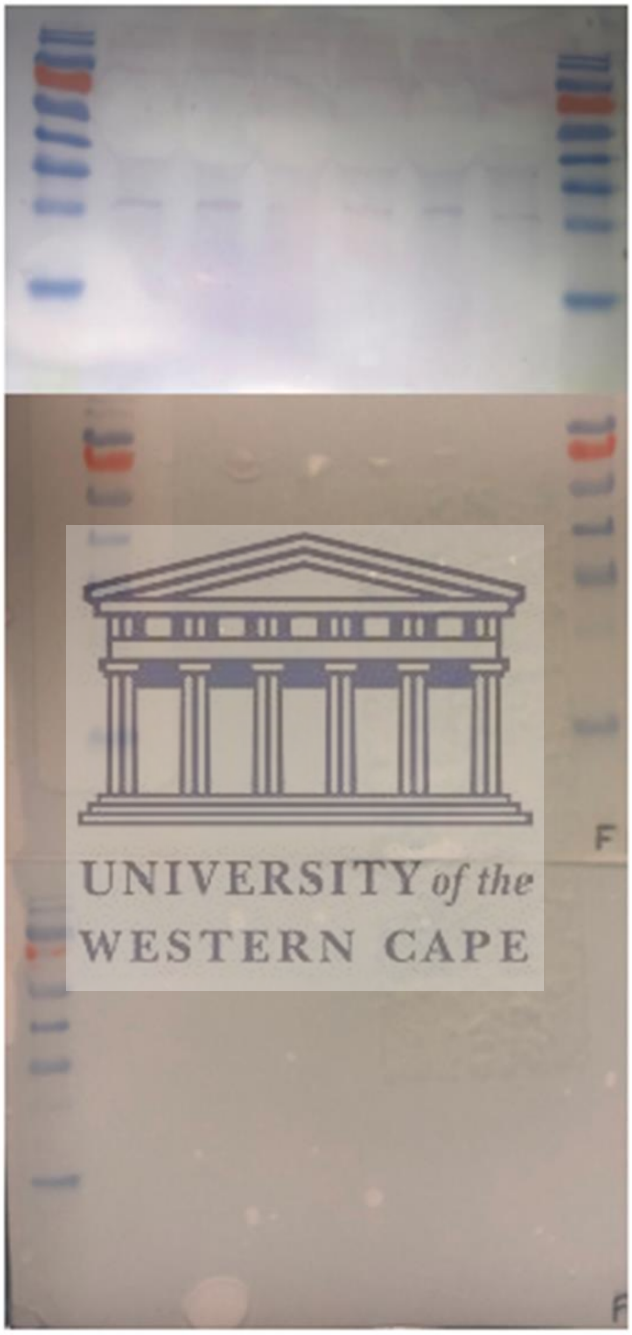

Image above contain Western Blot repetitions where ORF3-Baculovirus protein lysates were probed with the primary antibody at the dilution factors of 1:500, 1:200 and 1:100 respectively.