

2020

# Intrinsic Control Strategies for Herpesvirus-based Vaccine Vectors

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<http://hdl.handle.net/10026.1/16134>

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<http://dx.doi.org/10.24382/627>

University of Plymouth

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# UNIVERSITY OF PLYMOUTH

## **Intrinsic Control Strategies for Herpesvirus-based Vaccine Vectors**

By

**Hujaz Ismail Abdulrazzaq Alqirbi**

A thesis submitted to the University of Plymouth  
in partial fulfilment for the degree of

**DOCTOR OF PHILOSOPHY**

**School of Biomedical Sciences**

**July 2020**

*To my parents*

## Acknowledgments

After an intensive period of four years, today is the day: writing this note of thanks is the finishing touches on my thesis. It has been a period of intense learning for me, not only in the scientific arena but also on a personal level. Writing this dissertation has had a big impact on me. I would like to reflect on the people who have supported and helped me so much throughout this period.

Firstly, I would like to express my sincere gratitude to my Director of Studies Dr Michael Jarvis for the continuous support of my PhD study. His guidance helped me in all the time of research and writing of this thesis. I would also like to thank Dr Robert Belshaw for his guidance, critical comments, and invaluable advice during my study.

I would like to thank Dr Neil Avent for encouragement and support.

Besides supervisors, I would also like to thank all the members of Jarvis Lab specifically Dr Aisling Murphy and Dr Chantelle Norton for their insightful comments and encouragement for the hard question, which incited me to widen my research from various perspectives.

I would also like to express a warm gratitude for Dr William Vevers, Dr Paul Waines and all technician at Davy building for constantly being helpful, supportive, assisted me throughout my PhD journey.

I would like to thank Dr Craig Donaldson, the head of school of biomedical sciences for help, support and encouragement.

I would like to express my deepest thanks to Dr Patrick McMahon for his encouragement, help and support during my PhD journey.

I would also like to thank my parents and my brother for their love and support, whose love and guidance are with me in whatever I pursue. I would like to extend my thanks to my friends especially Dr Rabab Alkutbe and Dr Shaymaa Al-Juboori for their support. Finally, I would like to thank everyone who helped me directly or indirectly all the way through my study.

## Author's Declaration

At no time during the registration for the degree of Doctor of Philosophy has the author been registered for any other University award without prior agreement of the Doctoral College Quality Sub-Committee.

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Word count of main body of thesis: 57,160

Signed Hujaz Alqirbi

Date 7/7/2020

# Intrinsic Control Strategies for Herpesvirus-based Vaccine Vectors

Hujaz Ismail Abdulrazzaq Alqirbi

## Abstract

Herpesvirus-based vectors have been used in vaccine strategies to target zoonotic diseases such as Ebola and human monkeypox, through eliciting a highly immunogenic response. We are investigating strategies to attenuate the virus and control its ability to replicate and persist, while maintaining its ability to elicit protective immune responses. In this project we have investigated three distinct and innovative means to provide intrinsic control of herpesvirus-based vectors. The first strategy is based on deletion of a gene essential for viral latency to modify persistence *in vivo*. Studies by Thirion *et al* have shown that the bovine herpesvirus 4 (BoHV-4) ORF73 gene product is required for persistence through enabling episome tethering to mitotic chromosomes. The first aim of the project was to investigate the ability of a latency-defective BoHV-4  $\Delta$  ORF73 vector expressing heterologous antigens to induce immune responses. Four recombinant defective BoHV-4  $\Delta$  ORF73 vectors, each expressing different heterologous target antigens, were constructed. One of these constructs, which expressed a target antigen from *Mycobacterium bovis*, was tested *in vivo*. The results showed the attenuated version of BoHV-4  $\Delta$  ORF73 was able to induce an immune response against its heterologous antigen. The second attenuation strategy was based on modulation of viral replication fidelity through mutation of the viral DNA polymerase. Based on a substantial body of published work from other laboratories studying the impact of genome replication fidelity on RNA viruses, we

hypothesized that the genetic reduction in the fidelity of human cytomegalovirus (HCMV) replication would introduce an unsustainable level of mutations during replication of the virus, and thereby eventually result in 'genomic catastrophe' and demise of the virus. The aim of this second part of the thesis was to characterize a HCMV that has been modified through deletion of an aspartic acid residue 413 of domain II of exonuclease region in CMV DNA polymerase, designated HCMVUL54( $\Delta$ D413). Based on the proposed function of this domain in 'proof-reading' during virus DNA replication, it was hypothesized that this mutation would result in a decrease in the fidelity of the virus, leading to the accumulation of deleterious mutations and loss of fitness. Data presented in the thesis showed that HCMVUL54( $\Delta$ D413) had an accumulation of mutations consistent with an increased mutation rate and lower fidelity. The final attenuation strategy was based on engineering viruses in which the transgenes are flanked by identical regions. It was hypothesized that the presence of these flanking duplications will target intervening regions for genetic deletion. In contrast to the strategy based on fidelity and genomic catastrophic demise of the virus, this strategy aims to restore to recombinant virus to that of the wild type (WT) virus by facilitating excision of the heterologous transgene. Rather than intrinsic control of the CMV virus itself, this strategy is based on providing kinetic control over stability of the heterologous transgene within the recombinant virus. We hypothesized that the presence of a transgene between two homologues flanking regions would lead to its excision from the recombinant virus and restoration of the WT virus. We further hypothesized that the rate of loss of the heterologous transgene would be regulated by the length of the homologous flanking region.



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## List of abbreviations

AIHV-1	Alcelaphine gammaherpesvirus
BAC	Bacterial artificial chromosome
BCG	Bacillus Calmette–Guérin
BoHV-4	Bovine herpesvirus-4
<i>CB</i>	<i>Coxiella burnetii</i>
DIVA	Differentiation of vaccinated from infected
EBV	Epstein-Barr virus
GMO	Genetically modified organism
GMPs	Genome maintenance proteins
HCMV	Human cytomegalovirus
HSV	Herpes simplex virus
HVS	Herpesvirus saimiri
ICR	Intrachromosomal homologous recombination
Kan <sup>R</sup>	Kanamycin resistance
KSHV	Kaposi's sarcoma-associated herpesvirus
LANA	Latency-Associated Nuclear Antigen
LMICs	Low and middle-income countries
<i>Mb</i>	<i>Mycobacterium bovis</i>
MCMV	Mouse cytomegalovirus
MDBK	Madin–Darby bovine kidney
MHV-68	Murine gammaherpesvirus 68
<i>Mtb</i>	<i>Mycobacterium tuberculosis</i>
ORF	Open reading frame
PBMCs	Peripheral blood mononuclear cells
PPDs	Purified protein derivatives
RhCMV	Rhesus cytomegalovirus
RVFV	Rift Valley Fever virus

SICCT	Single comparative cervical tuberculin test
SOC	Super Optimal broth with Catabolite repression
TK	Thymidine kinase
WT	Wild type

## Chapter 1 Introduction

Vaccination has been considered as one of the most successful strategies to target infectious diseases. Viral vectors have been used recently in vaccine strategy. Using viral vectors including herpesviruses has an advantage over the traditional killed inactivated vaccine, it induced robust immune responses and increased cellular immunity against the pathogens. In spite of having the considerable success of vaccines based on the viral vectors in eradicating and preventing infectious diseases, using viral vectors in vaccine strategies raises concerns regarding safety as they are genetically modified organisms and might cause unpredictable harmful hazards. Intrinsic control for the viruses is not well developed compared with prokaryotic organisms. Many research required to investigate and develop new intrinsic control strategies to control the recombinant viral vector. The main thread of the thesis are twofold. Firstly, to investigate whether the conditionally attenuated recombinant bovine herpesvirus-4 (BoHV-4) can stimulate the immune response against its heterologous antigen. Second, to investigate new intrinsic attenuation strategies to control the recombinant cytomegalovirus (CMV) vector. In this chapter a background information on herpesviruses, viral vectors and the intrinsic control strategies are detailed.

### 1.1. Herpesviruses

The Greek word “herepin”, which means “to creep”, is the origin of the name “herpes” in herpesvirus. It is linked to the usual attribute of such viruses, being their capability of causing a latent and repeating infection within their host (Roizman, Campadelli-Fiume *et al.* 2011). We now know that herpesviruses infect a broad scope of organs and tissues in several species. Furthermore, the *Herpesviridae* family was elevated to the *Herpesvirales* order in 2009, subsequent to the discovery of molluscs and fish herpesviruses, consisting of a minimum of 135 members in three families: *Herpesviridae*, *Alloherpesviridae* and *Malacoherpesviridae*,

infecting a broad scope of hosts, ranging from birds and frogs to mammals and molluscs (Davison, 2010). Across the order, herpesviruses differ in genomic sequence, but their structure and genome organisation are similar.

All herpesviruses have the capability of replicating within the host cell nucleus as well as encoding the necessary enzymes for protein processing and synthesis in addition to causing latent infection subsequent to the initial productive infection. Herpesviruses are also able to reactivate at a later stage subject to certain circumstances (Roizman & Pellet, 2013). The virus particles range in diameter from 120-300 nm and contain a double-stranded linear DNA genome for which sizes vary from (~120-250 kb) according to the herpesvirus. An enveloped icosahedral capsid contains the DNA genome. There is proteinaceous material between the capsid and the lipid bilayer envelope, which is known as the tegument. The envelope carries glycoproteins, which mediate attachment to the host cell and virus entry.

#### 1.1.1. Classification of the *Herpesviridae* family

Classification was originally based on biological and physical properties, for example, cell tropism, growth cycle, host range, pathogenic features and virion morphology. In the age of genomics, classification is now according to sequence-based phylogeny and genomic organisation (Davison, 2010). The *Herpesviridae* family is split into the following three subfamilies: *alphaherpesvirinae*, *betaherpesvirinae*, and *gammaherpesvirinae*.

##### 1.1.1.1. *Alphaherpesvirinae*

*Alphaherpesvirinae* subfamily members are classified by a broad host range, rapid spread, and a short growth cycle as well as latency. Latency is mainly, but not exclusively, in sensory neurons (Roizman & Pellet, 2013). The *alphaherpesvirinae* subfamily is inclusive of the following genera: *simplexvirus* (e.g. herpes simplex virus 1 and 2), *varicellovirus* (e.g.

varicella-zoster virus, bovine herpesvirus 1, and feline herpesvirus 1), *Iltovirus* (e.g. gallid herpesvirus 1 and psittacid herpesvirus 1), and *mardivirus* (e.g. Gallid herpesvirus 2 (Marek's disease virus). Mardiviruses differ from other alphaherpesviruses in that they establish latency in CD4<sup>+</sup> T-cells and may lead to T-cell lymphomas (Jarosinski *et al.*, 2006).

#### 1.1.1.2. *Betaherpesvirinae*

The host range of betaherpesviruses is much narrower than alphaherpesviruses and is typified by slow spread, induction of cell enlargement (cytomegalia), latency establishment in myeloid progenitor cells, long growth cycle, and persistence in epithelial cells of kidneys and salivary glands (Mocarski, Shenk *et al.* 2013). The subfamily *Betaherpesvirinae* is inclusive of the genera *Muromegalovirus* (e.g. murine cytomegalovirus (MCMV)), *Roseolovirus* (e.g. human herpesvirus 6 and 7), *Cytomegalovirus* (e.g. human cytomegalovirus (HCMV)), and *Proboscivirus* (e.g. elephant endotheliotropic herpesvirus).

#### 1.1.1.3. *Gammaherpesvirinae*

Similar to betaherpesviruses, gammaherpesviruses also have a restricted host range. The *gammaherpesvirinae* subfamily is inclusive of the genera: *Lymphocryptovirus* (e.g. Epstein-Barr virus (EBV)), herpesvirus saimiri and bovine herpesvirus-4 (BoHV-4)), murine gammaherpesvirus 68 (MHV-68), *Rhadinovirus* (e.g. Kaposi's sarcoma herpesvirus (KSHV), *Percavirus* ((e.g. equine herpesvirus 2 (EHV-2), equine herpesvirus 2 (EHV-2)), and *Macavirus* (e.g. ovine herpesvirus 2 (OvHV-2)). The gammaherpesviruses are typified by primary productive infection in epithelial cells and subsequently by latency establishment in lymphocytes (Roizman & Pellet, 2013).

### 1.1.2. Herpesvirus structure

Each of the herpesvirus virions contains the following four principal structural elements: DNA, capsid, tegument and envelope. The genetic information of herpesviruses is comprised of a linear double-stranded DNA genome (120-250 kb). The DNA genome is contained within a capsid comprised of 161 capsomeres (150 hexons and 11 pentons) of 100 nm, ordered in icosahedral symmetry (triangulation number  $T=16$ ). The capsid consists of the following four conserved proteins: the major capsid protein, the dimer and monomer triplex proteins, and the small capsomere-interacting protein (Roizman & Pellet, 2013). The amorphous proteinaceous layer is positioned between the envelope and the capsid. It contains proteins which facilitate virus infection immediately subsequent to entry; for example, by inhibition of host protein synthesis, eluding cellular defences and stimulating viral gene expression. The tegument also performs functions in virus assembly and egress (Guo *et al.*, 2010; Kalejta, 2008; Roizman & Pellet, 2013). The envelope represents the outer virion layer and is principally comprised of the host-cell derived lipid membranes which contain virally-encoded glycoproteins that appear as short spikes embedded within the envelope in electron micrographs (Roizman, Campadelli-Fiume *et al.* 2011).

### 1.1.3. Cytomegalovirus

Cytomegalovirus (CMV) is a member of the *betaherpesvirus* subfamily, with which it shares genetic, physical and biological properties (Mocarski *et al.*, 2013). CMVs are present in a wide range of mammalian species, including chimpanzees, African green monkeys, rhesus macaques, bats, and humans (Mocarski *et al.*, 2013). A primary biological property that CMVs share with all other herpesviruses is the ability to establish and maintain a latent state within the host and then reactivate. Latency involves stable maintenance of the viral DNA genome within the nucleus as an episome, via low level expression of a small subset of viral genes



(Pass, 2014). CMVs also maintain a persistent low level replication, which may vary between the different CMV species, but is regarded as highly relevant for immunogenicity of the virus (Sylwester *et al.*, 2005).

Human cytomegalovirus (HCMV) has a large double-stranded (ds) DNA genome, which is approximately 230 kb in size and encodes more than 167 genes, making this the largest genome for any member of the *betaherpesvirus* subfamily. HCMV possesses a unique long (UL) region and a unique short (US) region, each of which is flanked by terminal and internal repeats. The ds DNA genome is enclosed by an icosahedral capsid composed of capsomer, which is then surrounded by an amorphous tegument protein coat. The tegument is encased in a glycoprotein-bearing lipid bilayer envelope (Mocarski *et al.*, 2013).

At least 25 membrane glycoproteins are found in the virion envelope (Vanarsdall & Johnson, 2012; Varnum *et al.*, 2004). Some of these proteins play an important role during viral attachment and entry such as glycoprotein B (gB), glycoprotein L (gL), glycoprotein H (gH), whilst others such as glycoprotein M (gM), glycoprotein N (gN) are important for assembly and maturation (Britt & Mach, 1996; Vanarsdall & Johnson, 2012). The glycoproteins are formed into a variety of complexes: glycoprotein complex I (gCI complex) is composed of homodimers of gB (Britt & Auger, 1986), glycoprotein complex II (gCII) is composed of a heterodimeric complex composed of gM:gN (Mach *et al.*, 2000), and gCIII complex is a heterotrimeric complex composed of gH:gL (Huber & Compton, 1997; Huber & Compton, 1998; Li, Nelson & Britt, 1997).

The gCI and gCIII complexes play an important role during attachment of the virus to the host cell surface receptor, while gCII has been reported to be important during maturation (Mocarski *et al.*, 2013). Glycoprotein O (gO) makes a complex with other glycoprotein in gCIII, which is involved in viral fusion and entry into fibroblasts. A related complex comprised of gH:gL: and gUL128-131 rather than gO is required for HCMV entry into epithelial cells and endothelial

cells (Ryckman, Chase & Johnson, 2008; Wille *et al.*, 2010). The gO protein has also been shown to function as a chaperone protein to promote export of the gH:gL from the endoplasmic reticulum.

#### 1.1.4. Bovine herpesvirus-4

Bovine herpesvirus-4 (BoHV-4) is a member of the *gammaherpesvirinae* subfamily. BoHV-4 was originally known as “bovid” because all viruses were isolated from members of the *Bovidae*: sheep, cattle and goats. There were many bovid herpesviruses typified according to the serology and restriction-enzyme analysis of DNA. These viruses were isolated from animals that displayed clinical indications of disease as well as from healthy creatures.

Initially (in 1963), Bartha *et al.* (1966) isolated BoHV-4 (prototype strain Movar 33-63), which displayed conjunctival and respiratory signs in calves. The virus was isolated at several locations throughout the world, where it was given a variety of names such as bovine herpesvirus 3, bovine herpesvirus 4, bovine herpesvirus 5 and bovine CMV. In order to avoid confusion, Bartha *et al.* (1987) gave the name “Bovine Herpesvirus-4” (BoHV-4) to all of these isolates as they all shared equal attributes. A decision was then made by the International Committee of Viral Taxonomy to adopt the official name of BoHV-4 in 2000 (Fauquet *et al.*, 2005).

BoHV-4 was originally categorised within the *Herpesviridae* family as a result of morphological attributes viewed by EM microscopy. As a result of its similarity to CMV, in regards to the presence of an inclusion body within the cytoplasm and nucleus of infected cells and slow replication cycle, BoHV-4 was originally included among *betaherpesvirinae* (Storz *et al.*, 1984).(Storz *et al.*, 1984) However, based on additional genomic structure and DNA sequence analysis, BoHV-4 was eventually categorised as belonging to *gammaherpesvirinae*. The BoHV-4 genome is comprised of a B-type structure (Roizman & Pellet, 2001) typified by

the presence of a long unique genome region (LUR) flanked by polyrepetitive DNA (prDNA) elements. Certain BoHV-4 genes are homologous to those of other herpesviruses; for example, HHV-8 and SaHV-2 (Bublot *et al.*, 1992; Goltz *et al.*, 1994). The virus contains a typical thymidine kinase gene (Kit *et al.*, 1986), which led to its classification to within the gamma-2 or rhadinovirus genus (Kit *et al.*, 1986). Today, over 40 BoHV-4 strains have been isolated that are classified into European (or Movar 33-63 like strain), African Buffalo or American strains (Dewals *et al.*, 2006).

#### 1.1.5. Herpesvirus life cycle

The herpesvirus life cycle is categorised into lytic and latent phases (Figure 1.1). Whether a virus establishes a lytic or latent infection within an infected cell in the natural host is principally dependent on the kind of cell infected and virus tropism. It is also possible for the virus to undergo lytic reactivation from latency following environmental triggers; for instance, during host immunosuppression, resulting in the production of progeny virus that can infect a new host.

##### 1.1.5.1 Lytic infection

The objective of the lytic or productive infection is production of infectious progeny virus with the ability of infecting other cells and, subsequently, spreading to other hosts. Lytic infection concerns the following events: virus attachment and entry into host cells, viral gene expression, viral DNA genome replication, virion assembly and egress.

###### 1.1.5.1.1 Attachment and entry

Virus attachment is the first stage of virus entry into the host cell. During this process, the viral envelope glycoproteins bind to cell receptors. Herpesviruses infect a wide range of cell types

by using multiple-cell surface receptors. Initial attachment for most herpesviruses involves viral glycoprotein gB interactions with cell surface heparin-sulfate proteoglycans (Roizman & Pellet, 2013). HCMV glycoproteins gB initially binds to the cell surface receptor, heparan sulfate. Subsequently, gB interacts with its non-heparin receptor leading to a more stable binding (Compton, Nowlin & Cooper, 1993). Epidermal growth factor (EGFR) and platelet derived growth factor alpha (PDGFR-alpha) receptors are recognised as two non-heparin gB binding receptors (Soroceanu, Akhavan & Cobbs, 2008).

Following attachment to host cell receptors, herpesviruses enter cells by fusion of the virus envelope with host cell membranes (Roizman & Pellet, 2013). This event leads to release of viral capsids and the associated tegument proteins into the cytoplasm. Transport of the capsid to the nuclear pore is facilitated by tegument proteins through utilisation of the cytosolic organelle transport network involving microtubules (Sinzger *et al.*, 2000). Following transport to the nucleus, DNA is released from the nucleocapsid and then enters the nucleus through the nuclear pore complex.

#### 1.1.5.1.2 Gene expression

The transcription of all herpesvirus genes occurs in the nucleus through utilisation of host cell RNA polymerase II and associated basal transcription machinery (Fortunato *et al.*, 2000). Gene expression may be categorised into three principal kinetic stages according to the kinetics of expression and sensitivity to inhibitors of protein translation and viral DNA synthesis. These kinetic classifications are as follows: immediate-early (IE or  $\alpha$ ), early (E or  $\beta$ ) and late (L or  $\gamma$ ) genes (Fortunato *et al.*, 2000). IE genes are expressed immediately following virus entry. Viral and cellular gene expression is controlled by IE gene products with a main purpose of optimising the environment for virus genome replication. Furthermore, products of these IE genes trigger a regulatory cascade, which then results in expression of E genes.

Expression of E genes depends on the availability and expression of IE gene products, but does not require viral DNA synthesis. According to time of expression, E genes can be divided into two subclasses,  $\beta 1$  (true-E) and  $\beta 2$  (E-L). Generally, E gene products encode non-structural proteins; for instance, proteins involved in virus DNA genome replication like viral DNA polymerase; UL54). Proteins such as MHC down-modulators, which are involved in immune evasion (for example, US2 and US11), are also expressed at this time (Jones & Sun, 1997; Wiertz *et al.*, 1996).

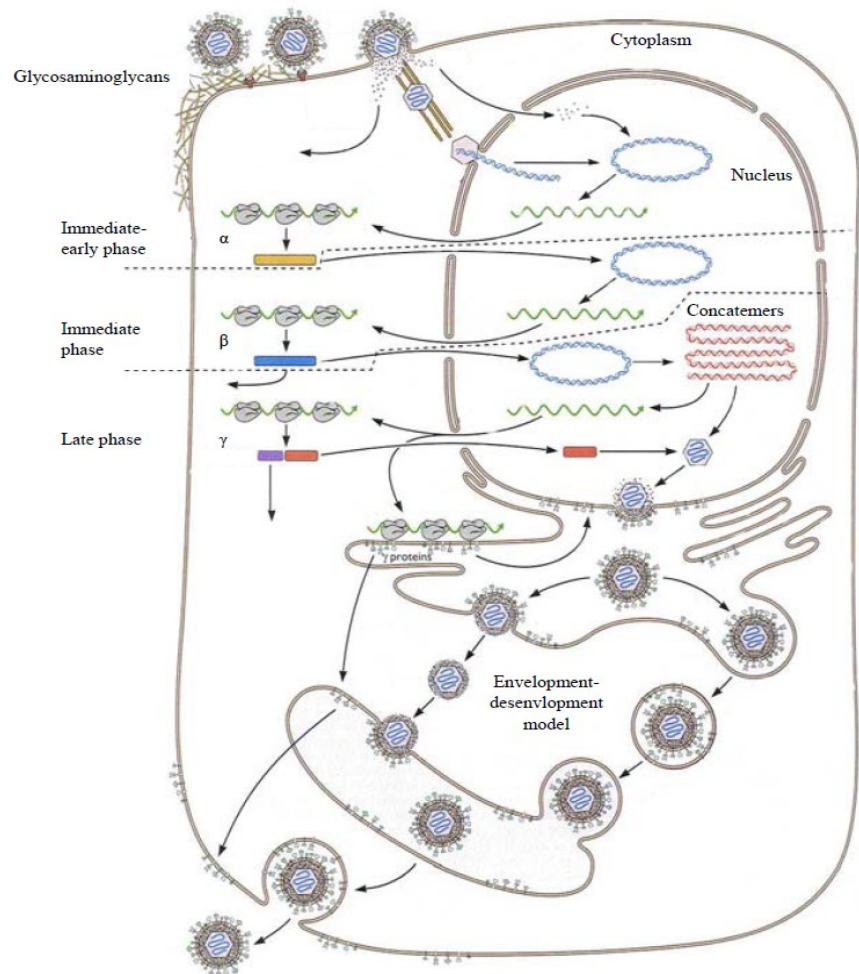
The final class of genes are L ( $\gamma$  phase) genes. L genes can similarly be divided into two classes:  $\gamma 1$  (partial-L) and  $\gamma 2$  (true-L). All L genes are influenced by viral DNA replication. Prior to DNA replication,  $\gamma 1$  genes are transcribed at low levels, but are amplified by viral DNA replication. In contrast, expression of  $\gamma 2$  genes has a total dependence on viral DNA replication. L genes encode for tegument and structural proteins, all of which are necessary for late virion assembly events and morphogenesis within the cytoplasm (AuCoin *et al.*, 2006; Silva *et al.*, 2003; Wing, Lee & Huang, 1996).

**A**

Attachment of the virus at cellular surface and fusion with the plasma membrane

Viral proteins synthesis and viral DNA replication

Viral membrane acquisition



**B**

Viral genome is under episomal form

LANA (maintenance protein encoded by ORF 73) is expressed

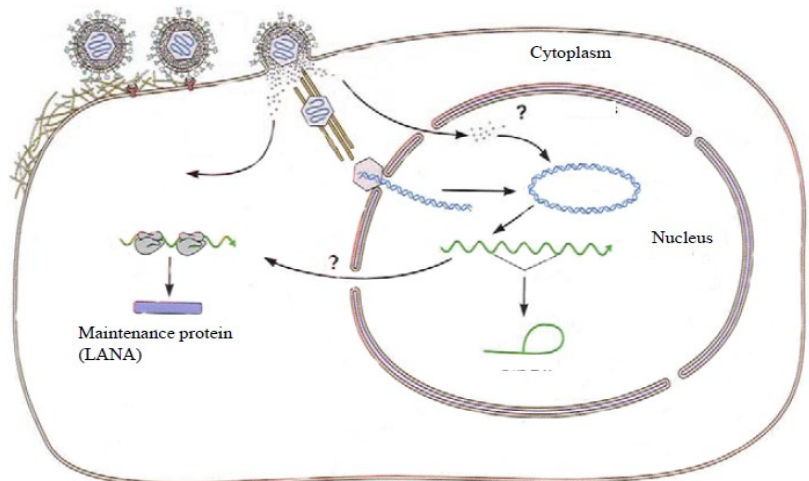


Figure 1.1: Replication cycle for herpesviruses. (A) the lytic state and (B) the latent state. From Flint *et al.*, 2000.

#### 1.1.5.1.3 Viral genome replication

Herpesvirus DNA replication, which depends on both host and viral proteins and the presence of required biosynthetic precursors, takes place within the nucleus. Similar to other viruses, herpesviruses are dependent to a certain level on the metabolic activity of the host cell for supply of energy and building blocks for viral replication. Cellular metabolic routes may be modulated by herpesviruses for facilitating viral replication. For example, herpes simplex virus (HSV) increases host pyrimidine biosynthesis (Vastag *et al.*, 2011), whilst HCMV manipulates the tricarboxylic acid cycle in order to improve lipid biosynthesis (Munger *et al.*, 2008; Vastag *et al.*, 2011; Yu *et al.*, 2011).

In terms of the mechanics of DNA replication, HCMV DNA synthesis commences with the formation of an intermediate ‘theta’ framework prior to switching to rolling circle replication. The replicating DNA architecture conforms to concatemeric structures which are usually created as a result of this type of replication. Concatameric DNA is also the probable source of linear encapsidated genome that then undergoes covalent circularisation following entry (McVoy & Adler, 1994; Mocarski *et al.*, 2013). HCMV DNA replication absolutely requires the activity of six core viral proteins, all of which are expressed with E kinetics. These core proteins comprise the replication fork machinery (Iskenderian *et al.*, 1996) and consist of UL44 (DNA polymerase-associated factor), UL54 (DNA polymerase), UL57 (single-stranded DNA-binding protein), UL70 (DNA primase-associated factor), UL102 (DNA helicase) and UL105 (DNA primase) (Iskenderian *et al.*, 1996; Pari & Anders, 1993) (Figure 1.2). In addition to core proteins, HCMV DNA replication also requires three IE regulatory proteins (encoded by UL36-38, IRSI/TRSI, and the major IE region spanning UL122-132) and two further nuclear-localised proteins expressed with E kinetics (UL84, UL112-113). As is the case with the six core proteins, blocking expression of any of these accessory protein sets averts viral replication (Ripalti *et al.*, 1995; Smith & Pari, 1995).

The viral DNA polymerase enzyme is comprised of pUL44 and pUL54. The pUL54 DNA polymerase contains both a 5' to 3' DNA synthesis function and a 3' to 5' exonuclease activity, which permits 'proofreading' of the genome. Processivity of DNA polymerase is increased by the pUL44 protein. DNA-dependent helicase, primase (HP) and ATPase activities are involved in this HP complex. Subsequent to the unwinding of the heteroduplex genome DNA, the HP complex produces RNA oligonucleotides to prime polymerase elongation on the lagging DNA strand. All dNTPs necessary for replication of the viral genome are provided by the host cell as unlike other herpesviruses subgroups, betaherpesviruses such as HCMV do not encode the required biosynthetic machinery for nucleotide biosynthesis (e.g., thymidine kinase) (Chee *et al.*, 1990). Cellular transcription, translation and genes involved in the DNA precursor biosynthesis are stimulated by HCMV (Estes & Huang, 1977; Song & Stinski, 2002). Virus DNA replication is further enhanced by a decrease in host cell DNA replication resulting from a virus-induced block in cell cycle progression (Fortunato *et al.*, 2000). This HCMV-mediated cell regulation provides an environment which is rich in the required precursors for replicating the viral genome, whilst at the same time reducing competition of the host DNA replication machinery to replicate its own cellular DNA.



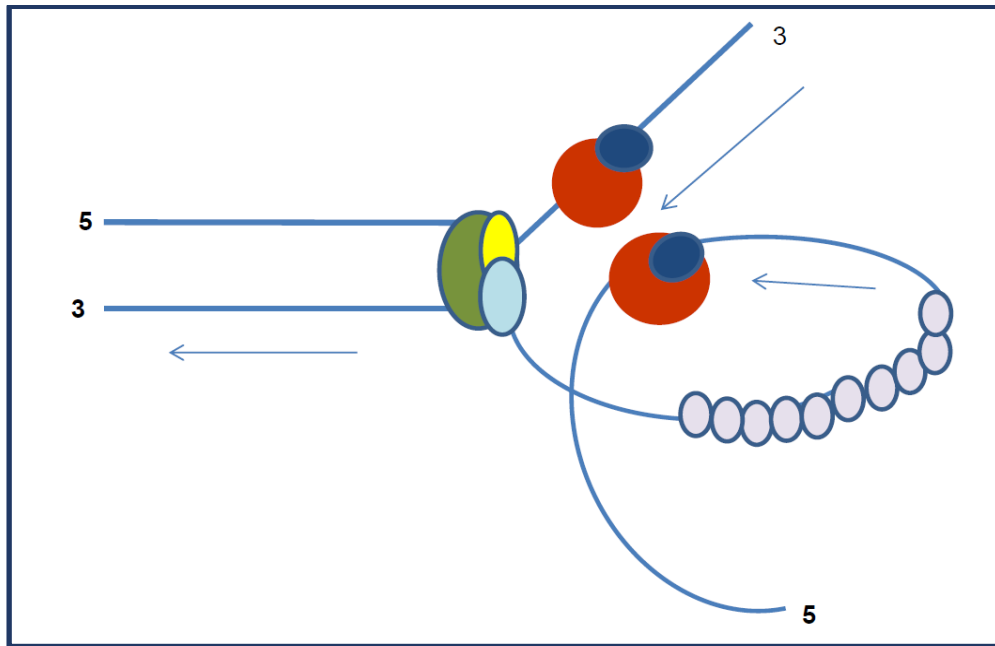


Figure 1.2: Schematic showing structure of the HCMV replication fork. UL54 (DNA polymerase, Brown), UL44 (DNA polymerase-associated factor, Dark Blue), UL57 (single-stranded DNA binding protein, Light Purple), UL70 (primase-associated factor, Yellow), UL102 (DNA helicase, Green) and UL105 (DNA primase, Light Blue) (Figure modified from Hirai, 1998).

HCMV DNA replication occurs inside the nucleus within a framework known as the ‘replication compartment’, which the virus induces and acts as a site for the accumulation of proteins involved in viral genome replication (Wilkinson & Weller, 2003). The initial site of ‘replication compartment’ formation occurs at cellular ND10 nuclear bodies’, which are also the site where circularized viral DNA genome localises subsequent to being released from the incoming nucleocapsid. Within the HCMV genome, DNA replication begins at a single viral lytic origins of replication (oriLyt) (Hamzeh *et al.*, 1990) positioned between UL57 and UL69 (Zhu, Huang & Anders, 1998). The oriLyt region, representing 3 to 4 kb positioned next to the single-strand DNA binding protein promoter (UL57) (Hamzeh *et al.*, 1990), is divided into two regions: essential region I and essential region II. Essential region I contains the bidirectional UL84/IE2-responsive promoter (Pari, 2008). Whilst CMVs have one ori-Lyt, others (e.g., EBV

and VZV) have two, or three (e.g., HSV-1 and HSV-2) (Roizman & Pellett, 2001). The preponderance of the evidence supports viral DNA replication by means of a rolling circle (theta,  $\theta$ -based) mechanism with similarities to bacteriophage DNA replication. Mechanistically, this mode of viral DNA synthesis leads to the production of the large head-to-tail genome concatemers (Zhu *et al.*, 1998), which act as the substrate for packaging/cleavage of unit length genome into the newly-synthesised capsid ( Figure 1.3).

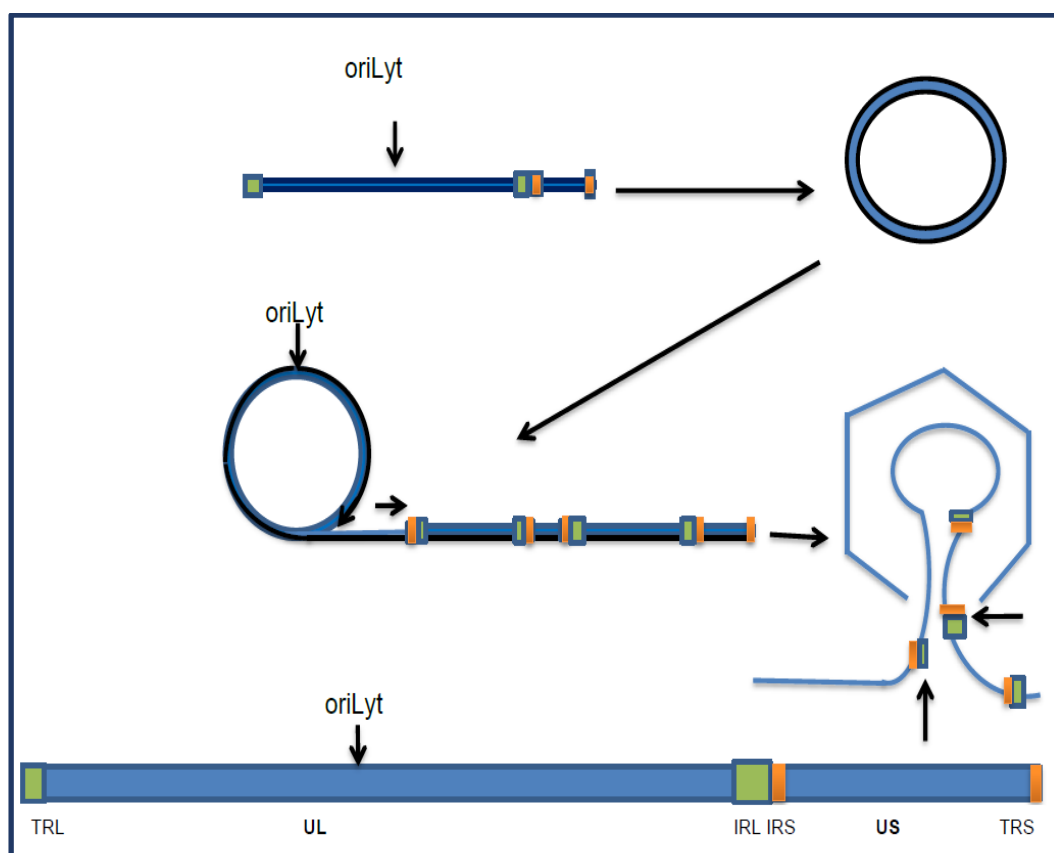


Figure 1.3: Mechanism of HCMV DNA replication. Replication is initiated from the replication origin (oriLyt) and involves concatemeric by rolling circle replication (Hirai, 1998).

#### 1.1.5.1.3.1 DNA polymerase

DNA polymerase is an important enzyme that is required for DNA replication and repair, this activity is essential for genome duplication and ensures high fidelity transfer of genetic information from one generation to another (Bebenek & Kunkel, 2004).

##### 1.1.5.1.3.1.1 Structure

Similar to all DNA polymerases, HCMV DNA polymerase is comprised of several domains that together resemble a 'right hand' in structure. The main domains are the thumb, palm and fingers. Some DNA polymerases have additional domains such as exonuclease and exonuclease N terminal domains, but these are not present in herpesvirus DNA polymerases. The thumb binds dsDNA, the palm contains the catalytic amino acid residues and binds dsDNA, and the fingers bind incoming nucleotides and interact with the single-stranded template (Patel & Loeb, 2001).

##### 1.1.5.1.3.1.2 Function

DNA molecules are comprised of two anti-parallel sugar-phosphate linked nucleotide chains. The chemical composition of each nucleotide is a nitrogenous base, sugar (deoxyribose) and phosphate group (Watson & Crick, 1953). During replication, the two antiparallel DNA chains unwind and each becomes a template for a new complementary strand (Watson & Crick, 1953). DNA polymerase is an essential enzyme in the process of synthesizing a new complementary strand from deoxyribonucleotide triphosphates (dNTPs).

The pathway showing incorporation of dNTPs during DNA replication is presented in Figure

1.4

**Starting Point:** Primer (P) binds to template DNA strand (T).

**Step 1:** Primer template (P/T) DNA binds to the DNA polymerase enzyme (E) to form E:P/T.

**Step 2:** E:P/T bind to dNTP resulting in an E:P/T:dNTP complex. At this step the DNA polymerase enzyme can distinguish between correct versus incorrect dNTP incorporation (Rothwell & Waksman, 2005).

**Step 3:** Activation of E:P/T:dNTP complex to form (E:P/T:dNTP\*).

**Step 4:** Formation of phosphodiester bond.

**Step 5:** Release of pyrophosphate (PPi) from the new complex.

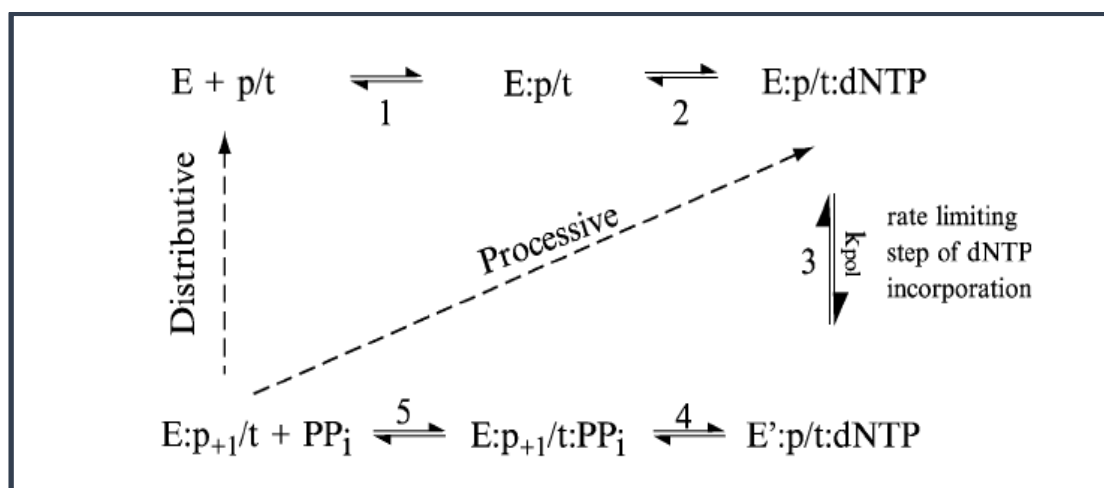


Figure 1.4: Kinetic pathway of nucleotide incorporation (adapted from (Rothwell & Waksman, 2005)).

According to the Watson and Crick model, thymine (T) binding to adenine (A), and guanine (G) binding cytosine (C) occurs by two and three hydrogen bonds, respectively (Watson & Crick, 1953). When an incorrect nucleotide is incorporated into the growing DNA stand, this binding leads to a change in geometry of the DNA structure and a second conformational change. As detailed in Figure 1.4, the second step is critical for fidelity of the DNA replication process, as this is the step at which DNA polymerase discriminates between correct and

incorrect nucleotides. Discrimination by polymerase between incorporation of the correct versus incorrect nucleotide appears to be highly efficient, with the difference in  $K_d$  between correct and incorrect nucleotides in the related DNA polymerase from bacteriophage T4 being 263-fold (Gillin & Nossal, 1976; Topal, DiGiuseppi & Sinha, 1980)

**DNA polymerase ‘proof-reading’:** Proof-reading 3’-5’ exonuclease activity is critical to prevent permanent incorporation of erroneous dNTPs during DNA replication. This activity is intrinsic to many DNA polymerases, and loss of this activity has a substantial impact on fidelity of DNA replication, leading to an increase in mutation frequency (Frey *et al.*, 1993; Kroutil *et al.*, 1996; Kunkel, Loeb & Goodman, 1984). Exonuclease activity has been shown to enhance replication fidelity by 10 to 100-fold for most DNA polymerases (Johnson & Johnson, 2001; Kunkel & Bebenek, 2000; Longley *et al.*, 2001; Song *et al.*, 2004). DNA polymerases of both T4 bacteriophage and herpesviruses are thought to partition the DNA primer between the polymerizing and exonuclease sites, which allows the enzyme to engage in idling turnover of excision and incorporation until the correct nucleotide is incorporated. These rounds of excision and incorporation are suggested to present a kinetic barrier of extension until the correct nucleotide is in place. Studies using T4 DNA polymerase have shown the ability of this enzyme to replicate with high fidelity and also indicate a high accuracy in nucleotide selection during polymerization and the balance of polymerization and exonuclease activity.

Importantly, these studies showed that only a minor change in this balance could lead to appearance of progeny with either a high (mutator) or low (anti-mutator) phenotype (Reha-Krantz, 1998; Reha-Krantz, 2010; Yang *et al.*, 2004).

#### 1.1.3.1.4 Virus assembly and egress

Virion assembly occurs both within the nucleus and cytoplasm (Kalejta, 2008). After nucleocapsid synthesis within the cytoplasm, nucleocapsid proteins in the form of

preassembled intermediates are translocated to the nucleus where they accumulate as nuclear inclusion bodies which produce the typical ‘owl’s eye’ appearance of CMV infected cells (Fons *et al.*, 1986). The newly-formed capsids are assembled within the nucleus on temporary scaffolds and then filled with newly-replicated viral genome in an energy-dependent process (Mettenleiter, Klupp & Granzow, 2009). The nuclear membrane is the first principal barrier in the course of the virus egress. As a result of their large size, viral nucleocapsids are unable to utilize nuclear pores for egress, which display no gross perturbation in the course of the viral movement across the nuclear membrane (Hofemeister & O'Hare, 2008). Instead, nucleocapsids transit the nuclear membrane by first becoming enveloped by budding at the inner nuclear membrane into the perinuclear space (primary envelopment). Capsids gain access to the cytoplasm by subsequent de-envelopment at the outer nuclear membrane (Johnson & Baines, 2011).

For primary HCMV envelopment, pUL50 and pUL53 form a core nuclear egress complex (NEC) by heterodimerisation at the nuclear rim prior to recruiting additional viral and cellular proteins. The main constituents of the HCMV multi-component NEC, determined by protein interaction studies and proteomic analyses (Milbradt *et al.*, 2014), are pUL97, p32/gC1qR, emerin, protein kinase C (PKC). The pUL97 is of specific significance, as its kinase activity is mainly responsible for nuclear lamina disassembly in the course of the late stage of HCMV replication. Previous studies indicated the association of NEC with pUL97, which occurs indirectly by means of binding to p32/gC1qR, which bridges pUL97 to the pUL50-pUL53 core NEC (Marschall *et al.*, 2005). Cellular protein kinases are also associated with the NEC, particularly PKC and cyclin-dependent kinase 1 (CDK1), as detected by pUL50-specific co-immunoprecipitation (Sonntag *et al.*, 2016; Sonntag *et al.*, 2017).

Recent studies have also shown that nuclear capsids interact with pUL53 and pUL97 in the course of capsid budding from the nucleus (Milbradt *et al.*, 2018). This is in addition to earlier

studies showing the involvement of pUL27 at this nuclear egress stage, and it has been suggested that an antagonistic relationship between pUL27 and pUL97 exists in terms of their respective activities in the course of infection. Furthermore, it has been demonstrated that pUL97-mediated phosphorylation of pUL27 ultimately disrupts p21Cip1 expression. Maribavir (MBV) inhibition of pUL97 leads to active pUL27, sustained increased p21Cip1 levels as well as CDK inhibition; consequently, virus release is disrupted. When active pUL27 is not present, p21Cip1 levels are elevated, and MBV-mediated loss of pUL97 activity is partly compensated by endogenous CDK1 (Bigley, Reitsma & Terhune, 2015).

Newly-formed virions which have entered the cytoplasm through de-envelopment are comprised of inner tegument proteins and the DNA containing nucleocapsids. Additional assembly stages include the acquisition of additional tegument proteins, as well as RNA (cytoplasmic tegumentation), followed by envelopment within the mature envelope (secondary envelopment) in the course of budding into cytoplasmic membranes, thereby producing an infectious virus. Mature enveloped virions are transported to the cell surface in cytoplasmic vesicles, which are released by fusion between cytoplasmic vesicles and the plasma membrane causing virus release (Guo *et al.*, 2010; Mettenleiter, 2006).

#### 1.1.5.2 Latent infection

One of the primary attributes of herpesviruses is their capability for establishing and sustaining latent infection within the natural host (discussed in more detail below). In cells that are latently infected, the viral genome assumes the form of a circular episomal molecule from which a considerably limited number of viral genes are expressed; consequently, no infectious virus is detected. The latent virus genome has the ability to reactivate and enter the lytic infection cycle to produce infectious virions under particular environmental conditions, such as

immunosuppression. Latent infection is established by different herpesviruses within different types of cells; for instance, HSV establishes latency in the neurons of sensory ganglia, while EBV establishes latency principally in B lymphocytes (Roizman & Pellet, 2013). Latency can be described as a complex process involving interactions between viral and host cellular factors. Normally, latent infection is established following a primary lytic infection and involves the following stages: (i) suppression of lytic infection and restriction of lytic gene expression while sustaining latent gene expression, (ii) improved survival of the latently-infected cells by evasion of the immune response and the prevention of apoptosis, and (iii) prevention of undesired reactivation whilst maintaining the ability of the virus ability to reactivate under suitable conditions (Bartel, 2004). Currently, there is no clear understanding of the precise molecular mechanisms behind the regulation of latency and reactivation.

For HCMV, CD14<sup>+</sup> monocytes and the CD34<sup>+</sup> myeloid progenitor cells are the main sites of latency (Mendelson *et al.*, 1996; Schrier, Nelson & Oldstone, 1985; Taylor-Wiedeman *et al.*, 1991). Only a limited set of transcripts is expressed during latency, and there is no virion production (Goodrum *et al.*, 2007; Hargett & Shenk, 2010; Poole *et al.*, 2013; Reeves & Sinclair, 2010; Rossetto, Tarrant-Elorza & Pari, 2013). A principal driver of lytic transcription suppression and latent infection maintenance is the remodelling of chromatin around the viral major immediate early promoter (MIEP) to a heavily repressive state (Murphy *et al.*, 2002; Reeves *et al.*, 2005a; Reeves *et al.*, 2005b; Reeves *et al.*, 2006; Reeves & Sinclair, 2010). The repressive state appears to be maintained by the absence of viral activators (such as pp71) and latency-associated virus-encoded repressors (such as HCMV RNA 4.9) (Noriega *et al.*, 2014; Rossetto, Tarrant-Elorza & Pari, 2013; Saffert, Penkert & Kalejta, 2010; Wright *et al.*, 2005). Reactivation of lytic infection from latency is driven by a number of different external and physiological stimuli, including stress, cellular differentiation and a number of pharmacological agents. For example, differentiation of progenitor cells into macrophages or



dendric cells modifies the nuclear environment, thereby causing changes in the conformation of chromatin, the lytic replication program and the MIEP (Reeves *et al.*, 2005a, Reeves *et al.*, 2005b). It has been experimentally demonstrated that histone deacetylase inhibitors like MC1568, trichostatin A and valproic acid alleviate the repressive chromatin structure, thereby enabling IE gene expression, at least transiently (Murphy *et al.*, 2002).

#### 1.1.5.2.3 Bovine herpesvirus 4 (BoHV-4) latency

Similar to other herpesviruses, BoHV-4 has been shown to be able to establish and then reactivate from latency (Castrucci *et al.*, 1987). The principle site of latency for BoHV-4 is lymphoid tissue, and specifically splenic mononuclear cells (monocyte/macrophage) (Lopez, Galeota & Osorio, 1996; Osorio & Reed, 1983). In comparison, the role of neuronal cells as a site of latency for BoHV-4 is unclear. Review of the literature suggests that although neuronal tissue can be a site of latency, it serves infrequently in this role. In a study of analyzing ganglia from 44 healthy cattle, BoHV-4 isolation was infrequent, being isolated from only two animals (Homan & Easterday, 1981). In a separate study, Egyed and Bartha (1998) studied the potential sites of BoHV-4 latency by PCR for 60 days following the experimental infection of calves following no detection of shed virus for two weeks. Viral DNA was detected in immune cells (lymph nodes, bone marrow, spleen) and also in nervous system cells (medulla oblongata, hippocampus, trigeminal ganglia, spinal cord). Consequently, they inferred that BoHV-4 has the ability to determine latency in the nervous and immune system, thereby uniting these positions on BHV-4 latency (Egyed & Bartha, 1998). Consistent with this model, Asano *et al.* (2003) reported that the BoHV-4 viral genome was detected by PCR in the lymph nodes and nervous tissues of the cattle that experimentally infected with BoHV-4(Asano *et al.*,2003).

In summary, both lymphoid and neuronal tissue appears to represent sites of BoHV-4 latency, with the former being the most common site.

#### 1.1.5.2.1 The role of genome maintenance proteins (GMPs) in herpesviruses latent infection

During latency, the viral genome persists within the nucleus as a circular episomal element, with the viral gene expression program being limited to non-coding RNAs and a small number of latency-associated proteins. The maintenance of genome has been most explored for the gammaherpesvirus subfamily, which includes BoHV-4. In this subfamily, genome maintenance proteins (GMPs) have been identified as a small subset of genes that are expressed in latently infected cells. GMPs are DNA binding proteins that have the ability to bind sequences within the viral genome, whilst simultaneously interacting with cell chromosome associated proteins, to guarantee the partitioning to daughter cells during mitosis. Although GMPs share little primary amino acid sequence similarity, they share conserved functions occupying necessary functions in latent infection (Sorel & Dewals, 2018).

*Lymphocryptovirus* GMPs are encoded by an open reading frame (ORF) BKRF1 and are named in accordance with the prototypical Epstein-Barr virus (EBV) nuclear antigen 1 (EBNA-1) (Table 1.1). For *Rhadinovirus*, *Percavirus* and *Macavirus* genera the GMPs are encoded by ORF73, and are named after the KSHV latency-associated nuclear antigen 1 (LANA-1). Functionally, EBNA-1, LANA and ORF73 have been shown to be necessary for efficient latency establishment of gammaherpesviruses. EBV EBNA-1 and KSHV LANA-1 are essential for episome persistence (Ballestas, Chatis & Kaye, 1999; Ballestas & Kaye, 2001; Sears *et al.*, 2003). HVS ORF73 has been shown to tether the viral genome to host chromosomes in order to facilitate episomal maintenance and host chromosomes by binding to terminal repeats DNA (Collins *et al.*, 2002; Verma & Robertson, 2003; White, Calderwood & Whitehouse, 2003). The ORF73-encoded protein of strain H26-95 of Macacine gammaherpesvirus 5 was similarly shown to bind to the viral episome and to be necessary for establishment of latency (DeWire & Damania, 2005; Wen, Dittmer & Damania, 2009).

Deletion of ORF73 from Alcelaphine gammaherpesvirus (AlHV-1) resulted in the lack of virus persistence *in vivo*, while the impairment of its expression had no impact on viral lytic replication (Palmeira *et al.*, 2013). A small number of studies have been conducted on MHV-68 ORF73; wherein it has been shown that ORF73 is again necessary in order to create and maintain latency (Fowler *et al.*, 2003; Moorman, Willer & Speck, 2003). Deletion of ORF73 from BoHV-4 has similarly been shown to impair viral persistence within a macrophage cell line *in vitro*, and within a permissive rabbit model *in vivo* (Thirion *et al.*, 2010). The critical function of GMPs in latency is further shown by the capability of LANA-1 to modulate cellular pathways implicated in cell growth and survival by targeting transcriptional regulators or co-regulators like p53, pRB, CBP and sin3A (Radkov *et al.*, 2000). Similarly to LANA-1, HVS ORF73 also represses the p53 and pRb functions; furthermore, it has been demonstrated that the expression of MHV-68 ORF73 leads to decreased p53 stabilisation (Borah, Verma & Robertson, 2004; Forrest *et al.*, 2007). LANA-1 also has the ability to control viral latency by repressing the replication and transcription activator protein (RTA), encoded by ORF50, which also functions as a transcriptional activator of early and late lytic genes (Lan *et al.*, 2004).

Table 1.1: Genome maintenance protein in gammaherpesviruses.

Genus/species		Common name (common abbreviation)	GMP
Lymphocryptovirus	Callitrichine gammaherpesvirus 3	Marmoset herpesvirus	ORF39
	Human gammaherpesvirus 4	Epstein-Barr virus (EBV)	EBNA1
	Macacine gammaherpesvirus 4	Rhesus lymphocryptovirus (rhLCV)	rhEBNA1
	Papiine gammaherpesvirus 1	Herpesvirus papio	baEBNA1
Rhadinovirus	Ateline gammaherpesvirus 3	Herpesvirus ateles strain 73 (AtHV-3)	ORF73
	Bovine gammaherpesvirus 4	Bovine herpesvirus 4, Movar virus, V. test virus	ORF73 (boLANA)
	Cricetid gammaherpesvirus 2	Rodent herpesvirus Peru	RHP73
	Human gammaherpesvirus 8	Kaposi's sarcoma-associated herpesvirus (KSHV)	LANA1
	Macacine gammaherpesvirus 5	Rhesus rhadinovirus	ORF73 (rhLANA)
	Murid gammaherpesvirus 4	Murine gammaherpesvirus 68 (MHV68)	ORF73 (mLANA)
	Murid gammaherpesvirus 7	Wood mouse herpesvirus	ORF73
	Saimiriine gammaherpesvirus 2	Herpesvirus saimiri (HVS)	ORF73 (sLANA)
Percavirus	Equid gammaherpesvirus 2	Equine herpesvirus 2 (EHV-2)	ORF73
	Equid gammaherpesvirus 5	Equine herpesvirus 5 (EHV-5)	ORF73
Macavirus	Alcelaphine gammaherpesvirus 1	Wildebeest-derived malignant catarrhal fever virus	ORF73 (aLANA)
	Alcelaphine gammaherpesvirus 2	Topi herpesvirus	ORF73
	Ovine gammaherpesvirus 2	Sheep-associated malignant catarrhal fever virus	ORF73 (oLANA)

#### 1.1.6. Herpesviruses as a vaccine vector

Viruses are seen as promising tools for development as vectors for vaccines and immunotherapeutics due to their ability to infect host cells and thereby increase immune recognition (Brave *et al.*, 2007; Ura, Okuda & Shimada, 2014). Many viruses have been explored for their use as vaccine vectors, which include: adenovirus (Ad) (Zhang & Seto, 2015), vaccinia virus (Rerks-Ngarm *et al.*, 2009), and yellow fever virus (Rollier *et al.*, 2011). Herpesviruses, including CMV and BoHV-4 have also been more recently studied towards this end (Donofrio *et al.*, 2013; Franceschi *et al.*, 2015; Hansen *et al.*, 2011; Tsuda *et al.*, 2015). Herpesvirus-based vectors have been shown to induce high T and B cell immunity, are able to superinfect the host by evading pre-existing vector-specific immunity. They also have large carrying capacity for heterologous genetic material, which is a limitation of many other virus vector platforms. Herpesviruses can also be manipulated by bacterial genetics following their cloning as a bacterial artificial chromosome (BACs) (Gillet *et al.*, 2005; Méndez, Rodríguez-Rojas & Del Val, 2019; Skenderi & Jonjić, 2012; Wagner, Ruzsics & Koszinowski, 2002). A number of herpesviruses-based vaccines have been approved for commercial use such as a live bovine herpesvirus-1 (BoHV-1) vaccine (Bovilis® IBR Marker Live, Intervet), which is used to protect cattle from virulent BoHV-1 (infectious bovine rhinotracheitis). VAXXITEK HVT+IBD is a herpesvirus of turkey (HVT)-based recombinant vaccine, which is used to protect chickens from infectious bursal disease (IBD) (Gumboro disease).

Live viral vectors have been shown to induce more robust immune responses than other vaccine modalities, such as killed microbial-based and recombinant protein-based vaccines. This effect appears to be considerably pronounced for herpesvirus-based vectors due to their persistence within the host. Due to their intracellular replication, immunity includes a heightened ability to induce cytotoxic T lymphocytes (CD8<sup>+</sup> T cells). CTLs play a critical role in the control of

intracellular pathogens and elimination of pathogen infected cells (Ura, Okuda & Shimada, 2014). CTLs recognize short peptide regions (T cell epitopes) in the context of the cellular major histocompatibility complex I (MHC-I). As this can potentially expose all regions of the virus proteome to immune recognition and effector responses (i.e, CTL-mediated killing), CTL responses may be able to target more conserved regions of the virus than antibodies (that recognize limited regions of the proteome, generally restricted to extracellular protein regions) (Berthoud *et al.*, 2011; Osterhaus, Fouchier & Rimmelzwaan, 2011).

Pre-existing immunity to the viral vector can be a problem for the use of some viral vector vaccines (for example, those based on Ad). However, herpesvirus-based vectors appear able to superinfect the host by evading pre-existing immunity, which has been most extensively studied for CMV (Hansen *et al.*, 2010). For CMV, this was shown to result from CMV encoding proteins US3, US2 ,US11 and US6 that down-modulate MHC-I expression, and involved subversion of CTL control (Hansen *et al.*, 2010). CMV was also shown to differ in the quality of T cells that it induces compared to other vaccine modalities by inducing effector rather than central memory biased responses (Kaech & Cui, 2012; Miller *et al.*, 2008). Effector memory cells reside in the extra lymphatic tissue such as lung and mucosae and respond rapidly to pathogens. In contrast, central memory are located in the secondary lymphatic tissue, have more proliferative capacity in response to pathogens, but are delayed in differentiation of their effector functions. As effector memory cells have an immediate effector function, and reside in the mucosae, immune responses induced by CMV are believed to be able to more rapidly operate at the site of initial pathogen infection and replication (Hansen *et al.*, 2011; Hansen *et al.*, 2013; Hansen *et al.*, 2009). CMV vectors have been largely regarded as poor inducers of antibody responses (Hansen *et al.*, 2011; Hansen *et al.*, 2009). However a more recent study suggested that high levels of antibodies could be induced by placing the target gene under

control of a promoter expressed with late (L) expression kinetics in relation to the virus replication cycle (Marzi *et al.*, 2016).

#### 1.1.6.1 CMV-based vaccine strategies

CMV-based vectors are being developed towards two distinct vaccine strategies: (i) conventional vaccination involving direct vaccination of individuals, (ii) disseminating vaccination with spread of the vaccine within a host population following initial inoculation of a small number of individuals (Murphy, Redwood & Jarvis, 2016; Tsuda *et al.*, 2015).

##### 1.1.6.1.1. Conventional CMV-based vaccines

CMV-based vectors have been used in vaccine strategies to target infectious diseases such as Ebola virus (Tsuda *et al.*, 2011; Tsuda *et al.*, 2015) and simian immunodeficiency virus (SIV), which has been used as a non-human primate (NHP) model for HIV/AIDS. CMV-based vaccine vectors were shown to induce durable pathogen-specific CD8<sup>+</sup> T cells supporting clearance of the pathogen via cell-mediated effector mechanisms (Hansen *et al.*, 2011; Hansen *et al.*, 2013; Tsuda *et al.*, 2015). Another study showed the ability of CMV vector encoding tetanus toxin fragment C to induce sustained levels of protective tetanus toxin antibodies in mice (Tierney *et al.*, 2012). Interestingly, in an Ebola virus NHP challenge model, a RhCMV-based vector induced primarily antibodies against the GP target antigen, which corresponded to (L) expression kinetics in relation to the virus replication cycle (Marzi *et al.*, 2016). CMV-based vaccine vector have also been reported to protect against *Mycobacterium tuberculosis* (Beverley *et al.*, 2014).

The ability of CMV-based vectors to induce durable immune response represented by specific CD8<sup>+</sup> T and or CD4<sup>+</sup> T cells against particular antigens has also been exploited towards development of therapeutic vaccines against cancer (Klyushnenkova *et al.*, 2012; Qiu *et al.*, 2015; Xu *et al.*, 2013). Together, these results show that CMV is a promising vaccine when

used as a conventional vaccine (i.e., direct individual inoculation) to prevent infectious disease and tumors (where there may be potential for therapeutic efficacy).

Despite these promising results showing that ability of CMV- based vaccine vectors to induce durable protective immune responses against the particular pathogen being targeted, there are a number of safety issues regarding the use of fully replicating CMV vectors in humans. Although generally benign, HCMV is considered a pathogen under certain circumstances, primarily associated with immunosuppression (Fowler *et al.*, 1992; Patrone *et al.*, 2003; Selik, Chu & Ward, 1995). The fully replication defective versions of CMV appear to elicit immune responses that, although not at the same level as those seen with the fully replicating virus, can induce substantial levels of CD8<sup>+</sup> T cells (Snyder *et al.*, 2011). This suggests that attenuation strategies may be utilized that increase the safety profile of CMV without having a major negative impact on immunogenicity and efficacy of the vaccine.

#### 1.1.6.1.2. Non-conventional self-disseminating CMV-based Vaccines

Zoonotic diseases are an emerging infectious disease threat to global human health, representing 60.3% of emerging infectious diseases, 71% of which originate from wildlife (Jones *et al.*, 2008). Examples of zoonotic diseases include pandemic and epidemic influenza A, rabies, Lassa fever, Middle East respiratory syndrome (MERS) and Ebola. Disseminating vaccines are an innovative strategy aimed at interrupting transmission of these pathogens from animals to humans and confer several advantages over conventional vaccines based on individual inoculation (see above), especially in the inaccessible harsh environments where many of these pathogens emerge. According to this strategy, a small proportion of animals within a given population are vaccinated against the target pathogen by using a replicating vaccine vector which has the capability to infect these animals, replicate, re-infect and then be transmitted throughout the host population (Murphy, Redwood & Jarvis, 2016).



The ability of CMV to infect, reinfect and establish a persistent infection among a range of different host species regardless of any pre-existing immunity makes this virus suited towards its development as a self-disseminating vaccine platform (Boppana *et al.*, 1999; Boppana *et al.*, 2001; Farroway *et al.*, 2005; Hansen *et al.*, 2009). CMV is thought to transmit between individuals via mucosal exposure to bodily fluids such as saliva, urine, genital secretions and breast milk (Mocarski *et al.*, 2007). This high level of transmission is one reason why CMV is considered as a vector amenable for development for use in disseminating vaccine strategies in also species specificity and it has the capacity for re infection. A number of laboratories have produced early studies applying this strategy to target Sin Nombre Virus (SNV) in the deer mouse reservoir (Rizvanov *et al.*, 2006; Rizvanov *et al.*, 2003) and Ebola virus in the great ape amplification host (Murphy, Redwood & Jarvis, 2016; Tsuda *et al.*, 2011; Tsuda *et al.*, 2015). However, none of these studies have used CMV in a self-disseminating format, using only direct inoculation to date (Murphy, Redwood & Jarvis, 2016).

#### 1.1.6.2. BoHV-4 as a viral vaccine vector

BoHV-4 is similarly being developed as a vaccine platform. BoHV-4 based vaccines have been shown to be immunogenic, inducing both antibody and T cell responses against its heterologous antigen (Donofrio *et al.*, 2006; Donofrio *et al.*, 2007b; Macchi *et al.*, 2018). Several studies have also shown that the immunity induced by BoHV-4 vectors can be protective immunity against multiple pathogens: monkeypoxvirus (Franceschi *et al.*, 2015), caprine herpesvirus type 1 (Donofrio *et al.*, 2013) and Crimean Congo hemorrhagic fever virus (Farzani *et al.*, 2019). A more recent study demonstrated the ability of a BoHV-4 based vaccine expressing bovine viral diarrhea virus (BVDV) glycoprotein E2 and the bovine herpesvirus 1 (BoHV-1) glycoprotein D to induce immune responses in cattle that previously infected with the wild type BoHV-4 virus, this study showed that BoHV-4 can superinfect (Williams *et al.*,

2019). Studies detailed in Chapter 3 use an attenuated version of BoHV-4 to examine its ability to induce immune response against *Mb*- bovis fusion protein (heterologous antigen).

#### 1.1.7. Control of genetically modified organisms (GMOs)

Since the 1970s, scientists have had deep concerns regarding the containment and control of recombinant GMOs. Biosafety issues linked to GMOs have served as the focus of conferences such as the Gordon research conference on nucleic acids (1973) and the 1975 Asilomar conference on recombinant DNA molecules (Berg *et al.*, 1974; Berg *et al.*, 1975). The attendees of these conferences recognized the potential hazards associated with recombinant DNA research, agreeing that such work should only proceed under appropriate containment conditions and that initial standards of protection should be greater with a view to modifying procedures as methods of containment were improved. In addition, it was recommended that the Director of the National Institutes of Health (NIH) give consideration to the formation of an appropriate committee in order that suitable guidelines could be implemented to control such work.

##### 1.1.7.1 Intrinsic control mechanisms

Attenuation is defined as the reduction of the infectivity or virulence of pathogenic microorganism. Several approaches have been used to attenuate microorganisms.

##### 1.1.7.1.1 Intrinsic control of prokaryote-based GMOs

**Mutant auxotrophic:** Construction of mutant auxotrophic organisms by introducing a deletional mutation of central metabolic pathway genes has been successfully applied for the control of the growth and pathogenicity of many virulent bacteria for use in the context of safe vaccines *in vivo* (Jacobs *et al.*, 2000; Mohamed *et al.*, 2016). These strategies have also been

used to decrease concerns relating to their release into the environment from vaccinated individuals. These defective (auxotrophic) organisms require metabolites that are unavailable in mammalian tissues and therefore have a reduced ability to grow *in vivo*. They can grow only *in vitro* under certain conditions and are dependent on complementation of an auxotrophy or other gene defect by supplementation with either the intact gene or the essential metabolite (Crawford *et al.*, 1996; Drazek *et al.*, 1995; Hoiseth & Stocker, 1981).

**Deletion of central metabolic pathways genes:** These approaches target genes involved in central metabolic pathway functions.

**Mutants auxotrophic for aromatic amino acids:** Aromatic amino acids are an essential component for bacterial growth and survival (Felgner *et al.*, 2016). Bacteria, unlike mammals, synthesize all their aromatic metabolites (Tzin, Galili & Aharoni, 2001). Several studies have shown that deletional mutations created in genes encoding key enzymes in the aromatic biosynthetic pathway (such as *aro A*, *aro D* and *aro C*) block the ability of these organisms to synthesise their aromatic component and this renders the bacteria unable to propagate in the mammalian host.

In 1980, Hoiseth and Stocker reported that the *Salmonella typhimurium* *aro A* mutant strain was able to grow *in vitro* only in media containing the aromatic amino acid (phenylalanine, tryptophan, tyrosine, *p*-amino-benzoic acid and 2,3-dihydroxybenzoate), was highly attenuated *in vivo* with reduced virulence for animals, and was unable to overcome the blockade in aromatic biosynthesis. A *Salmonella typhimurium* *aro A* mutant strain was immunologically able to protect all animals when used as an attenuated vaccine (Hoiseth & Stocker, 1981). Similar to *Salmonella typhimurium*, many bacteria have been attenuated using the same strategy such as *Bordetella pertussis* (Roberts *et al.*, 1990), *Pseudomonas aeruginosa* (Priebe *et al.*, 2002) and *Aeromonas hydrophila* (Moral *et al.*, 1998).

**Purine and pyrimidine auxotrophs:** The importance of *de novo* purine or pyrimidine biosynthesis in bacterial growth and pathogenesis has been shown in many studies (Pechous *et al.*, 2006; Yoshioka & Newell, 2016). Disruption of genes involved in either the purine or pyrimidine nucleotide biosynthetic pathway by introducing a deletional mutation in these genes has been shown to affect the growth of the bacteria *in vivo* and *in vitro*. These auxotrophic bacteria can grow normally only in medium which has the complement material. This method has been used to attenuate many intracellular bacteria by deletion of genes involved in purine biosynthesis, such as *Brucella melitensis* (deletion of pur E gene) (Drazek *et al.*, 1995), *Mycobacterium tuberculosis* (deletion of pur C gene, pur L gene) (Crawford *et al.*, 1996) and *Francisella tularensis* (deletion of Pur MCD gene (Pechous *et al.*, 2006)). Recently, Yoshioka and Newell reported that the disruption of genes involved in the *de novo* purine biosynthesis pathways in *Pseudomonas fluorescens* that uses biofilms leads to reduced biofilm formation and reduction in cell size in biofilms (Yoshioka & Newell, 2016).

**Mutants auxotrophic for thymidine synthetase:** This method was used to control or prevent uncontrolled proliferation of *Lactococcus lactis* in the intestine, *Lactococcus lactis* was used as a delivery vector to deliver the anti-inflammatory cytokines IL-10. Deletion of the thymidylate synthase gene (thy A), which is essential for growth of *Lactococcus lactis*, was replaced by the IL-10 gene (Steidler *et al.*, 2003).

**Conditional suicide systems:** Conditional suicide systems have been used in bacteria as well as in simple eukaryotes (i.e., *Saccharomyces cerevisiae*). *E. coli* has been subjected to control using this approach and many studies have been performed using this organism (see review by Moe-Behrens *et al.*, 2013). In this method, the engineered organism has a DNA ‘suicide cassette’ which contains a toxin-encoding gene controlled by an inducible promoter. The engineered organism can survive normally until exposed to an inducer promoter signal (Moe-Behrens, Davis & Haynes, 2013). In 1987, Molin and colleagues created the first conditional

suicide system in bacteria (*E. coli*) by designing a DNA cassette that contains the *hok* gene, which encodes a polypeptide that exhibits a lethal effect in bacteria. Expression of *hok* was active in the absence of tryptophan. Their aim was to design a system to enable replication of the bacteria in the permissive (tryptophan containing) environment, but death of the bacteria following release to the restrictive (tryptophan deficient) environment, where the *hok* gene is expressed leading to killing of the bacteria (Molin *et al.*, 1987).

An ‘induced lethality’ approach is a related, but alternative conditional suicide system. In this method, the engineered organism has a DNA ‘suicide cassette’, which contains a toxin-encoding gene controlled by an inducible promoter, with the engineered organism surviving until addition to an inducer promoter signal. Bej *et al.* (1988) placed expression of the *hok* gene under control of the *lac* promoter, which is regulated by IPTG. In the presence of IPTG, the *lac* promoter is induced and the toxic gene is expressed, leading to death. This strategy has also been used for other toxic genes such as *relf* and *gef*. In a series of studies, Knudsen and colleagues using the *relf* gene under the *lac* promoter reported a very efficient suicide system to control *E.coli* which was tested in soil and seawater, as well as in rats (Knudsen *et al.*, 1995). The same system that was used in *E.coli* was also used to control *Pseudomonas putida*.

A potential concern with all of these systems is that cell death is associated with release of bacterial genetic material which may recombine with environmental organisms (horizontal gene transfer). Therefore, other researchers have used a nuclease suicide system, which combines controlled killing of cells with the destruction of genetic material before its release from cells. This limits both the survival of genetically modified organisms and the transfer of recombinant DNA to other organisms. This system was used by Balan and Schenberg (2005) in *Saccharomyces cerevisiae*, where the suicide system was dependent on the intracellular production of a *Serratia marcescens* nuclease which is coded by the *nuc A* gene. This product is normally secreted into the medium but when it is produced in yeast cells it causes destruction

of the genetic material and kills the cells (Balan & Schenberg, 2005). Additional studies have used a conditional succinate system but these are based on different inducers of the toxic genes, such as heat (Ahrenholtz, Lorenz & Wackernagel, 1994), sucrose (Recorbet *et al.*, 1993) and arabinose (Liu, Winkler & Biegalke, 2009). Although some studies indicate the efficiency of these systems to control the genetically modified organism, other studies have highlighted the problem of using conditional succinate system methods to control the release of the genetically modified organism, wherein inactivation of expression of lethal genes may occur due to genetic mutation. Moreover, these systems do not provide intrinsic control as they rely on addition of an inducer agent to kill the GMO.

**Synthetic auxotrophic control:** Recently, Rovner and his colleagues have developed a new and highly innovative approach that provides for intrinsic control. In this system, synthetic auxotrophs were constructed whose growth was restricted by being dependent on exogenously supplied synthetic amino acids. To do this, all the TAG codons (stop codons) and Release Factor 1 were deleted from the entire *E. coli* genome. TAG was then converted to a sense codon via the introduction of an orthogonal translation system which contained an aminoacyl-tRNA synthetase/tRNA pair. The viability of the synthetic auxotroph was then dependent on the presence of the synthetic amino acid, which is absent from the normal environment. In the presence of the synthetic amino acid functional bacterial proteins (many of which are essential for bacterial replication) will be produced enabling viability. However, in the absence of the synthetic amino acids (i.e., within the normal environment) essential proteins will be truncated as a result of the stop codon TAG (Rovner *et al.*, 2015).

#### 1.1.7.1.2 Intrinsic control of eukaryote-based GMOs.

Compared to prokaryotic systems, intrinsic control strategies of eukaryotic organisms including eukaryotic viruses are far less developed.

**Attenuation by non-targeted genetic attenuation by passage:** By repeatedly passaging a virus in a foreign host such as cultured cells, embryonated eggs or a live animal host, viruses accumulate mutations that mediate attenuation. This approach has been historically used to attenuate many viruses such as measles, mumps, rubella (MMR), oral poliovirus vaccine (OPV), influenza, rotavirus, yellow fever and varicella. However, due to the inherent inability to ascribe attenuation to defined mutations, these strategies are presently being superseded by more targeted genetic mutation attenuation strategies (Lauring, Jones & Andino, 2010).

**Attenuation by targeted deletion of essential genes:** To date, mechanisms for Ad control are the most well developed, resulting in a replication-defective vector that has proved its safety in multiple animal experimental systems as well as numerous human clinical vaccine trials. This strategy targets an essential protein, E1, involved in virus replication (as well as non-essential immune evasion molecule, E3, in more recent vectors). Propagation of replication-defective Ad (i.e. with the E1 gene deleted) in cells that are able to supply E1 in *trans* is then used to produce vaccine stocks. An inherent problem with this approach has been contamination by a replicative component adenovirus (RCA) which is analogous to the WT virus; this presents a problem when the aim is to grow the replicative-defective virus for gene therapy applications. The replication-competent RCA problem was due to a double homologous recombination event between the defective vector and the E1 gene region that integrated in the HEK 293 (helper cells). This common issue has subsequently been circumvented by using an alternative cell line such as PER C6 that contains no region of homology between the E1 deleted virus and the helper cell line, thereby preventing homologous recombination (Fallaux *et al.*, 1998).

Herpesvirus attenuation systems have paralleled those approaches used for Ad, with essential genes required for all stages of the virus replication cycle being amenable to targeting. The gH glycoprotein is essential for entry/fusion of the virus during initial stages of infection. Boursnell *et al.* (1997) reported that a HSV mutant with a deleted glycoprotein H gene, termed a DISC

(disabled infectious single cycle) virus, is able to replicate for only one replication cycle and the resulting progeny are non-infectious and unable to establish a second replicative cycle (Farrell *et al.*, 1994). Several proteins required for viral DNA synthesis such as HSV UL5 and UL29 (ICP8) have also been targeted for deletion (Weller & Coen, 2012). Deletion of these two genes together from HSV was shown to result in a completely defective virus.

Uracil DNA glycosylase catalyses the removal of uracil residues from ds DNA, and is also essential for viral replication. In vaccinia virus (a large DNA poxvirus), deletion of uracil DNA glycosylase, an essential gene encoding an enzyme involved in removal of uracil residues from dsDNA, led to the virus becoming replication incompetent but still allowed infection and expression of early genes (Holzer & Falkner, 1997).

**Attenuation based on zinc finger nuclease targeting:** Zinc finger (ZF) domains mediate binding of proteins to specific nucleotide sequences of DNA (Iuchi, 2001). Several studies have used artificial zinc finger proteins to control eukaryotic viral replication (Dhanasekaran, Negi & Sugiura, 2006; Papworth *et al.*, 2003). Papworth and colleagues created zinc finger proteins fused to the KOX-1 transcription repression domain (ZF-KOX) to target the HSV-1 IE175k (ICP4) promoter. They chose the IE gene promoter in order to control replication of the virus at very early stages of the virus replication cycle. This approach was able to limit HSV replication in the infected cells and reduced the viral titre by 90% (Papworth *et al.*, 2003). In another study, two types of zinc fingers proteins were designed to target the human papilloma virus (HPV) origin of replication. Zinc fingers were designed to bind two regions within the replication origin of HPV resulting in inhibition of virus DNA replication (Mino *et al.*, 2006). Mino and his group subsequently fused a restriction enzyme nuclease to the ZF targeting domain, which was then able to cleave HPV DNA at the site of replication origin and reduce viral replication in culture cells (Mino *et al.*, 2008). Modifications of this strategy have been



used with similar results by other laboratories (Lauring, Jones & Andino, 2010; Wayengera, 2011).

### **Attenuation by codon deoptimization**

The importance of codon bias and codon pair bias for viral replication and pathogenesis has been studied for many viruses. In polioviruses (PV) Type 2 (Sabin) replacement of natural codons in the capsid gene with synonymous codons had an attenuating effect on the viral replication and infectivity (Burns *et al.*, 2006). These results were similar to earlier studies, wherein codon deoptimization of PV Type 1 (Mahoney) showed a similar marked attenuation (Mueller *et al.*, 2006). Increasing the frequency of codons in the PV capsid showed the same effect on viral fitness (Burns *et al.*, 2009). The mechanism of attenuation is proposed to result from a reduction in translational efficiency for the deoptimized virus (Burns *et al.*, 2009; Coleman *et al.*, 2008; Mueller *et al.*, 2006). Recently, codon deoptimization and codon pair strategy have been used to attenuate additional RNA viruses, such as influenza A virus (Nogales *et al.*, 2014), lymphocytic choriomeningitis virus (Cheng *et al.*, 2015), human respiratory syncytial virus (Le Nouën *et al.*, 2014) and dengue virus (Simmonds *et al.*, 2015).

### **Attenuation strategy based on utilizing host proteins involved in intrinsic immunity**

Nuclear ND10 bodies are host proteins that are involved in cellular intrinsic defence mechanisms (intrinsic immunity). These proteins can control the replication of CMV through repression of transcription of CMV immediate-early genes (IE), which are required for expression of the early gene (E), late gene (L) and replication of the viral genome (Everett *et al.*, 2006; Tang & Maul, 2003).

A number of studies have shown that HCMV tegument protein pp71 (coded by UL82) mediates the degradation of DAXX, which is one of the components of ND10 bodies protein (Preston & Nicholl, 2006; Saffert & Kalejta, 2006; Tavalai *et al.*, 2006). Absence of pp71 has

shown the ability to prevent DAXX host protein degradation, thus facilitating the control of CMV by host intrinsic immunity (repression of viral IE gene expression). Repression of expression of IE by host intrinsic immunity may enable establishment and maintenance of viral latency through silencing viral genes that are required for lytic replication (Penkert & Kalejta, 2012; Qin, Penkert & Kalejta, 2013; Tsai *et al.*, 2011). Recently, deletion of Rh110, an ortholog of HCMV UL82, from a RhCMV vector (strain 68-1) expressing SIV antigens resulted in a spread defective attenuated vector that was not shed in urine and was unable to spread from animal to animal (Marshall *et al.*, 2019). Importantly, the quality and quantity of the T cell immune response induced by the attenuated virus was identical to the wild type virus vector (Hansen *et al.*, 2019).

### **Attenuation strategy based on destabilization of essential viral protein**

This strategy has been used to conditionally control the replication of many microorganisms including viruses such as CMV (Dvorin *et al.*, 2010; Herm-Götz *et al.*, 2007; Muralidharan *et al.*, 2011; Russo *et al.*, 2009; Wang *et al.*, 2016). It is based on genetic fusion of a protein destabilization domain to proteins required for viral replication. The presence of the destabilization domain causes degradation of essential proteins through targeting to the proteasome. The essential protein is stabilized through the binding of a specific ligand, whereas, in the absence of ligand, the destabilized protein, including the product of the essential gene, is degraded (Banaszynski *et al.*, 2006). Currently, two destabilization domain (DD) systems are in use. The first system is based on the fusion of rapamycin-binding protein (FKBP) to viral genes such as pUL77 and pUL5, which are essential for the cleavage and packaging of the CMV genome (Borst *et al.*, 2013; Glaß *et al.*, 2009). This system has been used for CMV control through fusion to pUL48, a protein involved in intracellular capsid

transport, pUL94, a protein essential for cytoplasmic virus egress (Das *et al.*, 2014), IE1/IE2 and IE1/IE3, multifunctional transcriptional regulators (Glaß *et al.*, 2009). The stability of these viral proteins was dependent on the presence of synthetic ligands called Shield-1. Shield-1 binds to the FKBP destabilization domain and prevents degradation of the essential protein by the cellular proteasome (Banaszynski *et al.*, 2006). Wang *et al.* (2016) reported the efficiency of this system in control of HCMV replication (Wang *et al.*, 2016), which was subsequently moved into a Phase I clinical trial (Adler *et al.*, 2019). The second DD system is based on fusion of the *E. coli* dihydrofolate reductase (ecDHFR) using the stabilizing ligand trimethoprim, (TMP) (Iwamoto *et al.*, 2010). This system was used to control varicella zoster virus by fusion of the ecDHFR to ORF63, a gene required for virus replication (Mahalingam & Gildea, 2014).

#### 1.1.7.1.3 Intrinsic control of eukaryotic viruses

The release of recombinant replicating GMO vaccine vectors especially those designed to disseminate within the target population such as self-disseminating vaccine platforms raise obvious concerns regarding their possible environmental impact. Environmental impact includes the possibility for increased virulence induced by recombination of the vector with endogenous viruses. Risk level will depend on multiple factors, including the level of infection of the target populations by the WT version of the vector used in the platform as well as the level of pathogenesis associated with infection. However, for purposes of ensuring redundancy of safety and the small but present risk of unpredictable ‘black swan’ events when a GMO virus is transmitted and shed into human and animal populations, it is critical to begin to consider methods for robust intrinsic control sooner rather than later (Fisher, 2014; Myhr & Traavik, 2011; Skenderi & Jonjić, 2012).

In the case of CMV, such concerns have been countered by arguments citing its ubiquity of benign infection and host specificity, although further extensive study will be required before CMV-based vaccines can be applied to solving any real world problem (Hoenen, Groseth & Feldmann, 2012). The inherent requirement for a self-disseminating vaccine to move, at least for a period of time, through its host population limits the application of many of the strategies that have historically been used for virus attenuation (discussed above). A partial attenuation strategy such as codon deoptimization strategy would be a possibility, although the level of persistence of such a codon de-optimized version of a virus on a scale that would be required for attenuation of a virus as large as CMV may be problematic.

In order for the full potential of self-disseminating vaccines to stand any chance of being realized, it is critical that intrinsic attenuation methods able to provide regulatory agencies with the necessary assurance of safety be developed in parallel. In this project, we explore two distinct means of such control: one based on DNA virus fidelity and the other based primarily on the propensity of homologous regions of DNA to undergo recombination.

#### **Attenuation strategy based on modulation of viral replication fidelity.**

Modulation of fidelity is one of two innovative intrinsic control strategies that we are investigating. This is a novel approach that is based heavily in the RNA virus literature, wherein increasing mutation rate can result in genomic catastrophe. Before discussion of the approach as applied to CMV, a brief synopsis of the background of fidelity control and the impact of loss of control on virus fitness will be discussed.

**RNA virus fidelity:** In RNA viruses several studies report that the excessive accumulation of mutations in viral genome leads to viral death. This phenomenon, called ‘error catastrophe’, is defined by Crotty as ‘the phenomenon that occurs when the loss of genetic fidelity results in a lethal accumulation of error’ (Beaucourt *et al.*, 2011; Crotty, Cameron & Andino, 2001).

In 2001, Crotty *et al.* (2001) empirically studied this phenomenon in PV by using ribavirin as a mutagen. This study found that increasing the concentration of ribavirin led to an increase in mutation frequency (high genetic variability) which led to a decrease in the viability of the virus (Beaucourt *et al.*, 2011; Crotty, Cameron & Andino, 2001). Crotty demonstrated that high genetic variability, normally a major advantage for RNA viruses, can be used against the virus by increasing the genetic variability (the mutation rate) beyond tolerable levels and resulting in a 'genetic meltdown'. In addition to PV (Graci *et al.*, 2007), this phenomenon has been studied in many other RNA viruses where viral polymerases normally lack proof reading activity, such as hantavirus (Severson *et al.*, 2003), foot-and-mouth disease virus (Sierra *et al.*, 2000) and lymphocytic choriomeningitis (Grande-Perez *et al.*, 2005). Severson and his co-workers reported that when hantavirus was grown in the presence of ribavirin, the mutation frequency for viral RNA was higher than the mutation frequency of the virus that grew in the absence of ribavirin and also reported a substantial negative effect of ribavirin on the virus infectivity (Severson *et al.*, 2003). Another study found that growing foot-and-mouth disease virus in the presence of mutagenic base analogue 5-fluorouracil or 5-azacytidine resulted in a decrease of infectivity of the virus compared with virus grown in the absence of the mutagen, and they reported that the difference was noted in a single as well as multiple replication cycle (Sierra *et al.*, 2000).

#### 1.1.7.1.3.1 Intrinsic control strategy for CMV

In general, RNA viruses are characterised by an extremely high mutation rate compared to DNA viruses – the range of mutation rate is from  $10^{-8}$  to  $10^{-6}$  for DNA viruses and from  $10^{-6}$  to  $10^{-4}$  for RNA viruses (Sanjuan *et al.*, 2010). An inverse relationship was reported between the genome size and the mutation rate (Gago *et al.*, 2009), which was noticed also within the RNA virus group members. Belshaw *et al.* (2008) described that high mutation rate appears to restrict

RNA viruses to a small genome (describing RNA viruses as restless beasts pacing a small cage) (Belshaw *et al.*, 2008). The high mutation rate of these RNA viruses being a result of their polymerase lacking proof-reading activity. Belshaw *et al.*, (2008) reported that RNA viruses with larger genomes tend to have relatively larger polymerase, which they thought might reflect a higher copying fidelity. In agreement with this idea, coronaviruses with their large RNA genomes are the only RNA viruses that have proof-reading as their RNA-dependent RNA polymerase encodes for a 3'-5' exoribonuclease activity (Denison *et al.*, 2011).

DNA polymerase is an essential enzyme for DNA replication and contains two functional regions: a polymerization region which is involved in DNA synthesis and an exonuclease region which is involved in proof-reading activity. CMV DNA polymerase is highly conserved, with the HCMV DNA polymerase exonuclease region involved in proof-reading comprising three domains: I, II and III. However, to date our clearest understanding of the function of DNA polymerase comes not from CMV, but from HSV and Ad studies. Mutation of the exonuclease region of DNA polymerase from HSV (Hwang *et al.*, 1997b) and Ad (Uil *et al.*, 2011) was reported to have an effect on the replication fidelity of these viruses. For Ad, mutation of a critical residue within polymerase domain III had a modest mutator activity. This mutation was located in a region responsible for binding incoming dNTP, and is believed to have an effect on the selection of incoming nucleotides (Biles & Connolly, 2004; Uil *et al.*, 2011). Other studies using Ad revealed a functional interdependence of polymerization and exonuclease activity. Mutation of conserved exonuclease residues contributing to the metal ion binding in the exonuclease active site also affected the DNA replication function of these mutant viruses. This impact on DNA replication function of the enzyme is believed to result from these residues also being involved in strand displacement activity which is important for DNA polymerization (Esteban *et al.*, 1994; Soengas *et al.*, 1992; Uil *et al.*, 2011).

In HSV, conserved residues involved in exonuclease activity can, in many cases, be altered without affecting polymerase replication function. These genetic and biochemical studies have shown that HSV mutants encoding genetically modified DNA polymerase molecules have altered replication fidelity of the viral HSV DNA genome (Hall *et al.*, 1984; Hall *et al.*, 1985). A number of exonuclease mutations have been shown to lower fidelity as they were associated with an accumulation of mutations relative to the WT virus (Hwang *et al.*, 1997b; Kühn & Knopf, 1996). Song *et al.* (2004) found that an exo I mutant retained the WT level of activity of the polymerase and had the ability to extend from a mismatched base (as opposed to be caught in a futile cycling between incorporation and excision). Mutation of exonuclease domain III residues also altered the mutational spectra, decreasing the frequency of frame-shift mutations (Lu, Hwang & Hwang, 2002).

Loss of fidelity, is not always the outcome. Using a TK mutagenesis assay, viruses with a mutation within a conserved region of polymerase domain called PAA<sup>r5</sup> (Gibbs *et al.*, 1988) were found to have an anti-mutator phenotype (Hall *et al.*, 1984; Hall *et al.*, 1985; Hwang & Chen, 1995). Screening of several TK different mutants showed that WT polymerase generated TK mutants resulting from both base substitutions and frameshift mutation in an equal ratio. In contrast, the PAA<sup>r5</sup> mutant polymerase generated only frameshift changes. These results suggest that the WT DNA polymerase is less faithful than the anti-mutator PAA<sup>r5</sup> mutant polymerase. The PAA<sup>r5</sup> mutant polymerase has higher  $K_m$  of dNTPs than the WT molecule indicating that the mutant polymerase may have better selectivity of the incoming NTPs (Hall *et al.*, 1985; Huang *et al.*, 1999). Structural study of HSV polymerase based on the structure of RB69 polymerase proposed that the arginine residues 842 is located in a position which is close to the sugar of the dNTP. They suggested that mutation of arginine to serine in PAA<sup>r5</sup> mutant polymerase might affect the interaction between the PAA<sup>r5</sup> mutant polymerase and dNTP. Other studies using a mutant polymerase (L774F) with a mutation in a conserved region of

polymerase domain (VI) also had an anti-mutator phenotype (Hwang *et al.*, 2004). This study reported that the L774F mutant had altered sensitivity to the antiviral drug as increased resistant to Foscarnet. They also reported that the conserved VI region also coordinated with other regions within the polymerase domain to interact with incoming dNTP (Hwang *et al.*, 2004). Exonuclease domain III region is a conserved region of exonuclease function between both HSV and CMV. By mapping the functional regions of polymerization and exonuclease for these two viruses (Figure 1.5), the conserved  $\gamma$  region C of polymerization region overlaps with the Exo III region of exonucleases and conserved region IV of polymerase overlaps with the Exo II of the region of exonuclease (Hwang, 2011; Lurain & Chou, 2010). In 2008, Chou and Marousek observed the ability of the recombinant HCMV UL54 (HCMV UL54(D413A)) to replicate with mild attenuation, which was surprising as mutation in comparable residue in HSV D417A resulted in a replication-defective virus (Chou & Marousek, 2008).

HCMVUL54(D413A), virus was able to replicate at least until passage 15. After several passages, plaque sequencing revealed loss of this mutation and reversion to the WT in 37.5% of the eight plaques analyzed. Preliminary data since generated by Dr. Chou (OHSU) regarding genetic modification of HCMV, has shown that a mutation in an exonuclease region of DNA polymerase, which deletes aspartic acid residues codon 413 from HCMV gene (HCMV UL54( $\Delta$ 413)), may disrupt proof-reading activity and result in a mildly attenuated virus that displays increased resistance to the CMV antiviral drug maribavir – observations comparable to those observed in Dr. Chou's previous 2008 study. An increased rate of acquisition of resistance to CMV drug inhibitors indicates that HCMVUL54( $\Delta$ 413) may have an increased mutation rate consistent with a low fidelity phenotype. Importantly, HCMVUL54( $\Delta$ 413) appears to be genetically stable in terms of the  $\Delta$ 413 aspartic acid mutation, enabling this phenotype to be studied without reversion to WT.



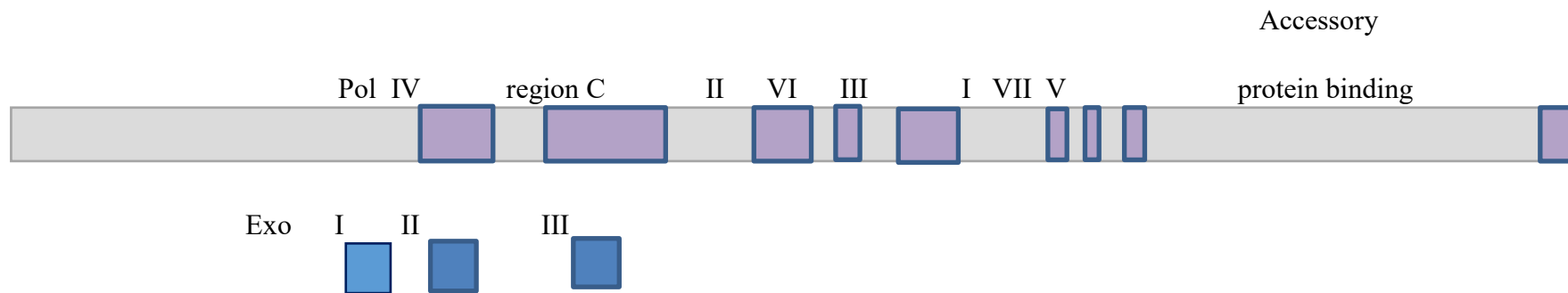


Figure 1.5: Schematic diagram showing the overlapping regions in herpesvirus DNA polymerase, polymerase region IV overlaps with the exonuclease domain II, polymerase region C overlaps with the exonuclease domain III. Polymerase domains (Purple) are IV, region C, II, VI, III, I, VII, V and exonuclease domains (Blue) are exo I, exo II, exo III (Lurain and Chou, 2010; Hwang *et al*, 2011).

## **Control strategy based on stability of the transgene**

Stability of the transgene within viral genome can affect virus replication. Previous studies showed that flanking the transgene by two identical sequences (a repeated flanking sequence) destabilizes the transgene. Rather than targeting the entire virus for deletion (i.e., through genomic catastrophe as described above), the antigenic transgene itself can be targeted for instability, with its loss from the virus resulting in generation of the WT virus already circulating in the population. The excision could be kinetically controlled depending on the size of the flanking repeated sequence. To achieve such destabilization of the target antigen transgene, we have adopted a strategy that is commonly used for marker removal from transgenic plants. Genetic transformation technology has been used to improvement of many crops. To efficiently distinguish between transformed and non-transformed cells, selectable marker genes such as antibiotic or herbicide resistant gene are used in plant transformation techniques (Bevan, Flavell & Chilton, 1983). However, the presence of these markers in transgenic plants rise concern regarding their possible impact on food safety and environment (Kwit *et al.*, 2011; Mason *et al.*, 2003; Miki & McHugh, 2004). Specifically, use of flanking repeated sequences has been used to effect targeted removal. This removal of transgene flanked by a repeated sequences, such as antibiotic resistant gene from genetically modified plants has been reported via intrachromosomal homologous recombination (ICR) (Zubko, Scutt & Meyer, 2000). Wagner and his colleagues also reported the excision of a bacterial artificial chromosome (BAC) from the recombinant genome of mouse CMV (MCMV) during viral reconstitution and the subsequent generation of a WT genome, as the BAC was flanked with two short identical viral sequences. The BAC vector sequence was gradually lost over several passages of the virus (Wagner, Koszinowski & Messerle, 1999).

In plants, the efficiency of the excision/recombination appears to depend on the size of the repeated sequence which flanks the transgene (Puchta & Hohn, 1991). For example, in tobacco

excision was not observed when the length of this sequence was 137 bases (Zoubenko *et al.*, 1994), while at 418 bases, excision occurred at a low frequency (Iamtham & Day, 2000). In *Chlamydomonas reinhardtii*, excision was not observed when the repeat length was reduced to 230 bases, while the 483 bp and 832 bp repeats allowed rapid excision of the transgene (Fischer *et al.*, 1996). We hypothesise that the flanking region will destabilize the target antigen within the herpesvirus vector leading to its excision from the recombinant virus and subsequent restoration of the WT virus after several replication cycles. Importantly, by losing the transgene, the GMO will 'drop down' to the WT status. For these studies we will engineer the genome of MCMV by using the LacZ gene as the transgene (Novak *et al.*, 2000). In this fashion, by using different lengths of repeated sequence to flank the LacZ gene we will be able to visually detect the impact of different lengths of identical repeating regions on maintenance of transgene. This could be the simplest and most elegant way to employ kinetic control to overcome the concerns relating to the use of recombinant virus vector by removing the transgene and restoring the WT virus. Ultimately, this may allow the transgene to be expressed for a kinetically defined period of time, after which the GMO loses the transgene, becoming WT in sequence.

#### 1.1.7.1.3.2 Intrinsic control strategy for BoHV-4

##### 1.1.7.1.3.2.1 Attenuation strategy based on deletion of thymidine kinase (TK) gene

The role of thymidine kinase in viral replication has been studied in many viruses including herpesviruses such as herpes simplex virus-1, herpes simplex virus-2, pseudorabies virus, bovine herpesvirus-1, bovine herpesvirus- 4 and bovine herpesvirus-5. Thymidine kinase is an enzyme that is involved in the metabolism of deoxyribonucleotides (dNTPs), and is required for viral replication in non-dividing cells. The enzyme catalyzes the phosphorylation of thymidine resulting in deoxy-thymidine monophosphate (dTMP), which is used during viral

DNA replication as substrate for the DNA polymerase. Importantly, this activity of TK is believed to be required for reactivation and growth of latent virus in non-dividing cells (Kit & Kit, 1987; Kit *et al.*, 1986; Smith, Young & Mattick, 1991).

Amino acid sequence analysis of different herpesviruses showed identity and similarity in many regions in the enzyme such as the active site and thymidine binding sites (Lomonte *et al.*, 1992). Similarly, several studies have also shown that mutation or deletion of TK from a variety of different herpesviruses affects viral persistence *in vivo*. Deletion of TK from HSV resulted in a decrease of the capacity of the recombinant virus to replicate in the trigeminal ganglia (the site for viral latency) (Chen *et al.*, 2004; Coen *et al.*, 1989; Katz, Bodin & Coen, 1990; Price & Khan, 1981; Tenser & Edris, 1987). It was also noted that mutation of TK in pseudorabies virus was associated with reduction of viral replication in non-dividing cells (Ferrari *et al.*, 2000; Moormann *et al.*, 1990). Similar findings were observed in rabbits inoculated with a TK-deleted strain of BoHV-5 (da Silva *et al.*, 2011; Silva *et al.*, 2010). Deletion of TK from BoHV-1 resulted in an attenuated virus which is unable to replicate in non-dividing cells (Kit & Kit, 1987).

TK deletion has been used to attenuate BoHV-4, wherein Donofrio and his group reported that BoHV-4 deleted TK attenuated *in vivo* (Donofrio *et al.*, 2009; Donofrio *et al.*, 2013; Donofrio *et al.*, 2007b; Franceschi *et al.*, 2015; Macchi *et al.*, 2018; Rosamilia *et al.*, 2016). The TK deleted BoHV-4 version was immunogenic, and induced humoral and cellular immune responses against several pathogens. One study showed that BoHV-4  $\Delta$  TK expressing caprine herpesvirus type 1 glycoprotein D (CpHV-1-gD) protected goats against CpHV-1 virus. In this study, it was indicated that the attenuated  $\Delta$  TK BoHV-4 vaccine had level of safety *in vivo*, with no shedding or spread of the virus from vaccinated animals (Donofrio *et al.*, 2013).

#### 1.1.7.1.3.2.2 Attenuation strategy based on deletion of gene coding for genome maintenance protein (GMP)

The importance of the GMP (ORF73) protein for viral latency has been studied in many gamma herpesviruses (see above). In murine herpesvirus 68 (MHV68) deletion of ORF 73 had an attenuating effect on viral replication *in vivo* (Fowler & Efstathiou, 2004; Fowler *et al.*, 2003). Fowler *et al* (2003) reported that MHV68  $\Delta$ ORF73 virus cannot persist *in vivo*: the viral genome could not be detected in the splenocytes of mouse infected with MHV68  $\Delta$ ORF73 virus at day 14 after infection while the genome of the wild type MHV68 virus was detected in the infected animals (Fowler *et al.*, 2003). The result of this study was similar to another recent study, wherein replacing a region containing ORF73 of MHV68 by viral transcription activator (RTA) showed a similar attenuation (Jia *et al.*, 2010). A number of studies have also shown that deletion of the ORF 73 region from alcelaphine herpesvirus 1 (AIHV-1) virus affects the capability of the virus to persist *in vivo* (Cunha *et al.*, 2016; Palmeira *et al.*, 2013).

Like other attenuated gamma herpesvirus, BoHV-4  $\Delta$ ORF73 virus is incapable of establishing a latent stage of infection. A previous study showed that the ORF73 protein is required for viral latency. A lack of ORF73 rendered BoHV-4 unable to persist *in vivo* but it did not impair viral replication *in vitro* (Thirion *et al.*, 2010). Persistence of BoHV-4 in monocytes and macrophages (antigen presenting cells) was reported as one of the characteristics that make BoHV-4 an attractive vaccine vector. However, we consider attenuation of the virus as a further characteristic for development of a vaccine vector (Donofrio *et al.*, 2006a; Dubuisson *et al.*, 1989; Egyed & Bartha, 1998; Naeem *et al.*, 1993; Osorio & Reed, 1983). In addition to safety, the immunogenicity of the attenuated viral vector is an important point that should be taken into account during developing vaccine vector. Thirion *et al.*, 2010 reported that the immune response of the attenuated version of BoHV-4 (BoHV-4  $\Delta$ ORF73) against its endogenous

antigen was comparable to the immune response induced by WT BoHV-4. To date, no studies have investigated the ability of the attenuated BoHV-4  $\Delta$ ORF73 vector to induce immune responses against a heterologous antigen.

## Aims and objectives

This thesis focuses on intrinsic attenuation strategies for herpesviruses vaccine vectors. The long-term aim of this project is to provide a means to control the genetically modified recombinant herpesviruses, either by controlling the persistence of the recombinant viruses or by enabling a kinetically controlled reversion of the recombinant back to the WT 'ground-state'. Success in one of these methods may lead to a safe and effective strategy to attenuate the herpesvirus vaccine vector platform for use either as a conventional or self-disseminating vaccine.

Aim 1. To test the ability of conditionally-attenuated bovine herpesvirus 4 (BoHV-4  $\Delta$ ORF73) vaccine vector to induce immune responses against an heterologous antigen, we constructed a BoHV-4  $\Delta$ ORF73 expressing a *Mycobacterium bovis* (*Mb*) fusion antigen.

Aim 2. To test the hypothesis that the  $\Delta$ 413 mutation in HCMV gene UL54 will result in a decrease in fidelity of the virus leading to the accumulation of other mutations. To achieve this aim, we established a genetic amplification, cloning and sequencing methodology to measure virus mutation rate. We then used this analysis to compare mutation rates of HCMV UL54( $\Delta$ 413) with the WT virus to determine whether there was an increase in mutation rate consistent with a low DNA polymerase fidelity.

Aim 3. To test the hypothesis that the positioning of a transgene between two identical flanking sequences can lead to excision of the transgene from the recombinant virus, resulting in restoration of the WT (non-GMO) virus. To achieve this aim we constructed a panel of genetically modified recombinant MCMV vectors expressing the beta-galactosidase (*LacZ*) gene as a target transgene flanked by different sized repeat regions.

## Chapter 2 Materials and methods

### 2.1 Bacteriology methods

#### 2.1.1 Bacteria and plasmids

Bacterial artificial chromosomes (BACs) and plasmids used in this study were maintained in *Escherichia coli* (*E. coli*) strains. The list of bacterial cells and plasmids are shown in Table 2.1. The EL250 *E. coli* strain was supplied by Dr. Donald L. Court (Centre of Cancer Research, National Cancer Institute Frederick, MD, USA). EL250 expresses E/T recombinases *exo*, *bet*, and *gam* genes under control of the temperature-sensitive repressor, *cI857*. Site-directed mutagenesis of BAC genomes requires a linear recombination fragment with terminal regions homologous to the site being targeted for mutagenesis within the BAC. Recombinants are selected on the basis of a selectable marker, which is flanked by *flpe* recombinase recognition sites (*frt* sites) (see below). The *exo* gene encodes a 5' to 3' exonuclease, which generates 3' overhangs within the terminal regions of homology of the recombination fragment. By binding to these 3' overhangs, the *bet* gene encodes ss-DNA binding proteins that promote recombination between the linear dsDNA and the target region within the BAC. The *gam* gene product prevents degradation of the recombinant fragments. The recombination system is induced by incubation at 42°C, which inactivates the *cI857* repressor controlling expression of the recombination proteins (at 32°C *cI857* is activated leading to repression of the recombination system). EL250 also contain an arabinose-inducible *flpe* gene. This gene is induced in the presence of arabinose leading to expression of *flpe* recombinase, which mediates recombination between two *frt* sites for removal of the selectable marker from recombinants.

Luria-Bertani (LB medium) or LB agar plates (Fisher Scientific, Leicestershire, UK) were used for growing *E. coli* strains. Medium was prepared (Appendix 1) and sterilized by autoclaving for 20 minutes at 121°C, pressure 15 lbs/in<sup>2</sup>. After cooling to 55°C, medium was mixed with the appropriate antibiotics. Inoculated LB plates were incubated inverted at 30°C in a standard



incubator (VWR INCU-Line-IL53, Leuven, Belgium). LB broth cultures were incubated overnight in a 30°C incubator with constant shaking at 230 rpm using orbital incubator (VWR, West Sussex, UK). Incubation of the EL250 bacteria at 30°C was critical as growth at >32°C could induce the E/T recombination system leading to genetic instability of the BAC genome.

Table 2.1: Bacterial strains, BACs and plasmids used in this research. The BACs contain Chloramphenicol (Cam) while the plasmids contain Kanamycin (Kan) or Carbenicillin (Carb) as a selectable marker.

Bacterial strains, BACs and plasmids	Antibiotic Selection	Growth Temp (°C)	Source or Reference
One shot <sup>TM</sup> TOP10 cells chemically competent <i>E. coli</i>	None	37	Invitrogen <sup>TM</sup>
pCR <sup>TM</sup> 4Blunt-TOPO® vector	Kan 50 µg/ml	37	Invitrogen <sup>TM</sup>
pCR <sup>TM</sup> Blunt II TOPO vector	Kan 50 µg/ml	37	Invitrogen <sup>TM</sup>
EL250	None	30	Dr. Donald L. CourtCentre of Cancer Research, National Cancer Institute Frederick, USA
BoHV-4 (BoHV-4 v.test G) BAC	Cam 17 µg/ml	30	Dr. Alain Vanderplasschen Laboratory of Immunology –Vaccinology, University of Liège, Belgium (Gillet <i>et al.</i> , 2005)
One Shot <sup>TM</sup> PIR1 Chemically Competent <i>E. coli</i>	None	30	Invitrogen <sup>TM</sup>
pCMV/EBOV-GP plasmid	Kan 50 µg/ml		Jarvis lab
pORF73Flank plasmid	Carb 100 µg/ml	30	GeneArt- Invitrogen
pWTF flank plasmid	Carb 100 µg/ml	30	GeneArt- Invitrogen
pMb-Fusion plasmid	Carb 100 µg/ml	30	GeneArt- Invitrogen
pRVFV-GN plasmid	Carb 100 µg/ml	30	GeneArt- Invitrogen
pCB-PIHspB1 plasmid	Carb 100 µg/ml	30	GeneArt- Invitrogen
SnaB1_C1_SInt_PvuII plasmid	Carb 100 µg/ml	30	GeneArt- Invitrogen
pCB-Com1 plasmid	Carb 100 µg/ml	30	GeneArt- Invitrogen
pCB-Mip plasmid	Carb 100 µg/ml	30	GeneArt- Invitrogen
MCMV BAC (pARK14)	Cam 17 µg/ml	30	Dr. Alec Redwood University of Western Australia
pEF1α/EBOV-GP plasmid	Kan 50 µg/ml	30	Jarvis Laboratory
Flanking region 250 nt plasmid	Carb 100 µg/ml	30	GeneArt- Invitrogen
Flanking region 25 nt plasmid	Carb 100 µg/ml	30	GeneArt- Invitrogen
Flanking region Zero nt plasmid	Carb 100 µg/ml	30	GeneArt- Invitrogen
pEQ176, Addgene plasmid 83943 (β-gal)	Carb 100 µg/ml	30	Addgene (Schleiss, Degnin & Geballe, 1991)

### 2.1.2 Storage of bacterial cultures

For short-term storage, plates of bacterial culture were kept in the fridge (4°C) after being wrapped in parafilm. For long-term storage, a single colony was inoculated into LB broth containing appropriate antibiotics. A volume of 750 µl of overnight culture was mixed with the same volume of 80% sterile glycerol (Fisher Scientific, Leicestershire, UK) and stored at -80°C. Stored bacterial cells were revived by scraping off an inoculum of the frozen cells followed by culturing in LB broth containing appropriate antibiotics.

### 2.1.3 Electroporation of herpesvirus BACs into EL250

BACs containing the herpesvirus genome (BoHV-4 or MCMV) were supplied in DH10B bacterial strains. BAC DNA was isolated from DH10B using the alkaline lysis method (Sambrook & Russell, 2001) and then electroporated into EL250 (see below).

#### 2.1.3.1 Preparation of electrocompetent bacteria (EL250)

EL250 were cultured in 3 ml of LB broth without antibiotic at 30°C overnight. One ml of the overnight culture was then added to 50 ml LB broth without antibiotics followed by incubation at 30°C for an additional 4 hours. Cells were harvested by transferring bacterial cultures into pre-chilled centrifuge bottles and centrifugation at 7,000 rpm for 15 minutes in a pre-cooled (4°C) centrifuge (Beckman Coulter, USA). Supernatant was removed and the bacterial pellet was washed by resuspension in 250 ml of 10% glycerol. Following centrifugation at 7,000 rpm for 15 minutes at 4°C, the supernatant was removed and the bacterial pellet was resuspended in the residual 10% glycerol. After transferring to a pre-chilled 1.5 ml Eppendorf tube, the bacterial pellet was then spun down and resuspended in 10% glycerol for electroporation.

#### 2.1.3.2 Transformation of electrocompetent bacteria (EL250)

Electroporation of EL250 was carried out using pre-cooled 0.2 cm cuvettes in a Bio-Rad Gene Pulser (BIO-RAD, Hertfordshire, UK). A total of 60  $\mu$ l EL250 bacteria and 3  $\mu$ l of BAC DNA were mixed gently in the cuvette by shaking followed by electroporation. Immediately after electroporation, 800  $\mu$ l of LB broth was added to the cuvette and mixed with the bacterial cells. The mixture was then transferred to 15 ml polypropylene snap-top tubes, and incubated at 30°C with shaking for 2 hours. After recovery, the bacterial cells were plated on LB agar containing appropriate antibiotics followed by incubation at 30°C for 24-48 hours. Single colonies were picked and BAC DNA was isolated and characterized by restriction digestion and DNA sequence analysis.

#### 2.1.4 Plasmid transformation

Plasmids synthesized by Invitrogen GeneArt Gene Synthesis (Thermo Fisher Scientific, UK) were supplied lyophilized and resuspended in 100  $\mu$ l EB buffer prior to use. Chemically competent One shot <sup>TM</sup>TOP10 bacteria (Invitrogen, Paisley, UK) were used for transformation. TOP10 bacteria were thawed on ice and then 4  $\mu$ l of plasmid DNA was added to the cells. The mixture was gently mixed and incubated on ice for 30 minutes. Bacteria were heat-shocked by incubation in a water bath at 42°C for 30 seconds, followed by recovery for 5 minutes on ice. Two hundred and fifty ml of Super Optimal broth with Catabolite repression (SOC) medium was then added and bacteria were incubated at 30°C with constant shaking at 230 rpm for 2 hours (VWR Orbital Shaker). Cells were plated onto LB agar plates containing the desired antibiotic, and incubated at 30°C for 24-48 hours. A single colony was picked and glycerol stocks were prepared.

## 2.2 Molecular Biology methods

### 2.2.1 Isolation and purification of nucleic acids

#### 2.2.1.1 Isolation of plasmid DNA

Bacteria carrying the desired plasmids were inoculated into 5 ml LB broth containing appropriate antibiotics. Cultures were incubated in a 30°C shaking incubator overnight at 230 rpm. Plasmids were isolated from bacteria using a Gene Elute™ HP plasmid Miniprep kit (Sigma-Aldrich, Dorset, UK), in accordance with the manufacturer's instructions. Briefly, bacteria were harvested by centrifugation at 3,500 rpm for 15 minutes at room temperature (Denley BR401, Hamshire, UK). The bacterial pellet was resuspended in 200 µl cell resuspension buffer and lysed with 200 µl of cell lysis buffer. The mixture was incubated at room temperature for 5 minutes. Cell lysates were neutralized with 350 µl neutralization buffer followed by precipitation by centrifugation at 14,000 rpm (Eppendorf Centrifuge 5418) for 10 minutes. Supernatants containing plasmid DNA were then added onto a spin column and centrifuged at 14,000 rpm (Eppendorf centrifuge, 5418) at room temperature for 1 minute. The flow-through was discarded and the spin column was washed with 500 µl washing buffer and centrifuged as above. The column was washed for a second time using 750 µl washing buffer 2 and bound DNA was eluted using 100 µl elution solution.

#### 2.2.1.2 Isolation of BAC DNA

Alkaline lysis was used to isolate BAC DNA from overnight bacterial cultures (Sambrook & Russell, 2001). Bacteria were harvested by centrifugation of overnight cultures at 3,500 rpm for 15 minutes at room temperature. The pellet was resuspended in 300 µl of P1 buffer (50 mM Tris base, 10 mM EDTA, adjusted with HCl to pH 8.0, 100 µg/ml RNAase; Appendix 2). Cells were lysed by adding 300 µl of P2 buffer (5 M NaOH, 10%SDS; Appendix 2) and mixing by inversion six times. The mixture was neutralized by addition of 300 µl of P3 buffer (3 M CH<sub>3</sub>CO<sub>2</sub>K) adjusted with glacial acetic acid to pH 5.5; Appendix 2) and mixing by inversion.

One ml of phenol/chloroform (Fisher Scientific Leicestershire, UK) was added and mixed on a rotator for 12 minutes. The mixture was then centrifuged at 14,000 rpm for 10 minutes to separate aqueous from organic phases. The upper aqueous phase was transferred to a 2 ml tube (Greiner Bio-One Ltd, Stonehouse, UK). One hundred  $\mu$ l of 3M sodium acetate (Appendix 2) was added and mixed by inversion. One ml of isopropanol was then added (Fisher Scientific, Leicestershire, UK), and the mixture was centrifuged at 14,000 rpm for 10 minutes at room temperature to precipitate the DNA. DNA pellets were washed with 500  $\mu$ l 70% ethanol (prepared using molecular grade water; both from Fisher Scientific, Leicestershire, UK) to remove excess salt, and centrifuged at 14,000 rpm for 10 minutes at room temperature. Ethanol was removed and pellets were dried at room temperature. DNA was resuspended in 50  $\mu$ l of EB buffer (10 mM Tris-HCl, pH 8.5).

#### 2.2.1.3 Isolation of viral DNA from virus stocks

Viral DNA was isolated from virus infected tissue culture cells by using QIAamp<sup>®</sup> MinElute<sup>®</sup> Virus Spin Kit (Qiagen, Manchester, UK). The virus stock was centrifuged at high speed (14,000 rpm) in a benchtop microfuge for one hour at room temperature. The pellet was resuspended in 200  $\mu$ l DPBS. Twenty-five  $\mu$ l of proteinase solution and 200  $\mu$ l of buffer AL were added, and vortexed to mix. The mixture was then incubated at 56°C for 15 minutes. Two hundred and fifty  $\mu$ l of 100% ethanol (Fisher Scientific, Leicestershire, UK) was added and mixed by vortexing and then incubated for an additional 5 minutes at room temperature. The mixture was applied onto a QIAamp MinElute column and centrifuged at 8,000 rpm for 1 minute at room temperature. Columns were sequentially washed with 500  $\mu$ l of buffer AW1, followed by 500  $\mu$ l of buffer AW2 and finally with 100% ethanol. To remove residual ethanol, columns were centrifuged at 14,000 rpm for 3 minutes and then incubated at 56°C for 3 minutes. Viral DNA was eluted from the column by adding 100  $\mu$ l of AVE elution buffer

(RNase-free water with 0.04% sodium azide) and incubated for 1 minute at room temperature, followed by centrifugation at 14,000 rpm for 1 minute.

### 2.2.2 DNA quantitation

A NanoDrop ND-1000 Spectrophotometer (Labtech International Ltd, UK) was used to determine the concentration of purified plasmid or PCR product based on absorbance at OD<sub>260</sub>. Two µl of each sample were used for quantification. DNA quality was evaluated based on 260/280 nm and 260/230nm ratios. High quality DNA corresponded to a ratio of 260/280 nm  $\geq$  1.8 and a ratio of 260/230nm between 1.8 and 2.2

### 2.2.3 Polymerase chain reaction (PCR)

#### 2.2.3.1 PCR primer design and preparation

All oligonucleotide primers used in this study were synthesized by Eurofins Genomics (Eurofins Genomics, Wolverhampton, UK) and supplied lyophilized. Primers were resuspended in EB buffer at a concentration of 50 pmol/µl. Primers used for cloning experiments were HPLC purified. Primer sequences are shown in Table 2.2 and Table 2.3.

#### 2.2.3.2 PCR

PCR was used for multiple purposes: i) amplification of genes of interest or regions within genomic DNA to generate fragments for cloning, ii) analysis of recombinant BACs, iii) amplification of recombinant gene fragments in BAC clones and reconstituted viruses prior to DNA sequencing of these regions, and for confirmation of the absence of wild type virus in recombinant virus stocks. PCR reactions were prepared as described in Appendix 3. A non-template control was included with every PCR experiment to control for contamination. PCR was performed using a PTC-100 Thermal Cycler (Bio-Rad, UK) (Appendix 3). PCR products were analysed on a 1% agarose gel.

### 2.3.3.2.1 Sequencing

Clonal stabs of bacterial clones to be sequenced were prepared in individual wells of a 96-well agar plate which contained LB agar containing Kan 50 µg/ml. Plates were then sent for DNA Sanger sequencing using TOPO M13 primer (LGC Genomics, Germany). Initially, a 96-well agar plate (sequencing kit) supplied by Eurofins Genomics (Germany) was used, but was then changed to LGC Genomics (and prepared 96-well plates by hand) as the sequence quality obtained from LGC Genomics was superior. SmartSeq kits (Eurofin Genomic, UK) were used for sequencing purified DNA samples. Primers used for sequencing are listed in Table 2.2, Table 2.3 and Table 2.4. Sequence data was analysed using MacVector software (Version 15.1.5).

Table 2.2: Oligonucleotides used for amplification of two regions within HCMV genome

Primer name	Sequence	Region
27UL1440F Forward primer	CTCGAGAAAGTCGCAGAAGG	<b>The first region</b>  A 1138 nt region within HCMV (strain AD169) genome located between position 33221 to 34359. This region is located within HCMV UL27 gene.
27UL302R Reverse primer	CCATGGTGTACATTCGCCGC	
UL73Forward primer	CTTCATCCTCTCCCGGATGC	<b>The second region</b>  A 979 nt region within HCMV (strain AD169) genome located between positions 105455 to 106434. This region contains UL73 gene and parts of UL72 and UL73 genes
UL73Reverse primer	CGTAACGAAACGCCGTA CAC	



Table 2.3: Oligonucleotides used for amplification and sequencing of BoHV-4 expression cassettes.

Primer name	Sequence
Outside ORF73 flanking for	ACACAACCCCAACAACCCA TT
Outside ORF73 flanking rev	AGTTGCTGCTCTGGTCTT CC
ORF73 flanking for	GCGCTGATGATGTTCTCGGGCTGTT
ORF73 flanking rev	GCGCGACATCTAGTGGTGGCTCGTA
BoHV-4 for seq. for	GGTCTATATAAGCAGAG CT
BoHV-4 for seq. rev	CTTAACGGCTGACATGGG
GN to promoter for	CCCGCCATTGACGTCA ATA
GN to promoter rev	TCGTTGTTGAGGTAGTGG GC
GN BST to flanking for	GTTCCGTTGTTGCCAGTG TT
GN BST to flanking rev	GGTCATGCCGTCGATGT AGT
GN ORF to flanking for	AAGATGCGTCTCGATGG CAA
GN ORF to flanking rev	CAGAGCAGGCAGAACTG TCA
TB to promoter for	TCATATGCCAAGTACGCC CC
TB to promoter rev	TCGACCCAGATTCCGACT CT
TB BST to flanking for	GGGGGTTTCCAAACAAG ACT
TB BST to flanking rev	CTTCAAATCTGCCGGCCA TG
TB ORF to flanking for	GGCTGTTTAGATGGCCCT GT
TB ORF to flanking rev	GGCCTGGTTCATCTGTGT CA
TB internal 2 for	TCAGCGGCTGGGATATC AAC

TB internal 2 for	AATCTCGGCTCCCAGAG ACT
CB Mip to promoter for	CCCGCCCATTGACGTCA ATA
CB Mip to promoter rev	TCGTGCTTTCTGAAGGCC TT
CB Com1 to promoter for	CCCGCCCATTGACGTCA ATA
CB Com1 to promoter rev	CCAGGGTCACATTTCCGT GA
CB ORF to flanking for	GGCTGTTTAGATGGCCCT GT
CB ORF to flanking rev	GTCCCGTTGATTTTGGTG CC

Table 2.4: Oligonucleotides used for amplification LacZ ( $\beta$ -gal) gene

Primer name	Sequence
Forward primer ( $\beta$ -gal)	GCGCGCTAGCAAGCTTACGTAGATCTCGAGC
Reverse primer ( $\beta$ -gal)	GCGCGCGGCCGCTTATTTTTGACACCAGACCA

#### 2.2.4 Agarose gel electrophoresis

PCR products and digested DNA samples were analysed on a 1% agarose gel. Gels were prepared using agarose 1% (W/V) dissolved in 1× Tris–Acetate–EDTA (TAE) (both supplied by Fisher BioReagents). Ethidium bromide (EtBr) (0.5  $\mu$ g/ml) was added to the gel to enable visualization of DNA under UV light. DNA samples were mixed with 6× loading dye and electrophoresed at 80 volts (V) for one hour in a horizontal tank (Bio-Rad) containing 1×TAE running buffer. A 1kb DNA ladder (Appendix 3) was included as a marker to determine the size of DNA. A gel documentation system (UVi Tech, Japan) was used to visualize DNA under UV light.

### 2.2.5 Agarose gel electrophoresis DNA purification

DNA bands of interest were excised from agarose gels using a razor blade on an UV table. Pure link<sup>®</sup> Quick Gel Extraction Kit (Invitrogen, Paisley, UK) was used to extract the DNA from the agarose gel slices. One volume of the gel slice was dissolved in three volumes of gel solubilisation buffer (L3) by melting in a heat block (50°C) for approximately 10 minutes until the gel piece was completely dissolved. The mixture was then incubated for an additional 5 minutes at 50°C. After addition of one volume of isopropanol, the mixture was mixed, applied onto a Quick Gel Extraction Column and centrifuged at 14,000 rpm for 1 minute. The flow-through was discarded. The column was washed with 500 µl washing buffer, and centrifuged again as described above. The column was centrifuged at a maximum speed for 2 minutes to remove the ethanol. The DNA was eluted from the column by adding 50 µl of Elution Buffer (E5) to the column, incubation for 1 minute at room temperature and then centrifugation at 14,000 rpm for 1 minute.

### 2.2.6 Spin-column DNA purification

PCR products were purified using a Pure Link<sup>™</sup> PCR Purification Kit (Invitrogen, USA). Eighty µl (four volumes) of Pure Link<sup>™</sup> binding buffer HC (B3) containing isopropanol was added to 20 µl (one volume) of PCR product. The mixture was applied onto a pure Link<sup>™</sup> spin column and centrifuged at 14,000 rpm for 1 minute (Eppendorf centrifuge 5418). The column was then washed with 750 µl washing buffer containing ethanol, and centrifuged again as above. DNA was eluted from the spin column by adding 50 µl buffer EB (10 mM Tris-Cl, pH 8.5) and incubated at room temperature for 1 minute, followed by centrifugation at 14,000 rpm for 2 minutes.

## 2.2.7 Cloning

### 2.2.7.1 TOPO cloning

The TOPO cloning system is a single step cloning strategy that enables direct insertion of blunt end PCR products into the TOPO vector without digestion or ligation. This technology depends of the topoisomerase I enzyme. The pCR<sup>TM</sup>4 Blunt TOPO plasmid is supplied as a linearized vector with vaccinia virus DNA topoisomerase I covalently bound to the 3' end of each DNA strand. This system enables cloning of blunt-ended PCR products into the vector due to attack by the 5' hydroxyl of the PCR product on the phospho-tyrosyl bond between the topoisomerase and the vector. This results in release of the topoisomerase and ligation of the PCR fragment into the vector. The vector contains the TOPO cloning site, which is the site of PCR product insertion, flanked by M13 forward and reverse priming sites for fragment sequencing. A Kan<sup>R</sup> gene enables selection of the plasmid. Two regions within HCMV genome were amplified by PCR from HCMV viral DNA samples and used for TOPO cloning. These samples represented viral DNA HCMV UL54 WT and HCMV UL54 ( $\Delta$ D413) from early and late *in vitro* passages (supplied by Dr. Sunwen Chou's laboratory (Division of Infectious Diseases, School of Medicine, Oregon Health and Science University, Portland, USA)). Using primers listed in Table 2.2, Kan-resistant clones were sequenced.

TOPO cloning was performed according to the manufacturer's instructions. Briefly, 4  $\mu$ l of purified PCR product was mixed with 1  $\mu$ l of salt solution (1.2M NaCl and 0.06M MgCl<sub>2</sub>) and 1  $\mu$ l of pCR<sup>TM</sup> Blunt II TOPO vector. The mixture was incubated for 30 minutes at room temperature, and then transformed into TOP10 cells. After incubation on ice for 30 minutes, the mixture was heat shocked at 42°C for 30 seconds to stimulate DNA uptake by the bacteria. After a recovery period of 5 minutes on ice, 250  $\mu$ l SOC medium was added. The transformation reactions were incubated with constant shaking at 230 rpm for 2 hours.

Transformed cells were plated onto a LB agar containing Kan 50 µg/ml, 40 mg/ml X-gal, followed by incubation at 37°C for 24 hours. Two different TOPO vectors were used for these studies: pCR<sup>TM</sup> 4 Blunt TOPO and pCR<sup>TM</sup> Blunt II TOPO. The pCR<sup>TM</sup>4 Blunt TOPO was recommended by the manufacturer for sequencing due to the shorter distance between the M13 sequencing primer site and insert. However, no significant difference was observed between the two vectors in terms of length and quality of the sequences obtained from cloned PCR products using these different vectors.

#### 2.2.7.2 Cloning using T4 DNA ligase

##### 2.2.7.2.1 Preparation of insert and vector

##### 2.2.7.2.1.1 Preparation of insert and vector used in construction of BoHV-4 expression cassettes with flanking viral homologous sequences

Synthetic target antigen open reading frames (ORFs) for the following were synthesized by Invitrogen GeneArt Gene Synthesis (Thermo Fisher Scientific, UK) (shown in Table 2.5): i) a *Mycobacterium bovis* (*Mb*) fusion protein consisting of six proteins, ii) a *Coxiella burnetii* (*CB*) fusion protein construct comprised of 2 proteins, iii) *CB* outer membrane protein, Com1, iv) *CB* outer membrane protein, Mip precursor, v) *CB* protein-export protein, SecB, vi) Rift Valley fever virus (RVFV) glycoprotein N. A synthetic region of BoHV-4 homologous to ORFs flanking ORF73 (ORF73Flank) was also synthesized for targeting of the above ORFs during recombination (see below). The plasmids carrying these ORFs were transformed into bacterial cells (see 2.1.4).

Table 2.5: Synthetic target antigens and the vectors used in construction of BoHV-4 expression cassettes with flanking viral homologous sequences.

Synthetic target antigen ORFs (Insert)	Parental Vector
<i>Mycobacterium bovis</i> ( <i>Mb</i> ) fusion protein	pCMV/EBOV-GP
ORF73Flank	pCMV/ <i>Mb</i> -Fusion
<i>Coxiella burnetii</i> (CB) fusion protein construct P1:HspB	pCMV/ <i>Mb</i> -FusionORF73Flank
CB outer membrane protein, Com1	pCMV/ <i>Mb</i> -FusionORF73Flank
CB outer membrane protein, Mip precursor	pCMV/ <i>Mb</i> -FusionORF73Flank
CB protein-export protein, SecB	pCMV/ <i>Mb</i> -FusionORF73Flank
Rift Valley fever virus (RVFV) glycoprotein N	pCMV/ <i>Mb</i> -FusionORF73Flank

Synthetic ORFs were cloned into the pCMV vector. This vector contains a pCMV promoter, a Kan<sup>R</sup> marker for selection, two *FRT* sites that flank the marker, and an *R6K* (suicide) origin of replication. Overnight cultures of the bacteria carrying the vector and target antigens were prepared, and plasmids purified using a Gene Elute™ HP plasmid Miniprep kit as described previously. The minipreped plasmids were digested (section 2.2.7.2.2), ligated and transformed into PIR1 bacteria (sections 2.2.7.2.3 & 2.2.7.2.4).

#### 2.2.7.2.1.2 Preparation of insert and vector used in construction of MCMV/LacZ expression cassettes flanked by different lengths of repeated sequences

Three synthetic flanking repeat regions were designed with 0, 25 and 250 nucleotides repeat sequences. These regions were synthesized by Invitrogen GeneArt Gene Synthesis (Thermo Fisher Scientific, UK). The LacZ ( $\beta$ -gal) gene was amplified from the pEQ176 plasmid by

PCR (see Table 2.4. for primers) using Q5 hot start high-fidelity DNA polymerase as detailed above. The amplified LacZ along with the various repeat sequence regions were cloned into the pEF1 $\alpha$ /EBOV-GP plasmid, which contains an EF1 $\alpha$  promoter, a Kan<sup>R</sup> marker that is used for selection, an *R6K* (suicide) origin of replication and the bovine growth hormone polyadenylation signal (BGHpA) site. Overnight cultures of pEQ176 plasmids, synthetic flanking regions and pEF1 $\alpha$ /EBOV-GP were grown and plasmids were extracted using a Gene Elute™ HP plasmid Miniprep kit as described previously. The amplified LacZ gene (which encodes  $\beta$ -gal), the synthetic flanking fragments and the vector were digested (section 2.2.7.2.2) and then ligated and transformed into PIR1 cells (section 2.2.7.2.3 and section 2.2.7.2.4).

#### 2.2.7.2.2 Digestion of DNA with restriction endonuclease enzymes

DNA was digested with the appropriate restriction enzymes at 37°C for 2 hours. Digestion was performed in a final volume of 40  $\mu$ l using 30  $\mu$ l of the DNA (plasmid or PCR product), 4  $\mu$ l of 10  $\times$  restriction buffer (50 mM potassium acetate, 20 mM Tris-acetate, 10 mM magnesium acetate, 100  $\mu$ g/ml BSA, pH 7.9), 1  $\mu$ l of High-Fidelity restriction enzyme (New England Biolab, UK) and 5  $\mu$ l molecular distilled water. For double digestion with compatible buffers, 1  $\mu$ l of each High-Fidelity (HF®) restriction enzyme was used with 4  $\mu$ l distilled water. Digests were incubated at 37°C for 2 hours. For double digests with incompatible buffers the DNA was digested with the first enzyme, buffer was exchanged by purifying the DNA using a PCR purification kit, and the DNA was then digested with the second enzyme. Restriction enzymes were heat-inactivated according to manufacturer's instructions when appropriate.

#### 2.2.7.2.3 Dephosphorylation and ligation of DNA fragments

Following restriction enzyme digestion, linearised vectors were dephosphorylated using Antarctic phosphatase (New England Biolab, UK) to remove phosphate residues from the 5' ends. This treatment helps to prevent re-ligation of the empty parental vector. Briefly, 1/10<sup>th</sup> volume of 10× Antarctic phosphatase reaction buffer (50 mM Bis-Tris-Propane HCl, 1 mM MgCl<sub>2</sub>, 0.1 mM ZnCl<sub>2</sub>, pH 6.0) and 1 µl of Antarctic phosphatase were added to the digested plasmid. The reaction was incubated at 37°C for 1 hour, followed by heat inactivation of Antarctic phosphatase at 75°C for 5 minutes.

T4 DNA ligase enzyme (New England Biolab, UK) was used to ligate digested DNA inserts into the linearised, dephosphorylated vector. Ligations were carried out by mixing 1 µl of insert, 1 µl of vector, 1 µl of T4 DNA ligase (New England Biolab, UK), 15 µl molecular distilled water and 2 µl (10×) ligation buffer (50 mM Tris-HCl, 10 mM MgCl<sub>2</sub>, 10 mM DTT, 1 mM ATP, pH 7.5). Reactions were incubated at room temperature for 30 minutes, followed by heat inactivation of the ligase at 65°C for 10 minutes. No insert controls were included with every experiment.

#### 2.2.7.2.4 Transformation of *E. coli* with DNA plasmids

Chemically competent PIR1 bacteria (Invitrogen, Paisley, UK) were used for transformation. PIR1 bacteria express the replication protein  $\pi$ . This protein activates the R6K $\gamma$  (suicide) origin of replication of pCMV and pEF1 $\alpha$ /EBOV-GP plasmids thereby enabling replication. PIR1 cells were defrosted on ice. Twenty-five µl of cells were transferred into pre-cooled 15 ml polypropylene tubes (Fisher Scientific, Leicestershire, UK). Four µl of the ligated reaction was added to the bacterial cells. The mixture was gently mixed and then incubated on ice for 30 minutes. Bacteria were heat-shocked in a water bath at 42°C for 30 seconds followed by incubation for 5 minutes on ice for recovery. Two hundred and fifty µl of SOC medium



(Invitrogen, Paisley, UK) was then added to the transformation reaction and incubated in a 30°C incubator with constant shaking at 230 rpm for 2 hours using a shaking incubator (VWR, West Sussex, UK). Transformed cells were plated onto a LB agar plate containing Kan 50 µg/ml. Plates were incubated at 30°C for 24-48 hours. Colonies were then picked, and plasmid DNA was extracted using Mini preps kit (Sigma, UK) and screened by restriction digest analysis. Glycerol stocks of selected clones were made as detailed above and stored at -80°C.

#### 2.2.8 E/T (Red) homologous recombination

This technique was established for use to genetically modify herpesvirus BAC DNA by Wagner and Koszinowski (Wagner & Koszinowski, 2004). This technique enables a recombination DNA fragment to be designed to target any region in the viral BAC genome that has homologous sequence of 40 bp or more. Generally, recombination fragments contain the homology flanking regions, desired gene for insertion and a selectable marker (Kan<sup>R</sup>) flanked by *FRT* sites. Electroporating the recombination fragment into EL250 cells containing the viral BAC genome followed by induction of the E/T recombinase allows homologous recombination.

##### 2.2.8.1 Preparation of DNA recombinant fragment for recombineering

Recombination expression cassettes were generated as described (Section 2.2.7.2). Prior to recombination, expression cassettes are linearised by restriction enzyme digestion, and purification using a PCR purification kit (Invitrogen, USA).

##### 2.2.8.2 Homologous recombination ('Recombineering') of the virus BAC genome

EL250 bacteria containing viral BACs were grown overnight at 30°C in 3 ml LB broth (Cam 17 µg/ml). One ml of the overnight culture was transferred to 50 ml LB broth with (Cam 17 µg/ml) and incubated at 30°C for 4 hours. Cultures were then incubated at 42°C for 17 minutes

in a shaking water bath to induce the recombination system followed by rapid cooling on ice for 15 minutes. Bacteria were harvested by centrifugation at 7,000 rpm for 15 minutes in a pre-cooled (4°C) centrifuge (Beckman Coulter, USA). Bacterial pellets were washed twice by resuspension in 10% (V/V) glycerol (Molecular Biology Grade) (Sigma, UK).

EL250 bacteria containing the BAC of interest were mixed with the recombinant fragment in pre-cooled 0.2 cm cuvettes, and electroporated using BIO-RAD MicroPulser (Ec3 program). After electroporation, 800 µl of LB broth (without antibiotics) was added to the bacteria and the suspension was transferred to 15 ml polypropylene snap-top tubes, incubated at 30°C with shaking for 2 hours. Bacteria were then plated on LB agar with Cam 17 µg/ml and Kan 25 µg/ml at 30°C for 24-48 hours. The colonies were picked and BAC DNA was isolated for analysis by restriction digest.

#### 2.2.9 Removal of the Kanamycin (selection marker) from the recombinant clones

The Kan<sup>R</sup> gene was removed by flp-mediated recombination induced by arabinose. Briefly, 5 ml LB (Cam 17.5 µg/ml) was inoculated with 100 µl of overnight culture. After shaking incubation at 30°C for 4 hours, 50 µl of 10% arabinose solution (Fisher Scientific, Leicestershire, UK) was added to the culture and incubated for 1 hour to induce flp recombinase. Five hundred µl from this culture was added to 4.5 ml LB (Cam 17 µg/ml) and incubated at 30°C shaking for an hour. After 1 hour, serial dilution was performed and 50 µl of 1x10<sup>-4</sup> dilution was plated on LB agar plates containing Cam 17 µg/ml. Plates were incubated at 30°C for 24-48 hours. Fifteen colonies were picked and each colony was replica plated on LB agar plates containing both Cam 17 µg/ml and Kan 25 µg/ml, and on plates containing only Cam 17 µg/ml. Plates were incubated at 30°C for 48 hours. Kan-sensitive colonies were identified and then BAC DNA was isolated from these colonies and characterized by restriction digest analysis.

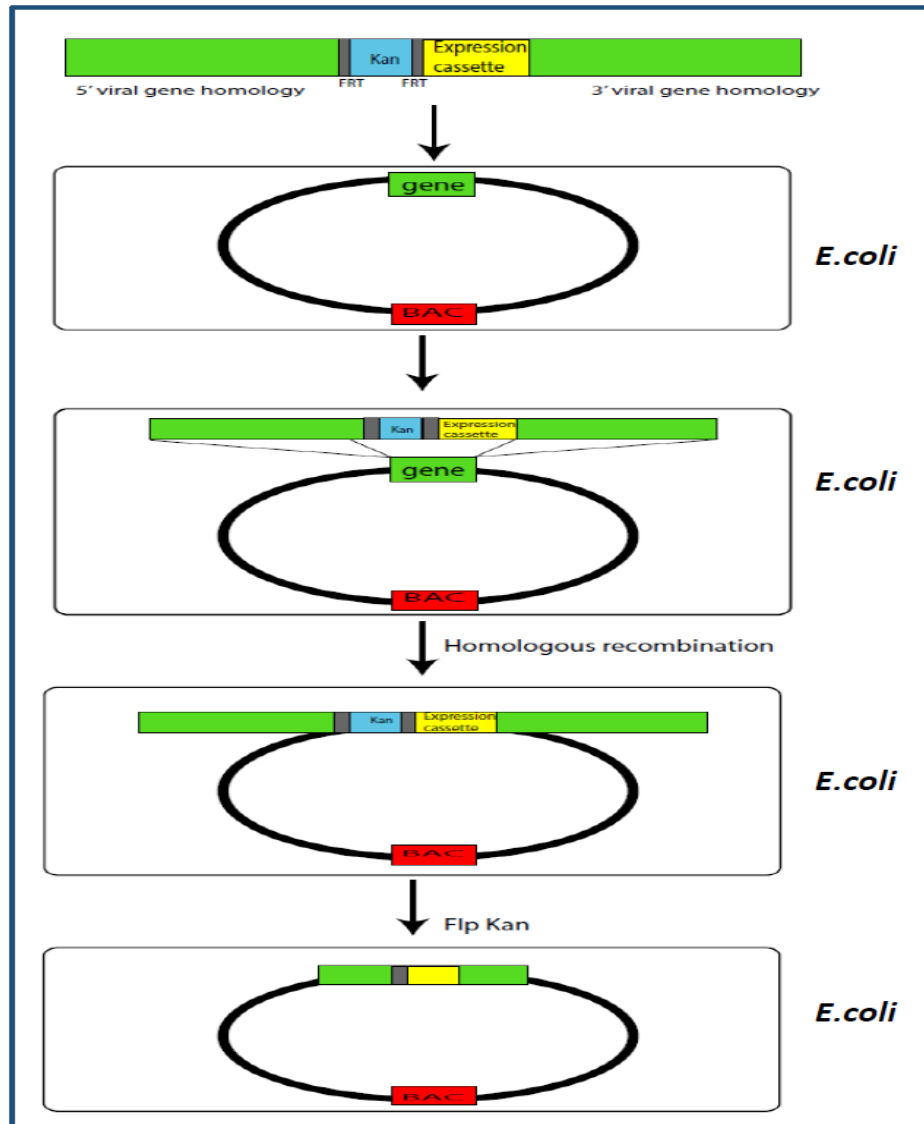


Figure 2.1: Schematic of E/T (Red) homologous recombination ('recombineering') strategy used for construction of recombinant BoHV-4 vaccine vectors and MCMV based flanking attenuated vectors. Recombinant fragments consist of linear vectors carrying a selectable marker (Kan<sup>R</sup>) flanked by FRT sites and desired expression cassette (bTB expression cassette, CB Com1 expression cassette, CB Mip expression cassette, RVFV Gn and Lac Z expression cassette) are constructed by cloning. The recombination fragment also contains synthetic sequence flanking the site targeted for insertion of the expression cassette within the viral BAC. Recombination fragments are electroporated into EL250 *E. coli* strains expressing  $\alpha$ ,  $\beta$ , and  $\gamma$  proteins that required for homologous recombination. Following selection of recombinant BoHV-4 BACs clones on the basis of kanamycin resistance, the selectable marker is removed by FLP mediated-excision. The desired expression cassette and a single FRT 'scar' remain in the BoHV-4 genome. The Kan<sup>R</sup> marker remains in the recombinant MCMV BACs clones as it could be used as a selectable marker in eukaryotic cells. BAC, bacterial artificial chromosome (Red box); FRT, FLP-recombinase recognition site from *Saccharomyces cerevisiae* (grey box); Kan<sup>R</sup>, kanamycin resistance marker (Blue box); and M, desired expression cassette (yellow box).

## 2.3 Maintenance of eukaryotic cell lines

Tissue culture was carried out in a Class II biological safety cabinet (Scanlaf Mars, UK). Madin–Darby bovine kidney (MDBK) cells used in this study were incubated at 37°C in a Binder CO<sub>2</sub> incubator (Binder, Germany) under 95% humidity in the presence of 5% CO<sub>2</sub>. Cells were visualised using an Olympus inverted microscope (MoTic AE2000, UK).

### 2.3.1 MDBK cell line

MDBK cells were supplied by Dr. Alain Vanderplasschen, Laboratory of Immunology-Vaccinology, Faculty of Veterinary Medicine, Belgium. MDBK cells were cultured in minimum essential medium (MEM; Gibco, Paisley, UK) supplemented with 10% fetal bovine serum, 1% penicillin/streptomycin (PSG; 100 unit/ml and 100 µg/ml) and 1% non-essential amino acids (NEAA).

### 2.3.2 Sub-culturing of MDBK cells

MDBK cells were passaged by trypsinisation. When the cells reached 80% confluent growth, medium was removed from the flask and cell monolayers were washed with 1× Dulbecco's phosphate buffered saline (DPBS). Pre-warmed Trypsin/EDTA (0.25% Gibco, Paisley, UK) was added to the cells monolayer and incubated at 37°C for 2-5 minutes in the incubator. After the cells had detached, fresh fully supplemented medium was added to inactivate the trypsin. The cells were collected and centrifuged at 1,200 rpm for 10 minutes at room temperature. The supernatant was removed and the cell pellet was re-suspended with fresh fully supplemented medium. Cells were counted by using a haemocytometer when necessary to determine plating density.

### 2.3.3 Counting cells using a haemocytometer

A haemocytometer is a counting chamber which contains nine  $1\text{ mm}^2$  squares. Each square represents  $1 \times 10^{-4}$  ml volume. Ten  $\mu\text{l}$  of the cell suspension were applied to the haemocytometer. Four squares of the chamber were counted using an Olympus inverted microscope (MoTic AE2000, UK). The average number of the cells was calculated. Cell concentration in cells/ml was calculated by multiplying the average number of cells per  $\text{mm}^2$  by  $1 \times 10^4$ . Cells were then diluted according to required seeding density.

### 2.3.4 Storage of cells

Cells were stored by cryopreservation media. Briefly, cells were harvested by trypsinization as described above. The cell suspension was centrifuged at 1,000 rpm for 5 minutes at room temperature, followed by resuspension in freezing medium containing 20% dimethyl sulfoxide (DMSO; Sigma-Aldrich, Dorset, UK) and 80% fetal bovine serum (FBS; heat-inactivated at  $56^\circ\text{C}$  for 30 minutes). One ml of cell suspension containing  $2 \times 10^5$  cells per ml were transferred to cryovials. Cells were then frozen in a freezing pod container (Mr. Frosty; Fisher Scientific, Leicestershire, UK) at  $-80^\circ\text{C}$  overnight. Cells were stored at either  $-80^\circ\text{C}$  or in liquid nitrogen (for longer storage). For revival, frozen cells were rapidly thawed in a  $37^\circ\text{C}$  water bath and then transferred to 5 ml media. The cells were centrifuged at 1,000 rpm for 10 minutes at room temperature, supernatant was removed and the pellet was resuspended in 10 ml media and transferred into a T25 flask. Cells were incubated at  $37^\circ\text{C}$  in the presence of 5%  $\text{CO}_2$ .

## 2.4 Virology

### 2.4.1 Reconstitution of viral progeny from recombinant BoHV4 BAC DNA in MDBK cells (method 1)

Recombinant and WT viruses were reconstituted by transfection of viral BAC DNA into MDBK cells using a Lipofectamine<sup>TM</sup>3000 transfection kit (L3000-008, Invitrogen). MDBK were seeded on 6-well cell culture treated plates at a density  $5 \times 10^5$ / well. On the day of transfection when monolayers were 70-90% confluent, the media was removed and replaced with 2 ml of MEM supplemented with 1% FBS and 1% NEAA without antibiotics.

The following mixture from A and B was made according to the following Table 2.6.

Table 2.6: The components that used in transfection

Component	Volume per well
<u>A: Lipomix</u>	
5 $\mu$ l of Lipofectamine 3000	5 $\mu$ l
Opti-MEM Reduced Serum Medium (Opti-MEM; Gibco, Paisley, UK)	120 $\mu$ l
<u>B: DNA mix:</u>	
Viral BAC DNA	2 $\mu$ l
P3000 reagent	10 $\mu$ l
Opti-MEM Reduced Serum Medium (Opti-MEM; Gibco, Paisley, UK)	113 $\mu$ l

After 5 minutes incubation at room temperature, the total 250  $\mu$ l mixture volume was added to each well. The plate was gently rocked to ensure coverage the mixture on the surface of the monolayer cells, followed by centrifugation at 1,000xg for 30 minutes at room temperature.

After an incubation period of 5 hours at 37°C with 5% CO<sub>2</sub>, the liquid was removed from each well and media was replaced with fully supplemented media (MEM with 10% fetal bovine serum, 1% penicillin/streptomycin (100 unit/ml and 100 µg/ml) and 1% NEAA). The plate was then returned to the incubator and examined daily for plaque formation under the light microscope. The green channel (488 nm) of the Zoe microscope (BioRad) was also used to detect virus as the BoHV-4 BAC cassette contains a functional GFP gene. When full CPE was observed in transfected cells, cells and supernatant were collected and centrifuged at 1000 × g for 10 minutes at room temperature. Supernatant was aliquoted into 1 ml volumes and stored at -80°C to be used as virus seed stocks from which to prepare BoHV-4 concentrated stocks. Remaining pellets were used for western immunoblotting.

#### 2.4.2 Reconstitution of viral progeny from recombinant BoHV4 BAC DNA in MDBK cells (method 2)

Recombinant and WT viruses were reconstituted by transfection of viral BAC DNA into MDBK as detailed above (section 2.4.1). After incubation for 5 hours at 37°C with 5% CO<sub>2</sub>, the media was removed from each well and the cell monolayers overlaid with a 1.2% sea plaque agar (Lonza, Slough, UK) (Appendix 4) mixed with 2× DMEM. The mixture was made by mixing 12.5 ml of melted 1.2% sea plaque agar with 12.5 ml of 2× DMEM (500 ml sterile H<sub>2</sub>O, 13.38 g DMEM powder, 7.5% sodium bicarbonate, 10% FBS; Life Technology, Paisley, UK and PSG), followed by addition of 2 ml to each well. The plates were left at room temperature until the overlay agar had solidified and then returned to the incubator and examined daily for plaque formation as described above. BoHV-4 plaques were picked using a sterile 1 ml pipette tip (Dutscher, Essex, UK) and transferred to a 2 ml microcentrifuge tube containing 200 µl fresh medium and stored at -80°C. The medium containing the virus plaques was used to infect MDBK cells, from which were prepared seed stocks that were used to prepare the BoHV-4 concentrated stocks.

#### 2.4.3 Production of concentrated BoHV4 Virus Stocks

Ten T175 tissue culture flasks were seeded with MDBK cells and incubated until the cells reached 80-90% confluence. Before infection of monolayers with virus, media was removed from flasks and 20 ml fresh media was added. One ml of virus seed stock was added to 49 ml aliquot of media and mixed carefully by pipetting up and down. Five ml of diluted virus was then added to each flask. Flasks were gently rocked to distribute the virus-inoculated media evenly over each cell monolayer. Flasks were inspected daily for cytopathic effect (CPE). When full CPE was observed, the cells and the supernatant were harvested and stored at  $-80^{\circ}\text{C}$  overnight. The supernatant was thawed and centrifuged at 3,800 rpm ( $4,600 \times g$ ) for 20 minutes at room temperature. Thirty-one ml of clarified supernatant was added to Oakridge nalgene-style tubes (Cat. No. 1053-8341, Fisher scientific, Leicestershire, UK), underlayered with a 4 ml 20% (V/V) sorbitol cushion (1mM  $\text{MgCl}_2$ ; 5mM Tris-HCl; pH7.4; Appendix 4) and then centrifuged in high speed centrifuge (Beckman Coulter, Fisher Scientific, UK) at 24,000 rpm ( $3,200 \times g$ ) for 80 minutes at room temperature. The supernatant was removed and virus pellets were resuspended in 2 ml of 2% FCS in DPBS and then aliquoted in cryovials. Concentrated virus stocks were stored at  $-80^{\circ}\text{C}$ .

#### 2.4.5 Titration of virus by $\text{TCID}_{50}$

A  $\text{TCID}_{50}$  assay was used to titre the concentrated viral stocks. MDBK cells were seeded in 96 well plates at a density of  $1 \times 10^5$  cells per well in a 100  $\mu\text{l}$  volume. The following day, serial dilutions of viral stock were prepared and added to each well in a final volume of 100  $\mu\text{l}$ . Plates were incubated at  $37^{\circ}\text{C}$  for 7-10 days, and the number of CPE-positive wells were counted using the immunofluorescence (Zoe) or light microscope. The fraction of CPE-positive wells in each row was calculated and then the dilution fraction of CPE-positive wells (Reed &



Muench, 1938). Calculation viral titer was according to the following formula, which is based on the Spearman-Kärber method.

$$\text{Viral titer (PFU/ml)}=10^{(x+0.8)}$$

where x = the sum of fraction of CPE-positive wells

#### 2.4.6 Western immunoblotting

Cells infected with recombinant viruses were harvested at 100% CPE, centrifuged at  $1000 \times g$  for 10 minutes at room temperature, and pellets were resuspended in Laemmli 2x buffer (Bio-Rad Laboratories, USA) with 2-mercaptoethanol (55mM) (Thermo Fisher scientific, UK). Samples were homogenized using QiaShredders (Qiagen, Hilden, Germany) and heated to 70°C for 10 minutes prior to electrophoresis. Fifty  $\mu\text{l}$  of Cell Lysates were electrophoresed on Mini-PROTEAN TGX Precast Gels (Bio-Rad Laboratories, USA) with 1x TGX running buffer Appendix 5 (Bio-Rad Laboratories, Germany) at 130V for 60 minutes. Precision Plus Protein Kaleidoscope Standards were loaded along with the samples. Proteins were transferred to nitrocellulose membranes (Trans-Blot Turbo transfer pack, Bio-Rad Laboratories, USA) using a Bio-Rad Trans-Blot Turbo Transfer System for 7 minutes (Semi-dry western blotting). Membranes were blocked with 5% (W/V) non-fat milk (2.5 g skimmed milk powder in 50 ml 1x TBST) for 2 hours at room temperature or at 4°C overnight. Membranes were incubated with anti-V5 antibody (Novex, Thermo Fisher Scientific, USA) in 5% (W/V) non-fat milk at room temperature for 1 hour. After incubation, primary antibody was removed by washing the membranes in TBST three times for 5 minutes each. Secondary peroxidase-conjugated anti-mouse antibody diluted 1:5000 in 5% (W/V) non-fat milk in TBST was added to the membrane and incubated for one hour at room temperature. Excess secondary antibody was removed by washing the membranes in TBST three times for a period of 5 minutes per wash. Membranes

were exposed to super signal west femto maximum sensitivity substrate reagent (Thermo Fisher scientific, USA) for 2 minutes at room temperature.

#### 2.4.7 Animal models

To detect the immune response induced by attenuated BoHV-4 $\Delta$ ORF73/*Mb*, studies were carried out in rabbits. Twelve animals were divided into two groups (see Figure 2.2). Group 1 (eight animals) were inoculated with BoHV-4 $\Delta$ ORF73/*Mb*. Group 2 (four animals) were inoculated with BoHV-4 WT (control animals). Rabbits were inoculated subcutaneously (s.c.) with  $1 \times 10^8$  pfu followed by a boost using an identical inoculum at 4 weeks. During this study, one of the animals that were inoculated with the BoHV-4 WT died. At week 12 post inoculation, animals were humanely euthanized and blood was collected. The animal study was performed in collaboration with Dr. Alain Vanderplasschen Laboratory. All ethical approvals were obtained by the local ethics committee of the University of Liège (Belgium).

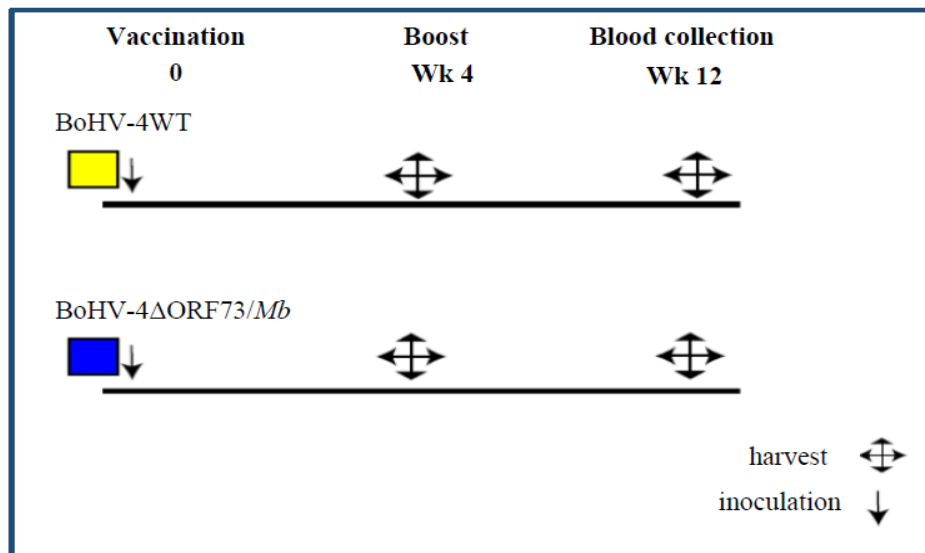


Figure 2.2: Schematic showing BoHV-4 $\Delta$ ORF73/*Mb* vaccinated rabbit groups. Rabbits were immunized using  $1 \times 10^8$  pfu of the indicated BoHV-4 $\Delta$ ORF73/*Mb* vector (s). Rabbits were then given a booster dose of an identical inoculum at week 4. Control group received BoHV-4 WT ( $1 \times 10^8$  pfu). At week 12, blood was collected from the animals.

#### 2.4.8 Enzyme-linked immunosorbent assay (ELISA)

ELISAs were carried out to detect anti-*Mycobacterium bovis* (*Mb*) antibody in the serum of the vaccinated rabbits as follows. Fifty  $\mu\text{l}$  of *Mb* antigens that were supplied by Martin Vordermeier (Edward Jenner Institute for vaccine research, UK) as recombinant proteins (Rv3804 (Ag85A), Rv0287 (TB9.8), Rv0288 (TB10.4) and Rv3615c) were coated onto 96-well ELISA plates (Nunc MaxiSorp™ flat-bottom from Thermo fisher). After a one hour incubation at 37°C the plates were washed five times with phosphate buffered saline Tween 20 (PBST). A total of 200  $\mu\text{l}$  of the blocking buffer was added to each well and incubated for two hours at room temperature, after which the plates were washed five times. Fifty  $\mu\text{l}$  of the tested rabbit serum diluted in blocking buffer was added to wells. After a one hour incubation at 37°C the plates were washed five times with PBST and then 50  $\mu\text{l}$  of alkaline phosphatase antibody produced in goat anti-rabbit IgG (whole molecule) (Sigma) diluted in blocking buffer was added and incubated for one hour at room temperature. Plates were then washed five times with PBST and then 100  $\mu\text{l}$  of Substrate (Alkaline Phosphatase Yellow (pNPP) Liquid Substrate System for ELISA, Sigma) was added to each well. (note: Rv3615c was used as non-vaccine *Mb* control protein)

#### Statistics

A chi square Test was used in statistics. P-values  $\leq 0.05$  were considered significant and displayed by (\*) in the figures.

## Chapter 3 Development of bovine herpesvirus-4 deleted for ORF73 (BoHV-4 $\Delta$ ORF73) as a conditionally-attenuated vaccine platform against zoonoses: *Mycobacterium bovis*, *Coxiella burnetii* and Rift Valley fever virus.

### 3.1 Introduction

The main hypothesis being tested is whether bovine herpesvirus-4 (BoHV-4), based on a number of criteria (see below), is suitable for development as a vaccine vector platform for targeting of zoonotic diseases (Franceschi *et al.*, 2015). Recombinant BoHV-4 has been shown to be capable of inducing an immune response against pathogen targets in all common domestic ruminant animals tested [goats (Donofrio *et al.*, 2013; Rosamilia *et al.*, 2016) and sheep (Donofrio *et al.*, 2007b)], as well as pigs (Donofrio *et al.*, 2011), rabbits (Donofrio *et al.*, 2006b; Donofrio *et al.*, 2007b), chickens (Donofrio *et al.*, 2008a) and mice (Donofrio *et al.*, 2018; Macchi *et al.*, 2018). In these animals, BoHV-4 has been shown capable of eliciting both humoral and cellular immune responses against encoded heterologous pathogen target antigens, including: peste des petits ruminants virus hemagglutinin envelope glycoprotein (Macchi *et al.*, 2018), Ebola virus glycoprotein (GP) (Rosamilia *et al.*, 2016), monkeypoxvirus glycoproteins, A29L, M1R and B6R (Franceschi *et al.*, 2015), caprine herpesvirus type 1 glycoprotein D (Donofrio *et al.*, 2013), bovine herpesvirus 1 glycoprotein D (Donofrio *et al.*, 2006b), bovine viral diarrhoea glycoprotein E2 (Donofrio *et al.*, 2007b; Donofrio *et al.*, 2011), bluetongue virus glycoprotein VP2 (Franceschi *et al.*, 2011) and Crimean Congo hemorrhagic fever virus nucleocapsid protein (Farzani *et al.*, 2019).

In the wild, BoHV-4 is naturally found in cattle (Li *et al.*, 2005a; Markine-Goriaynoff *et al.*, 2003; Thiry *et al.*, 1992), African buffalo (Dewals *et al.*, 2006), sheep and goats (Moreno-Lopez *et al.*, 1989). The frequency of infection in cattle is high. In one study measuring infectious virus, 16-18% of animals were shown to be infected (Wellenberg *et al.*, 1999). In a study based on serology, antibodies to BoHV-4 Ab were detected in 70% of animals (Dewals

*et al.*, 2005). In a study of African buffalo, 25% of animals were infected with 94% of the animals tested having antibodies to the virus (Rossiter *et al.*, 1989b). Infection in smaller ruminant species is similarly high, with frequencies of infection of 26.5% and 13.6% in sheep and goats, respectively (Kálmán & Egyed, 2005).

BoHV-4 is not consistently associated with overt disease; the virus being characterized as an infectious agent for which there is no consistent symptoms associated with disease, although evidence suggests some association with postpartum endometritis (see below) (Frazier *et al.*, 2001; Monge *et al.*, 2006). The virus has been isolated from healthy animals (Rossiter *et al.*, 1989a), as well as from animals showing a variety of mild clinical signs, such as skin lesions (House *et al.*, 1990), mammary dermatitis (Reed, Langpap & Anson, 1977), conjunctivitis, pneumonia, inflammation of the upper respiratory tract (Mohanty, Hammond & Lillie, 1971), enteritis (Eugster, 1979), chronic metritis (Wellemans *et al.*, 1984; Wellemans *et al.*, 1983) and postpartum metritis (Parks & Kendrick, 1973). However, there is no evidence that BoHV-4 is the etiologic agent for any of these diseases. BoHV-4 has also failed to induce any clinical signs of disease in experimental cattle and sheep infected with the virus (Bartha, Juhasz & Liebermann, 1966).

The strongest evidence for disease association is associated with a subset of BoHV-4 strains causing postpartum metritis in cattle that may function as a secondary agent during bacterial infection (Donofrio *et al.*, 2007a; Frazier *et al.*, 2001; Frazier *et al.*, 2002; Monge *et al.*, 2006). The requirement for bacterial co-infection for disease is believed to result from the bacterially-induced secretion of prostaglandin (PGE<sub>2</sub>) that, along with tumour necrosis factor alpha (TNF- $\alpha$ ) and bacterial lipopolysaccharide (LPS), stimulates BoHV-4 replication. In this model, heightened viral replication leads to further damage and inflammation of the endometrial cells (Donofrio *et al.*, 2004; Donofrio *et al.*, 2008b; Jacca *et al.*, 2013). Although not consistently

associated with disease, conditional attenuation of the BoHV-4 vector is attractive for increased safety, as well as increased environmental control.

Many approaches have been used to attenuate BoHV-4 (see Introduction). These strategies include: i) deletion of a 1kb region within a late gene coding for a polyadenylated RNA, ii) deletion and insertional inactivation of the thymidine kinase (TK) gene, and iii) deletion of ORF73.

**Deletion a region within gene that coding for a polyadenylated RNA:** The role of BoHV-4 polyadenylated RNA in viral replication and spread is not known however, study has shown that deletion about 1kb within the region that coding for the polyadenylated RNA resulting attenuated virus slowly spread *in vitro* (Capocefalo *et al.*, 2013).

**Deletion/inactivation of TK gene:** Although the precise mechanism by which deletion of the non-essential TK gene would result in attenuation *in vivo* is not known, studies have shown that a TK deleted version of BoHV-4 (BoHV-4 $\Delta$ TK) could not be recovered from the body secretions and blood of vaccinated animals (Donofrio *et al.*, 2013). This study also showed that BoHV-4 $\Delta$ TK did not spread between animals, indicating that presumably due to the lower levels of replication, this method can be used to control BoHV-4 within the environment.

**Deletion of ORF73:** Thirion *et al* (2010) have shown that deletion of a gene designated open-reading frame (ORF) 73 (ORF73) from BoHV-4 results in a conditionally-attenuated virus that is able to replicate normally *in vitro*, but is unable to persist *in vivo*. The protein expressed by ORF73 has been shown to be required for tethering of the viral episome to mitotic chromosomes, which is required for viral latency. This role of the ORF73 product was initially suggested by homology to proteins from other herpesviruses that have been more extensively studied (Calderwood *et al.*, 2005; Fowler *et al.*, 2003; Renne *et al.*, 2001). Amino acid sequence analysis of BoHV-4 ORF73 protein shows the N- and C-regions to be homologous to proteins from other herpesviruses that are associated with latency, including Latency-Associated

Nuclear Antigen (LANA-1) of Kaposi's sarcoma-associated herpesvirus (KSHV), mLANA of MHV-68 and other ORF73 orthologues (Thirion *et al.*, 2010).

The best-characterized ORF73 protein in herpesviruses is LANA-1 of KSHV, also termed human herpesvirus 8 (HHV-8). LANA-1 is an essential protein for establishing and maintaining the latent viral episome within the nucleus. It has been shown that LANA-1 binds to histone H1 to mediate tethering of the viral episome to host chromosomes during division of the latency infected cells (Ballestas, Chatis & Kaye, 1999; Barbera *et al.*, 2006; Cotter II & Robertson, 1999; Ye *et al.*, 2004). LANA-1 is also involved in modulation of multiple cellular pathways involved in cell growth and survival by targeting transcriptional regulators or co-regulators like p53, pRB, CBP and sin3A (Friborg Jr *et al.*, 1999; Krithivas *et al.*, 2000; Lim *et al.*, 2001). A critical role for ORF73 (referred to as mLANA) in latency was also identified in MHV-68. Similar to LANA-1 of KSHV-1, multiple studies have indicated that the mLANA protein was required for maintaining the MHV-68 genome as an extrachromosomal episome in the nuclei of the dividing cells. These studies showed that genetic inactivation of ORF73 in MHV-68 resulted in viruses that were unable to establish latency after intranasal (Fowler *et al.*, 2003; Moorman, Willer & Speck, 2003) and intraperitoneal inoculation (Paden *et al.*, 2010), or when inoculated into immunocompromised mice (Paden *et al.*, 2010).

Prior to my work, only antibody (Ab)-based immunity against the whole BoHV-4 virus had been examined in an ORF73-deleted BoHV-4 virus. In this earlier work, Ab response to BoHV-4 $\Delta$ ORF73 was detected in rabbits by ELISA and were comparable to those induced by WT BoHV-4 (Thirion *et al.*, 2010). In my thesis, a BoHV-4 vector conditionally attenuated by deletion of ORF73 (BoHV-4 $\Delta$ ORF73) was developed as a vaccine platform for use in ruminants against both bacterial and viral zoonotic pathogens. Specifically, the BoHV-4 $\Delta$ ORF73 vector was developed to target the zoonotic bacteria (*Mycobacterium bovis* and *Coxiella burnetii*) and zoonotic virus (Rift Valley fever virus). By targeting these agents in

animals involved in transmission to humans, this work will develop important tools not only to prevent disease in livestock animals, but also to prevent transmission of these pathogens from animals to humans.

The aim of this chapter was therefore to test the hypothesis that *a recombinant BoHV-4 conditionally attenuated by deletion of ORF73 is able to induce immune responses against encoded heterologous antigens*. Due to ease of analysis, antibody response was used as the read-out for immune response induction. A number of heterologous antigens were selected to be expressed by BoHV-4 $\Delta$ ORF73. Those antigens were selected based on their immunogenic criteria, as well as their potential impact as an effective vaccine against the pathogen expressing these antigens. The conditionally attenuated BoHV-4 virus that expressing *Mb* fusion proteins (BoHV-4 $\Delta$ ORF73*Mb*) was constructed and then sent with the wild type BoHV-4 virus (WT BoHV-4) to the Laboratory of Dr. Alain Vanderplasschen (University of Liège, Belgium), where the animals were vaccinated with the constructed vaccine and then the serum samples were sent to the Jarvis lab where the antibody responses were detected by ELISA.

**Criteria for selection of *Mycobacterium bovis* (*Mb*) antigens.** The first pathogen to be targeted was *Mycobacterium bovis* (*Mb*). Although not a zoonotic risk in developed countries due to changes in food handling (primarily pasteurization of milk), *Mb* is a major commercial problem impacting the UK cattle industry and remains a zoonotic pathogen in developing countries. The *Mb* antigen used in this study was a fusion protein of *Mycobacterium bovis* that comprised of Rv3620, Rv1733, Rv2389, Rv3804 (Ag85A), Rv0287 (TB9.8) and Rv0288 (TB10.4). These *Mb* antigens were selected based on a number of criteria. First, they are known to be immunogenic in both humans and cattle, such that in a ‘one health’ environment information gained from our studies in *Mb* could inform development of *Mycobacterium tuberculosis* (*Mtb*) vaccines for use in humans. Second, previous studies had shown that



vaccines containing these antigens had afforded a level of protection against *Mycobacterium tuberculosis* (TB) in an animal challenge system (Bertholet *et al.*, 2010; Coppola *et al.*, 2015; Dean *et al.*, 2014; Hansen *et al.*, 2018; Ma *et al.*, 2016; Teng *et al.*, 2015). Third, these antigens are expressed over the entire life cycle of *Mb*, which is important for designing a multiphasic vaccine (to target multiple phases of *Mb* infection). Finally, these antigens were selected to maintain differentiation of vaccinated from infected (DIVA) for the purified protein derivatives (PPD) single comparative cervical tuberculin test (SICCT), which is the internationally accepted method for bovine TB assessment in live animals (none of the selected antigens are present in the PPD antigen preparation) (Prasad *et al.*, 2013)). *Mb*-infected cattle had been shown to respond to all of these antigens at substantial, but differing levels (courtesy of Dr Vordermeier, unpublished data).

**Rv3620:** This is a small secreted protein and member of the EsX virulence family. Recombinant Rv3620 protein induces interferon gamma and tumor necrosis factor in peripheral blood mononuclear cells (PBMCs) from PPD+ healthy animals (Bertholet *et al.*, 2008). Specifically, Rv3620 was selected based on a previous study that had shown that guinea pigs immunized with ID93TB vaccine, which is comprised of four fusion TB antigens including Rv3620, had robust immune response to both ID93 and its components, but they were not sensitive to PPDs (Baldwin *et al.*, 2014). Bertholet *et al* (2010) showed that ID93 vaccine, which is comprised of four fusion TB antigens including Rv3620, when administered with synthetic monophosphoryl lipid A (MPLA) adjuvant enhanced the immunogenicity in mice and protection in a guinea pig *Bacillus Calmette–Guérin* (BCG) boost challenge model (Bertholet *et al.*, 2010).

**Rv1733:** The function of this antigen is unknown but is thought to be associated with latency. Immunogenicity of this antigen has been reported for both humans and mice (Black *et al.*, 2009; Schuck *et al.*, 2009). Rv1733 has been shown to induce interferon gamma in CD4 T cells

and also specific antibody responses (Coppola *et al.*, 2015). Rv1733c, the *Mtb* latency antigen, was recognized during latency in infected individuals to provide protection in mice both pre- and post-challenge models (Coppola *et al.*, 2015)

**Rv2389 (RpfD):** Resuscitation-promoting factor D is a secreted protein, with a hormone-like activity that also plays an important role in the activation of bacterial replication from dormancy (Davies *et al.*, 2008). RpfD induces a stronger immune response in latently infected individuals than in those undergoing active infection (Arroyo *et al.*, 2018; Riaño *et al.*, 2012). In a recent study, vaccination with rhesus CMV (RhCMV) vectors expressing eleven TB antigens including Ag85A, RV1733 and Rv2389 (RpfD) in rhesus macaques significantly decreased the load of TB in the lung of the infected animals and protected 41% of the vaccinated animals (Hansen *et al.*, 2018).

**Rv3804 (Ag85A):** This is a protein secreted during primary infection, which also plays an important role in synthesis of the glycolipid of mycobacterial cell wall and bacterial survival during the dormant state (Dewi, 2017; Elamin *et al.*, 2011; Wiker & Harboe, 1992). Immunization of cattle with Ag85A expressed by modified vaccinia virus Ankara following an Ag85A plasmid DNA vector prime resulted in induction of antigen-specific interferon gamma secreting CD4<sup>+</sup> and CD8<sup>+</sup> T cells (Taracha *et al.*, 2003). Study of humans also indicated that Ag85A is highly immunogenic. Specifically, Ag85A expressed by a recombinant human type 5 adenovirus induced T cells responses in both BCG<sup>+</sup> and BCG<sup>-</sup> human volunteers (Smaill *et al.*, 2013). Several other studies have shown that vaccines containing Ag85A can enhance protection against *Mtb*. WH121, a vaccine containing Ag85A in a polyfusion construct, gave persistent protection when used independently, as well as when used in a BCG boost model (Ma *et al.*, 2016). Another study showed that an adenovirus based vaccine expressing Ag85A or a fusion construct comprised of Ag85A, Rv0287(TB9.8), Rv0288(TB10.4) used to boost BCG vaccinated cattle enhanced the protection in these animals compared with animals that

were vaccinated with BCG alone (Dean *et al.*, 2014). Similarly, a replication-deficient chimpanzee adenovirus expressing Ag85A (ChAdOx1.85A) alone or used together with modified vaccinia Ankara expressing Ag85A (MVA85A) has been shown to protect mice (Stylianou *et al.*, 2015)

**Rv0287 (TB9.8):** An extracellular protein that is secreted by actively replicating bacteria. This antigen is highly immunogenic, with recombinant Rv0287 antigen inducing interferon gamma via a CD4<sup>+</sup> Th1 cell response. Previous studies reported the capability of Rv0287 to induce *in vitro* cellular immune responses in infected calves and humans (Al-Attayah *et al.*, 2004; Coler *et al.*, 2009; Mustafa *et al.*, 2006). Cattle vaccinated with Bacillus Calmette Guerin (BCG) and boosted with adenovirus expressing fusion protein comprised of four *Mb* antigens including Rv0287 showed a level of protection compared to non-vaccinated animals. The bacterial load in lymph nodes decreased in vaccinated animals. The protection was also evaluated by gross pathology (Dean *et al.*, 2014).

**Rv288 (TB10.4):** This is an extracellular protein secreted by actively replicating bacteria. It was selected based on a previous study that had reported that Rv288 induced strong immune response (interferon gamma) in the cattle infected with *Mycobacterium* (Aagaard *et al.*, 2003). However, in animals that were considered to be sensitized to environmental *Mycobacterium* (either vaccinated or previously infected with *Mb*), TB10.4 did not induce interferon gamma (Aagaard *et al.*, 2003; Vordermeier, Gordon & Hewinson, 2011). Rv288 has also been shown to induce a cellular immune responses in humans (Geldenhuys *et al.*, 2015; Schuck *et al.*, 2009). Vaccinated mice with a vaccine called CTT3H, comprised of five immunodominant antigens including TB10.4, provided effective protection against *Mtb* aerosol challenge (Teng *et al.*, 2015).

### **Criteria for selection of *Coxiella burnetii* (CB) antigens**

*CB* was the second pathogen that was selected to be targeted with a BoHV-4 ORF73 based vaccine vector. *CB* is an intracellular zoonotic bacteria that causes Q fever and similar to *Mb*, the protective antigens are not known. A fusion protein comprised of P1 (bacterial outer membrane protein) and HspB (heat shock protein B) antigens was the first antigen selected for expression by BoHV-4 ORF73. Both antigens are known to be immunogenic (Vodkin & Williams, 1988; Williams *et al.*, 1990), inducing humoral and cell-mediated immunity. However, the quality and the quantity of the immune response induced by these antigens are not similar, with levels of antibodies in mice immunized with HspB higher than those immunized with P1. Selection of the P1:HspB as a fusion construct was based on a previous study that had demonstrated this fusion protein to induce stronger immune responses than those induced by the individual P1 or HspB antigens (Li *et al.*, 2005b). The fusion protein was also shown to provide an efficient level of protection in mice against *CB* (Li *et al.*, 2005b).

The first step in constructing a recombinant BoHV-4 vector is cloning of the target antigen into a BoHV-4 genome targeting plasmid. However, cloning of the *CB* P1:HspB fusion protein into the genome targeting pCMV vector was unsuccessful presumably due to bacterial incompatibility issues. As an alternative approach, three additional antigens were selected for insertion within the BoHV-4 vector: Com1 (27kDa outer membrane protein), Mip (Outer membrane protein Mip precursor) and SecB (protein-export protein SecB). These antigens were selected for a number of reasons. First, these antigens are immunogenic. Specifically, it has been shown that recombinantly expressed versions of all three proteins are capable of inducing humoral immune responses when measured by ELISA (Vigil *et al.*, 2011; Zhang & Samuel, 2003). These antigens were also shown to efficiently activate mouse bone marrow derived dendritic cells (BMDCs) (antigen presenting cells), with mice receiving BMDC pulsed with these antigens stimulating CD8<sup>+</sup> T cells (Wei *et al.*, 2011; Xiong *et al.*, 2012). These

antigens were also able to induce interferon gamma production in CD4 Th1 cells (Wei *et al.*, 2011; Xiong *et al.*, 2012). Adoptive transfer of dendritic cells from mice vaccinated with these proteins partially protected animals against *CB* (bacterial loads were decreased in vaccinated animals) (Wei *et al.*, 2011; Xiong *et al.*, 2012), which shows that immunity is associated with protection. Com1 and Mip were subsequently used to construct BoHV-4 vectors, with SecB cloning running into similar issues as observed for the HspB:P1 fusion.

### **Criteria for selection of Rift Valley fever virus (RVFV) antigen**

The third pathogen selected to be targeted by a BoHV-4 ORF73-based vaccine vector was RVFV. RVFV is a zoonotic pathogen that primarily affects livestock ruminants (cattle, goats and sheep) and can also be lethal in humans. RVFV glycoprotein N (Gn) was selected for expression by BoHV-4 $\Delta$ ORF73. RVFV Gn is an immunogenic antigen that induces strong RVFV neutralizing antibody responses in animals. Gn is also DIVA (Faburay *et al.*, 2014; Kortekaas *et al.*, 2010b; Papin *et al.*, 2011; Warimwe *et al.*, 2016). Selection of Gn was based primarily on its ability to afford full protection in a sheep challenge model (Faburay *et al.*, 2016).

### 3.2 Results

The aim of this project was to test the ability of conditionally-attenuated BoHV-4 $\Delta$ ORF73 expressing defined heterologous antigens to induce specific immune response. BoHV-4 represents a promising vaccine vector for many of the ruminant animals such as cattle, sheep and goats that are involved in transmission of zoonotic pathogens such as *CB*, RRVFV and *Mb* to humans. Although *Mb* is no longer a zoonotic agent in developed countries (due to pasteurization), this obligate intracellular bacteria is a major agricultural pathogen, and still causes substantial zoonotic disease in developing countries. In this regard four BoHV-4 vaccine-based vectors were constructed, these vaccine vectors were designed to express antigens from these zoonotic pathogens.

The first concern was the generation of a suitable expression cassette, which was then to be integrated into BoHV-4  $\Delta$ ORF73 genome to replace the ORF73 gene. The rationale for integration of the expression cassette to replace the endogenous ORF73 locus was to further increase the safety of the BoHV-4  $\Delta$ ORF73 vector. Specifically, deletion of ORF73 during generation of the recombinant vaccines reduced the possibility for repair of ORF73 occurring through recombination of the vaccine with wild type (WT) virus. In certain vaccine vector constructs, the expression cassette was also inserted into the WT BoHV-4 genome without gene deletion. The BoHV-4 v. test G BAC was used to create all BoHV-4 $\Delta$ ORF73 and WT BoHV-4 based vectors (Gillet *et al.*, 2005). E/T linear homologous recombination methods were used to construct these vectors (Muyrers *et al.*, 2004). All bacterial strains, plasmids and BACs are listed in Table 3.1

Table 3.1: Bacterial strains, plasmids and BACs used in this study.

Bacterial strains, plasmids and BAC	Antibiotic Selection	Growth Temp(C°)	Source or Reference
One shot™ TOP10 cells chemically competent <i>E. coli</i>	None	37	Invitrogen™
EL250	None	30	Dr. Donald L. Court Centre of Cancer Research, National Cancer Institute Frederick, USA
BoHV-4 (BoHV-4 v.test G) BAC	Cam 17 µg/ml	30	Dr. Alain Vanderplasschen  Laboratory of Immunology – Vaccinology, University of Liège, Belgium  (Gillet <i>et al.</i> , 2005)
One Shot™ PIR1 Chemically Competent <i>E. coli</i>	None	30	Invitrogen™
pCMV/EBOV-GP plasmid	Kan 50 µg/ml		Jarvis Laboratory
pORF73Flank plasmid	Carb 100 µg/ml	30	GeneArt- Invitrogen
pWTF flank plasmid	Carb 100 µg/ml	30	GeneArt- Invitrogen
pMb-Fusion plasmid	Carb 100 µg/ml	30	GeneArt- Invitrogen
pCB-P1HspB1 plasmid	Carb 100 µg/ml	30	GeneArt- Invitrogen
SnaB1_C1_SInt_PvuII plasmid	Carb 100 µg/ml	30	GeneArt- Invitrogen
pCB-Com1 plasmid	Carb 100 µg/ml	30	GeneArt- Invitrogen
pCB-Mip plasmid	Carb 100 µg/ml	30	GeneArt- Invitrogen
pRVFV-Gn plasmid	Carb 100 µg/ml	30	GeneArt- Invitrogen

### 3.2.1 Construction of conditionally attenuated and WT BoHV-4 vaccine vectors expressing a *Mb* protein fusion.

To construct BoHV-4 vectors expressing a *Mb* fusion protein required initial design of the *Mb* synthetic protein fusion. This fragment was comprised of six antigens: Rv3620, Rv1733, Rv2389, Rv3804 (Ag85A), Rv0287 (TB9.8) and Rv0288 (TB10.4) based on a set of selection criteria (see above). In addition to the synthetic gene encoding the *Mb* fusion protein, two synthetic homology regions were positioned to flank the *Mb* expression cassette. These flanking regions are homologous to the site of insertion of the *Mb* expression cassette within the BoHV-4 genome and are required for E/T-based recombination. Homology flanking regions were designated: i) pORF73Flank plasmid and ii) pWTFlank plasmid, and designed to insert the *Mb* expression cassette to replace ORF73, or at an intergenic *BstEII* site for construction of ORF73-deleted and WT vectors, respectively. Expression vectors were linearized prior to E/T-based insertion at the desired position within BoHV-4 genome by using E/T homologous recombination (Figure 3.1). A schematic of the cloning strategy is shown in Figure 3.2, Figure 3.3 and Figure 3.4.



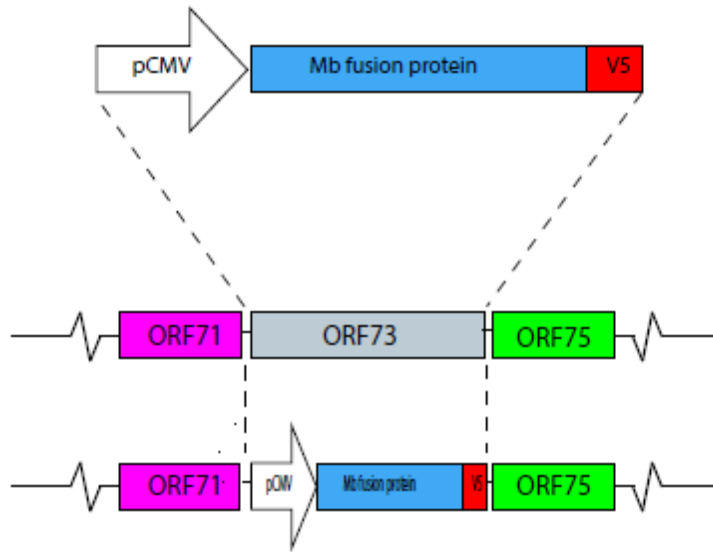


Figure 3.1: Schematic illustrating construction of BoHV-4 $\Delta$ ORF73/*Mb* vector. The *Mb* fusion protein expression cassette was inserted within BoHV-4 genome to replace the endogenous ORF73 by using E/T-recombination. Linearized vector carrying the Kan<sup>R</sup> marker and *Mb* expression cassette was electroporated into *E. coli* (strain EL250), a bacterial strain containing E/T recombinases. Following removal of the Kan<sup>R</sup> marker, the BoHV-4  $\Delta$ ORF73/*Mb* BAC was transfected into MDBK cells to reconstitute BoHV-4  $\Delta$ ORF73/*Mb* virus.

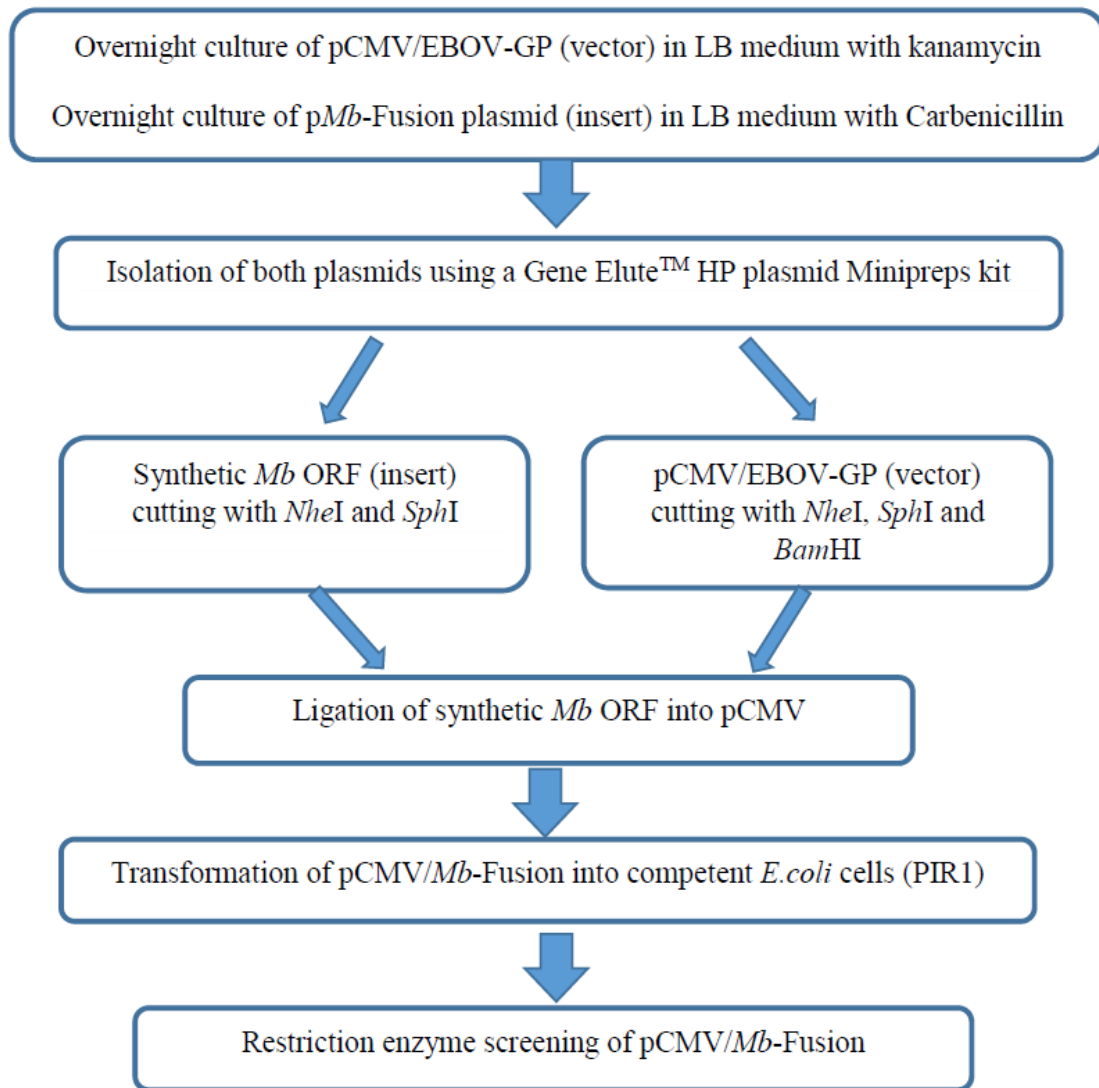


Figure 3.2: A flow-chart showing cloning *Mb* synthetic ORF into pCMV vector.

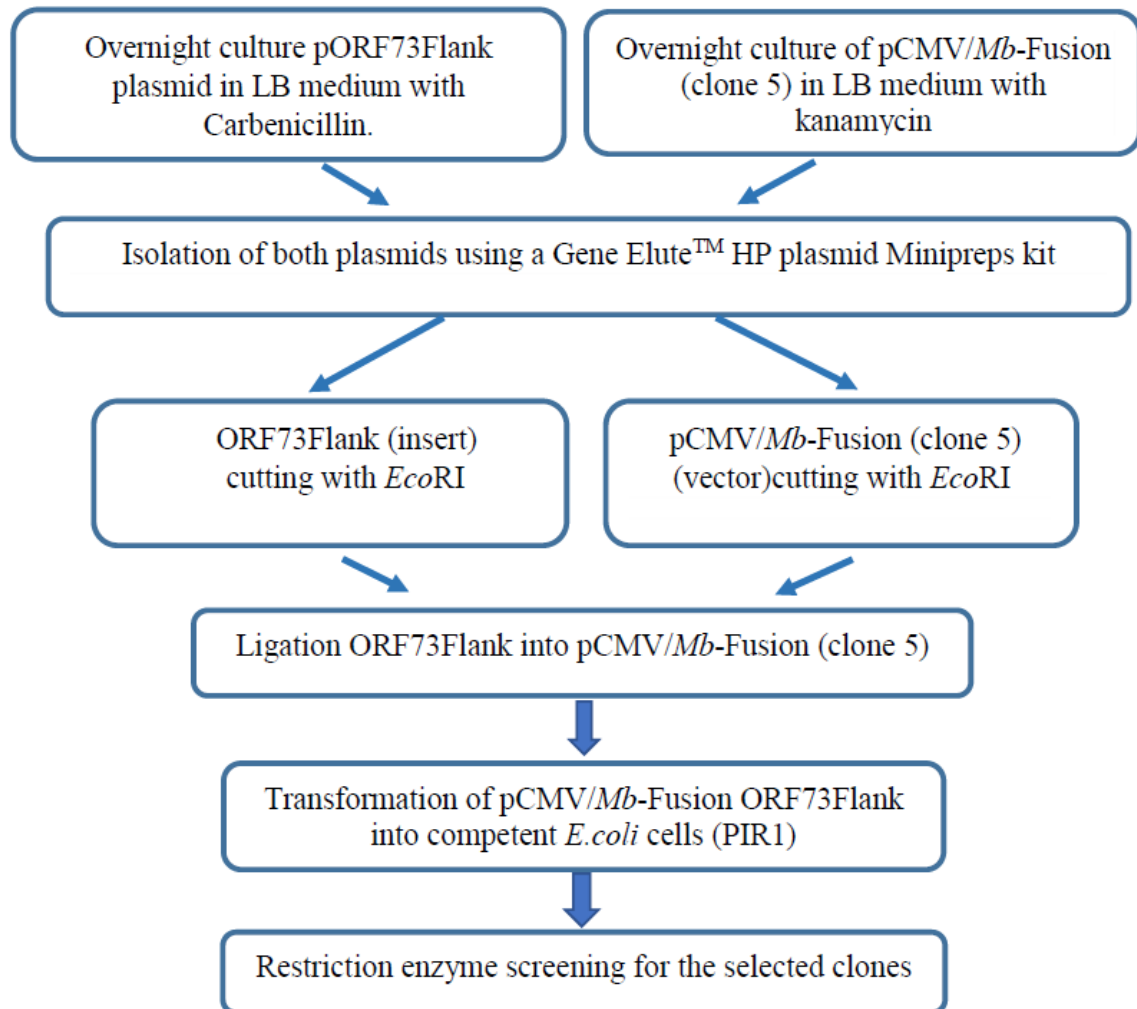


Figure 3.3: A flow-chart showing cloning flanking regions ORF73F flank into pCMV/Mb vector.

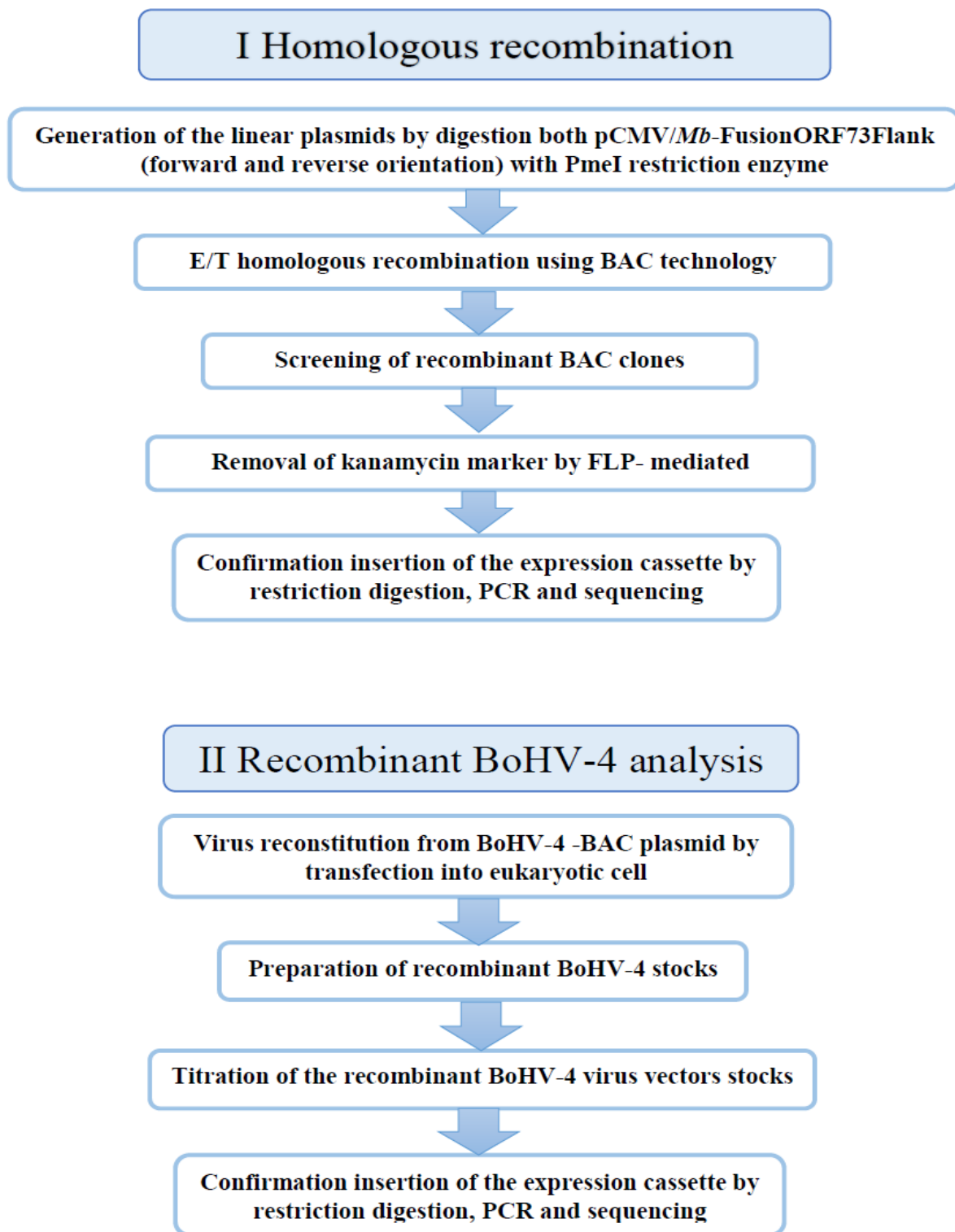
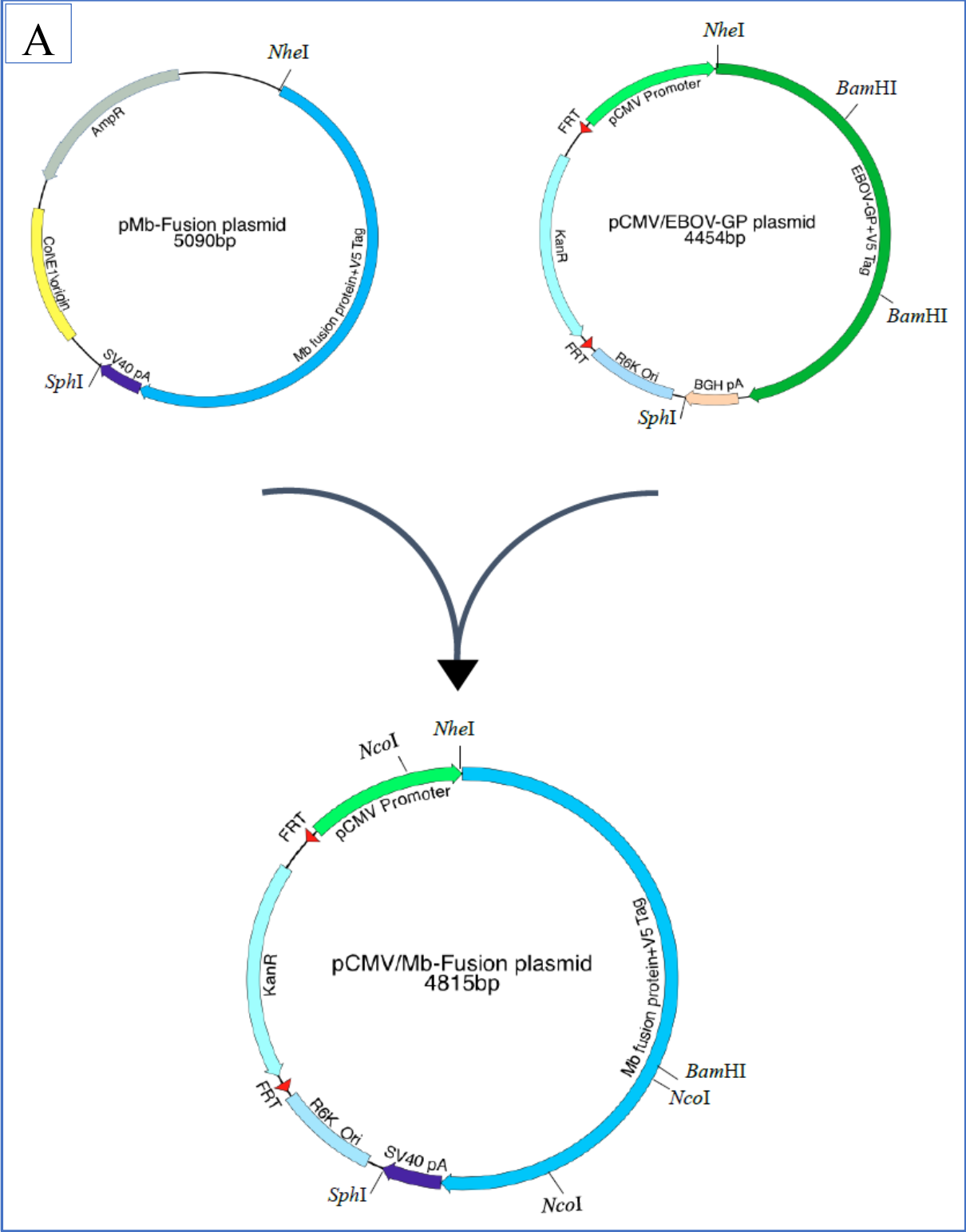


Figure 3.4: A flow-chart of construction of the BoHV-4 vector vaccine

### 3.2.1.1 Generation of *Mb* antigen expression cassette with flanking viral homology regions.

A plasmid containing the *Mb* expression cassette flanked by virus regions homologous to the site of insertion was generated. Following restriction enzyme-based linearization, this pCMV/*Mb*-FusionORF73Flank plasmid served as a source for generation of the recombination fragment required for insertion of the *Mb* expression cassette into the BoHV-4 genome by E/T recombination. This plasmid contains an immediate-early (IE) human cytomegalovirus promoter regulating *Mb* gene expression, a downstream simian virus 40 (SV40) polyadenylation site (PolyA), together with the necessary BoHV-4 homology regions flanking the site of insertion. The plasmid also contains a short (34 bp) FRT (FLP recognition target) recombination sequence-flanked Kan<sup>R</sup> selectable marker and a R6K $\gamma$  ‘suicide’ origin of replication. The use of a ‘suicide’ plasmid vector allows replication of the plasmid only in bacteria expressing the replication protein  $\pi$ , thereby preventing replication of the plasmid in EL250 bacteria, which lack the  $\pi$  protein, which avoids false-positive Kan-resistant clones following E/T-based recombination. Figure 3.5 shows a flow-chart diagram of the entire construction process.



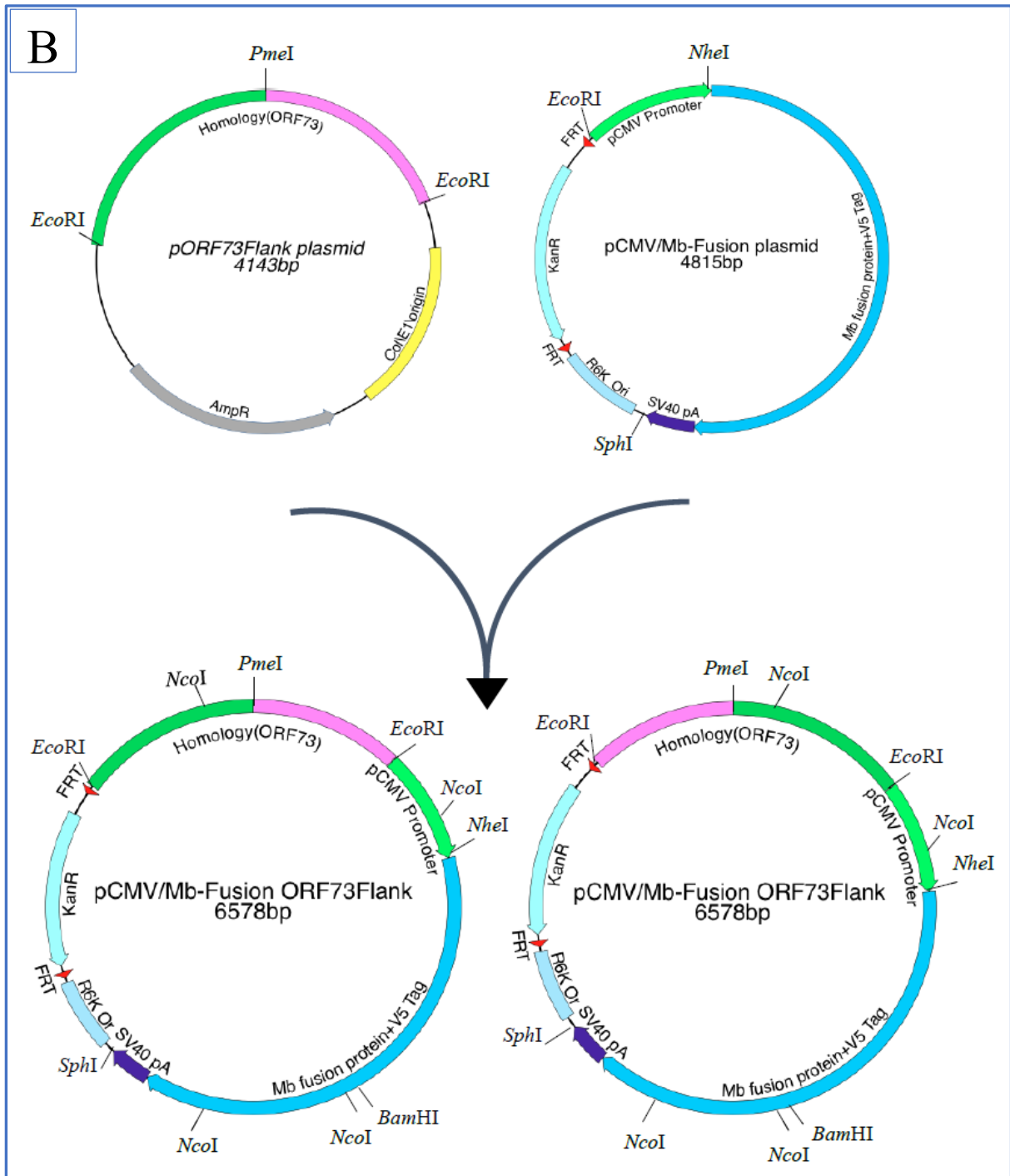


Figure 3.5: Schematic illustrating cloning strategy for generation of *Mb* Fusion protein expression cassette with viral homology flanking BoHV-4 ORF73. (A) The first step in the construction was cloning *Mb* synthetic ORF into pCMV (suicide-based vector) containing a Kan<sup>R</sup> gene flanked by FRT sites as well as *NheI* and *SphI* (for insertion of the *Mb* synthetic ORF). (B) The second step was cloning the flanking regions ORF73Flank into pCMV/*Mb*-Fusion at an *EcoRI* restriction enzyme site. As this cloning step is not directional, it results in two pCMV/*Mb*-Fusion ORF73Flank plasmid versions, either in forward or reverse orientation. These two plasmids were then used in construction of BoHV-4  $\Delta$ ORF73 *Mb* forward/reverse.

#### 3.2.1.1.1 Cloning of synthetic *Mb* ORF into the pCMV DNA plasmid vector

The synthetic *Mb* ORF contains six *Mb* antigens followed by a V5 tag sequence, SV40 pA, and restriction sites (*NheI* and *SphI*) required for insertion into the pCMV/EBOV-GP plasmid (to replace the EBOV-GP gene). The plasmid containing the *Mb* construct (p*Mb*-Fusion plasmid) was generated synthetically by GeneArt. The p*Mb*-Fusion plasmid was digested with *NheI* and *SphI* to release the *Mb*-Fusion gene. The pCMV/EBOV-GP plasmid used as the source of the pCMV vector contains the Ebola virus (EBOV) glycoprotein (GP) gene. The cloning strategy required excision of the EBOV GP gene prior to insertion of the *Mb* synthetic ORF into the pCMV vector. The pCMV/EBOV-GP vector was digested with *NheI*, *SphI* and *BamHI*. The EBOV GP gene contains internal *BamHI* sites, and *BamHI* digestion was used to fragment the GP EBOV gene.

As shown in Figure 3.6 in lane 10, four bands were observed following restriction enzyme digestion: the upper band of approximately 2.1 kbp corresponded to the pCMV linearized vector and the lower bands (971 bp, 751 bp and 629 bp) were the EBOV-GP digested fragments. The *NheI* x *SphI* digested synthetic *Mb* ORF 2712 bp fragment was then ligated into the 2103 bp pCMV vector containing the 'suicide' R6K $\gamma$  origin of replication, generating pCMV/*Mb*-Fusion. The ligated DNA was transformed into competent PIR1 *E.coli*. Transformed cells were plated onto LB agar plates with Kan 50 $\mu$ g/ml. To determine whether the synthetic *Mb* ORF had been inserted into the pCMV vector to replace the EBOV GP, 9 colonies were picked from the plates and grown overnight in LB broth with Kan 50 $\mu$ g/ml. Plasmid DNA was isolated from these clones using a plasmid Miniprep kit and analysed by restriction enzyme digest using *NcoI* and *BamHI*. As shown in Figure 3.7A, clone 5 has the anticipated three bands after digestion with *NcoI*: an upper band of approximately 2561 bp, a middle band of approximately 1687 bp in size, and a lower band of 567 bp. *BamHI* digestion linearized clone 5 as expected and is shown in Figure 3.7 B.



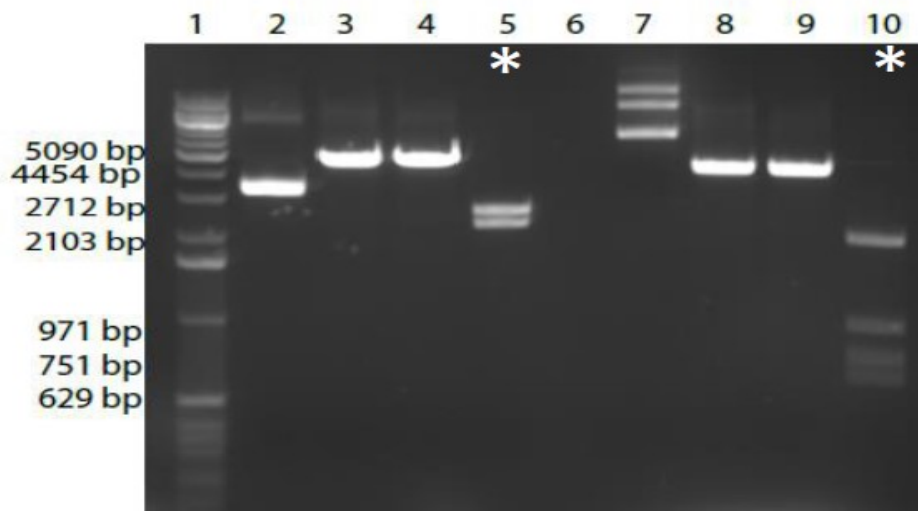


Figure 3.6: Gel electrophoresis showing *NheI* and *SphI* digestion of synthetic *Mb* ORF-plasmid (GeneArt) and *NheI*, *SphI* and *BamHI* digestion of pCMV/EBOV-GP (vector) plasmid during cloning of pCMV/*Mb*-Fusion. Lane 1: DNA ladder (1 Kb DNA extension ladder), Lane 2: uncut synthetic *Mb* ORF-plasmid (GeneArt), Lanes 3, 4 and 5 show the restriction digestion of synthetic *Mb* ORF-plasmid (GeneArt), single digested *NheI* (lane 3), *SphI* (lane 2) and double digested *NheI*, *SphI* (lane 5). DNA fragment of 2712 bp indicating synthetic *Mb* ORF (lane 5). The uncut vector (lane 7) was compared to single digested *NheI* (lane 8), *SphI* (lane 9) and triple digested *NheI*, *SphI* and *BamHI* (lane 10), synthetic *Mb* ORF 2712 bp was the cloned in to *NheI*, *SphI* sites of pCMV (vector). The multiple bands that appear in lane 2 and lane 7(uncut plasmids) represent supercoiled, relaxed circle and linear (maybe not linear) DNA. The DNA bands have the same size but migrate differently based on distinct superhelical structure.

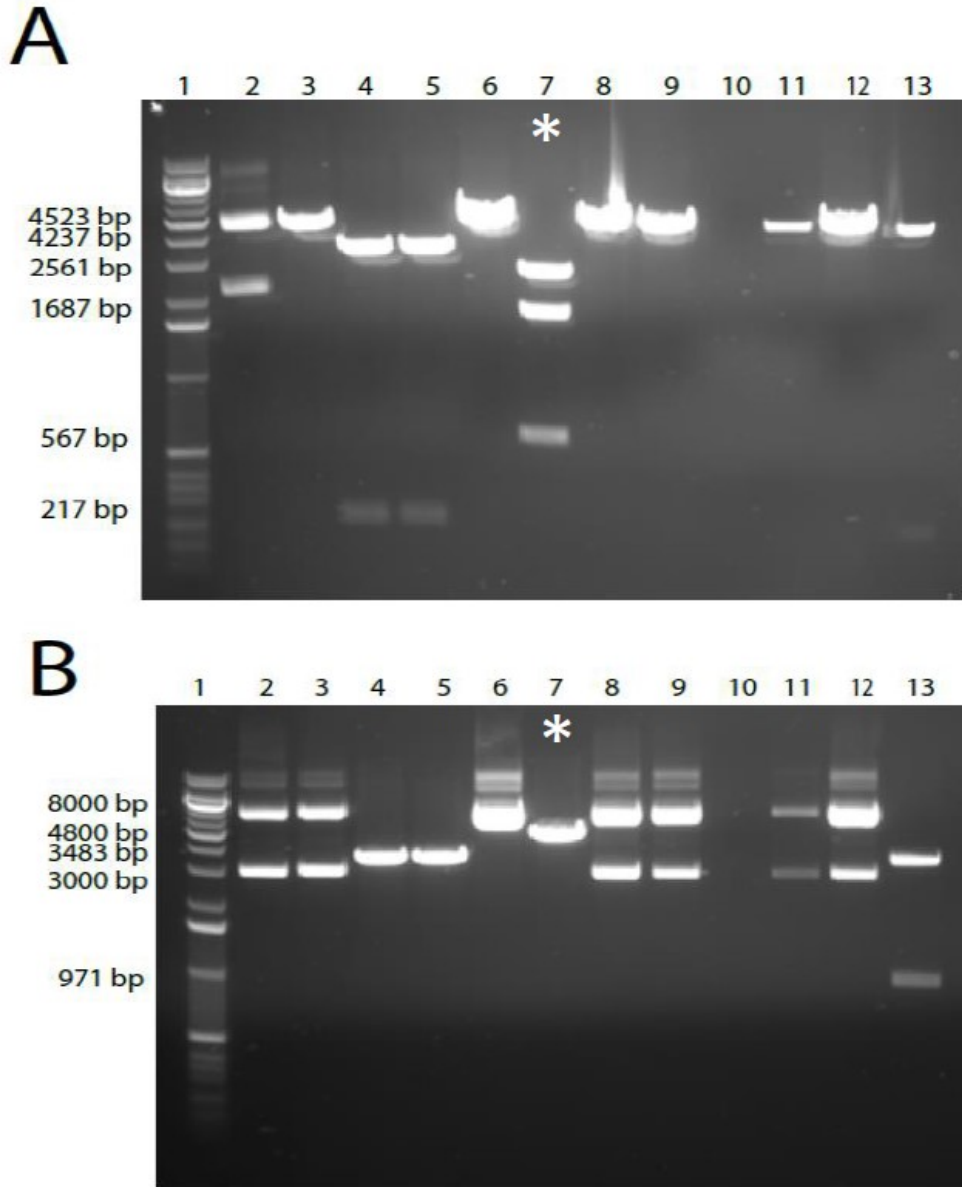


Figure 3.7: Restriction enzyme screening of pCMV/*Mb*-Fusion clones. Lane 1: DNA ladder (1 Kb DNA extension ladder). Lane 2: shows uncut pCMV/*Mb*-Fusion clone. Lanes 3-9, 11 and 12: nine screened clones. Lane 13: shows parental plasmid pCMV/EBOV-GP. (A) plasmids digested with *Nco*I . Lane 7 (clone 5) show confirmed of insertion of *Mb* into pCMV following digestion with *Nco*I lane 7. Three bands are observed; upper band of approximately 2561 bp. Middle band of approximately 1687 bp and lower the lower bands of approximately 567 bp. The size of parental EBOV digested band with *Nco*I are 4237 bp and 217 bp. (B) plasmids digested with *Bam*HI. Lane 7 (clone 5) show confirmed of insertion of *Mb* into pCMV following digestion with *Bam*HI lane 7 (clone 5), the plasmid was linearized after digested with *Bam*HI. The size of parental pCMV/EBOV-GP digested band with *Bam*HI are 3484 bp and 971 bp. The yellow asterisk at lane 7 represents the clone that was selected (clone number 5).

### 3.2.1.1.2 Cloning of *Mb* antigen into pCMV/ ORF73Flank to generate a *Mb* antigen expression cassette with virus homology flanking ORF73

The next step was to genetically flank the *Mb* expression cassette with regions of BoHV-4 flanking the ORF73 site of insertion, which is necessary for E/T based recombination of the cassette to within the BoHV-4 BAC genome. A plasmid containing two flanking regions homologous to ORF71 and ORF75 separated by a unique single *PmeI* site required for linearization and a single *EcoRI* restriction site to enable insertion into the pCMV/*Mb*-Fusion were synthesized using an AmpR carrier vector from GeneArt (Thermo Fisher Scientific, UK). *PmeI* was selected based on its absence from flanking homology regions and from the pCMV plasmid and pathogen ORFs being targeted (see below).

The pORF73Flank plasmid, containing homology to ORF71 and ORF75 was designed to target the *Mb* expression cassette from pCMV/*Mb*-Fusion into the BoHV-4 genome to replace the ORF73 locus. The pORF73Flank plasmid contained 791 nucleotides homologous to ORF71 - Bo13 (nts 98230 to 99021) and 964 nucleotides homologous to ORF75 (nts 99784 to 100748). These homologous regions flanking the ORF73 locus are required to target the E/T homologous recombination machinery to the desired site within the BoHV-4 BAC. It had been shown that 36 nucleotides sequences is the minimum size needed for E/T recombination (Wagner *et al.*, 1999; Wagner, Ruzsics & Koszinowski, 2002). The *EcoRI* fragment containing the ORF71- /ORF75 flanking regions was excised from pORF73Flank with *EcoRI* digestion and then cloned into pCMV/*Mb*-Fusion, also digested with *EcoRI*. After ligation the plasmid was transformed into competent PIR1 *E. coli* cells and plated onto LB agar plate containing Kan 50µg/ml and incubated at 30°C. The following day 10 single colonies were picked from the plates and grown overnight with Kan 50µg/ml. Plasmid DNA was extracted and purified using a plasmid Miniprep kit (see section 2.2.1.1) and analysed by restriction enzyme digest. *NcoI*

was used to confirm that the fragment containing the ORF71/ORF75 flanking regions had been cloned into pCMV/*Mb*-Fusion. Ten clones were screened using *Nco*I restriction enzyme digestion. Results shown in Figure 3.8 indicate that five of the 10 clones contain the ORF71/ORF75 flanking regions correctly inserted into the pCMV/*Mb*-Fusion plasmid, as four bands was observed (plasmid designated pCMV/*Mb*-FusionORF73Flank).

Surrounding genes and promoters can influence expression of the heterologous gene following their insertion into the herpesvirus genome (Gillet *et al.*, 2005). Restriction enzyme analysis was used to determine the orientation of the expression cassette in relation to the flanking ORF71/ORF75 regions prior to use in homologous recombination. Orientation of the expression cassette within the vector (the recombinant fragment) was determined based on the size of the digested plasmid with *Nco*I. Clones 1 and 7 had four bands associated with a forward (*Mb*-Fusion ORF translated from leading strand) orientation (2775 bp, 1687 bp 1549 bp and 567 bp). Clones 6, 9 and 10 showed a 3342 bp, 1687 bp, 982 bp and 567 bp band corresponding to a reverse (*Mb*-Fusion ORF translated from lagging strand) orientation. The *Nco*I restriction digestion showed clones 2, 3, 5 and 8 to be the parental pCMV/*Mb* (2561 bp, 1687 bp and 567 bp). Clone 4 was also incorrect showing an extra about 1500 bp in size. Two clones were selected: clone 1 (forward orientation) and clone 6 (reverse orientation). These clones were used for construction of BoHV-4 $\Delta$ ORF73*Mb* (forward orientation) and BoHV-4 $\Delta$ ORF73*Mb* (reverse orientation).

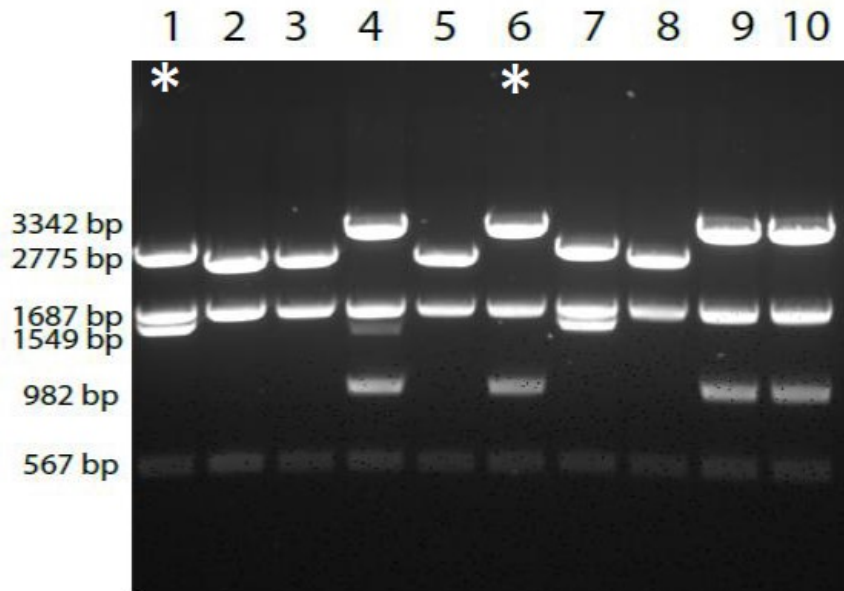


Figure 3.8: Restriction enzyme screening of pCMV/*Mb*-FusionORF73Flank using *Nco*I. Lane 1-10 show digestion of ten clones using *Nco*I. Lane 1, 6, 7, 9 and 10 (clone 1, 6, 7, 9 and 10) show confirmed insertion of the ORF73 flanking regions into pCMV/*Mb* following digestion with *Nco*I four bands are observed. Determination the orientation of the expression cassette within the vector based on the size of these bands. Clone 1 and 6 were in forward orientation. The size of pCMV/*Mb*-FusionORF73Flank (forward orientation) digested bands with *Nco*I are 2775 bp, 1687 bp, 1549 bp and 567 bp. Clone 6, 9 and 10 in reverse orientation. The size of pCMV/*Mb*-FusionORF73Flank (reverse orientation) digested bands with *Nco*I are 3342 bp, 1687 bp, 982 bp and 567 bp. Lane 2, 3, 5 and 8 shows parental plasmid pCMV/*Mb* digested bands. The size of parental pCMV/*Mb* digested bands with *Nco*I are 2561 bp, 1687 bp and 567 bp. The yellow asterisk at lane 1 represents the clone that was selected (forward orientation); the yellow asterisk at lane 6 represents the clone that was selected (reverse orientation).

### 3.2.1.2 Construction of recombinant BoHV-4 $\Delta$ ORF73*Mb* by E/T homologous recombination

The two independent BoHV-4 $\Delta$ ORF73*Mb* virus vectors differing in orientation of the *Mb*-Fusion expression cassette in respect to surrounding BoHV-4 genes were constructed by E/T-based recombination by using pCMV/*Mb*-FusionORF73Flank clones 1 and 6 as a source of the recombination fragments. To derive the recombination fragment, pCMV/*Mb*-FusionORF73Flank clones 1 and 6 were linearized by digestion with *Pme*I at 37°C for 2 hours. Digested plasmids were then purified using a Pure Link™ PCR Purification Kit. These

linearized fragments containing the *Mb* expression cassette and Kan<sup>R</sup> marker were then transformed by electroporation into EL250 electrocompetent cells that express recombinase enzymes required for the process of recombination (described in section 2.2.8.2). Recombinant clones were selected on the basis of Kan and Cam resistance. Five recombinant clones were selected for further characterization. Overnight cultures for these clones were performed and then the recombinant BACs DNA were isolated by alkaline lysis (Sambrook & Russell, 2001) as described in section 2.2.1.2.

#### 3.2.1.2.1 Restriction digestion of BoHV-4 $\Delta$ ORF73*Mb* BAC DNA

Restriction digest analysis was carried out to ensure that the recombinant BACs clones had retained intact genomes. Briefly, selected recombinant clones were grown overnight followed by purification of BACs by using the alkaline lysis method. BAC DNA was digested with *EcoRI* followed by then separation and visualization by DNA gel electrophoresis. As shown in Figure 3.9, the resulting *EcoRI* digested fragments of BoHV-4 $\Delta$ ORF73*Mb* BAC were compared with *EcoRI* digested fragments of WT BoHV-4 BAC. The results showed absence of large genome rearrangement.

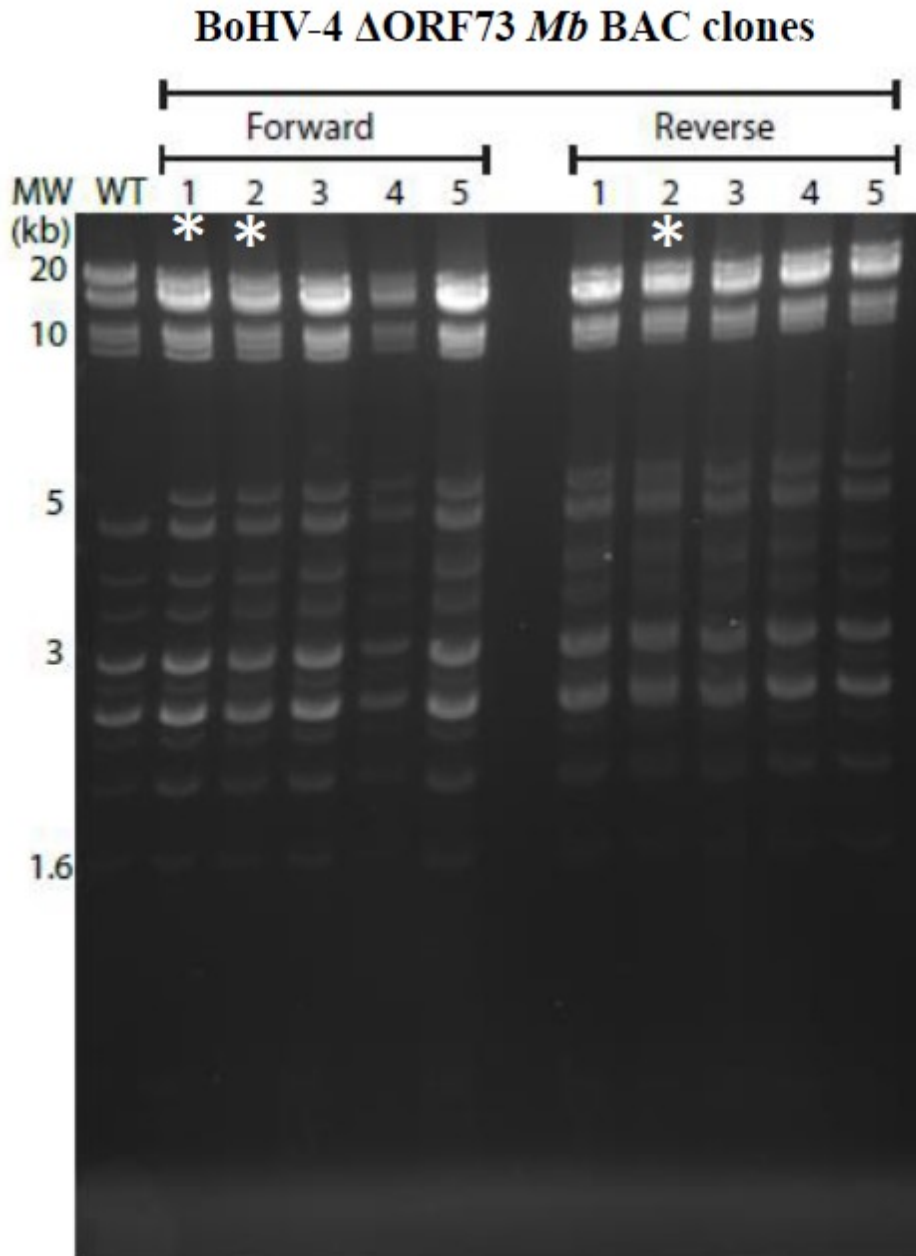


Figure 3.9: Characterization of the BoHV-4  $\Delta$ ORF73 *Mb* Forward/Reverse BAC genome by restriction enzyme digestion. BoHV-4 -WT and five independent clones from BoHV-4  $\Delta$ ORF73 *Mb* Forward and BoHV-4  $\Delta$ ORF73 *Mb* Reverse BACs were digested with *Eco*RI (restriction enzyme). *Eco*RI digested fragments were then separated according their molecular weight by electrophoresis at 45 V for 18-20 hours and compared with the wild type.

#### 3.2.1.2.2 Excision of kanamycin cassette from BoHV-4 $\Delta$ ORF73Mb

In order to remove the Kan<sup>R</sup> from the BoHV-4 $\Delta$ ORF73Mb BAC recombinants, three clones were selected: clone 1 and clone 2 in forward orientation and clone 2 in reverse orientation (Figure 3.9). The Kan<sup>R</sup> marker used for selection of recombinant BoHV-4 $\Delta$ ORF73Mb BACs is flanked by two FRT sites (FLP recognition target), a recombination sequence recognition sequence of 34 bp in size (Zhang *et al.*, 1998), which enables subsequent Kan<sup>R</sup> marker removal by induction of FLP recombinase from *Saccharomyces cerevisiae*. Induction of FLP-recombinase in the EL250 via arabinose induction mediates recombination between two FRT sites leading to excision of the Kan<sup>R</sup> gene leaving a single FRT ‘scar’ (Wagner *et al.*, 1999). The Kan<sup>R</sup> marker was removed from BoHV-4 $\Delta$ ORF73Mb BAC clones by FLP-mediated recombination. The resulting clones were screened for loss of Kan<sup>R</sup> by replica plating on Cam/Kan and Cam plates. Clones that grew on Cam but not on Cam/Kan plates were selected and further characterized. See Figure 3.10.

To confirm the insertion of the recombinant fragment and then excision of the Kan<sup>R</sup> cassette from recombinant BAC genomes, PCR analysis was performed using ORF73 flanking forward and reverse primers (see section 2.2.3.2). These primers were located outside ORF73 genome within the ORF73 flanking regions at 38-134 bps distant from the site of insertion of the recombinant fragment that contains the expression cassette, R6K $\gamma$  origin of replication and Kan<sup>R</sup> marker. The ORF73 flanking primers were used to amplify the inserted fragments. PCR was performed on recombinant BoHV-4 $\Delta$ ORF73Mb BAC DNA pre-FLP (clone 2) and post-FLP (clone 2-6) and the WT BoHV-4 BAC DNA (Figure 3.11 ). The size of the amplified PCR products 5008 bp (lane2) and 4004 bp (lane3) indicated insertion of the recombinant fragment (pre-FLP) and excision of the Kan<sup>R</sup> cassette from the recombinant fragment in (post-FLP). The reduction in size by 1000 bp after FLP-mediated removal of the Kan<sup>R</sup> cassette was consistent with excision of Kan<sup>R</sup>. Excision of Kan<sup>R</sup> was confirmed by direct DNA sequencing (Figure



3.12), showing the single predicted FRT sequence followed by ORF75, and absence of the Kan<sup>R</sup> gene and the second FRT site. For sequencing, PCR products were spin column purified (see section 2.2.6). Ten sequencing primers were designed, five in forward orientation and another five primers in reverse orientation to ensure sequence coverage of the entire region in both directions. Two mutations were detected, the first in the pCMV promoter and a second in the suicide' R6K $\gamma$  origin of replication gene. Neither of these mutations prevented expression of *Mb* fusion construct (see below).

### BoHV-4 $\Delta$ ORF73 Mb BAC clones

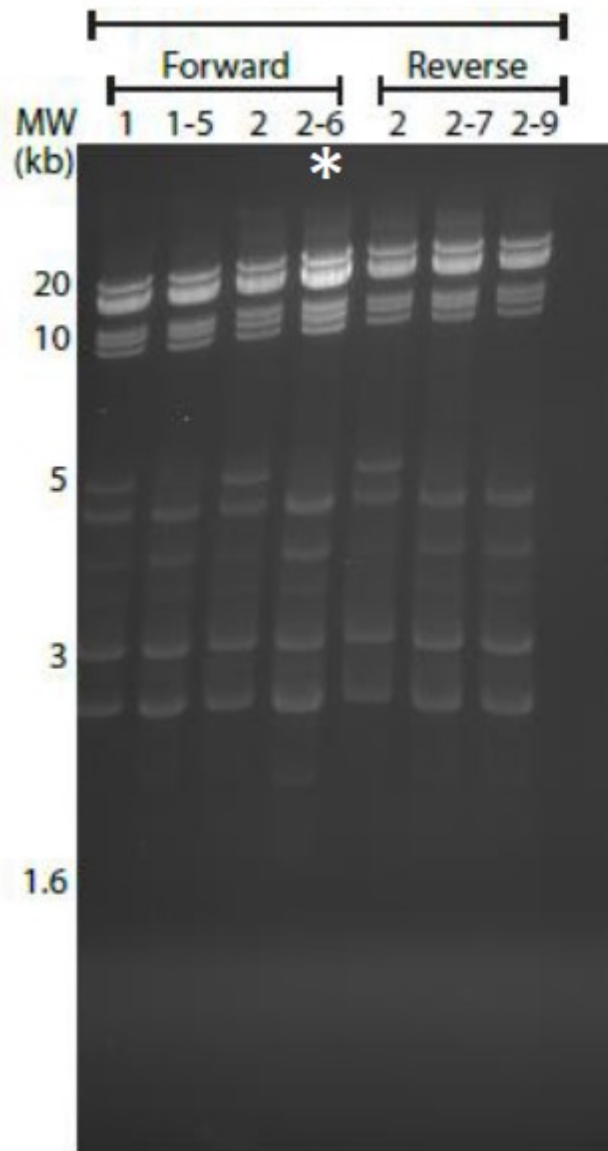


Figure 3.10: Characterization of the post-FLP BoHV-4  $\Delta$ ORF73 Mb Forward/Reverse BAC genome and WT BoHV-4 Mb BAC by restriction enzyme digestion. BoHV-4  $\Delta$ ORF73 Mb Forward/Reverse pre- FLP( Forward clone 1 and 2, Reverse clone 2) and post-FLP(Forward clone 1-5 and 2-6, Reverse clone 2-7 and 2-9) and WT BoHV-4 Mb pre- FLP( Forward clone 4, Reverse clone 2) and post-FLP(Forward clone 4-13 and 4-20, Reverse clone 2-4 and 2-5) BACs were digested with *Eco*RI ( restriction enzyme). *Eco*RI digested fragments were then separated according their molecular weight by electrophoresis at 45 V for 18-20 hours.

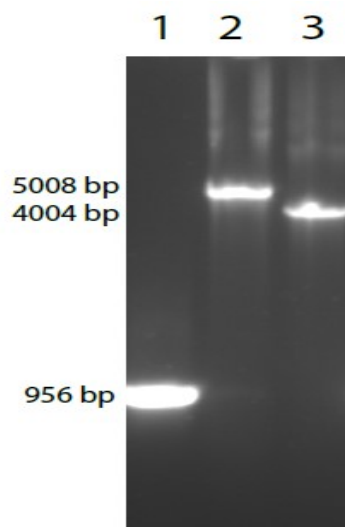


Figure 3.11: Confirmation of insertion of the *Mb* expression cassette within BoHV-4 and the Kan<sup>R</sup> marker removal from the post-FLP BoHV-4 $\Delta$ ORF73*Mb* BAC genome by PCR. DNA from clone 2 and clone 2-6 forward orientation of recombinant BoHV-4 $\Delta$ ORF73*Mb* BACs (pre and post FLP) were screened by PCR using ORF 73 flanking primers, lane 2 and lane 3 respectively. PCR products were separated on a 1% agarose gel for 60-90 minutes at 75 V, and visualized by staining with EtBr (0.5  $\mu$ g/ml). PCR of pre- (as shown in lane2) and post-(as shown in lane 3) BoHV-4 $\Delta$ ORF73*Mb* yielded PCR products of approximately 5000 bp and 4000 bp, respectively. Lane1 shows PCR product of the wild type BoHV-4.

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CTTCTGAATTCGATGCGCGAATTCCTTTGGCAGAAAGAA

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Figure 3.12: Confirming presence *Mb* expression cassette in BoHV-4 $\Delta$ ORF73*Mb* BAC genome via Sanger DNA sequencing. PCR was performed on post-FLP BoHV-4 $\Delta$ ORF73*Mb* BAC clones clone (2-6) followed by DNA sequencing. Five primers in forward orientation and another five in reverse orientation were used in sequence the amplified region because the size of the region about 40004 bp. The pink represent upstream homologous region (ORF71), light green represent pCMV promoter, dark blue represent *Mb* Fusion protein, black represent V5 and SV40 pA, light blue represent suicide Ori, the red represent FRT and the dark green ORF75.

### 3.2.1.3 Reconstitution of BoHV-4 $\Delta$ ORF73Mb vaccine in permissive eukaryotic cells

Genomic DNA of herpesvirus is infectious, meaning that introduction of the viral genome contained within a BAC into BoHV-4 permissive eukaryotic cells will result in reconstitution *de novo* of progeny virus (Gillet *et al.*, 2005; Roizman & Pellett, 2001). To reconstitute BoHV-4 $\Delta$ ORF73Mb viruses, BAC DNA from selected clones was introduced into Madine Darby bovine kidney (MDBK) cells through transfection by using Lipofectamine<sup>TM</sup>3000 (L3000-008, Invitrogen). MDBK cells were monitored daily for signs of virus-associated cytopathic effect (CPE), including development of virus plaques. Cultures were also assessed for GFP expression as the BAC cassette contains a GFP gene (Gillet *et al.*, 2005; Storz *et al.*, 1984).

All viruses could be reconstituted, with GFP-positive virus plaques being visible in cell monolayers at day 5-7 after transfection suggesting that expression of the Mb-Fusion target antigen was not toxic and prohibitive to BoHV-4 reconstitution and replication. BoHV-4 $\Delta$ ORF73Mb and WT BoHV-4 plaques were picked and used to prepare seed stocks and concentrated virus stocks.

### 3.2.1.4 Expression of the Mb-Fusion protein in BoHV-4 $\Delta$ ORF73Mb

The Mb-Fusion protein was tagged with a V5 epitope at the carboxyl terminus in order to facilitate detection of protein expression. Western blot analysis using BoHV-4 $\Delta$ ORF73Mb infected MDBK cell lysates was used to determine whether recombinant viruses expressed the Mb-Fusion protein. Using a V5 epitope tag specific monoclonal antibody, a V5 signal of the predicted 87kDa size was detected in lysates from all BoHV-4 $\Delta$ ORF73Mb recombinants. Following two serial passages, seed stocks and then final purified stocks were prepared, and expression was again confirmed by western immunoblot (Figure 3.13).

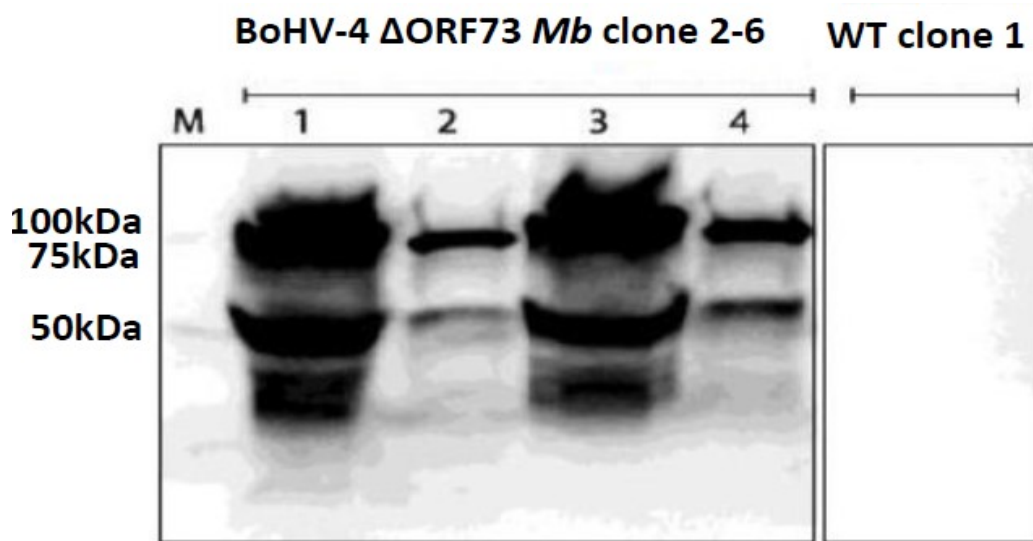


Figure 3.13: BoHV-4 *Mb* expression. MDBK cells were infected with two independent concentrated virus stocks of BoHV-4  $\Delta$ ORF73 *Mb* clone 2-6. Monolayer were harvested at 100% CPE. Cell lysates (1) & (2) undiluted and 1:10 dilution of stock A, and (3) & (4) undiluted and 1:10 dilution of stock B respectively, were analysed by western blot using anti-V5 antibody to detect the V5 tagged *Mb* fusion protein (87 kilo Daltons). Smaller bands presumably represent proteolytic fragment (section 2.4.6).

#### 3.2.1.5 Confirmation of the absence of BoHV-4 WT from recombinant BoHV-4 $\Delta$ ORF73 *Mb*

Characterization of BoHV-4 $\Delta$ ORF73*Mb* virus stocks was critical prior to use in subsequent animal experiments. BoHV-4 $\Delta$ ORF73*Mb* (clone 2-6) virus stock was analyzed to ensure *Mb* expression cassette sequence was intact and mutations had not been acquired during the reconstitution process that affected expression of the *Mb* fusion protein. It was also necessary to confirm the absence of BoHV-4 WT contamination. PCR amplification was carried out using viral DNA extracted from concentrated stocks (see 2.2.1.3). This was followed by direct DNA sequencing of PCR products. PCR amplification reactions for BoHV-4 $\Delta$ ORF73*Mb* clone (2-6) and BoHV-4 WT were performed using the same primers used previously to confirm excision of Kan<sup>R</sup> from recombinant BoHV-4 BAC (ORF73 flanking primers) (see Table 2.3). Results showed bands of the expected sizes of 956 bp and 4004 bp for BoHV-4 WT lane (1), and recombinant BoHV-4 $\Delta$ ORF73*Mb* (lane 2), respectively (Figure 3.14). These results

confirmed the presence of the *Mb* expression cassette. These results also showed the absence of BoHV-4 WT contamination of recombinant stocks. Sequence data confirmed the presence of the full-length *Mb* fusion protein in the correct genomic position, with the absence of mutations (Figure 3.15).

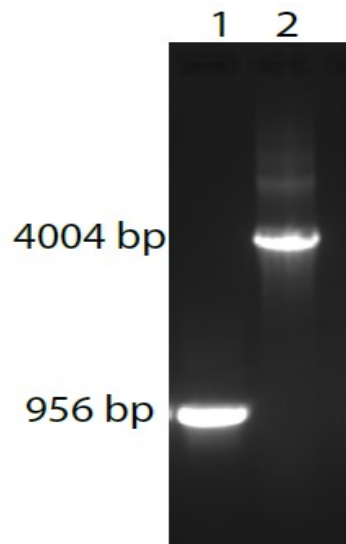


Figure 3.14: Confirming presence of the *Mb* expression cassette in BoHV-4 $\Delta$ ORF73*Mb* virus clone (2-6) by PCR. DNA was isolated from BoHV-4 $\Delta$ ORF73*Mb* concentrated stock and from BoHV-4 WT virus, using a QIAamp kit and PCR was carried out using ORF73 flanking primer. PCR products were separated on a 1% agarose gel for 60-90 minutes at 75 V. Lane (1) show 956 bp band of the BoHV-4 wild type virus. The presence of a 4004 bp band in recombinant virus lane (2) confirmed that the recombinant fragment containing *Mb* expression cassette was present, and the absence of the BoHV-4-WT sized band (956 bp) in the BoHV-4 $\Delta$ ORF73*Mb* virus confirmed the absence of WT contamination of recombinant.

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AATCCCTTTGGCAGAAAGAA

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Figure 3.15: Conformation presence of *Mb* expression cassette within BoHV-4ΔORF73*Mb* virus genome via Sanger sequencing. PCR was performed on the DNA extracted from concentrated virus stocks clone (2-6). PCR products were sequenced. The pink represent upstream homologous region (ORF71), light green represent pCMV promoter, dark blue represent *Mb* Fusion protein, black represent V5 and SV40 pA, light blue represent suicide Ori, the red represent FRT and the dark green ORF75.



### 3.2.2 Construction of conditionally-attenuated BoHV-4 $\Delta$ ORF73 vectors expressing *CB* antigens Com1 and Mip, and RVFV Gn.

Similar to *Mb*, *CB* is an obligate intracellular bacterial zoonotic pathogen that infects both humans and ruminant animals and has historically been a cause of major outbreaks in many developing and developed countries such as UK (Van Woerden *et al.*, 2004), Netherlands (Delsing, Kullberg & Bleeker-Rovers, 2010; Karagiannis *et al.*, 2007; Schimmer *et al.*, 2008), Portugal (Cruz *et al.*, 2018) and in Iraq among member of US military (Anderson *et al.*, 2005; Faix *et al.*, 2008; Raoult, 2009). RVFV, whilst being a bunyavirus, shares host target (ruminants) and clinical pathology with *CB*. The aim of this next body of work was to construct separate additional BoHV-4 vectors individually expressing *CB* and RVFV antigens as prototype vectors that could be broadly used in all ruminant species. The possibility for oral administration is a key potential advantage over current injectable vaccines, which can potentially spread these diseases if used during an outbreak.

As indicated above, the initial step in construction of BoHV-4 vectors is creation of a recombination plasmid containing an expression cassette of the target antigen flanked by homology regions to direct insertion to within the desired region of the BoHV-4 genome. Repeated attempts at cloning of the synthetically generated *CB* P1HspB1 ORF into the pCMV vector proved unsuccessful, with none of the screened clones by restriction enzyme digestion showing the predicted DNA band sizes. An alternative strategy was therefore developed to clone the P1HspB1 ORF directly into the pCMV/*Mb*-FusionORF73Flank (forward and reverse) to replace the *Mb*-Fusion gene. The P1HspB1 ORF was excised from the pCB-P1HspB1 plasmid using *NheI* and *SphI* restriction enzymes, and then ligated into both forward and reverse versions of the 3.9 kb fragment representing the vector from a *NheI*, *SphI* and *BamHI* digest of pCMV/*Mb*-FusionORF73Flank plasmids; *BamHI* was included to remove the

*Mb* ORF, which contains *Bam*HI sites. More than 500 colonies were observed for the recombinant plates, whereas the number of clones on non-insert control plates was 39. However, screening of over 50 colonies by restriction enzyme digestion showed none of the clones to contain a band of the correct size corresponding to the *CB* P1HspB1 ORF.

It was hypothesized that one or both of the synthetic genes P1 or HspB1 was toxic or otherwise incompatible with cloning into the recombination expression vector in bacteria, resulting in an inability to clone the P1HspB1 synthetic ORF into *Mb* backbone plasmids. Two strategies were used in an attempt to overcome this problem. The first strategy was to insert a synthetic intron (called CI) within the *CB* P1HspB1 ORF to inhibit the expression of the *CB* P1HspB1 in *E. coli*. The intron is a sequence of non-coding DNA that prevents expression of the gene in prokaryotic cells when it is inserted within its sequences. In eukaryotic cells, the intron does not affect expression as it is removed during splicing from the pre mRNA during transcription in the nucleus. This strategy was used previously to block the expression of Cre-recombinase that was required for excision of a floxed BAC cassette from a recombinant herpesvirus genome (Smith & Enquist, 2000).

The second strategy was to amplify the P1 gene and the HspB1 gene then clone each one separately. Two sets of primers were designed: the first set to amplify the P1 gene and the second set for amplification of the HspB1 gene. After a GCGC anchor region, each primer contained restriction enzyme sites to enable insertion into pCMV/ORF73Flank vector. The p*CB*-P1HspB1 plasmid was used as the template. PCR products were then visualized on a 1% (w/v) agarose DNA gel. The results indicated that the P1 and HspB1 gene had been amplified, resulting in production of a single, well defined DNA band at the expected molecular size for P1 (971 bp) and for HspB1 (1889 bp) as shown in Figure 3.16. PCR products were spin column purified, digested and then ligated separately into digested pCMV/*Mb*-FusionORF73Flank vectors in order to insert P1 or HspB1 in place of the synthetic *Mb* ORF. No colonies were

identified for HspB1. Approximately 90 colonies grew on the P1 plates, but none of them were positive for the P1 insert (clones contained only the vector plasmid). The repeated inability to clone the P1 gene and the HspB1 gene into the pCMV expression plasmid was consistent with a toxic effect of P1 and HspB1 proteins in bacterial cells. This toxicity may occur at the DNA level, but is generally associated with repeat regions, of which none were observed in either HspB1 or P1. It could also potentially occur at the level of the protein, as the pCMV promoter has been shown to be active in bacterial cells (Lewin *et al.*, 2005).

As an alternative to the HspB1 and P1 strategy, three additional *CB* antigens (SecB, Com1 and Mip) were selected for expression in the BoHV-4  $\Delta$ ORF73 vectors. All three antigens had been shown to be immunogenic in previous studies (Wei *et al.*, 2011; Xiong *et al.*, 2012; Zhang & Samuel, 2003). Specifically, mice receiving bone marrow-derived dendritic cells stimulated with these antigens induced interferon gamma producing Th1 cells. Com1 and Mip also induced CD8<sup>+</sup> CTL cells. Th1 responses were also shown to be associated with enhanced clearance of *CB* (Wei *et al.*, 2011; Xiong *et al.*, 2012). Cloning of SecB into pCMV/ORF73Flank forward and reverse vectors proved unsuccessful, while Com1 and *CB* Mip were successfully cloned into the recombination vectors (detailed below).

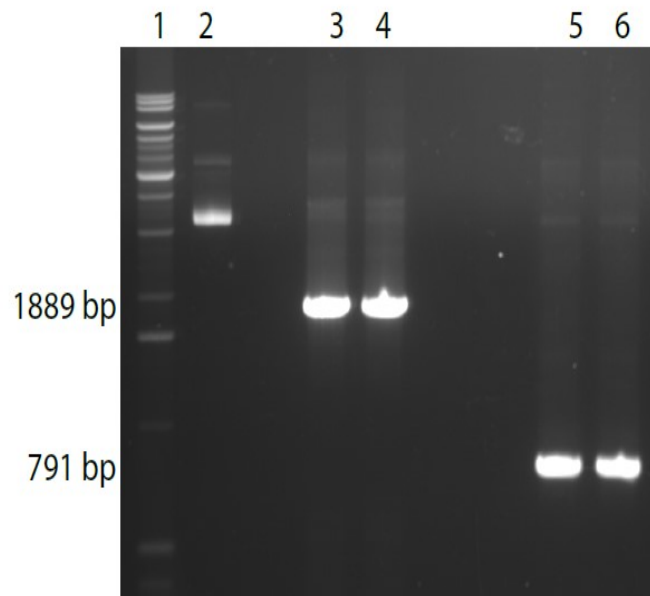


Figure 3.16: DNA gel showing PCR amplification of *CB* HspB1 gene and *CB* P1 from pCB-P1HspB1 plasmid. PCR products were separated on a 1% agarose DNA gel and visualized by using EtBr (0.5 $\mu$ g/ml). The single DNA band at 1889 bp (lane 3 and 4) and 791bp (lane 5 and 6) show amplification of the desired HspB1 gene, P1 gene respectively. DNA ladder (1 Kb DNA extension ladder) (lane1), negative PCR control (lane 2).

### 3.2.2.1 Generation of *CB*- Com1/ Mip expression cassettes with virus homology flanking ORF73

In order to construct the recombinant fragments containing the *CB* Com1/Mip expression cassettes flanked by BoHV-4 homologous sequence to target the ORF 73 region within BoHV-4 BAC genome, pCMV/*CB*-Com1ORF73Flank and pCMV/*CB*-MipORF73Flank were generated individually (see Figure 3.17, Figure 3.18 and Figure 3.19). Having already cloned the pCMV/*Mb*-FusionORF73Flank (forward and reverse) vectors made the construction of *CB* recombination plasmids relatively straightforward. The synthetic *CB*- Com1 ORF and *CB*- Mip ORF were removed from their backbone plasmids (pCB-Com1 plasmid and pCB-Mip) using

*NheI* and *SphI*. Both pCMV/*Mb*-FusionORF73Flank (forward and reverse) vectors, being used as a source of the pCMV/ ORF73Flank component, were digested with *NheI*, *SphI* and *BamHI* to release the fragment of the synthetic *Mb* ORF. The synthetic Com1 ORF and Mip ORF were then ligated individually into the linearized pCMV/ORF73Flank vectors. The ligated DNA was then transformed into competent PIR1 *E.coli* cells, and plated onto agar containing Kan 50 µg/ml. After 24-48 hours the colonies were picked and screened by restriction enzyme digestion *NheI* and *SphI* digestion was used for screening. As shown in Figure 3.20, all clones tested yielded the 1000 bp (insert) and 3900 bp (vector backbone) bands as expected for pCMV/*CB*-Com1ORF73Flank forward and reverse. For additional confirmation, clones were digested with *HinDIII* Figure 3.21, double-digested with *SnaBI* and *MfeI*, and triple digested with *PmeI*, *HpaI* and *NcoI* (data not shown). All clones showed the expected band sizes. These results demonstrated the successful cloning of Com1 into both pCMV/ORF73Flank forward and reverse vectors.

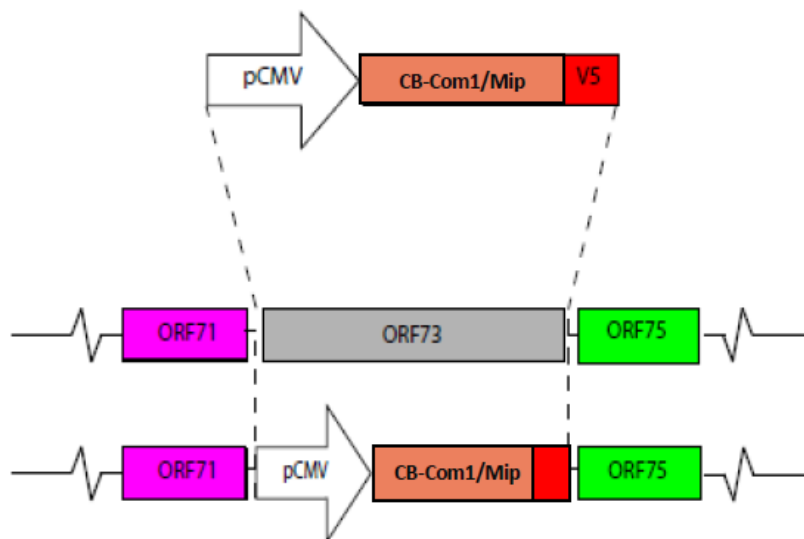


Figure 3.17: Schematic illustrating construction of BoHV-4 $\Delta$ ORF73 CB-Com1/Mip vector. The CB-Com1/Mip expression cassette were inserted individually within BoHV-4 genome to replace the endogenous ORF73 by using E/T-recombination. Linearized vector carrying the Kan<sup>R</sup> marker and CB-Com/Mip expression cassette were electroporated into *E. coli* (strain EL250), a bacterial strain containing E/T recombinases. Following removal of the Kan<sup>R</sup> marker, the BoHV-4  $\Delta$ ORF73 CB-Com1/Mip BACs were transfected individually into MDBK cells to reconstitute BoHV-4  $\Delta$ ORF73CB- COM1/Mip viruses.

Results shown in Figure 3.22 indicate insertion of the Mip ORF into pCMV/ORF73Flank. Screening of selected clones with *NheI* and *SphI* yielded the expected 970 bp band (insert) and 3900 bp (vector backbone). Clones were also screened also with *HincII*, *XmaI* and *SfoI* (data not shown).

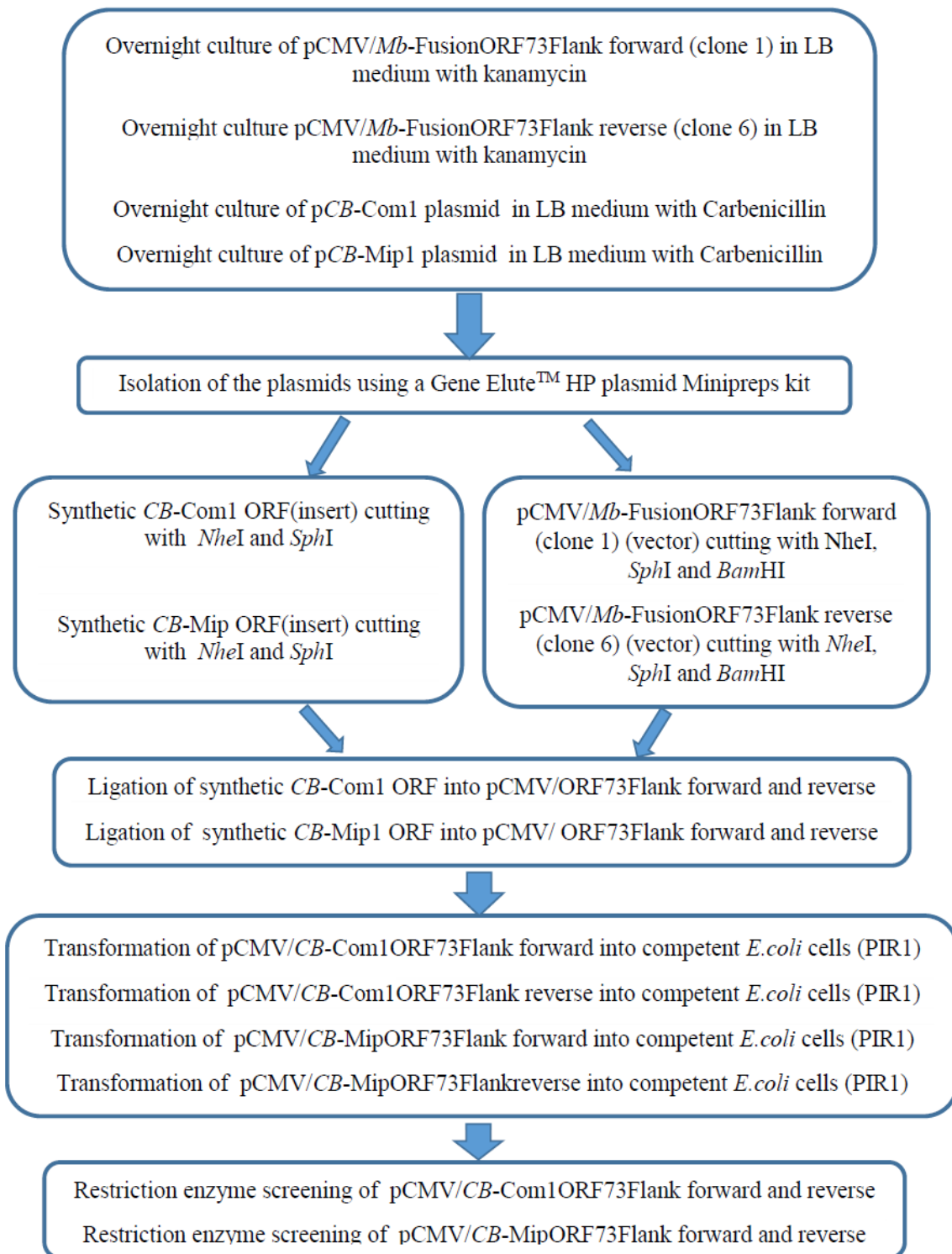
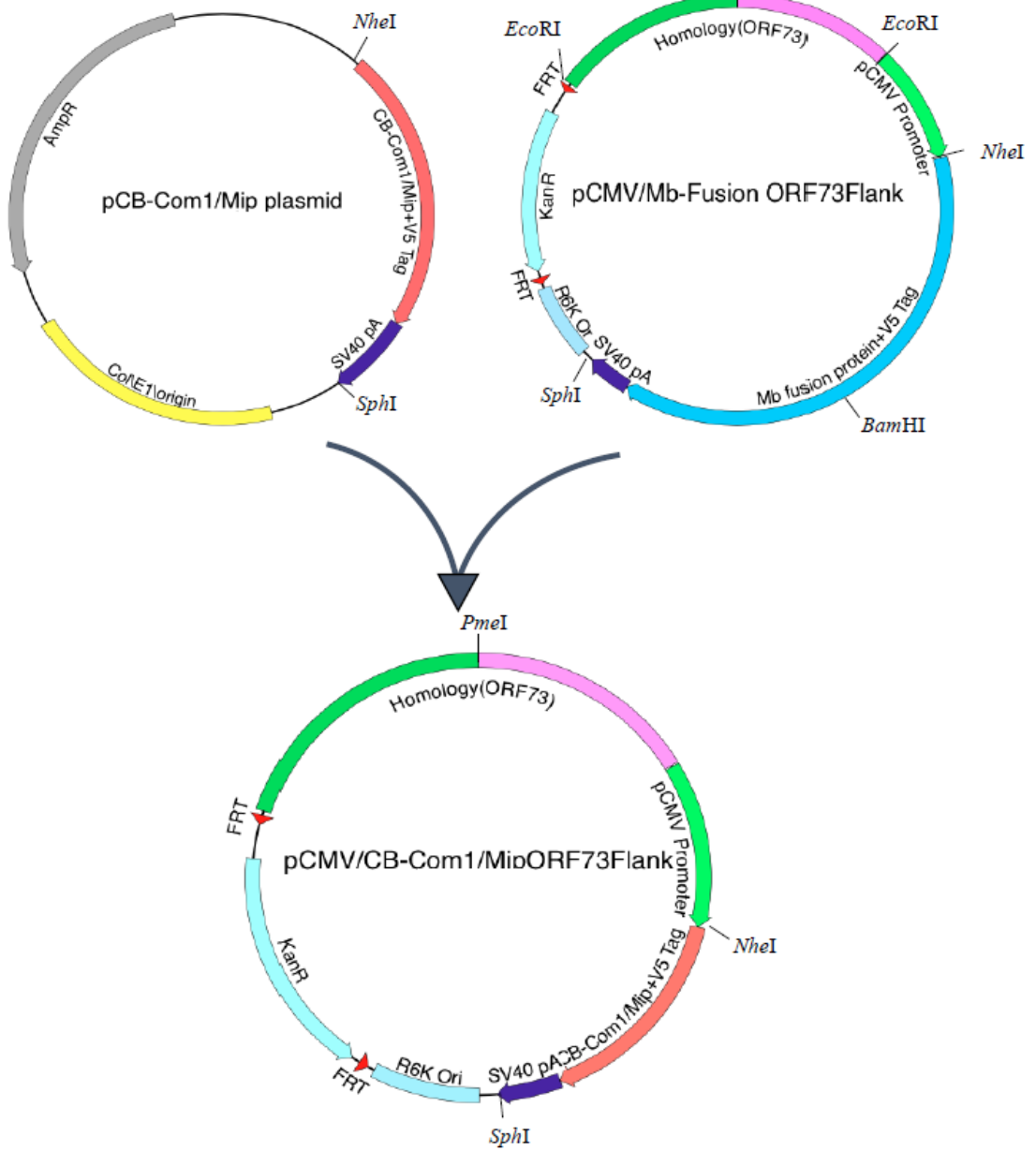


Figure 3.18: A flow-chart showing cloning synthetic ORF *CB*-Com1 ORF / synthetic *CB*-Mip ORF into pCMV/ORF73F flank forward and reverse vectors.

A





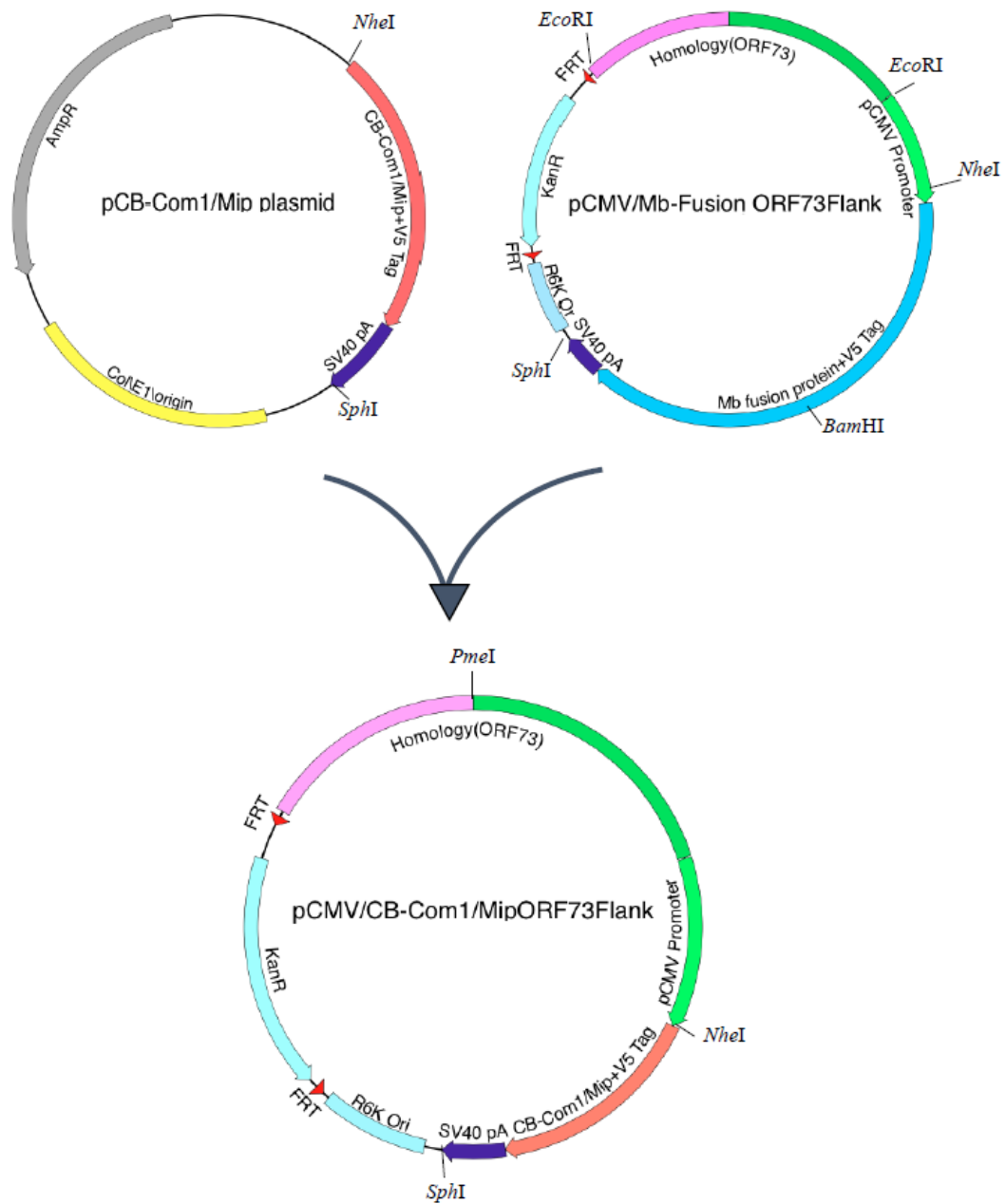
**B**

Figure 3.19: Schematic illustrating cloning strategy for generation of *CB* antigens expression cassettes with viral homology flanking BoHV-4 ORF73. (A) Cloning *CB-Com1* and *CB-Mip* individually into pCMV/ ORF73Flank forward orientation (suicide-based vector) containing the ORF 73 flanking regions, a Kan<sup>R</sup> gene flanked by FRT sites as well as *NheI* and *SphI* (for insertion of the *CB* antigens). (B) Cloning *CB-Com1* and *CB-Mip* individually into pCMV/ ORF73Flank reverse orientation (suicide-based vector) containing the ORF 73 flanking regions, a Kan<sup>R</sup> gene flanked by FRT sites as well as *NheI* and *SphI* (for insertion of the *CB* antigens). These plasmids were then used in construction of BoHV-4 ΔORF73 *CB-Com1* Forward/Reverse and BoHV-4 ΔORF73 *CB-Mip* Forward/Reverse .

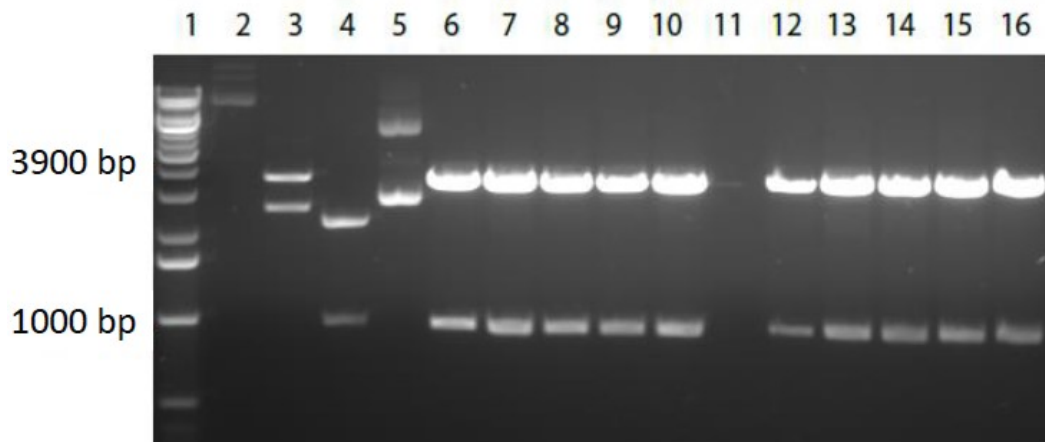


Figure 3.20: Restriction enzyme screening of pCMV/*CB-Com1*ORF73Flank clones. Lane1: DNA ladder (1 Kb DNA extension ladder). Lane 2 and 3 show uncut and cut pCMV/*Mb-FusionORF73Flank* (the parental plasmid). Lane 4 shows synthetic *CB-Com1* ORF digested plasmid. Lanes 6-10 (five screened clones (forward orientation)). Lanes 12-16 (five screened clones (reverse orientation)). Plasmids digested with *NheI* and *SphI* show confirmed of insertion of synthetic *CB-Com1*ORF into pCMV/ORF73Flank. Following digestion two bands observed; upper bands 3900 bp corresponds to pCMV/ORF73Flank and the lower bands 1000 bp corresponds to synthetic *CB-Com1* ORF.

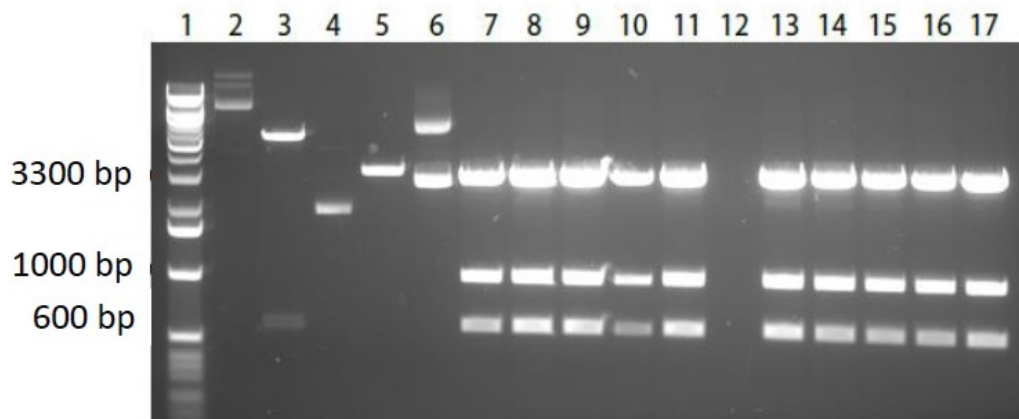


Figure 3.21: Restriction enzyme screening of pCMV/*CB-Com1*ORF73Flank clones. Lane1: DNA ladder (1 Kb DNA extension ladder). Lane 2 and 3 shows uncut and cut pCMV/*Mb-FusionORF73Flank* (the parental plasmid). Lane 4 and 5 show uncut and cut synthetic *CB-Com1* ORF plasmid. Lanes 7-11 (five screened clones (forward orientation)). Lanes 13-17 (five screened clones (reverse orientation)). Plasmids digested with *HinDIII* show confirmed of insertion of synthetic *CB-Com1* ORF into pCMV/ORF73Flank. Following digestion the three expected bands observed; upper bands 3300 bp, middle band 1000 bp and the lower bands 600 bp.

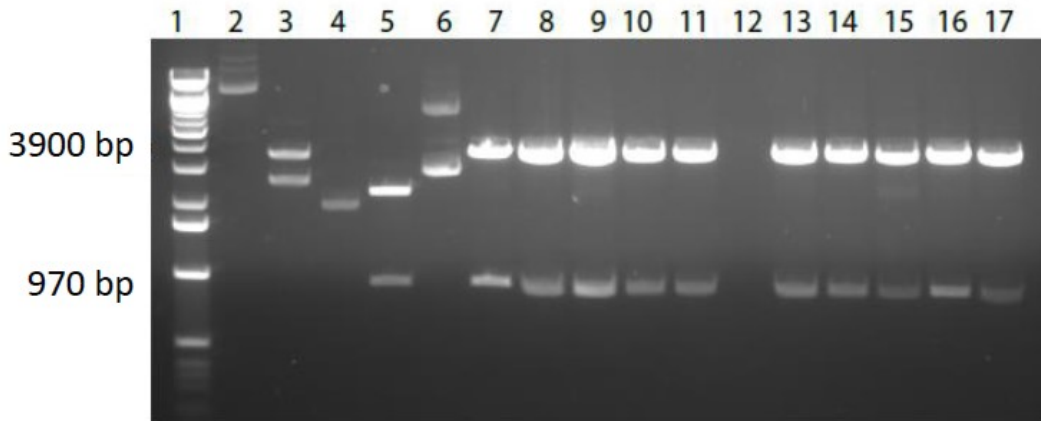


Figure 3.22: Restriction enzyme screening of pCMV/*CB*-MipORF73Flank clones. Lane 1 DNA ladder (1 Kb DNA extension ladder). Lane 2 and 3 shows uncut and cut pCMV/*Mb*-FusionORF73Flank (the parental plasmid). Lane 4 and 5 show uncut and cut synthetic *CB* Mip ORF plasmid. Lanes 6-11 (five screened clones (forward orientation)). Lanes 12-17 (five screened clones (reverse orientation)). Plasmids digested with *NheI* and *SphI* show confirmed of insertion of synthetic *CB* Mip ORF into pCMV/ORF73Flank. Following digestion two bands observed; upper bands 3900 bp corresponds to pCMV/ORF73Flank and the lower bands 970 bp corresponds to synthetic *CB* Mip ORF.

### 3.2.2.2 Generation of RVFV-Gn glycoprotein expression cassette with virus homology flanking ORF73

In previous sections, generation of *CB* antigen expression cassettes was performed by cloning the synthetic *CB* ORFs into pCMV/ ORF73Flank vectors containing the pCMV promoter and BoHV-4 homology flanking ORF73 required for E/T recombination. A similar approach was taken to clone a recombination plasmid expressing RVFV Gn Figure 3.23. Gn is the surface glycoprotein of RVFV and plays an important role in virus attachment and initiate infection. It has also been shown to be immunogenic, with induction of protective neutralization antibodies against RVFV (Faburay *et al.*, 2016). A synthetic region comprised of the Gn antigen fused to a V5 epitope tag with a down-stream SV40 poly-adenylation sequence and the necessary restriction sites (*NheI* and *SphI*) for insertion of the synthetic fragment into pCMV/ ORF73Flank was designed and synthesized by GeneArt Invitrogen. As before, pCMV/*Mb*-FusionORF73Flank (forward and reverse) vectors were used as the source of the

pCMV/ORF73Flank component (see Figure 3.24 and Figure 3.25) using *NheI*, *SphI* and *BamHI*. To determine whether the RVFV Gn synthetic ORF had been correctly inserted pCMV/ORF73Flank forward and reverse plasmids, DNA extracted from five individual clones were analysed by *NcoI* restriction digestion. The first three clones were also analysed by *BbsI* and *MscI* restriction digestion. As shown in Figure 3.26, Figure 3.27 and Figure 3.28 all clones showed bands of the expected size.

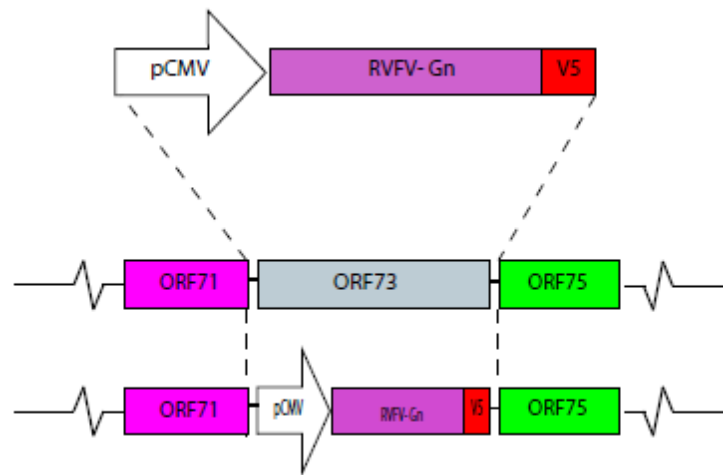


Figure 3.23: Schematic illustrating construction of BoHV-4 $\Delta$ ORF73 RVFV-Gn vector. The RVFV-Gn expression cassette was inserted within BoHV-4 genome to replace the endogenous ORF73 by using E/T-recombination. Linearized vector carrying the Kan<sup>R</sup> marker and RVFV-Gn expression cassette was electroporated into *E. coli* (strain EL250), a bacterial strain containing E/T recombinases. Following removal of the Kan<sup>R</sup> marker, the BoHV-4  $\Delta$ ORF73 RVFV-Gn BAC was transfected into MDBK cells to reconstitute BoHV-4  $\Delta$ ORF73 RVFV-Gn virus.

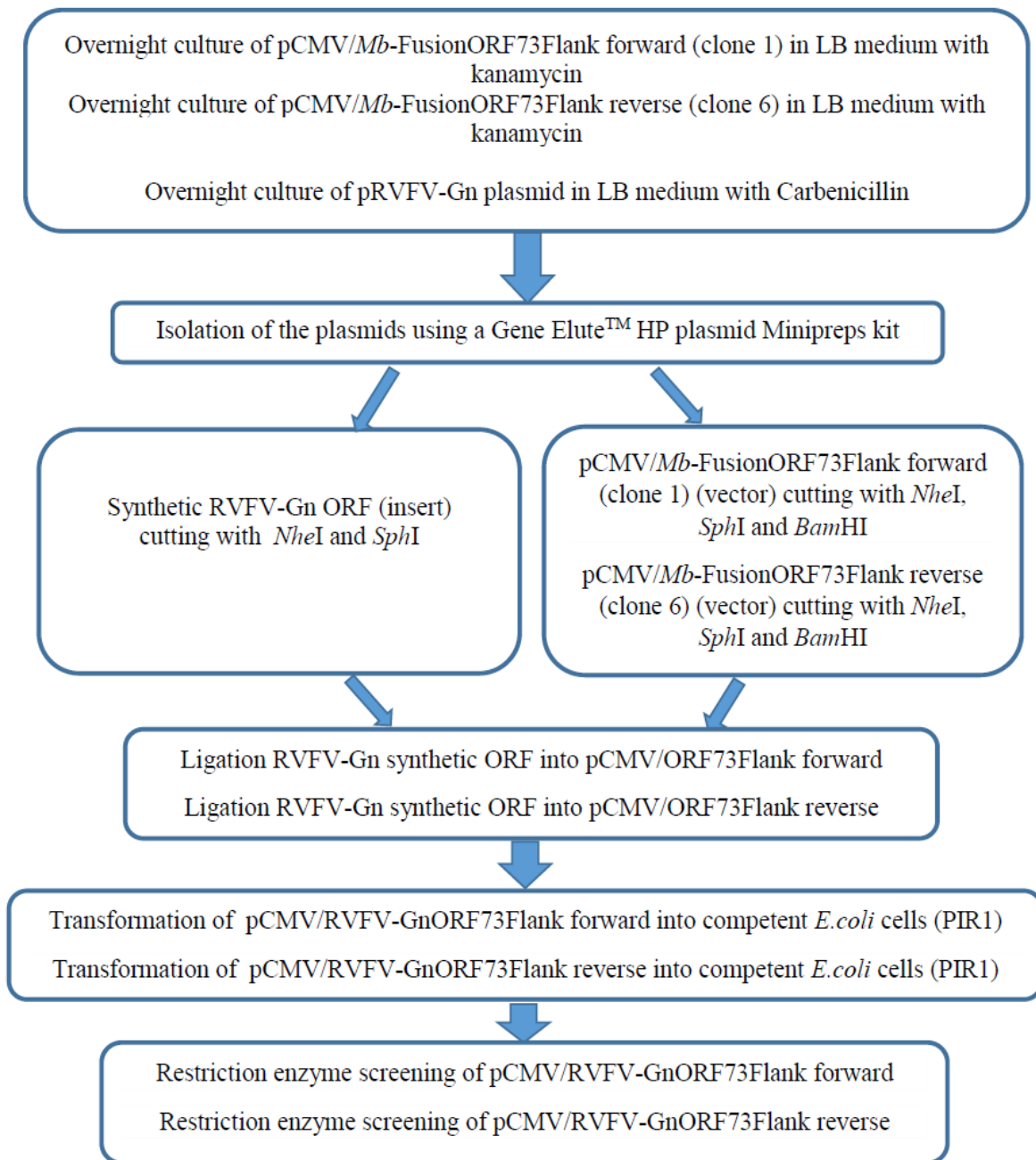
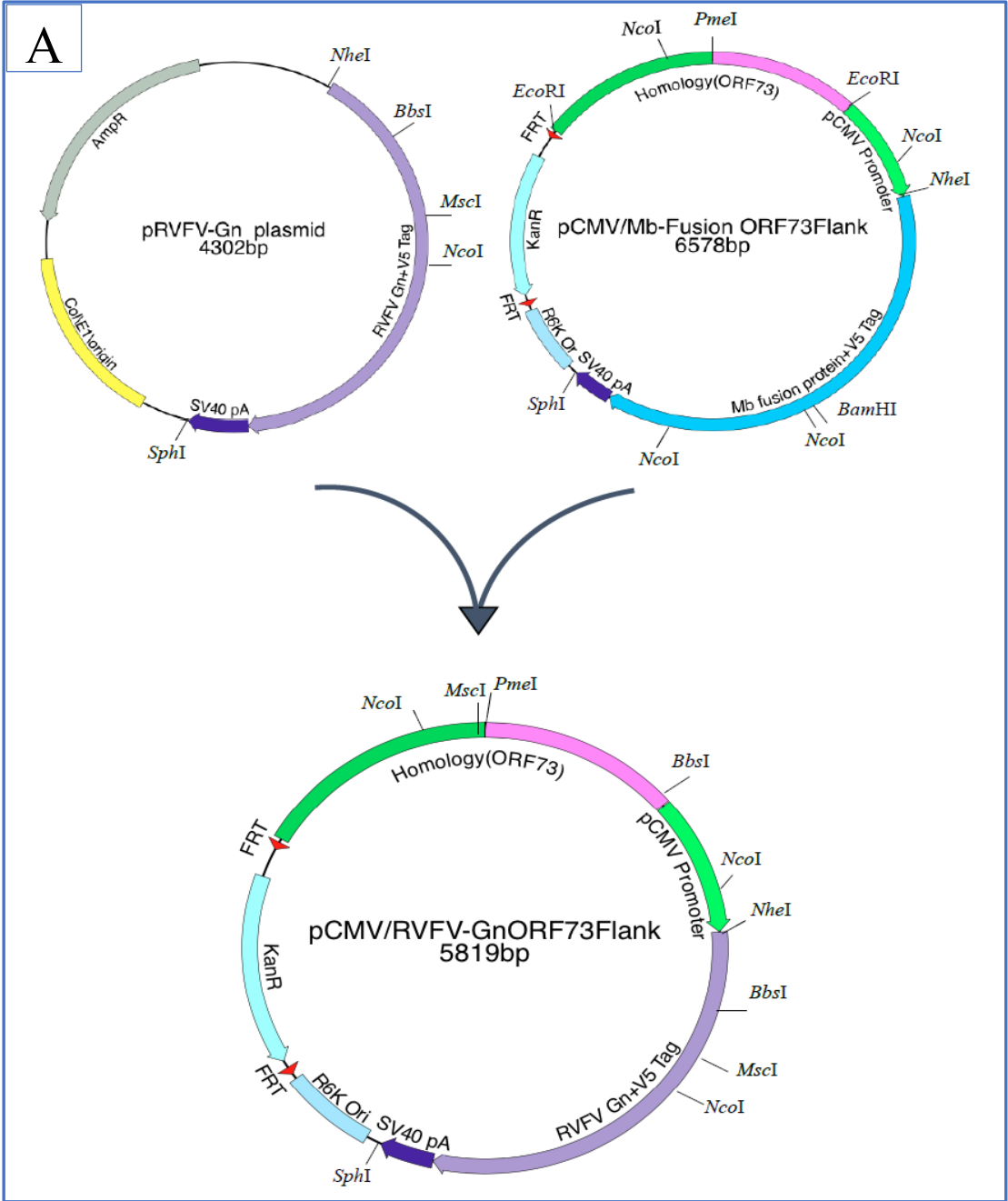


Figure 3.24: A flow-chart showing cloning synthetic RVFV-Gn ORF into pCMV/ORF73Flank forward and reverse vectors.



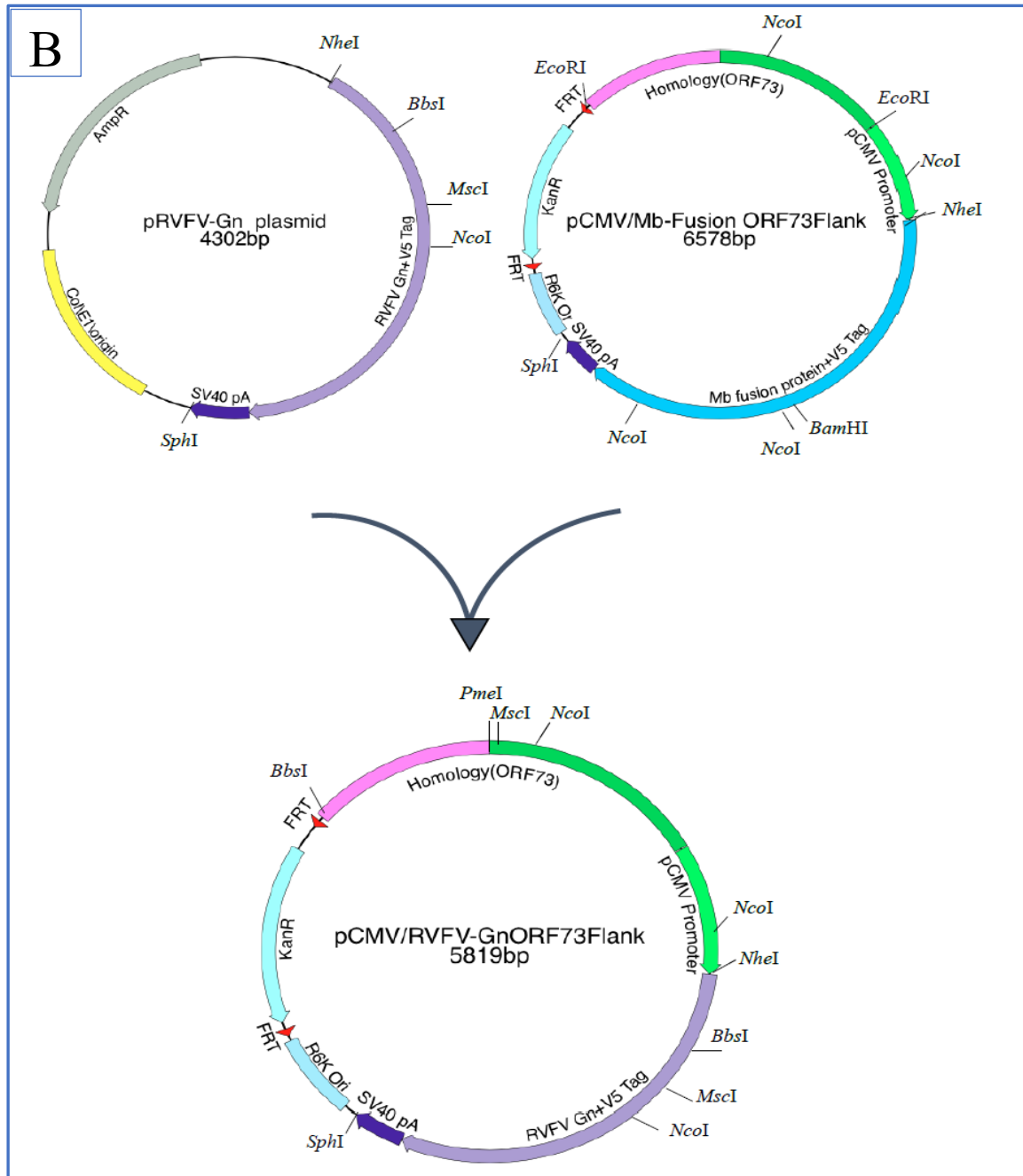


Figure 3.25: Schematic illustrating cloning strategy for generation of RVFV-Gn antigen expression cassette with viral homology flanking BoHV-4 ORF73. (A) Cloning RVFV-Gn into pCMV/ ORF73Flank forward orientation (suicide-based vector) containing the ORF 73 flanking regions, a Kan<sup>R</sup> gene flanked by FRT sites as well as *NheI* and *SphI* (for insertion of the RVFV-Gn antigen). (B) Cloning RVFV-Gn into pCMV/ ORF73Flank reverse orientation (suicide-based vector) containing the ORF 73 flanking regions, a Kan<sup>R</sup> gene flanked by FRT sites as well as *NheI* and *SphI* (for insertion of the RVFV-Gn antigen). These two plasmids were then used in construction of BoHV-4  $\Delta$ ORF73 RVFV-Gn Forward/Reverse.

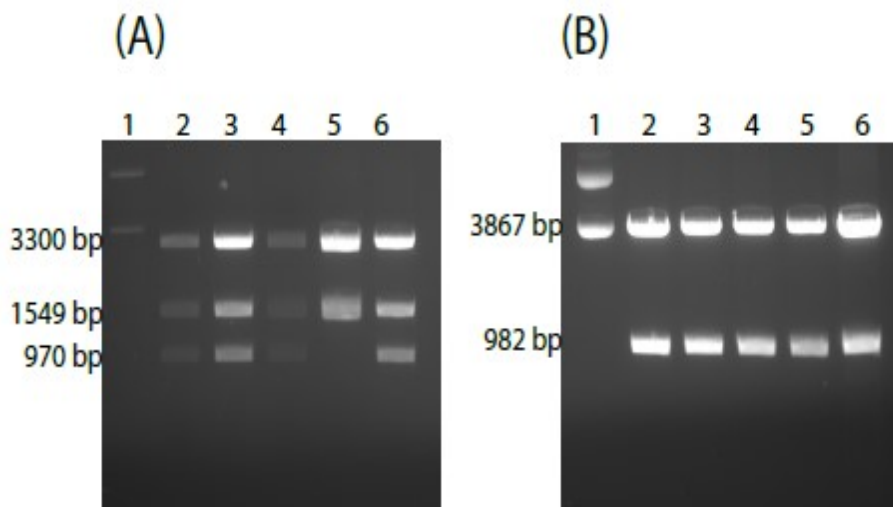


Figure 3.26: Restriction enzyme screening of pCMV/RVFV-GnORF73Flank clones using *NcoI*. (A) Lane1 shows uncut pCMV/RVFV-GnORF73Flank (forward orientation). Lanes 2-6 show confirmed pCMV/RVFV-Gn ORF 73Flank clones (forward orientation) following digestion with *NcoI* lanes (2-6). Three bands are observed as expected; upper band 3300 bp middle bands 1549 the lower bands 970 bp. (B) Lane1 shows uncut pCMV/RVFV-GnORF73Flank (reverse orientation). Lanes 2-6 show confirmed pCMV/RVFV-GnORF73 Flank clones (reverse orientation) following digestion with *NcoI* lanes (2-6). Two bands are observed; upper bands 3867 bp the lower bands (982 bp and 970 bp).

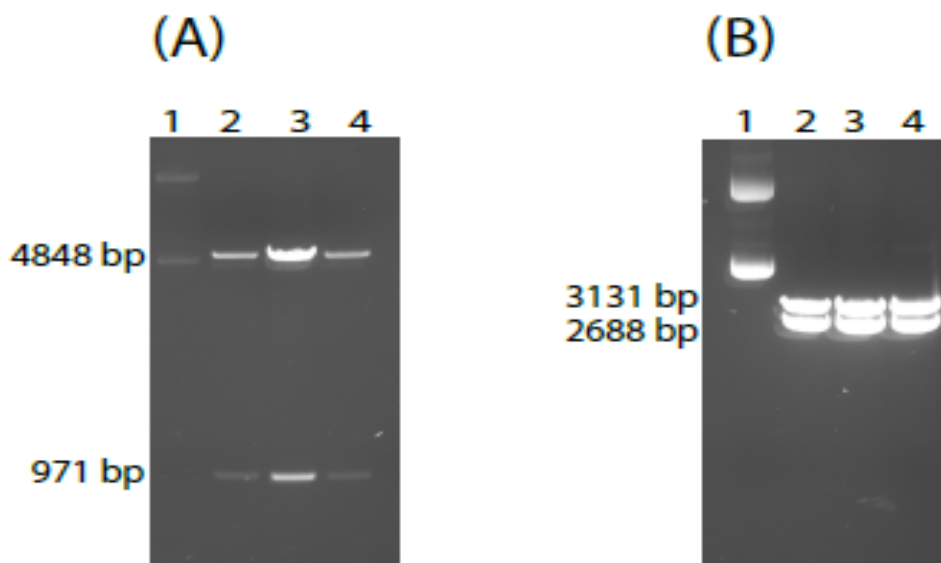


Figure 3.27: Restriction enzyme screening of ER1 Flank ER1 ORF73 pCMV/RVFV-GnORF73Flank clones using *BbsI*. (A) Lane1 shows uncut pCMV/RVFV-GnORF73Flank (forward orientation). Lanes 2-4 show confirmed pCMV/RVFV-GnORF73Flank clones (forward orientation) following digestion with *BbsI* lanes (2-4). Two bands are observed as expected; upper bands 4848 bp the lower bands 971 bp. (B) Lane1 shows uncut pCMV/RVFV-GnORF73Flank (reverse orientation). Lanes 2-4 show confirmed pCMV/RVFV-GnORF73Flank clones (reverse orientation) following digestion with *BbsI* lanes (2-4). Two bands are observed; upper bands 3131 bp the lower bands 2688 bp.



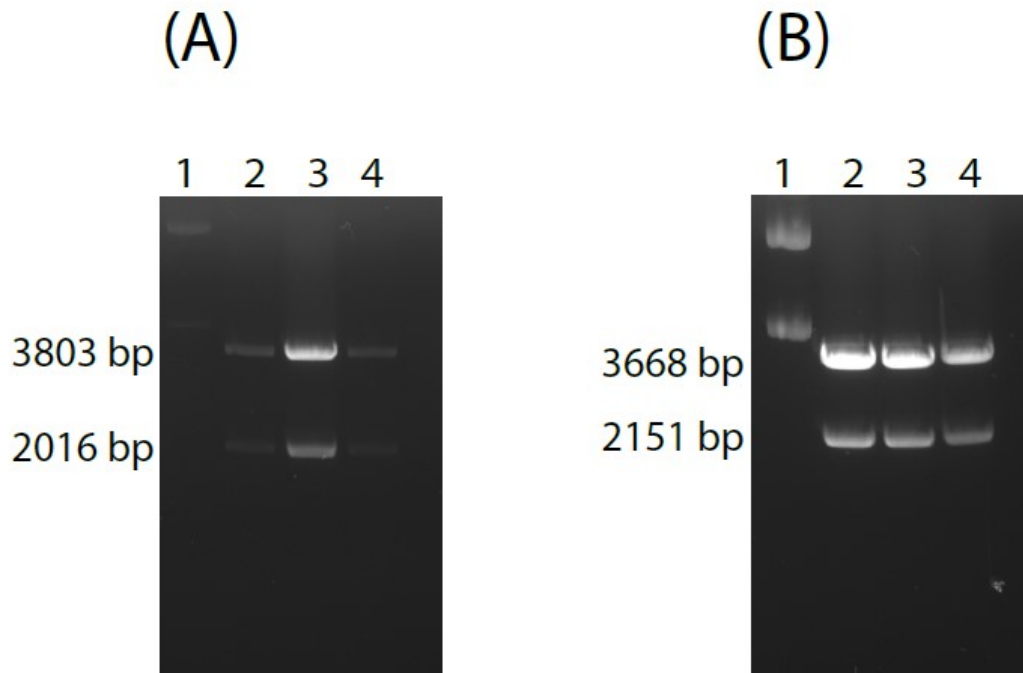


Figure 3.28: Restriction enzyme screening of pCMV/RVFV-GnORF73Flank using *MscI*. (A) Lane1 shows uncut pCMV/RVFV-GnORF73Flank (forward orientation). Lanes 2-4 show confirmed pCMV/RVFV-GnORF73Flank clones (forward orientation) following digestion with *MscI* lanes (2-4). Two bands are observed as expected; upper bands 3803 bp the lower bands 2016 bp. (B) Lane1 shows uncut pCMV/RVFV-GnORF73Flank (reverse orientation). Lanes 2-4 show confirmed pCMV/RVFV-GnORF73Flank clones (reverse orientation) following digestion with *MscI* lanes (2-4). Two bands are observed; upper bands 3668 bp the lower bands 2151 bp.

### 3.2.2.3 Construction of recombinant BoHV-4 $\Delta$ ORF73 vectors expressing *CB* antigens (Com1 and Mip) and RVFV-Gn by E/T homologous recombination

E/T homologous recombination methods were used to construct i) two independent BoHV-4  $\Delta$ ORF73 vectors expressing the *CB* antigens (BoHV-4 $\Delta$ ORF73*CB*-Com1 and BoHV-4 $\Delta$ ORF73*CB*-Mip), and ii) two independent BoHV-4  $\Delta$ ORF73 RVFV-Gn vectors (forward or reverse orientation). Prior to use, all recombination plasmids were linearized by digestion with *PmeI*. E/T homologous recombination was then used to insert these linearized recombination fragment between Bo13 and ORF75 regions within the BoHV-4 BAC, resulting in deletion of ORF73. Recombinant BAC clones were selected based on their resistance to both Kan and Cam, followed by *EcoRI* digestion to ensure clones maintained genomic integrity (Figures 3.29, 3.30).

#### 3.2.2.4 Excision of kanamycin cassette from BoHV-4 $\Delta$ ORF73CB-Com1, BoHV-4 $\Delta$ ORF73CB-Mip and BoHV-4 $\Delta$ ORF73RVFV- Gn BAC DNA

Following confirmation that BoHV-4 $\Delta$ ORF73CB-Com1, BoHV-4 $\Delta$ ORF73CB-Mip and BoHV-4 $\Delta$ ORF73RVFV-Gn BAC genome were genetically intact, the next step was to remove the FRT-flanked Kan<sup>R</sup> marker by arabinose induction of the EL250 FLP recombinase. To confirm that Kan<sup>R</sup> was removed from recombinant BACs, 12 colonies from each construct were replica-plated onto Cam/Kan and Cam only plates (see section 2.2.9). The clones from which the Kan<sup>R</sup> marker had been removed were unable to grow in LB agar containing Kan<sup>R</sup>. In order to confirm the absence of genetic rearrangements that could have happened during Kan<sup>R</sup> removal, DNA was extracted from selected recombinant BAC clones and analysed by *Eco*RI restriction digestion (Figure 3.31 and Figure 3.32). The banding patterns produced by the *Eco*RI digested recombinant BoHV-4 BACs confirmed the genomic integrity of the recombinant BoHV-4 $\Delta$ ORF73 (CB-Com1, CB-Mip and RVFV-Gn) BACs following Kan<sup>R</sup> excision by FLP recombinase.

PCR was performed to confirm excision of the Kan<sup>R</sup> marker from the recombinant BAC genomes. ORF73 flanking primers were used to amplify the site of expression cassette insertion region on both pre-and post-FLP recombinant BACs (Figure 3.33). PCR of the pre-FLP BoHV-4 $\Delta$ ORF73CB-Com1 BAC (clone 4) and BoHV-4 $\Delta$ ORF73CB-Mip BAC (clone 4) yielded bands of 3366 bp and 3271 bp respectively, consistent with presence of the Kan<sup>R</sup> gene within the recombinant fragment. PCR products of the corresponding post-FLP BoHV-4 $\Delta$ ORF73CB-Com1 and BoHV-4 $\Delta$ ORF73CB-Mip BAC clones yielded expected bands of 2362 bp and 2267 bp, respectively, with the reduction in size reflecting the loss of the Kan<sup>R</sup> gene (1004 bp).

To confirm sequence integrity of the expression cassette after Kan<sup>R</sup> excision, PCR products of post-FLP BoHV-4 $\Delta$ ORF73CB-Com1BAC (clone 4-1) and post-FLP BoHV-4 $\Delta$ ORF73CB-Mip BAC (clone 4-2) were sequenced as detailed above. The sequence data indicated that the

expression cassettes had been inserted within the desired region of the BoHV-4 genome, and that the Kan<sup>R</sup> gene had been excised (Figure 3.34 and Figure 3.35). Similarly, PCR of the pre-FLP BoHV-4 $\Delta$ ORF73 RVFV-Gn (clone 1) and corresponding post-FLP BoHV-4 $\Delta$ ORF73 RVFV-Gn (clone 1-1)( Figure 3.36) yielded bands of approximately 4249 bp and 3262 bp, respectively, again with the reduction in size reflecting the loss of the Kan<sup>R</sup> gene. DNA sequencing indicated that the expression cassette was inserted within the desired site of the BoHV-4 genome with Kan<sup>R</sup> gene excision (Figure 3.37).

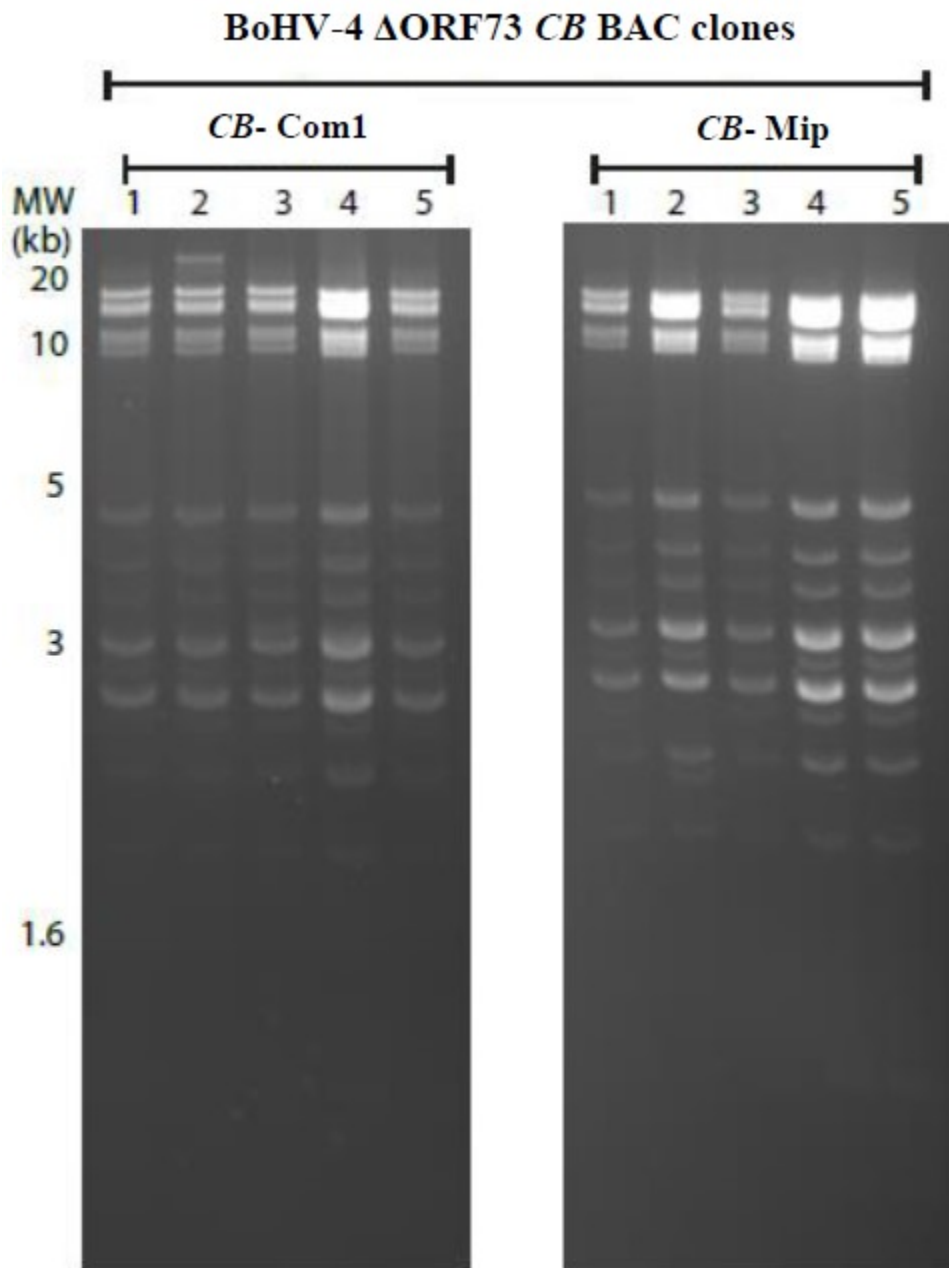


Figure 3.29: Characterization of the BoHV-4  $\Delta$ ORF73 CB-Com1/ CB-Mip Forward BAC genome by restriction enzyme digestion. Five independent clones from BoHV-4  $\Delta$ ORF73 CB-Com1 Forward and BoHV-4  $\Delta$ ORF73 CB-Mip Forward BACs were digested with *Eco*RI (restriction enzyme). *Eco*RI digested fragments were then separated according their molecular weight by electrophoresis at 45 V for 18-20 hours.

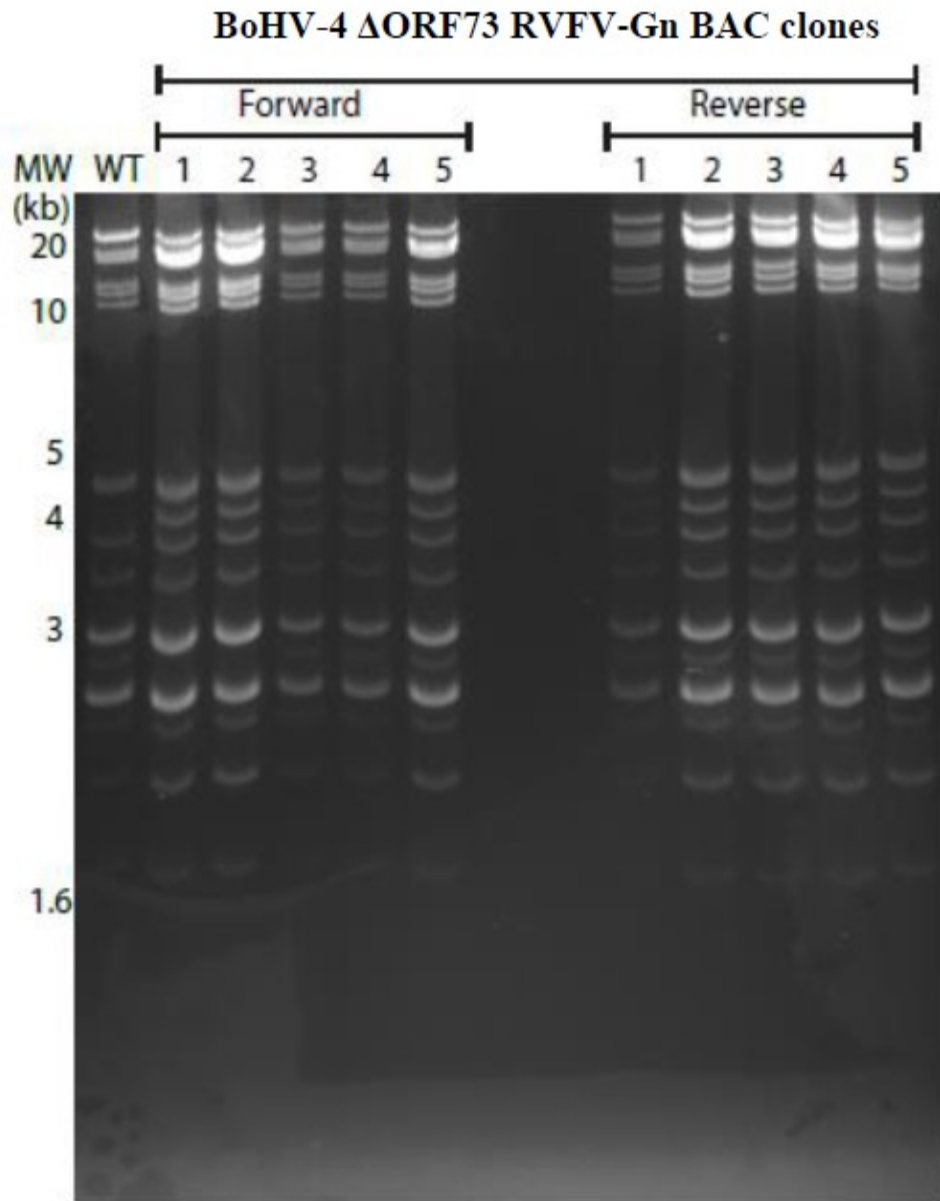


Figure 3.30: Characterization of the BoHV-4  $\Delta$ ORF73 RVFV-Gn Forward/Reverse BAC genome by restriction enzyme digestion. BoHV-4 -WT and five independent clones from BoHV-4  $\Delta$ ORF73 RVFV-Gn Forward and BoHV-4  $\Delta$ ORF73 RVFV-Gn Reverse BACs were digested with *Eco*RI (restriction enzyme). *Eco*RI digested fragments were then separated according their molecular weight by electrophoresis at 45 V for 18-20 hours and compared with the wild type.



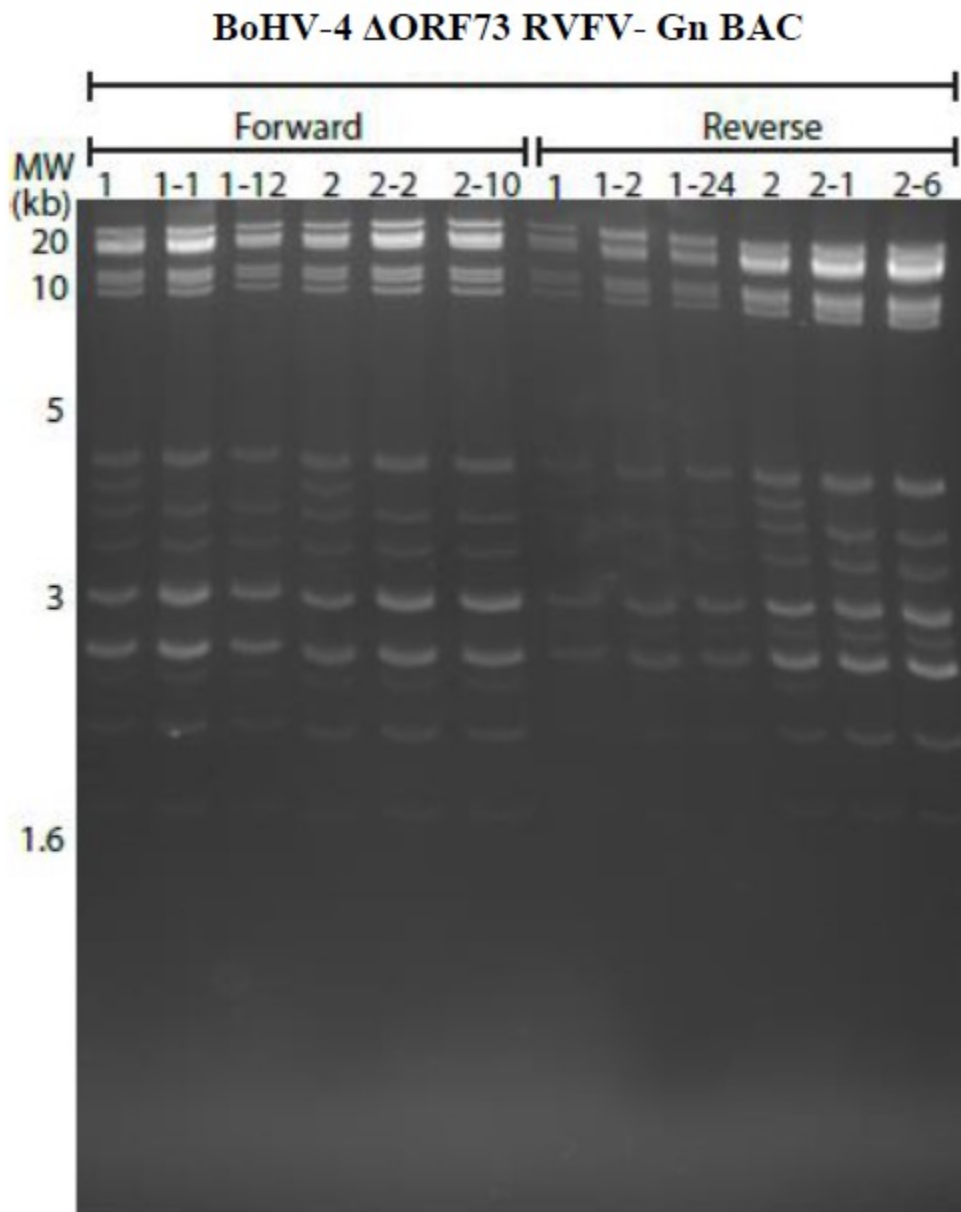


Figure 3.32: Characterization of the post-FLP BoHV-4  $\Delta$ ORF73 RVFV-Gn Forward/Reverse BAC genome by restriction enzyme digestion. BoHV-4  $\Delta$ ORF73 RVFV-Gn Forward/Reverse pre- FLP ( Forward clone 1 and 2, Reverse clone 2 and clone 2) and post-FLP (Forward clone 1-1, 1-12, 2-2 and 2-10; Reverse clone 1-2, 1-24, 2-1 and 2-6). BACs were digested with *Eco*RI (restriction enzyme). *Eco*RI digested fragments were then separated according their molecular weight by electrophoresis at 45 V for 18-20 hours.

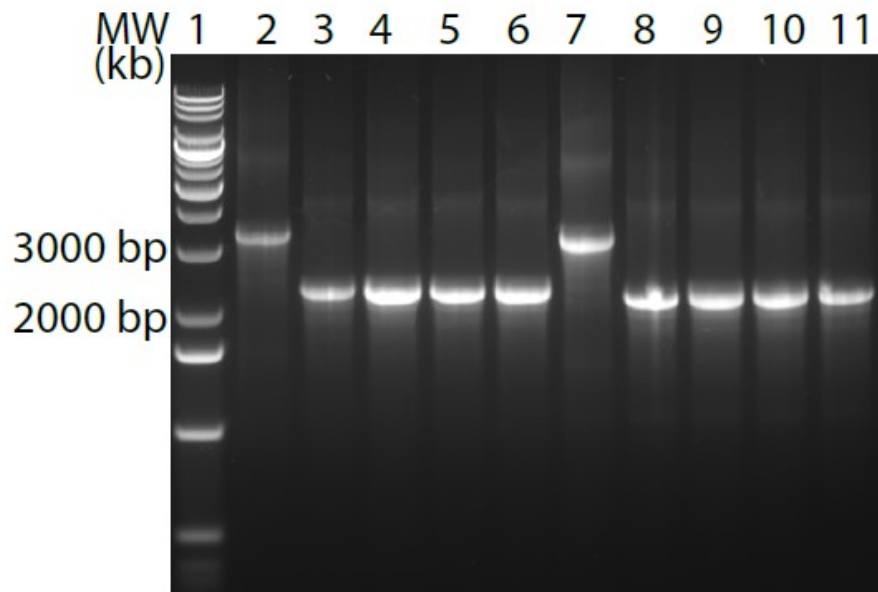


Figure 3.33: Confirmation of excision of the Kan<sup>R</sup> marker from BoHV-4ΔORF73CB-Com1 and BoHV-4Δ ORF 73CB-Mip BACs genome by PCR. BAC DNA from BoHV-4ΔORF73CB-Com1 and BoHV-4ΔORF73CB-Mip BACs (Pre-Flp) and four post-Flp clones of each recombinant BAC were screened by PCR using ORF73 flanking primers. PCR products were separated on a 1% agarose gel for 60-90 minutes at 75 V, and visualized by staining with 0.5 μg/ml EtBr. PCR pre-FLP BoHV-4ΔORF73CB-Com1 clone 4 (lane 2) and post- BoHV-4ΔORF73CB-Com1 clone 4-1(lane 3), clone 4-3 (lane 4), clone 4-6 (lane 5) and clone 4-13(lane 6) yielded PCR products of 3366 bp and 2362 bp, respectively. PCR pre-FLP BoHV-4ΔORF73CB-Mip clone 4 (lane 7) and post- BoHV-4ΔORF73CB-Mip clone4-2 (lane 8), clone 4-3(lane 9), clone 4-9 (lane 10) and clone 4-10 (lane 11) yielded PCR products of 3271 bp and 2267 bp, respectively. Molecular size marker (MW) in bp is indicated.



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Figure 3.34: Confirmation of presence of *CB-Com1* expression cassette within BoHV-4 BACs via Sanger sequencing. PCR were performed on post-FLP BAC clones 4-1. Pink represent upstream homologous region (ORF71), light green represent pCMV promoter, yellow represent *CB-Com1*, black represent V5 and SV40 pA, light blue represent suicide Ori, the red represent FRT and the dark green ORF75.

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Figure 3.35: Confirmation of presence of *CB-Mip* expression cassette within BoHV-4 BACs via Sanger sequencing. PCR were performed on post-FLP BAC clones 4-2. Pink represent upstream homologous region (ORF71), light green represent pCMV promoter, brown represent *CB-Mip*, black represent V5 and SV40 pA, light blue represent suicide Ori, the red represent FRT and the dark green ORF75.

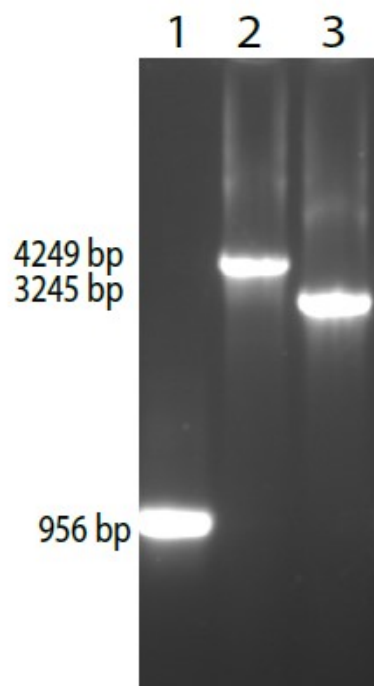


Figure 3.36: Confirmation of excision of the Kan<sup>R</sup> marker from BoHV-4 $\Delta$ ORF73RVFV-Gn BAC genome by PCR. DNA from recombinant BoHV-4 $\Delta$ ORF73RVFV-Gn BACs clone 1 (pre FLP) and clone 1-1 (post FLP) forward orientation were screened by PCR using ORF 73 flanking primers. PCR products were separated on a 1% agarose gel for 60-90 minutes at 75 V, and visualized by staining with EtBr (0.5  $\mu$ g/ml). PCR of pre- (as shown in lane2) and post- (as shown in lane3) BoHV-4 $\Delta$ ORF73RVFV-Gn yielded PCR products of approximately 4249 bp and 3262 bp, respectively. Lane1 shows PCR product of the wild type BoHV-4.

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Figure 3.37: Confirmation of presence of RVFV-Gn expression cassette within BoHV-4 BACs via Sanger sequencing. PCR were performed on post-FLP BAC clones 1-1. Pink represent upstream homologous region (ORF71), light green represent pCMV promoter, purple represent RVFV-Gn, black represent V5 and SV40 pA, light blue represent suicide Ori, the red represent FRT and the dark green ORF75.

### 3.2.2.5 Reconstitution of BoHV-4 $\Delta$ ORF73CB-Com1, BoHV-4 $\Delta$ ORF73CB-Mip and BoHV-4 $\Delta$ ORF73RVFV-GN vaccine in permissive eukaryotic cells

Introducing herpesvirus genomic DNA within the BAC into permissive eukaryotic cells is sufficient for *de novo* reconstitution of progeny virus. To reconstitute the recombinant viruses, BAC DNA was introduced into MDBK cells via transfection by using Lipofectamine<sup>TM</sup>3000 (L3000-008, Invitrogen) (see section 2.4.2). Three independent clones of each BAC construct expressing CB-Com1, Mip or RVFV Gn were selected for reconstitution. MDBK cells were assessed daily for signs of virus-associated CPE and development of virus plaques. Cultures were also assessed for GFP expression (Gillet *et al.*, 2005; Storz *et al.*, 1984).

All recombinant BACs were successfully reconstituted as viruses. Virus plaques were visible in MDBK monolayers beginning at 5 to 7 days after transfection, with the presence of GFP in the BoHV-4 BAC plasmid backbone allowing for ease of detection of reconstituted viruses. Plaques were picked as described in 2.4.2 and serially passaged prior to preparation of seed stocks and concentrated virus stocks.

### 3.2.2.6 Expression of CB antigens in BoHV-4 $\Delta$ ORF73CB-Com1 and BoHV- $\Delta$ ORF73CB- Mip and glycoprotein N in BoHV-4 $\Delta$ ORF73RVFV-Gn

CB and RVFV antigens were tagged with V5 in order to enable antigen expression to be easily determined by using western analysis and an epitope-specific antibody. Figures 3.38 and 3.39 indicated expression of CB and RVFV antigens, with V5 signals of predicted sizes for Com1 (27kDa), Mip (25kDa) and Gn (56kDa). Viral DNA was extracted and sequenced (Figure 3.40, Figure 3.41 and Figure 3.42)

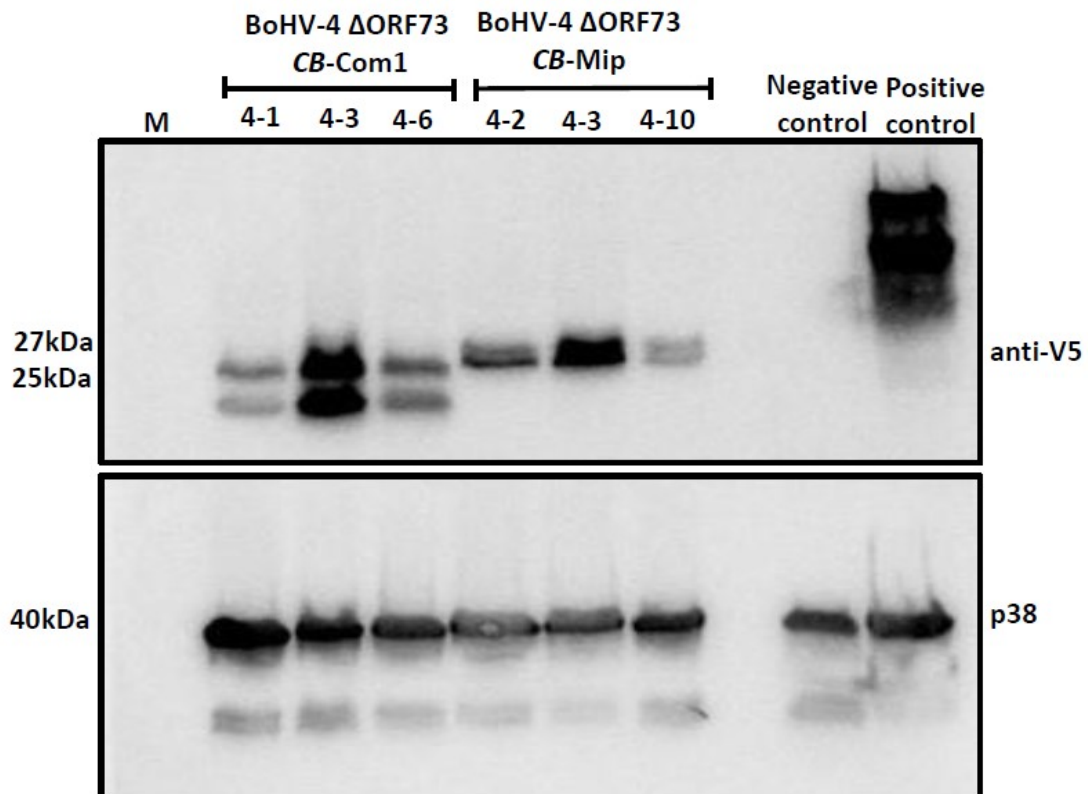


Figure 3.38: *CB* expression. Monolayers of MDBK cells were infected individually with BoHV-4ORF73 *CB-Com1* and BoHV-4ORF73 *CB-Mip*. Monolayers were harvested at 100% CPE and cell lysates were analysed by western blot. Anti-V5 antibody was used to detect the V5 tagged Com1 proteins (27kDa) and Mip proteins (25kDa). The negative control was BoHV-4 WT and the positive control was BoHV-4 ΔORF73 *Mb* clone 2-6. P38 was used as a loading control.

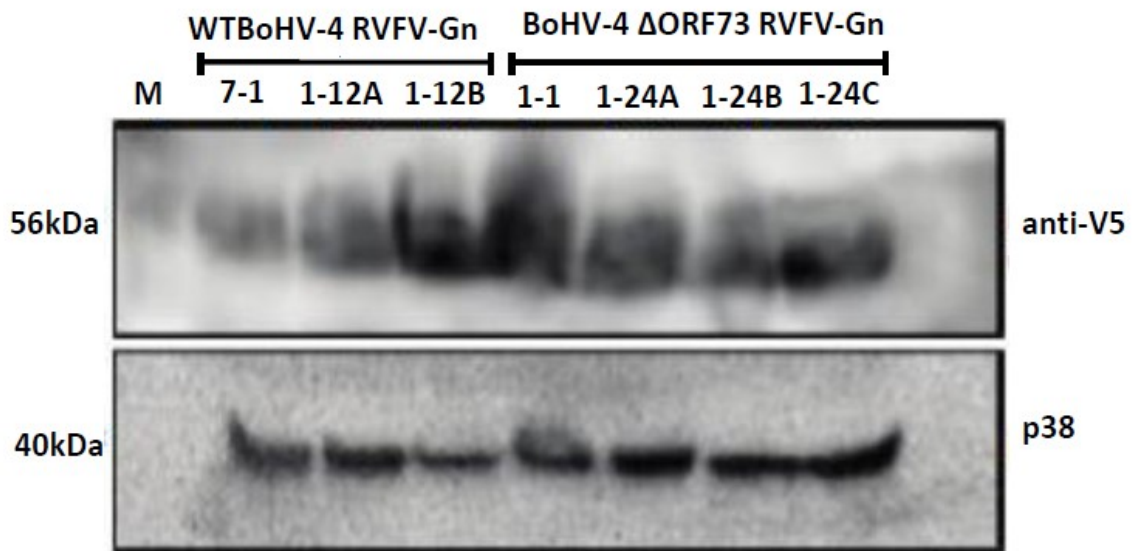


Figure 3.39: RVFV-Gn expression. Monolayers of MDBK cells were infected with WT BoHV-4 RVFV-Gn and BoHV-4  $\Delta$ ORF73 RVFV-Gn clones. Monolayer were harvested at 100% CPE. Cell lysates, were analysed by western blot. Anti-V5 antibody was used to detect the V5 tagged Gn protein. P38 used as a loading control.

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Figure 3.40: Confirmation presence of *CB-Com1* expression cassette within BoHV-4 virus genome via Sanger sequencing. PCR was performed on the DNA extracted from concentrated virus stocks clone (4-1). PCR products were sequenced. Pink represent upstream homologous region (ORF71), light green represent pCMV promoter, yellow represent *CB-Com1*, black represent V5 and SV40 pA, light blue represent suicide Ori, the red represent FRT and the dark green ORF75.



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Figure 3.41: Confirmation presence of *CB-Mip* expression cassette within BoHV-4 virus genome via Sanger sequencing. PCR was performed on the DNA extracted from concentrated virus stocks clone (4-2). PCR products were sequenced. Pink represent upstream homologous region (ORF71), light green represent pCMV promoter, brown represent *CB-Mip*, black represent V5 and SV40 pA, light blue represent suicide Ori, the red represent FRT and the dark green ORF75.

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AACCATAGAACATATCATGAAGACATTCAAAAATATTCAAATCAGGTGGAATTCGTTGACATTGATT
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```

Figure 3.42: Confirmation of presence of RVFV-Gn expression cassette within BoHV-4 virus genome via Sanger sequencing. PCR were performed on the DNA extracted from concentrated virus stocks clone (1-1). PCR products were sequenced. Pink represent upstream homologous region (ORF71), light green represent pCMV promoter, purple represent RVFV-Gn, black represent V5 and SV40 pA, light blue represent suicide Ori, the red represent FRT and the dark green ORF75.

### 3.2.3 Construction of WT Bovine herpesvirus expressing various antigens (*Mb* fusion protein and RVFV-Gn)

Individual WT BoHV-4 vectors expressing *Mb* fusion protein and RVFV-Gn were constructed with the aim of determining whether the deletion of ORF73 would affect the ability of the virus vector to stimulate the immune system (these *in vivo* studies are beyond the scope of the present work). Vectors were generated using a strategy comparable to that used for construction of the conditionally-attenuated  $\Delta$ ORF73 BoHV-4 detailed above. The synthetic flanking regions (Bo14 to Bo15) separated by a single *PmeI* site and flanked by an *EcoRI* for insertion into the pCMV suicide-based vector were designed to target the expression plasmid without removal of any BoHV-4 sequence. The synthetic flanking regions were cloned into the pCMV/*Mb*-Fusion vector clone to generate pCMV/*Mb*-FusionWTFlank. The orientation of the *Mb* expression cassette was also determined and two vectors in different orientation (forward and reverse) were selected and used as a source to generate *CB*-Com1, *CB*-Mip and RVFV-Gn expression cassette with pWTFlank plasmids.

The pCMV/*CB*-Com1WTFlank (forward and reverse), pCMV/*CB*-MipWTFlank (forward and reverse) and pCMV/RVFV-GnWTFlank (forward and reverse) were used to generate the linearized recombination fragments by using *PmeI* restriction digest as detailed above. Following E/T recombination, recombinant clones were selected by plating on LB agar containing Kan and Cam. BAC DNA from recombinant clones was then prepared for further analysis as detailed above. WT BoHV-4 *Mb*-Fusion, and WT BoHV-4 RVFV-Gn BACs were successfully constructed. Restriction digestion was also carried out to ensure expression cassettes have been inserted in the WT BoHV-4 BAC and to confirm genomic stability of the recombinant BACs clones. The WT BoHV-4 *Mb* BAC clones and WT BoHV-4 RVFV-Gn BAC clones were digested with *EcoRI*. The resulting *EcoRI* digested fragments were separated according their molecular weight by electrophoresis and were compared with the *EcoRI*

digested fragments of WT BoHV-4 BAC. After that the Kan<sup>R</sup> gene was removed from the recombinant BAC clones and then the selected BAC clones were transfected into MDBKs cells and the expression of the *Mb* and RVFV Gn were analysed by western blot. Cloning of WT BoHV-4 *CB-Com1* and WT BoHV-4 *CB-Mip* BACs was unsuccessful, with restriction digestion analysis showing consistent loss of large segment of the BoHV-4 BAC genome.

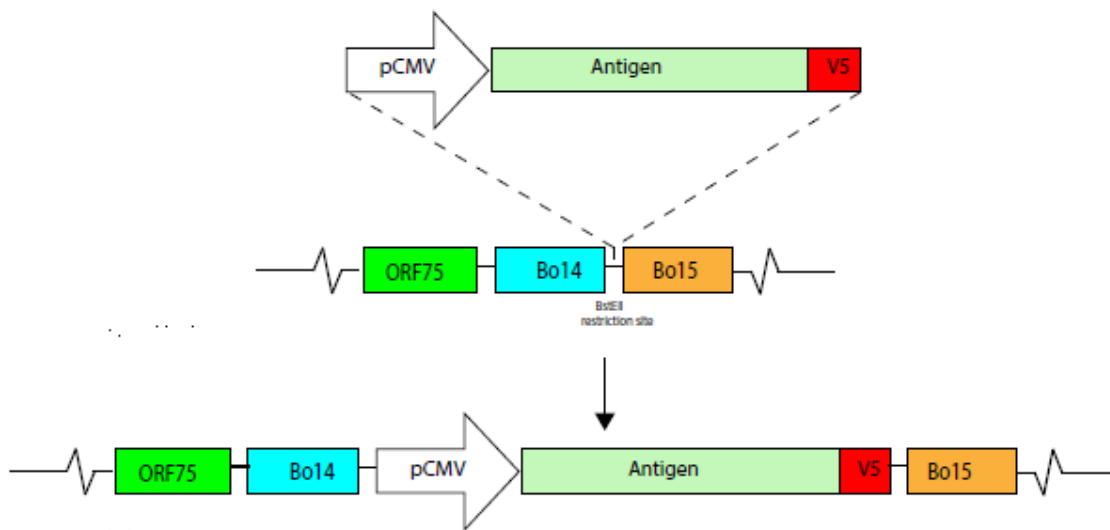


Figure 3.43: Schematic illustrating the construction of WT BoHV-4 vector expressing various antigens. The antigen expression cassette was inserted within BoHV-4 genome into *BstEII* restriction site using E/T-recombination. Linearized vector carrying the Kan<sup>R</sup> marker and *Mb* expression cassette was electroporated into *E. coli* (strain EL250), a bacterial strain containing E/T recombinases. Following removal of the Kan<sup>R</sup> marker. Following removing the Kan<sup>R</sup> marker, the recombinant BACs was transfected into MDBK cells to reconstitute WT BoHV-4 expressing *Mb* Fusion protein, *CB-Com1* and *CB-Mip*.

### 3.2.4 Immune responses in rabbits inoculated with BoHV-4 $\Delta$ ORF73 *Mb*-fusion protein

A rabbit model was used to test the hypothesis that a BoHV-4  $\Delta$ ORF73 vector can induce an immune response against its encoded heterologous target antigen. Eight rabbits were vaccinated subcutaneously with  $1 \times 10^8$  pfu and then at week 4 these animals were boosted using identical inoculum. The control group (four rabbits) received a comparable vaccine regimen using WT BoHV-4. *Mb*-directed antibodies measured by ELISA were then used as a readout for a *Mb*-specific immune response. Only four antigens were available for analysis by ELISA. These antigens were provided as recombinant proteins Rv3804 (Ag85A), Rv0287 (TB9.8) and Rv0288 (TB10.4). Rv3615c was used as non-vaccine *Mb* control protein. As shown in Figure 3.44, antibodies were detected against the *Mb* antigens encoded by the BoHV-4  $\Delta$ ORF73 *Mb* vector by ELISA in 3 of 8 animals (37.5%) of animals. Responses were recorded against different antigens in different animals indicating that multiple regions of the recombinant *Mb* fusion protein are being recognised by the rabbit host immune response. The number of responders presumably represents an underestimate, as only three of the six antigens contained within the fusion protein were available as recombinant proteins. In order to confirm that the attenuated version of BoHV-4  $\Delta$ ORF73 *Mb* vector not persist in the vaccinated animals, PCR was performed to target BoHV-4 ORF16. The results Figure 3.45 showed the absence of detectable virus in the spleen by 12 weeks post-vaccination.

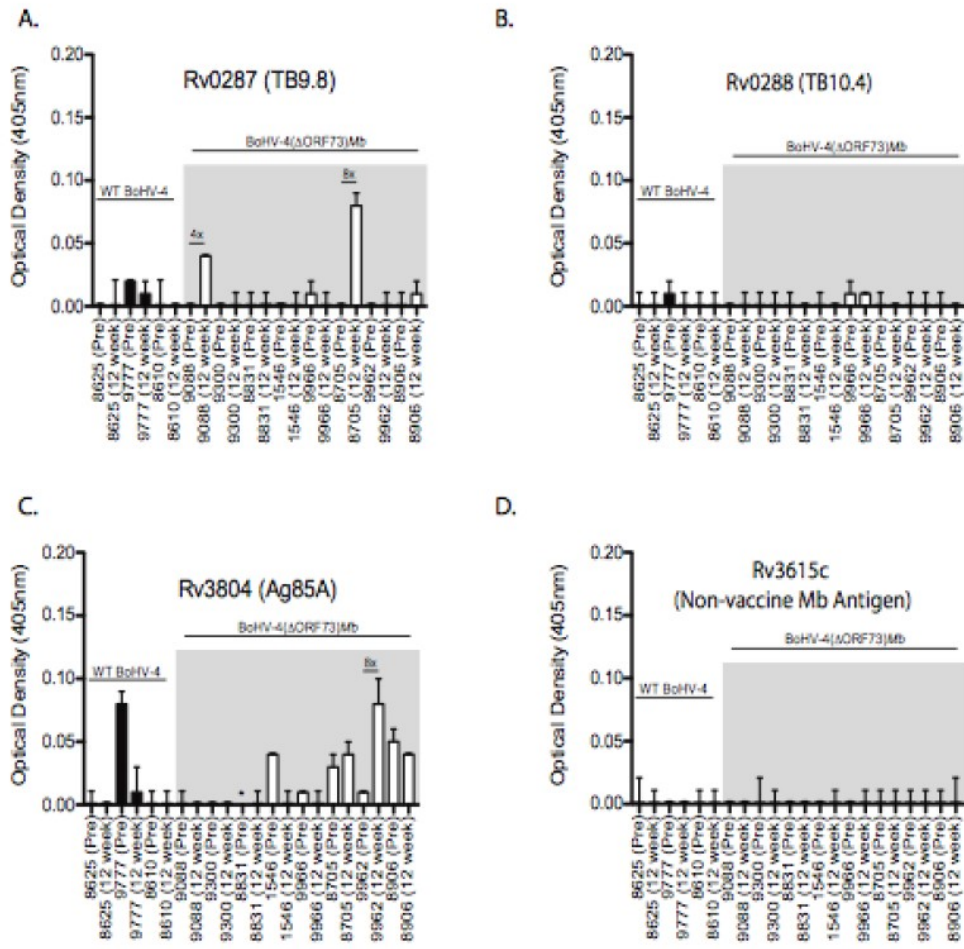


Figure 3.44: ELISA showing responses against *Mb* proteins from BoHV-4  $\Delta$ ORF73 *Mb* vaccinated rabbits. Rabbits (n=8) were vaccinated with BoHV-4  $\Delta$ ORF73 *Mb* and then boosted 4 weeks later with  $1 \times 10^8$  pfu via the sub-cutaneous route. Control animals (n=3) received an identical regimen, but using wild-type (WT) BoHV-4. At 12 weeks, animals were humanely euthanised, at which time blood was collected. ELISAs using recombinant *Mb* proteins present within the vaccine fusion protein (A: Rv0287, B: Rv0288 and C: Rv3804) and D: Rv3615c (as a non-vaccine *Mb* antigen) were performed. All sera was tested at a dilution of 1:200 using a rabbit IgG-specific antibody coupled to alkaline phosphatase at a dilution of 1:750. ELISAs developed for 30 minutes before reading at 405nm. Samples were analysed in triplicate and results show absorbance levels after subtraction of average animal-matched sera response against the Rv3615c control antigen. All fold level changes were < 2-fold compared to matched pre-bleed sera. Positive sera were arbitrarily defined as sera 4-fold above pre-bleed values and corresponded to three animals: Rabbit 9088, Rv0287 (4-fold); Rabbit 8705, Rv0287 (8-fold); and Rabbit 9962, Rv3804 (8-fold). \*Pre-bleed sera from rabbit 8831 with >100-fold consistent reactivity against Rv3804 was removed from analysis against this antigen.

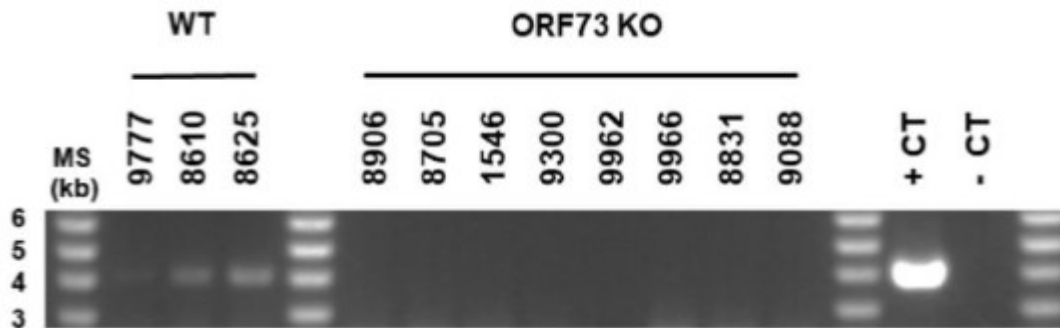


Figure 3.45: BoHV-4  $\Delta$ ORF73 *Mb* is attenuated *in vivo*. Rabbits vaccinated as described above were analysed for the presence of persistent vaccine in spleen at termination of the study at week 12 post-initial vaccination by using PCR targeting BoHV-4 ORF 16. The absence of PCR signal indicates the loss of the attenuated BoHV-4  $\Delta$ ORF73 *Mb* vaccine within 8 weeks of vaccination (rabbits were boosted at week 4). This is in contrast to control animals with WT BoHV-4, where the virus persisted (Data from Alain Vanderplasschen Laboratory).

Our ELISA results showed that the conditionally attenuated BoHV-4 (BoHV-4  $\Delta$ ORF73 *Mb*) that expressed *Mb* fusion proteins can induce an immune response against *Mb* (a heterologous pathogen target antigen). This result provides a ‘proof-of-concept’ for using the attenuated BoHV-4 (BoHV-4  $\Delta$ ORF73) based vectors in developing safe vaccines to target zoonotic and epizootic microbes including *CB* and RVFV, therefore the BoHV-4  $\Delta$ ORF73 *CB* and BoHV-4  $\Delta$ ORF73-RVFV-Gn were constructed.

### 3.3 Discussion

Zoonotic diseases represent the majority (60.3%) of the emerging infectious diseases which have been shown significantly affect human health. These zoonotic diseases are caused by pathogens emerging from wildlife and agricultural animal population and cause serious diseases in human (Jones *et al.*, 2008). Bacteria represent the majority of these pathogens (54.4%), with viruses (25.4%) and protozoa, fungi and helminths comprising the rest (Jones *et al.*, 2008). *Mb* and *CB* are both zoonotic bacteria causing diseases considered as emerging or re-emerging in different part in the world (Arricau-Bouvery & Rodolakis, 2005; Esteban *et al.*, 2005). Rift Valley fever is caused by a viral pathogen (RVFV) that is also reported to cause reemergent disease in different areas (Nyakarahuka *et al.*, 2019; van Vuren *et al.*, 2019).

Vaccination is considered as an important innovation that has significant impact on preventing many of diseases that afflict both humans and animals by eliciting the immune response of the host against the pathogens. Developing a vaccine to target these pathogens in animals can interrupt transmission of these pathogens from animal to human populations (Meeusen *et al.*, 2007). Developing animals vaccine has an advantage over human vaccine development, with the average time required for developing an animal vaccine being 5-7 years while for a human vaccine it is 15-20 years (Bregu *et al.*, 2011). This difference is, in large part, due to the lower stringency of approval requirements for animal vaccines. For this reason, the WHO has suggested developing a vaccine to target the zoonotic coronavirus MERS-CoV in the camel reservoir rather than in humans as this will be faster than developing a human vaccine (Modjarrad *et al.*, 2016).

In this chapter, we showed the ability of conditionally attenuated BoHV-4 base vaccine vector to induce immune response against the *Mb*-fusion protein (heterologous antigen) as the first step toward developing a safe, and inexpensive vaccine to target emerging zoonotic diseases



in ruminant species. Viral vector-based vaccines such as BoHV-4 are a relatively new form of vaccination that provides many advantages over traditional vaccine technologies. The viral vectors have been engineered to carry a gene from the pathogen that could be expressed in the host and induce both cellular immune response (by activating CD8<sup>+</sup> T cells) and humoral (by activating CD4<sup>+</sup> T cells) that contribute to controlling the pathogen either directly by killing the infected cells or by inducing antibodies (Liu, 2010a; Liu, 2010b). The vector-based vaccine technology could overcome some challenges related with developing a conventional vaccines against emergent infectious diseases; a major advantage is that it does not require cultivation of the emerging pathogen, which is required for any killed vaccine, as it is based on an engineered viral vector that express antigens of the pathogens (Afrough, Dowall & Hewson, 2019; Draper & Heeney, 2010; Rauch *et al.*, 2018). It also has no capacity for reversion to the virulent form of the pathogen, which is an inherent concern with live attenuated vaccine strategies.

In both of these regards, BoHV-4 base vector is an attractive vaccine vector. It is generally benign, and several studies have shown its ability to express heterologous genes of many different pathogens, some of which were associated with specific protective immunity (Donofrio *et al.*, 2013; Franceschi *et al.*, 2015). However, use of replicating viral vectors even as apparently benign as BoHV-4, in a vaccine application raises concern related to safety, not only for the animal, but also within the environment. Attenuation of the viral vector is therefore an important aspect of any vaccine. Two conditional attenuation strategies have been developed for BoHV-4 vaccine vectors. The first strategy is based on deletional inactivation of the TK gene (Donofrio *et al.*, 2002; Donofrio *et al.*, 2013; Donofrio *et al.*, 2007b; Franceschi *et al.*, 2011; Franceschi *et al.*, 2015; Franceschi *et al.*, 2014; Macchi *et al.*, 2018; Rosamilia *et al.*, 2016). The second conditional attenuation approach is based on deletion of the ORF73 gene. In addition to these two strategies, the Donofrio group reported a BoHV-4 attenuation strategy

based on deletion of 1.7 kb of a late gene that encodes a polyadenylated RNA. The resulting virus was attenuated with regards to replication and spreading *in vitro*. They reported for the first time that the deletion affects virus replication (Capocefalo *et al.*, 2013). This attenuation mechanism was not well characterized and only tested *in vitro*.

In addition to BoHV-4, TK inactivation has been used to attenuate many herpesvirus such as herpes simplex virus (Chen *et al.*, 2004; Coen *et al.*, 1989; Katz, Bodin & Coen, 1990; Price & Khan, 1981; Tenser & Edris, 1987), Pseudorabies virus (Ferrari *et al.*, 2000; Moormann *et al.*, 1990), BoHV-5 (da Silva *et al.*, 2011; Silva *et al.*, 2010) and BoHV-1 (Kit & Kit, 1987). Deletion of TK affects the metabolism of the deoxyribonucleotides that are required for viral DNA synthesis and inhibits genome replication in proliferative cells due to a limitation in levels of phosphorylated nucleic acid precursors. Deletion of TK from BoHV-4 resulted in a replicative defective virus which was restricted in its replication to the site of inoculation. It also was not secreted into body fluids of vaccinated animals and did not spread between animals (Donofrio *et al.*, 2013). However, there is a possibility that the virus vector can be taken up from the site of inoculum by macrophages and transferred by lymphatic route to lymph nodes and other organs of the vaccinated animals (Donofrio *et al.*, 2013). This raises concerns especially when the vaccine vector is to be used to vaccinate animals such as cattle, sheep or goats in which their products (meat or milk) will be consumed from human. The concern is that this might lead to transfer of the genetically modified vaccine vector to humans. Moreover, several studies showed that TK deleted mutant herpesviruses could establish latent infections (Cadore *et al.*, 2013) that can reactivate (Whetstone *et al.*, 1992).

The second conditional BoHV-4 attenuation strategy is based on deletion of the ORF73 region. Earlier work based on homology to orthologous proteins of other herpesviruses suggested that N- and C- terminal regions of ORF73 might be implicated in episome maintenance and viral/cellular gene regulation, and thereby the protein play a role in viral latency due to episome

tethering to mitotic chromosome during replication. Consistent with this model, deletion of ORF73 region from BoHV-4 was shown to result in a virus which can replicate normally *in vitro* but could not persist *in vivo* (Thirion *et al.*, 2010). The requirement for ORF73 for maintenance of the BoHV-4 genome during latency led the authors of Thirion *et al* 2010 to propose deletion of ORF73 as a method for attenuation (Thirion *et al.*, 2010). A similar strategy has been used successfully to attenuate murine gammaherpesvirus, MHV-68 (Fowler & Efstathiou, 2004).

Until the present work, the impact of deletion of ORF73 on BoHV-4 immunogenicity had only been explored in terms of the effect on endogenous BoHV-4 proteins. In order to extend this work to the development of BoHV-4 vaccine vectors, we sought to determine the impact of ORF73 deletion on immunogenicity of heterologous proteins expressed by BoHV-4. To this end, we constructed a series of ORF73-deleted BoHV4 vectors expressing target antigens from the zoonotic pathogens *Mb*, *CB* and RVFV. The main finding of this chapter was the demonstration of the ability of a BoHV-4 vector deleted for ORF73 to induce immune responses against the heterologous antigen from *Mb*. *Mb*, the causative agent of bTB, remains a significant zoonotic pathogen in developing countries. It is also a cause of serious economic problems in many developing as well as developed countries.

Since around 2000, scientific efforts have been focused on developing a vaccine to target *Mycobacterium bovis* in cattle. BCG is one of the earliest vaccines to be developed. It is a live attenuated vaccine made from a strain of *Mb* that has been attenuated by serial growth on oxen bile glycerine potato medium. The efficacy of BCG has been shown to be highly variable, ranging from between 0 and 80% depending on many factors, including the age of the animals and method of vaccine delivery. A further fundamental problem of BCG is the difficulty to distinguish the vaccinated animal from an infected animal (DIVA) when using the standard single comparative cervical tuberculin SICCT test. In addition to BCG, many additional types

of *Mb* vaccines have been investigated, including killed *Mycobacterium* vaccines, *Mycobacterium* protein vaccines, DNA vaccines and viral-based vaccine. However, currently BCG is the only vaccine available to target *Mb*. A number of publications showed that BCG vaccine cannot provide long-lasting immunity in cattle. We aimed to develop a new more effective and cheap vaccine to target *Mb* in cattle based on an attenuated version of the BoHV-4 vaccine vector.

In this chapter BoHV-4  $\Delta$ ORF73 was engineered to express an *Mb* fusion protein that was composed of six *Mb* antigens (Rv3620, Rv1733, Rv2389, Rv3804 (Ag85A), Rv0287 (TB9.8) and Rv0288 (TB10.4be DFIVA). These antigens were selected based on a number of criteria based on immunogenicity; potential efficacy and DIVA (see Introduction). The immunogenicity of the attenuated BoHV-4ORF73 *Mb* was demonstrated in a rabbit model. An important reason for using rabbits is due to this species being permissive for BoHV-4 infection, where the virus establishes a persistent infection resembling infection in cattle (Osorio & Reed, 1983; Osorio, Reed & Rock, 1982; Osorio, Rock & Reed, 1985). In the present study, using antibodies against *Mb* antigens as the measure of immunity, BoHV-4  $\Delta$ ORF73 *Mb* induced responses were detected by ELISA in three of eight animals (37.5%). The number of responders presumably represents an underestimate, as only three of the six antigens contained within the fusion protein were available as recombinant proteins.

This level of response is comparable to numbers seen in previous studies using outbred populations following vaccination with other recombinant *Mb* or *Mycobacterium tuberculosis* (*Mtb*) vaccines, or during *Mb* and *Mtb* infection. A study by Ma *et al* (2016) showed that recognition of distinct antigens within a *Mtb* fusion protein construct ranged between 0 and 30.8% in a group of active and latently *Mtb* infected human individuals on the basis of IFN $\gamma$  release (Ma *et al.*, 2016). Another study showed that only 50% of antigens (7 of 14) were detected following vaccination mice with a MVA vector expressing a *Mtb* polyprotein fusion.

Both of these antigens were detected using a sensitive ELISPOT assay rather than antibody, which was used as a marker for immunity in the present study (Leung-Theung-Long *et al.*, 2015).

In vaccines comprised of a single or few *Mb* or *Mtb* antigens, the number of responders can also vary considerably (Taracha *et al.*, 2003). A number of studies showed that using adjuvants such as IC31® adjuvant and liposomal AS01E increase the number of responder. In a recent human trial using the IC31® adjuvant the number of responders increased from 11% (1 of 9) to 50% (4 of 8) in 17 *Mtb* naïve individuals who were vaccinated with a recombinant fusion protein consisting of Ag85B and ESAT-6 (H1) (Hussein *et al.*, 2018). Another study showed that using liposomal AS01E as adjuvant with recombinant fusion of 2 *Mtb*-derived proteins enhanced the immunity, with all individuals seroconverted after 2 vaccine doses (Penn-Nicholson *et al.*, 2015).

In summary, the levels of response that were observed are comparable to those seen for other vaccine platforms expressing TB antigens. Use of an adjuvant with BoHV-4  $\Delta$ ORF73 *Mb* vaccine also might increase the number of the responders. It will be interesting for future study to determine whether an using adjuvant could enhance the immune response induced by BoHV-4  $\Delta$ ORF73 *Mb* vaccine.

The CD4 T responses are known to be critical for control of TB through interferon gamma. This is due to the activation of macrophage where *Mb* resides (Flynn, 2004). Both the antibodies and T cells responses are important for control the *Mb*, *CB* and RVFV. Antibodies are involved in opsonization and phagocytosis in addition to the killing of extracellular *Mtb* (Abebe & Bjune, 2009; Achkar & Prados-Rosales, 2018; Counoupas, Triccas & Britton, 2019). Neutralizing antibodies are known to control RVFV (Faburay *et al.*, 2016; Kortekaas *et al.*, 2010a; Wang *et al.*, 2019; Warimwe *et al.*, 2016). Ab-mediated humoral immunity induces

protective immunity against *CB* (Shannon & Heinzen, 2009; Zhang *et al.*, 2013; Zhang *et al.*, 2007). The CD8 T cells also play an important role in protection against TB during chronic infection (van Pinxteren *et al.*, 2000). MHC-I-restricted antigen-specific CD8 T cells play an important role in controlling replication of *CB* through their ability to produce interferon gamma and induce death of infected cells. For controlling primary *CB* infection, it has been shown that both MHC-I-restricted antigen specific CD8 T cells and MHC-II-restricted antigen specific CD4 T cells are important (Buttrum *et al.*, 2018). CD8 T cell responses protect immune-competent mice against RVFV infection (López-Gil *et al.*, 2013). Antibodies were used in present study as an easily measured marker of immunity. Detection IgG antibodies, indicating the presence of class isotype switching and induction of biologically significant *Mb*-specific CD4 T cell responses. T cells will be analysed in future study.

Another further possible explanation for responses not being observed in all animals could be due to the BoHV-4  $\Delta$ ORF73 *Mb* still containing the BAC vector sequence. The presence of this sequence combined with the *Mb* expression cassette results in an additional 15kb within the viral genome. The overlength genome could affect the replication of the virus and thereby affect the ability of the virus to stimulate the immune system (Adler, Messerle & Koszinowski, 2001; Bett, Prevec & Graham, 1993). Indeed, the presence of the additional genetic material from the BAC cassette for MCMV, which had no effect on *in vitro* replication, was shown to negatively impact replication *in vivo* in mice. If immunogenicity is correlated to the level of viral vector replication, then the presence of the BAC could have resulted in further attenuation of BoHV-4  $\Delta$ ORF73 *Mb* below that necessary for induction of immunity in all animals.

Finally, as indicated above BCG immunity in cattle wanes after 12-24 months. Prior to our work, the response at least against total BoHV-4 endogenous proteins by the ORF73-deleted virus was shown to persist for at least 63 days. Duration of response was not an endpoint in the present study. However, our data extended upon this work by showing responses out to 84 days

post-initial vaccination (56 days post-boost). The impact of the BAC cassette on immunogenicity and durability of responses will be important aspects to look at in future studies.

Since *Mb* still causes significant disease in developing countries construction of less expensive vaccines such as one based on BoHV-4 could play a role in the eradication of tuberculosis in these low and middle-income countries (LMICs) where the resources of detection and control the infection are unavailable. Infection in livestock was reported in 70% of African countries (Awada *et al.*, 2018; De Garine-Wichatitsky *et al.*, 2013). In Cameroon, infection was detected in 22% of the cattle slaughtered at one of the country's main abattoirs (Awah-Ndukum *et al.*, 2016), and comparable levels of infection were reported in other regions of the country (Egbe *et al.*, 2016) and across the African continent (Demelash *et al.*, 2009). *Mb* also represents a significant zoonotic threat, with an estimated 3.1% of human TB cases being caused by *Mb* infection. Most of the cases were reported LMIC such as Nigeria (3.9%), Uganda (7%), Tanzania (16% ) (El-Sayed *et al.*, 2016) and Indian (Bapat *et al.*, 2017). The low-cost of production of conditionally attenuated BoHV-4 vaccines makes them suited to development for use in LMICs.

Even in developed countries such as the UK, cost is an important concern. In the UK, approximately 41% of cattle are located in the high-risk area (The Cattle Book 2008, GOV.UK). It has been shown that the farmers realized the risk of infecting the cattle and they have a willingness to pay (WTP) about £17 per dose / per animal / per year for a vaccine (Bennett & Balcombe, 2012). The estimated cost of production of a BoHV-4 *Mb* vaccine can be based on the £3 wholesale cost per dose of the commercially available Bovilis® IBR Marker Live (Merck Animal Health). BoHV-4 is similar to Bovilis® IBR Marker as both of them are conditionally-attenuated and the requirements for producing this BoHV-4 based vaccine are

also similar. The marginal revenue for a BoHV-4 vaccine will be about £18 per dose/per animal/per year, which is within the acceptable cost range.

Speed of vaccine construction is important to control diseases caused by newly emerging pathogens. In the recent WHO R&D blueprint for emerging infectious diseases, the committee confirmed the need for reducing the time between the announcement of a public health emergency and the availability of vaccines in order to save a greatest possible number of lives (Mehand *et al.*, 2018). Prior to BAC technology, homologous recombination in eukaryotic cells required introducing the gene of interest and the selectable marker into the target gene through eukaryotic cellular recombination machinery. The selection of the recombinant virus from the wild type virus using this method required multiple rounds of selection and is time consuming especially for a slow growing virus such as herpesviruses (Manning & Mocarski, 1988; Spaete & Mocarski, 1987). Other methods were based on sub-cloning viral genomes as a set of overlapping cosmid clone in *E. coli*, including the cosmid that contains the genetic material that is targeted. After genetic modification in the cosmid clone, recombinant viruses needed to be reconstituted by cotransfecting the clone that contains the modified genetic material together with the remaining cosmid set in eukaryotic cells. Reassembling the entire full-length genome in the eukaryotic also needs many recombinant events (Brune, Messerle & Koszinowski, 2000). Importantly, use of BAC technology and gene synthesis enables construction of herpesvirus-based vaccines in a short time, the time from synthesis of newly identified virus sequences in the field to production of vaccine seed stocks has now been reduced to 4 weeks. This rapid turnaround completely aligns with use of the BoHV-4 vector to target emerging diseases in animal populations.

Although using BAC technology has overcome the problems that related to the time that required for construction the recombinant viruses there still some problems that could delay the construction such as the toxicity. The toxicity of the antigens slowed down the construction



of a *CB* vaccine in this study. Initially, HspB and P1 *CB* antigens were selected as a candidate antigens to be expressed by the BoHV-4  $\Delta$ ORF73 vector because a previous study showed that these antigens provide a level of protection against *CB* (Li *et al.*, 2005b). The P1 and HspB fusion protein, which is comprised of the structural outer membrane protein 1(P1) and heat-shock protein B(HspB) was synthesised by GeneArt Synthetic. Cloning these fusion proteins into pCMV/ ORF73Flank and pCMV/ WTFlank were unsuccessful. The reason why the P1:HspB fusion protein could not be cloned into this vector is probably due to the toxic effect of this gene to the PIR1 bacterial cells (Kimelman *et al.*, 2012). Our results were clearly consistent with both P1 and HspB gene being toxic to *E.coli* PIR1 bacteria. However, this result has not previously been described during cloning these genes as fusion or single antigens into bacterial cells such as *E. coli* M15 cells or LMG194 cells, respectively (Li *et al.*, 2005b; Varghees *et al.*, 2002). Unpublished data indicated that successful cloning of HspB required modification of a region of these two antigens (unpublished personal communication).

More than four months was taken by attempts at cloning of the *CB* P1HspB gene. In response to this issue, an alternative strategy of cloning multiple antigens in parallel was adopted. Three antigens, *CB* Com1, *CB* Mip and *CB* SecB were selected based on their ability to induce interferon gamma producing Th1 CD4 T cells and induce protective immune response in animal models (Wei *et al.*, 2011; Xiong *et al.*, 2012). Such a delay in construction the vaccine would have undermined the ability to respond rapidly to the new emerging pathogens. Therefore, during any outbreak a number of antigens should be selected in parallel and used in developing the vaccine. *CB* Com1 and *CB* Mip were successfully cloned, which indicates a need to develop antigens in parallel.

The cloning of *CB*-Com1 and *CB*-Mip into pCMV/ ORF73Flank (forward and reverse orientations) and pCMV/ WTFlank (forward and reverse orientations) vectors were successful.

The herpesvirus vectors are distinguished from other vaccines in their need for replication. Although this is regarded as beneficial, it also presents potential problems if the target antigen is refractory to herpesvirus replication. Similarly, gene regions flanking the expression cassette have been shown to affect expression in an orientation-specific fashion. To this end, the target antigen was cloned in both orientations, again with a strategy of providing redundancy and preempting known bottlenecks to vector construction. Clearly, this would be more of an issue for construction of a vaccine targeting a previously unknown pathogen emerging through ruminants (Note CEPI guidelines for the construction of a vaccine within 16 weeks). Finally, prior to synthetic gene technology, PCR was used to amplification of the gene of interest and the flanking regions during the construction of the expression cassette. Using synthetic gene removes another bottleneck that could result from unsuccessful amplification of these regions by PCR.

In this study, the BoHV-4 based vaccine vector expressing *CB* antigens was constructed to be used as a vaccine to target *CB* in ruminant animals. Currently, an inactivated vaccine (Coxevac, CEVA) was reported as a most effective vaccine in animals. It is licensed for goats and cattle in Europe. This vaccine reduces the incidence of an abortion caused by *CB* and it was noted that this vaccine also reduces *CB* shedding in vaginal mucus. However, it could not protect previously infected animals (Arricau-Bouvery *et al.*, 2005; Dijkstra *et al.*, 2012; Guatteo *et al.*, 2008; Hogerwerf *et al.*, 2011; Rousset *et al.*, 2009). Another disadvantage of this vaccine is that it is not DIVA, such that it is not possible to distinguish between the previously infected (naturally infected) and the vaccinated animals by ELISA. The vaccine, being an inactivated form of pathogenic bacteria, also requires the cultivation of virulent *CB*. Using a vaccine-based vector like BoHV-4 could be effective especially to target an intracellular pathogen such as *CB* as it could stimulate both T and B cells, which play a critical role in controlling *CB* infection (Buttrum *et al.*, 2018; Xiong *et al.*, 2016). Due to time limits in this study, B and T cells immune

responses were not measured. However, in future work both responses would be relevant for assessment for *CB* and *Mb* vaccines. As only defined antigens of the pathogen are being targeted, the immune response induced by BoHV-4-based vaccines will be able to be distinguished from the immune response induced by infection (eg., these vaccines are DIVA). The immune response induced to particular antigens expressed by BoHV-4 vaccine vector may be higher and of different B and T cell bias than the immune response induced by inactivated vaccine, but as it is unknown whether the attenuated version of BoHV-4 induces a similar level of the immune response to the wild type BoHV-4. It will therefore be interesting for future study to compare the immune response that is induced by both versions against a particular antigen.

The third vaccine constructed was designed to target RVFV, a mosquito-borne virus that can cause serious and lethal disease in both humans and animals (Flick & Bouloy, 2005; Gerdes, 2004). So far there is no RVFV vaccine licensed for human use. For livestock use, multiple vaccines are available: live attenuated vaccines based on Smithburn, SAV (Smithburn, 1949) and Clone 13; and formalin-inactivated vaccine. These vaccines are commercially available (Onderstepoort Institute in South Africa; Kenya Veterinary Vaccine Producing Institute and Veterinary Serum and Vaccine Research Institute, Egypt) and have participated significantly in controlling the infection in some endemic areas in Africa (Pepin *et al.*, 2010). However, there are still some drawbacks to using them. The Smithburn vaccine was reported to cause abortion and teratogenicity in pregnant animals (Botros *et al.*, 2006; Hunter, Erasmus & Vorster, 2002). Clone 13 was also recently shown to be teratogenic (Makoschey *et al.*, 2016). A major further limitation of these attenuated live vaccines is the possibility for reversion to virulence (Faburay *et al.*, 2017). In addition to the safety concerns associated with production of inactivated vaccines, these vaccines are also poorly immunogenic, needing many doses to be effective and they have short durability (WHO, 1983).

Recently, a number of studies showed that the virus vector-based vaccine could be a promising approach to target RVFV in animals (Busquets *et al.*, 2014; Kortekaas *et al.*, 2010a; Papin *et al.*, 2011; Said *et al.*, 2017; Soi *et al.*, 2010; Warimwe *et al.*, 2016).

These vaccines have many advantages such as inducing long-duration immunity. However some viruses may have problems for boosting caused by anti-vector immunity (homologous boosting) also the possibility of pre-existing immunity to the vector. BoHV-4 could be a good candidate to use as a vaccine vector to target RVFV. In the first part of this study, boosting rabbits with BoHV-4  $\Delta$ ORF73 Mb at 4 weeks led to increase the level of antibody responses against the delivered heterologous Mb fusion antigens, a very recent study showed that BoHV-4 can superinfect (Williams *et al.*, 2019).

To produce a vector-based vaccine for RVFV with a good safety profile, the recombinant BoHV-4 that lacked ORF 73 gene was created to express RVFV antigen. Gc and Gn antigens are the major neutralizing antibody targets of RVFV. They represent the two viral glycoproteins encoded by RVFV and required for entry. Therefore, the Gn was selected to be expressed by both the the latency defective BoHV-4(BoHV-4  $\Delta$ ORF73) and the wild type WT BoHV-4. BoHV-4  $\Delta$ ORF73 RVFVGn and the wild type BoHV-4 RVFVGn viruses were successfully constructed using E/T homologous recombination. In summary, a viral vector expressing only a defined target antigen such as BoHV-4 is an alternative strategy that avoids DIVA aspects of attenuated RVFV vaccines, and high-level production of the pathogen as needed for killed vaccines. The BoHV-4 vaccines also potentially addresses the further issue of spreading RVFV infection during an outbreak that can happen with parenterally administered vaccines. The potential for oral administration of BoHV-4 vaccines potentially overcomes this risk. In conclusion, our findings have demonstrated the ability of conditionally attenuated BoHV-4  $\Delta$ ORF73 Mb vector to induce immune response against the heterologous antigens. This result indicated that Mb fusion proteins could induce an immune response against the heterologous

antigen in rabbit and this is critical for using the rabbits in the next round of studies as an animal model to study the efficacy of this vaccine. Future studies will be able to exploit the ability of BoHV-4 to induce both humoral and cellular immune response against other zoonotic pathogens such as *CB* and RVFV.

## Chapter 4 Estimation of the mutation rate for human cytomegalovirus exonuclease mutant HCMVUL54( $\Delta$ 413) using a molecular cloning based strategy

### 4.1 Introduction

Proof-reading 3'-5' exonuclease activity of viral DNA polymerase is critical in preventing incorporation of erroneous nucleotide triphosphates (NTPs) during replication. This activity is intrinsic to many DNA polymerases, and its loss can have a substantial impact on fidelity of DNA replication, leading to an increase in mutation frequency both *in vitro* and *in vivo* (Frey *et al.*, 1993; Kroutil *et al.*, 1996; Kunkel, Loeb & Goodman, 1984). Preliminary data generated in the Chou Laboratory has shown that a mutation in exonuclease region II of DNA polymerase, which deletes the aspartic acid residue codon 413 from the HCMV gene UL54 (HCMVUL54( $\Delta$ 413)), may disrupt proof-reading activity and result in a mildly attenuated virus that displays increased resistance to the CMV antiviral drug Maribavir. These observations were consistent with results from an earlier 2008 study using HCMVUL54( $\Delta$ 413), wherein the virus had an increased rate of acquisition of resistance to several CMV drug inhibitors. After several passages, plaque sequencing revealed loss of this mutation and reversion to the wild type (WT) (Chou & Marousek, 2008). Importantly, HCMVUL54( $\Delta$ 413) appears to be genetically stable, enabling this phenotype to be studied without reversion to WT.

The aim of this part of the project was to characterize a HCMV that has been modified through deletion of an aspartic acid residue 413 of domain II of exonuclease region in CMV DNA polymerase, designated HCMVUL54( $\Delta$ 413). Based on the proposed function of this domain in 'proof-reading' during virus DNA replication, it is hypothesized that this mutation will result in a decrease in fidelity of the virus, leading to the accumulation of mutations. Our data show

that the virus has an accumulation of mutations consistent with an increased mutation rate and lower fidelity.

#### 4.2 Strategy for measuring mutation rate by molecular cloning

To determine whether HCMVUL54( $\Delta$ 413) has an increased mutation rate, we have established a protocol to measure the mutation frequency using a molecular cloning-based strategy similar to one used for measuring the mutation frequency of RNA viruses (Beaucourt *et al.*, 2011; Rozen-Gagnon *et al.*, 2014). This strategy has been used for many RNA viruses to screen and identify viruses with mutation in RNA polymerase that affect error rate or fidelity during replication. Following PCR amplification, PCR products were cloned into a TOPO cloning vector, followed by transformation and isolation of individual TOPO clones, with each clone containing a single amplified copy of the targeted region. Multiple individual TOPO clones were then sequenced to provide sequence information for the population of sequences present within the targeted region. The number of mutations in low and high passage virus was then used to measure the rate of mutation accumulation compared to the WT virus. There are some basic assumptions inherent with this method. First, all sequences are assumed to amplify from the viral DNA genome at a comparable efficiency and therefore will provide an unbiased representation of the original viral DNA population. Second, mutations will accumulate during passaging of the HCMVUL54( $\Delta$ 413) virus *in vitro*.

From the mutation frequency we measured the mutation rate depending on the number of replication cycles. Viral DNA was extracted from a low passage (DNA from cells transfected with BAC clone of HCMVUL54( $\Delta$ 413)), and from high passages (passage 15 and passage 23) of the virus of HCMVUL54( $\Delta$ 413) as well as from high passage (passage 23) of the wild type virus that lacked the deletion (HCMVUL54WT). Two independent regions within the viral genome were amplified using Q5 hot start high-fidelity DNA polymerase (New England

Biolabs, USA), following the supplied guideline conditions. Q5 has high fidelity ( $5 \times 10^{-7}$ ) (Potapov & Ong, 2017) and was selected in order to make the PCR-based background error rate as low as possible. The first amplified region was a 1138nt region within the UL27 gene, located between positions 33221 to 34359 of the HCMV (AD169) genome (GenBank Acc#: X17403.1). The second was a 979nt region that spanned the UL72-74 open-reading frames (ORFs), located between positions 105455 to 106434 of the HCMV genome (Figure 4.1). UL27 was selected based on the following criteria: UL27 is non-essential for *in vitro* HCMV replication (Yu, Silva & Shenk, 2003), the gene is highly variable (at least between different isolated HCMV clinical isolates) (Chou, 2009) and mutations within UL27 have previously been used as a marker of mutation, being linked to Maribavir resistance (Chou *et al.*, 2004). Although UL73 is essential for *in vitro* HCMV replication (Dal Monte *et al.*, 2004), this region has been shown to be highly variable, with variability approaching 50% between clinical samples (Pignatelli *et al.*, 2004). UL74 was also reported as a variable region (Pignatelli *et al.*, 2004; Rasmussen *et al.*, 2002). In contrast, UL72 was reported as being non-variable (Pignatelli *et al.*, 2004). We designed a set of primers to target these regions (Table 2.2). The first set was designed to amplify a 1138nt region within HCMV genome which is located between nucleotides position 33221 to 34359. This region is within the HCMV UL27 gene. The second set was designed to amplify a 979nt region located between nucleotides position 105455 to 106434. This region is comprised of HCMV UL73 which lies between UL72 and overlaps by 29nt with the UL74 region (see Figure 4.1). Following amplification, the PCR products of these regions were cloned into a TOPO vector (Invitrogen, USA). Insertion of the amplified regions into the TOPO vector was confirmed based on insert size for putative positive clones (based on a blue-white screen) using restriction enzyme digestion. PCR colony screening was also used in screening the colonies. The schematic representative of cloning strategies used in this study is shown in Figure 4.1



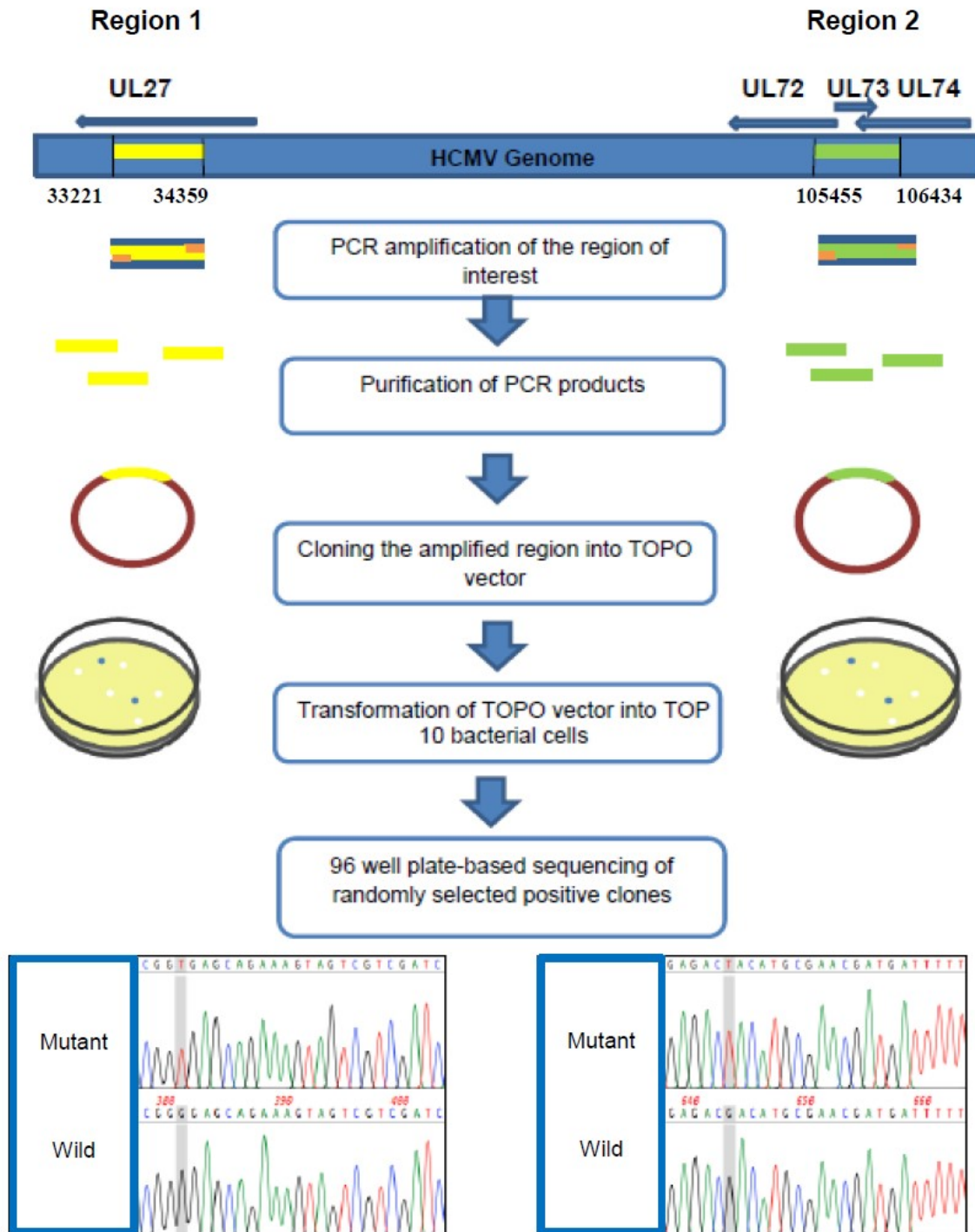


Figure 4.1: Overview of cloning strategy. Two different regions were amplified using Q5 hot start high-fidelity DNA polymerase. The PCR product was cloned into a TOPO vector then transformed into TOP10 bacteria. Bacterial clones were then sequenced by Sanger sequencing.

## 4.3 Results

### 4.3.1 Detection of HCMVUL54( $\Delta$ 413) mutations by Sanger sequencing

Two HCMV recombinant viruses were used in this study: (1) HCMVUL54( $\Delta$ 413), a putative low fidelity mutant with a deletion of aspartic acid residue 413 in domain II of the exonuclease region, and (2) HCMVUL54WT, a WT control. These viruses were engineered in Dr. Sunwen Chou's laboratory, and were passaged 23 times. Viral DNA was then extracted from early and late *in vitro* passages (passage 15 and 23) from HCMVUL54( $\Delta$ 413) and from late *in vitro* passages (passage 23) from HCMVUL54WT. The DNA was then supplied by Dr. Sunwen Chou's laboratory (Division of Infectious Diseases, School of Medicine, Oregon Health and Science University, Portland, USA) to our laboratory, where the different DNA samples were amplified and cloned into TOPO vector.

A summary of the results is shown in Table 4.1. Two independent experiments were performed comprised of separate PCR amplification of the targeted genome regions (either UL27 or UL73) and TOPO-based cloning of the PCR products. Sequences were then aligned to the HCMV AD169 reference sequence (Acc#: X17403.1) using Macvector software 14.5.3, USA. A region of 858 bp in length with consistent high quality sequence for the UL27 region (33,491 to 34,349nt) and a comparable 850 bp length region for UL73, part of UL72 and part of UL73 (105,580 to 106,430nt), were used for mutation analysis (Figure 4.2). Sequences that gave reads shorter than these indicated sizes were deleted from the data set. Reads of poor quality sequence with multiple overlapping peaks were re-sequenced and added to the data set (see Table 4.2). Mutations were defined as real only when present on both strands of a sequenced TOPO clone.

#### 4.3.1.1 Detection of mutations in UL27 Gene Locus

For the UL27 gene, a total of 834 clones were sequenced: 123 of HCMVUL54( $\Delta$ 413) (low passage), 356 of HCMVUL54( $\Delta$ 413) (high passage (p15)), 109 of HCMVUL54( $\Delta$ 413) (high passage (p23)) and 246 HCMVUL54WT (high passage (p23)). Mutations were only observed within the HCMVUL54( $\Delta$ 413) (high passages p(15) and p(23)) with a total of 29 mutations located in this region (see Table 4.3 and 4.4). Eleven mutations were found in clones of HCMVUL54( $\Delta$ 413) high passage (p23). Eighteen mutations were found in clones of HCMVUL54( $\Delta$ 413) high passage (p15) (six present in each of three independent PCR reactions for this region). No mutations were identified in TOPO clones from either the HCMVUL54( $\Delta$ 413) (low passage) or HCMVUL54WT (high passage). 212 clones of HCMVUL54WT (high passage) were sequenced. These clones from three independent experiments. 70 clones from the first experiment (total of 60060nt (70 x 858)) were sequenced, 48 clones from the second experiment (total of 41184nt (48 x 858)) were sequenced and 94 clones from the third experiment (total of 80652nt (94 x 858)) were sequenced (see table 4-1). Twenty-two of the 29 mutations in HCMVUL54( $\Delta$ 413) were non-synonymous. In UL27 two independent mutations were found in four individual clones. In the first clone a non-silent (non-synonymous) D351E mutation and a synonymous mutation located at position 33491 region was observed. In the second clone two non-synonymous mutations were present, one at L228M and the second at A272S. In the third clone two non-synonymous mutations were present, one at H334R mutation and the second at A272S. In the fourth clone a non-synonymous L372M mutation and a synonymous mutation located at position 33503 region was observed. The distribution of the mutations in Region 1 (the UL27 region) is shown in Figure 4.3.

We also found a region within UL27 region where 14 mutations out of 29 were detected in the region from position 33491 to 33619 HCMV (UL27 codon 374-389). This region was previously reported as a conserved region within the UL27 (Hantz *et al.*, 2009).

#### 4.3.1.2 Detection of mutations in UL73 Gene Locus

A total of 486 clones were sequenced: 129 of HCMVUL54( $\Delta$ 413) (low passage), 124 of HCMVUL54( $\Delta$ 413) (high passage p(15)), 108 of HCMVUL54( $\Delta$ 413) (high passage p(23)) and 125 HCMVUL54WT (high passage). Twenty mutations were identified. Twelve mutations were found in clones of HCMVUL54( $\Delta$ 413) high passages (p23) (5 in UL72 and 7 in UL73). Five mutations were found in clones of HCMVUL54( $\Delta$ 413) high passages (p15) (three in UL73 and two in UL74), while three mutations were found in clones of HCMVUL54( $\Delta$ 413) low passage (two in UL73 and one in UL74) (see Table 4.5 and Table 4.6). No mutations were identified in TOPO clones from HCMVUL54WT. 118 clones of HCMVUL54WT were sequenced (total of 100300nt (118 x 850)) were sequenced.

Sixteen of 20 mutations were non-synonymous. The distribution of the mutations in Region 2 of UL73, Region 2 of UL74 and Region 2 of UL72 are shown in Figure 4.4 through 4.6

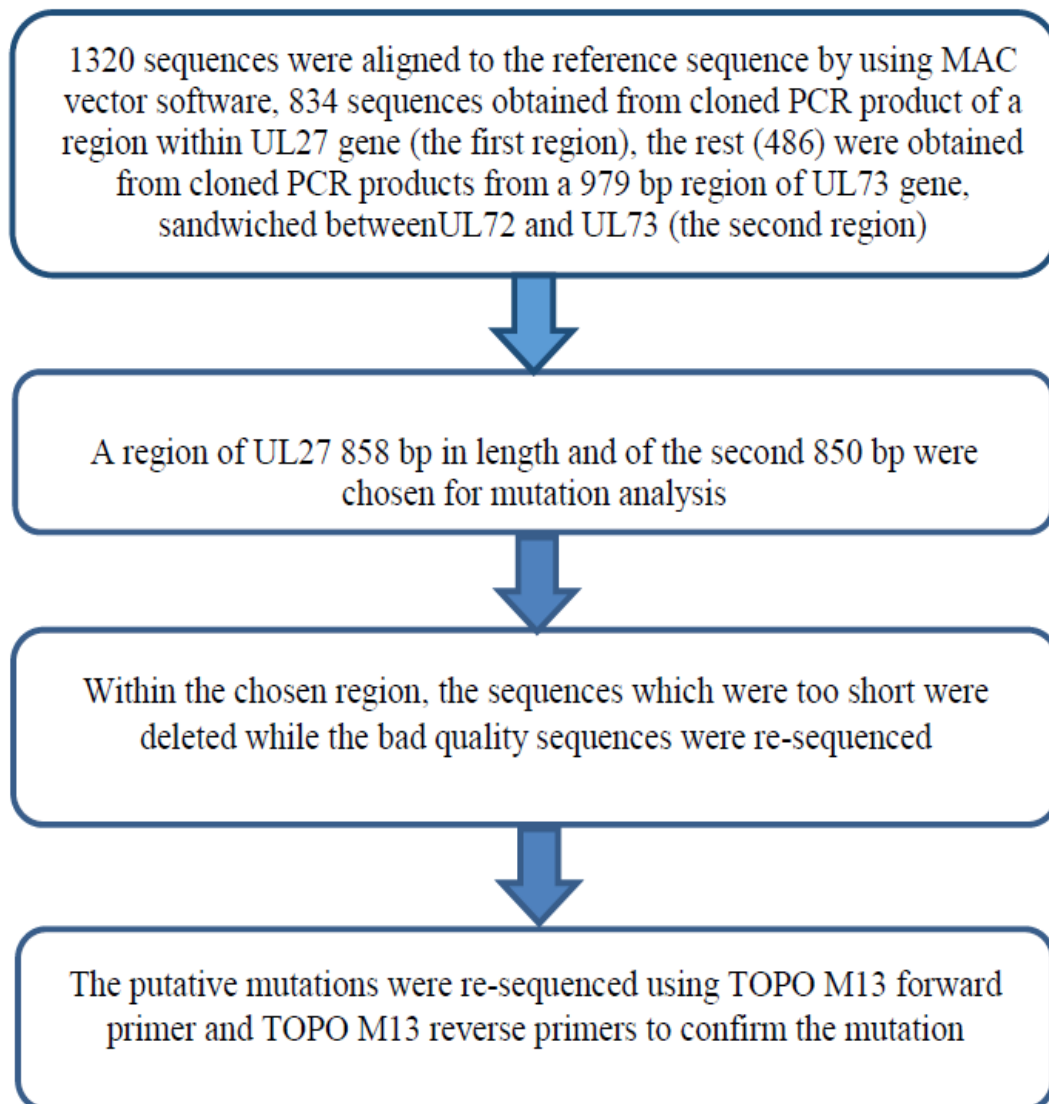


Figure 4.2: Flow diagrams of mutational analysis strategy.

Table 4.1: Summary of the data showing the number of mutations found in clones of two regions of HCMVUL54 ( $\Delta$ 413) in low and high passages (passage 15 and passage 23) and WT high passage (passage 23) (n = number of sequenced clones). Region 1 (a region within UL27 gene) and region 2 (a region of UL73 gene, positioned between UL72 and UL73).

	Low passages		High passage (p15)				High passage (p23)			
	Region 1	Region 2	Region 1 PCR 1	Region 1 PCR 2	Region 1 PCR 3	Region 2	Region 1 PCR 1	Region 1 PCR 2	Region 1 PCR 3	Region 2
HCMV UL54 WT										
							70(n) (858 bp)	48(n) (858 bp)	94(n) (858 bp)	118(n) (850 bp)
							0	0	0	0
HCMV UL54( $\Delta$ D413)	118(n) (858 bp)	118(n) (850 bp)	100(n) (858 bp)	116(n) (858 bp)	94(n) (858 bp)	118(n) (850 bp)	105(n) (858 bp)			103(n) (850 bp)
	0	3	6	6	6	5	11			12

Table 4.2: Summary of sequenced clones.

DNA samples/ regions	Number of sequenced clones	Number of poor sequences	Number of good quality sequences which are used in mutational analysis
Clones of Region 1 within HCMV UL54( $\Delta$ 413) genome of low passage virus	123	5	118
Clones of Region 1 within HCMV UL54( $\Delta$ 413) genome of high passage virus (p15)	356	46	310
Clones of Region 1 within HCMV UL54( $\Delta$ 413) genome of high passage virus (p23)	109	4	105
Clones of Region 1 within HCMV UL54 WT genome of high passage virus (p23)	246	34	212
Clones of Region 2 within HCMV UL54( $\Delta$ 413) genome of low passage virus	129	11	118
Clones of Region 2 within HCMV UL54( $\Delta$ 413) genome of high passage virus (p15)	124	6	118
Clones of Region 2 within HCMV UL54( $\Delta$ 413) genome of high passage virus (p23)	108	5	103
Clones of Region 2 within HCMV UL54 WT genome of high passage virus (p23)	125	7	118
Total	1320	118	1202

Table 4.3: Details of synonymous and non-synonymous mutations found in HCMVUL54( $\Delta$ 413)(passage 15).

DNA samples	Region 1	Nucleotide position	Gene	Codon change	Amino acid substitution	Transition/Transversion
HCMVUL54( $\Delta$ 413) (passage 15)/ replicate 1	33491 - 34349	33844	UL27	272	Alanine- Serine	Transversion
HCMVUL54( $\Delta$ 413) (passage 15)/ replicate 1	33491 - 34349	33589	UL27	357	Leucine- Methionine	Transversion
HCMVUL54( $\Delta$ 413) (passage 15)/ replicate 1	33491 - 34349	33539	UL27	373	Synonymous (Leucine)	Transition
HCMVUL54( $\Delta$ 413) (passage 15)/ replicate 1	33491 - 34349	33575	UL27	361	Aspartate-Glutamate	Transversion
HCMVUL54( $\Delta$ 413) (passage 15)/ replicate 1	33491 - 34349	33527	UL27	377	Tryptophan- Cysteine	Transversion
HCMVUL54( $\Delta$ 413) (passage 15)/ replicate 1	33491 - 34349	34083	UL27	192	Threonine- Methionine	Transitions
HCMVUL54( $\Delta$ 413) (passage 15)/ replicate 2	33491 - 34349	33538	UL27	374	Proline-Threonine	Transversion
HCMVUL54( $\Delta$ 413) (passage 15)/ replicate 2	33491 - 34349	33491	UL27	389	Synonymous (Tyrosine)	Transitions
HCMVUL54( $\Delta$ 413) (passage 15)/ replicate 2	33491 - 34349	33605	UL27	351	Aspartate - Glutamate	Transversion
HCMVUL54( $\Delta$ 413) (passage 15)/ replicate 2	33491 - 34349	33617	UL27	347	Synonymous (Alanine)	Transversion
HCMVUL54( $\Delta$ 413) (passage 15)/ replicate 2	33491 - 34349	33976	UL27	228	Leucine- Methionine	Transversion
HCMVUL54( $\Delta$ 413) (passage 15)/ replicate 2	33491 - 34349	33844	UL27	272	Alanine- Serine	Transversion
HCMVUL54( $\Delta$ 413) (passage 15)/ replicate 3	33491 - 34349	33844	UL27	272	Alanine- Serine	Transversion
HCMVUL54( $\Delta$ 413) (passage 15)/ replicate 3	33491 - 34349	33608	UL27	352	Glutamine-Histidine	Transversion
HCMVUL54( $\Delta$ 413) (passage 15)/ replicate 3	33491 - 34349	33693	UL27	322	Leucine-Proline	Transition
HCMVUL54( $\Delta$ 413) (passage 15)/ replicate 3	33491 - 34349	33538	UL27	374	Proline-Threonine	Transversion
HCMVUL54( $\Delta$ 413) (passage 15)/ replicate 3	33491 - 34349	33844	UL27	272	Alanine- Serine	Transversion
HCMVUL54( $\Delta$ 413) (passage 15)/ replicate 3	33491 - 34349	33657	UL27	334	Histidine-Arginine	Transition



Table 4.4: Details of synonymous and non-synonymous mutations found in HCMVUL54( $\Delta$ 413) (passage 23).

DNA samples	Region 1	Nucleotide position	Gene	Codon change	Amino acid substitution	Transition/Transversion
HCMVUL54( $\Delta$ 413) (passage 23)	33491 - 34349	33584	UL27	358	Synonymous (Glycine)	Transversion
HCMVUL54( $\Delta$ 413) (passage 23)	33491 - 34349	34319	UL27	113	Synonymous (Serine)	Transversion
HCMVUL54( $\Delta$ 413) (passage 23)	33491 - 34349	34059	UL27	200	Glycine-Valine	Transversion
HCMVUL54( $\Delta$ 413) (passage 23)	33491 - 34349	33784	UL27	292	Asparagine -Aspartate	Transitions
HCMVUL54( $\Delta$ 413) (passage 23)	33491 - 34349	33913	UL27	249	Proline- Serine	Transitions
HCMVUL54( $\Delta$ 413) (passage 23)	33491 - 34349	33579	UL27	360	Alanine - Valine	Transitions
HCMVUL54( $\Delta$ 413) (passage 23)	33491 - 34349	33503	UL27	385	Synonymous (Glycine)	Transversion
HCMVUL54( $\Delta$ 413) (passage 23)	33491 - 34349	33544	UL27	372	Leucine- Methionine	Transversion
HCMVUL54( $\Delta$ 413) (passage 23)	33491 - 34349	33882	UL27	33882 delA at codon 259	Synonymous (Valine)	
HCMVUL54( $\Delta$ 413) (passage 23)	33491 - 34349	33829	UL27	277	Tyrosine-Aspartate	Transversion
HCMVUL54( $\Delta$ 413) (passage 23)	33491 - 34349	33863	UL27	265	Phenylalanine -Leucine	Transversion

Table 4.5: Details of synonymous and non-synonymous mutations found in HCMVUL54( $\Delta$ 413) (low passage and passage 15).

DNA samples	Region 2	Nucleotide position	Gene	Codon change	Amino acid substitution	Transition/Transversion
HCMVUL54( $\Delta$ 413)( low passage)	105580-106430	105858	UL73	41	Threonine - Asparagine	Transversion
HCMVUL54( $\Delta$ 413)( low passage)	105580-106430	105801	UL73	22	Asparagine- Serine	Transitions
HCMVUL54( $\Delta$ 413)( low passage)	105580-106430	106353	UL74	391	Glutamate-Aspartate	Transversion
HCMVUL54( $\Delta$ 413) (passage 15)	105580-106430	106283	UL74	415	Isoleucine- Leucine	Transversion
HCMVUL54( $\Delta$ 413) (passage 15)	105580-106430	106244	UL74	428	Leucine- Isoleucine	Transversion
HCMVUL54( $\Delta$ 413) (passage 15)	105580-106430	105790	UL73	18	Glutamate-Aspartate	Transversion
HCMVUL54( $\Delta$ 413) (passage 15)	105580-106430	105972	UL73	79	Arginine- Leucine	Transversion
HCMVUL54( $\Delta$ 413) (passage 15)	105580-106430	105989	UL73	85	Phenylalanine- Isoleucine	Transversion

Table 4.6: Details of synonymous and non synonymous mutations found in HCMVUL54( $\Delta$ 413) (passage 23).

DNA samples	Region 2	Nucleotide position	Gene	Codon change	Amino acid substitution	Transition/Transversion
HCMVUL54( $\Delta$ 413) (passage 23)	105580-106430	105636	UL72	29	Threonine - Isoleucine	Transitions
HCMVUL54( $\Delta$ 413) (passage 23)	105580-106430	105819	UL73	28	Threonine - Isoleucine	Transitions
HCMVUL54( $\Delta$ 413) (passage 23)	105580-106430	105686	UL72	12	Synonymous (Glutamine)	Transitions
HCMVUL54( $\Delta$ 413) (passage 23)	105580-106430	105840	UL73	35	Serine- Tyrosine	Transversion
HCMVUL54( $\Delta$ 413) (passage 23)	105580-106430	105712	UL72	4	Methionine -Leucine	Transversion
HCMVUL54( $\Delta$ 413) (passage 23)	105580-106430	105973	UL73	79	Synonymous (Arginine)	Transitions
HCMVUL54( $\Delta$ 413) (passage 23)	105580-106430	105856	UL73	40	Synonymous (Serine)	Transitions
HCMVUL54( $\Delta$ 413) (passage 23)	105580-106430	105710	UL72	4	Methionine - Isoleucine	Transitions
HCMVUL54( $\Delta$ 413) (passage 23)	105580-106430	105820	UL73	28	Synonymous (Threonine)	Transversion
HCMVUL54( $\Delta$ 413) (passage 23)	105580-106430	105942	UL73	69	Serine- Tyrosine	Transversion
HCMVUL54( $\Delta$ 413) (passage 23)	105580-106430	105644	UL72	26	Phenylalanine- Leucine	Transversion
HCMVUL54( $\Delta$ 413) (passage 23)	105580-106430	105930	UL73	65	Threonine - Asparagine	Transversion

To measure mutation frequency we used the method of Beaucourt (Beaucourt *et al.*, 2011). Briefly, the numbers of single nucleotide polymorphisms (SNPs) that are different from the reference strain were identified. This number was then divided by the total number of nucleotides sequenced and presented as the average number of mutations per nucleotides (this represents the mutation frequency). The total number of nucleotides sequenced is the arithmetic product of the number of clones multiplied by the length of the region sequenced (Beaucourt *et al.*, 2011). To calculate the mutation rate for the virus ( $\lambda$ ), the mutation frequency is then divided by the total number of replication cycles (see Table 4.7).

#### Calculation of mutation rate ( $\lambda$ ) for HCMVUL54( $\Delta$ 413)

The mutation rate ( $\lambda$ ) = Total number of mutations / total number of nucleotide copying events (N)

The number of nucleotide copying events is the length of the sequence x number of the sequenced clones x the number of replication cycles

Total number of nucleotide copying events (N) for Region 1 is A+B+C+D

$$A = \text{Region 1 replicate 1 (passage 15)} = 858 \times 100 \times 15 = 1287000 = 1.287 \times 10^6$$

$$B = \text{Region 1 replicate 2 (passage 15)} = 858 \times 116 \times 15 = 1492920 = 1.49292 \times 10^6$$

$$C = \text{Region 1 replicate 3 (passage 15)} = 858 \times 94 \times 15 = 1209780 = 1.20978 \times 10^6$$

$$D = \text{Region 1 (passage 23)} = 858 \times 105 \times 23 = 2072070 = 2.07207 \times 10^6$$

$$\begin{aligned} \text{The total number of nucleotide copying events (N)} &= (1.287 \times 10^6) + (1.49292 \times 10^6) \\ &\quad + (1.20978 \times 10^6) + (2.07207 \times 10^6) \\ &= 6.06186 \times 10^6 \end{aligned}$$

The mutation rate for HCMVUL54 ( $\Delta$ 413) region 1 = Total number of mutations / total number of nucleotide copying events (N)

$$\begin{aligned} &= 29 / (6.06186 \times 10^6) \\ &= 4.78 \times 10^{-6} \end{aligned}$$

Total number of nucleotide copying events (N) for Region 2 is A + B

$$A = \text{Region 2 (passage 15)} = 850 \times 118 \times 15 = 1504500 = 1.5045 \times 10^6$$

$$B = \text{Region 2 (passage 23)} = 850 \times 103 \times 23 = 2013650 = 2.01365 \times 10^6$$

$$\begin{aligned} \text{The total number of nucleotide copying events (N)} &= 1.5045 \times 10^6 + 2.01365 \times 10^6 \\ &= 3.51815 \times 10^6 \end{aligned}$$

The mutation rate for HCMV UL54 ( $\Delta 413$ ) region 2 = Total number of mutations / total number of nucleotide copying events (N)

$$= 17 / (3.51815 \times 10^6)$$

$$= 4.83 \times 10^{-6}$$

By pooling all the mutations, my mutation rate is in effect a single observation so it is not possible to put confidence intervals on it.

Table 4.7: Mutation rate for HCMVUL54( $\Delta$ 413)

	Region 1			Region 2		
	HCMVUL54( $\Delta$ 413) passage 15			HCMVUL54( $\Delta$ 413) passage 23	HCMVUL54( $\Delta$ 413) passage 15	HCMVUL54( $\Delta$ 413) passage 23
	PCR 1	PCR 2	PCR 3			
Number of non Synonymous mutations	5	4	6	7	5	8
Number of Synonymous mutations	1	2	0	4	0	4
Mutation frequency	$6/(858 \times 100) = 6.9 \times 10^{-5}$	$6/(858 \times 116) = 6.0 \times 10^{-5}$	$6/(858 \times 94) = 7.4 \times 10^{-5}$	$11/(858 \times 105) = 1.22 \times 10^{-4}$	$5/(850 \times 118) = 4.9 \times 10^{-5}$	$12/(850 \times 103) = 1.37 \times 10^{-4}$
Mutation rate	$4.78 \times 10^{-6}$			$4.58 \times 10^{-6}$		

#### 4.3.2 Analysis of synonymous and non-synonymous mutations

In order to determine whether the detected mutations were under selection, analysis of synonymous (Syn) and non-synonymous (Non-syn) mutations were performed in the two selected regions (Region 1 and Region 2) within the HCMV genome. Twenty nine mutations were detected in Region 1, 22 of which were non-synonymous and 7 mutations were Syn. In a typical protein-coding gene, 75% of the mutations are expected to be Non-syn and 25% to be Syn (Graur and Li, 2001). Therefore 21.75 mutations (75%) were expected to be Non-syn and 7.25 mutations (25%) were expected to be Syn. Twenty mutations were detected in Region 2, 16 of which were Non-syn and 4 were Syn. The expected number of Non-syn and Syn mutations was 15 and 5, respectively. These small differences from the observed were not significant in a chi-squared test ( $p = 0.704954$ ).

In addition to the previous method, the expected number of non-Syn mutations in the absence of selection was calculated by counting the number of all possible mutations in the actual codons that are non-Syn compared to the number that are Syn. For example, in Table 4.8 the A272S codon GCA (WT) changed to TCA (Mut). GCA codes for Ala, and change of the first nucleotide in this codon could lead to three different amino acids TCA (Ser), CCA (Pro) and ACA (Thr). This means that there are 3 possible changes, all of which are non-Syn. Similarly, changing the second nucleotide in this codon would also lead to three different amino acids GTA (Val), GAA (Glu) and GGA (Gly). Changes in the last nucleotide of this codon did not change the amino acid: GCT, GCC, and GCG, with all coding for Ala. There are thus 6 non-Syn and 3 Syn possibilities (Nei & Gojobori, 1986). As shown in Table 4.8, in Region 1 among all mutated codons there were a total of 190 possible non-Syn and 70 Syn changes. The percentage of all possible non-Syn mutations =  $190 / (190 + 70) = 73.07\%$ . The expected number of non-Syn mutations is  $(73.07 \times 29) / 100 = 21$ . The percentage of all possible Syn mutations =  $70 / (190 + 70) = 26.92\%$ . The expected number of Syn mutations is  $(26.92 \times 29)$

$/ 100 = 8$ . The chi-square result for the difference between the observed and expected numbers was not significant in a chi-squared test ( $p = 0.76$ ).



Table 4.8: The possibility of non –synonymous and synonymous mutations in the actual codons that detected in HCMVUL54 ( $\Delta$ 413) Region 1

Mutation	Codon	Observation	Possibilities						Total	
			Non- synonymous			Synonymous			Non- synonymous	Synonymous
A272S	GCA(WT) TCA(Mut)	Non - synonymous	3	3	0	0	0	3	6	3
L357M	CTG(WT) ATG(Mut)	Non- synonymous	2	3	0	1	0	3	5	4
33539 or 1119 Codon (373)	CTC(WT) CTT(Mut)	Synonymous	3	3	0	0	0	3	6	3
D361E	GAC(WT) GAA(Mut)	Non- synonymous	3	3	2	0	0	1	8	1
W377C	TGG(WT) TGT(Mut)	Non- synonymous	3	3	3	0	0	0	9	0
T192M	ACG(WT) ATG(Mut)	Non- synonymous	3	3	0	0	0	3	6	3
P374T	CCC (WT) ACC (Mut)	Non- synonymous	3	3	0	0	0	3	6	3
33491 Codon (389)	TAC(WT) TAT(Mut)	Synonymous	3	3	2	0	0	1	8	1
D351E	GAC (WT) GAG (Mut)	Non- synonymous	3	3	2	0	0	1	8	1
Codon 247	GCC(WT) GCA(Mut)	Synonymous	3	3	0	0	0	3	6	3
L228M	CTG(WT) ATG(Mut)	Non- synonymous	2	3	0	1	0	3	5	4
A272S	GCA(WT) TCA(Mut)	Non - synonymous	3	3	0	0	0	3	6	3
A272S	GCA(WT) TCA(Mut)	Non - synonymous	3	3	0	0	0	3	6	3
Q352H	CAG(WT) CAT(Mut)	Non- synonymous	3	3	2	0	0	1	8	1

L322P	CTG(WT) CCG(Mut)	Non-synonymous	2	3	0	1	0	3	5	3
P374T	CCC (WT) ACC (Mut)	Non-synonymous	3	3	0	0	0	3	6	3
A272S	GCA(WT) TCA(Mut)	Non-synonymous	3	3	0	0	0	3	6	3
H334R	CAC (WT) CGC (Mut)	Non-synonymous	3	3	2	0	0	1	8	1
Codon 358	GGC(WT) GGA(Mut)	Synonymous	3	3	0	0	0	3	6	3
Codon 113	TCA(WT) TCT(Mut)	Synonymous	3	3	0	0	0	3	6	3
G200V	GGG(WT) GTG(Mut)	Non-synonymous	3	3	0	0	0	3	6	3
N292D	AAC(WT) GAC(Mut)	Non-synonymous	3	3	2	0	3	1	8	1
P249S	CCC(WT) TCC(Mut)	Non-synonymous	3	3	0	0	0	3	6	3
A360V	GCC(WT) GTC(Mut)	Non-synonymous	3	3	0	0	0	3	6	3
Codon 385	GGC(WT) GGA(Mut)	Synonymous	3	3	0	0	0	3	6	3
L372M	CTG(WT) ATG(Mut)	Non-synonymous	3	3	0	0	0	3	6	3
33882 delA at codon 259	GTT(WT) GTT(Mut)	Synonymous	3	3	0	0	0	3	6	3
Y277D	TAC(WT) GAC(Mut)	Non-synonymous	3	3	2	0	0	1	8	1
F265L	TTT(WT) TTA(Mut)	Non-synonymous	3	3	2	0	0	1	8	1
Total									190	70

Table 4.9: The possibility of non –synonymous and synonymous mutations in the actual codons that detected in HCMVUL54 ( $\Delta$ 413) Region 2

Mutation	Codon	Observation	Possibilities						Total	
			Non- synonymous			Synonymous			Non- synonymous	Synonymous
T41N(UL73)	ACT(WT) AAT(Mut)	Non-synonymous	3	3	0	0	0	3	6	3
N22S(UL73)	AAC(WT) AGC(Mut)	Non-synonymous	3	3	2	0	0	1	8	1
E391D(UL74)	GAA(WT) GAT(Mut)	Non-synonymous	3	3	2	0	0	1	8	1
I415L(UL74)	ATA(WT) CTA(Mut)	Non-synonymous	3	3	1	0	0	2	7	2
L428I(UL74)	CTA(WT) ATA(Mut)	Non-synonymous	2	3	0	1	0	3	5	4
E18D(UL73)	GAG(WT) GAT(Mut)	Non-synonymous	3	3	2	0	0	1	8	1
R79L(UL73)	CGA(WT) CTA(Mut)	Non-synonymous	2	3	0	1	0	3	5	4
F85I(UL73)	TTT(WT) ATT(Mut)	Non-synonymous	3	3	2	0	0	1	8	1
T29I(UL72)	ACC(WT) ATC(Mut)	Non-synonymous	3	3	0	0	0	3	6	3
T28I(UL73)	ACC(WT) ATC(Mut)	Non-synonymous	3	3	0	0	0	3	6	3
Codon 12 (UL72)	CAA(WT) CAG(Mut)	Synonymous	3	3	2	0	0	1	8	1
S35Y(UL73)	TCT(WT) TAT(Mut)	Non-synonymous	3	3	0	0	0	3	6	3
M4L(UL72)	ATG(WT) CTG(Mut)	Synonymous	3	3	3	0	0	0	9	0
Codon 79 (UL73)	CGA(WT) CGG(Mut)	Synonymous	3	3	0	0	0	3	6	3
Codon 40(UL73)	TCA(WT) TCG(Mut)	Synonymous	3	3	0	0	0	3	6	3
M4I (UL72)	ATG(WT) ATA(Mut)	Non-synonymous	3	3	3	0	0	0	9	0

Codon 28(UL73)	ACC(WT) ACA(Mut)	Synonymous	3	3	0	0	0	3	6	3
S69Y(UL73)	TCT(WT) TAT(Mut)	Non-synonymous	3	3	0	0	0	3	6	3
F26L(UL72)	TTC(WT) TTA(Mut)	Non-synonymous	3	3	2	0	0	1	8	1
T65N(UL73)	ACT(WT) AAT(Mut)	Non-synonymous	3	3	0	0	0	3	6	3
Total									137	43

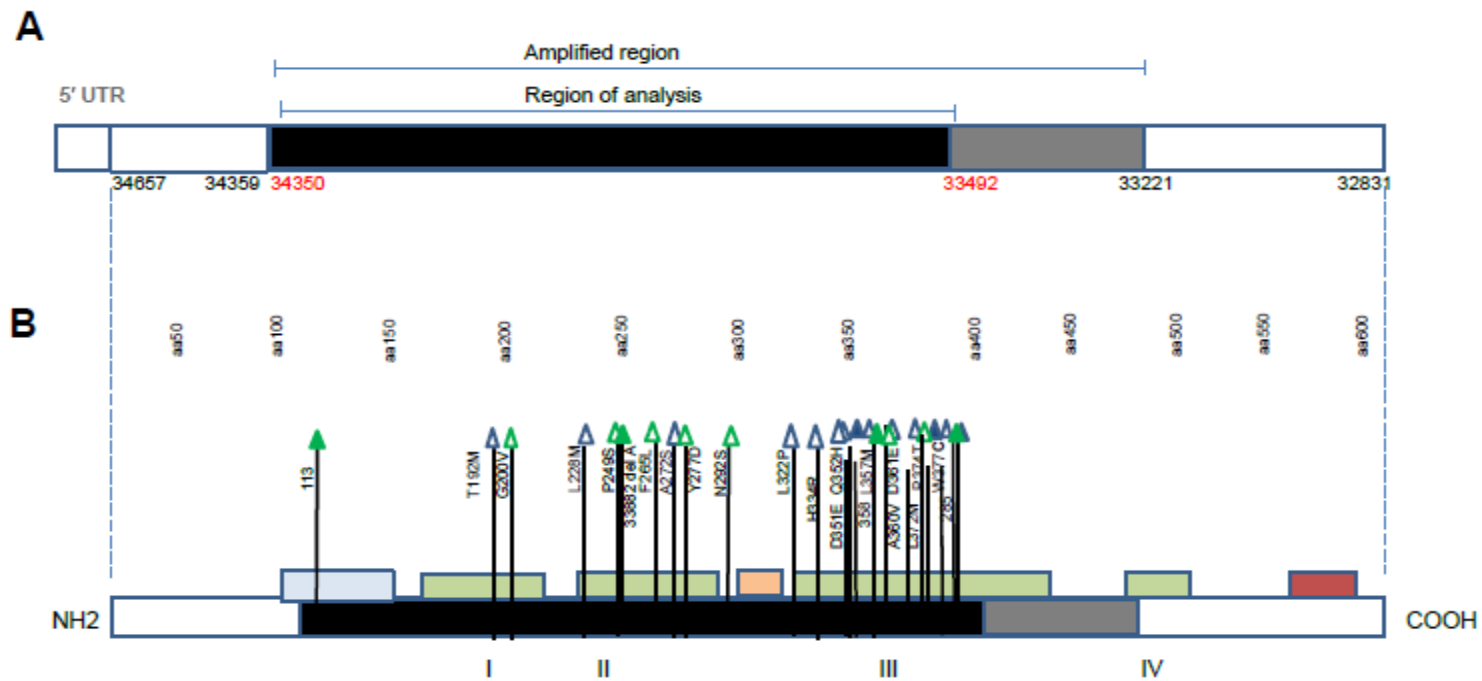


Figure 4.3: Distribution of the mutations that detected within the UL27 gene of HCMV UL54 high passages (P15) and (P23).

A272S mutation was detected in four sequenced clones (derived from independent replicates). The P374T mutation was detected in two sequenced clones (derived from independent replicates).  $\Delta$  refer to non-synonymous mutations and  $\blacktriangle$  refers to the synonymous mutations (found in HCMV UL54( $\Delta$ D413) high passages (P15)).  $\blacktriangle$  refer to non-synonymous mutations and  $\triangle$  refers to the synonymous mutations (found in HCMV UL54( $\Delta$ D413) high passages (P23)),  $\square$  N-ter conserved region,  $\square$  putative flexibility region,  $\square$  nuclear localization signal,  $\square$  conserved regions (adapted from Hantz *et al.*, 2009).

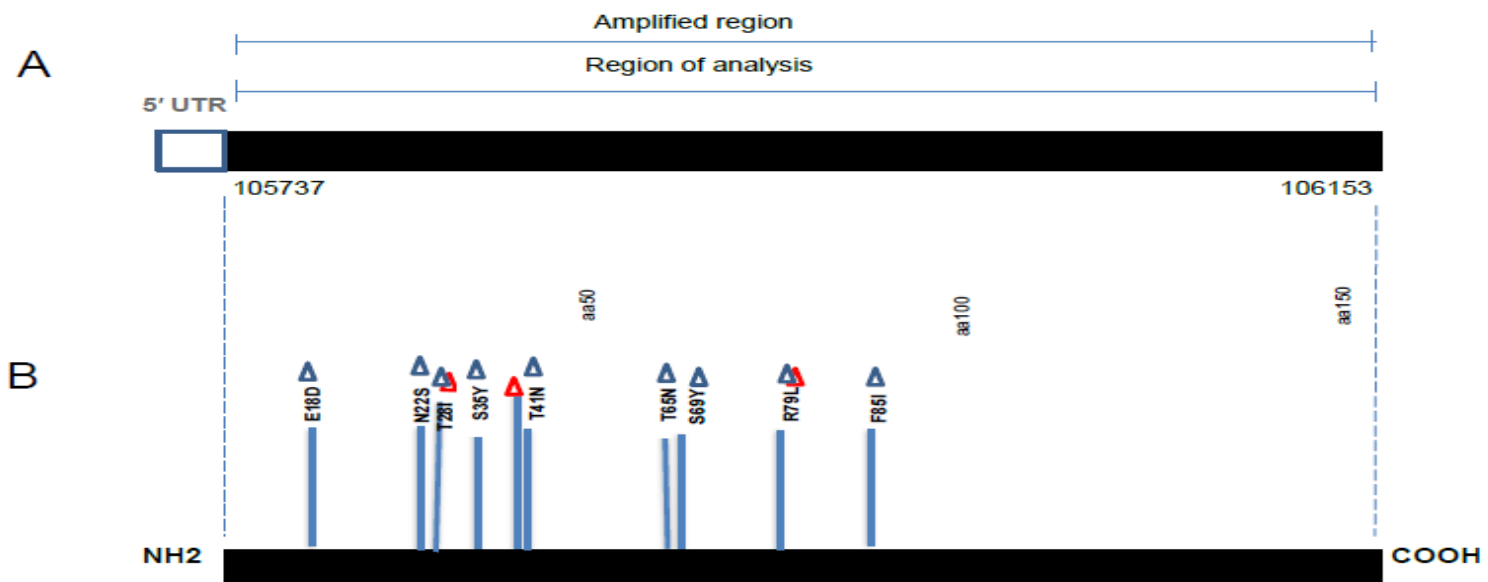


Figure 4.4: Distribution of the twelve identified mutations detected in the UL73 gene of HCMVUL54( $\Delta$ D413).

$\Delta$  refer to non synonymous mutations  $\triangle$  refer to synonymous mutations

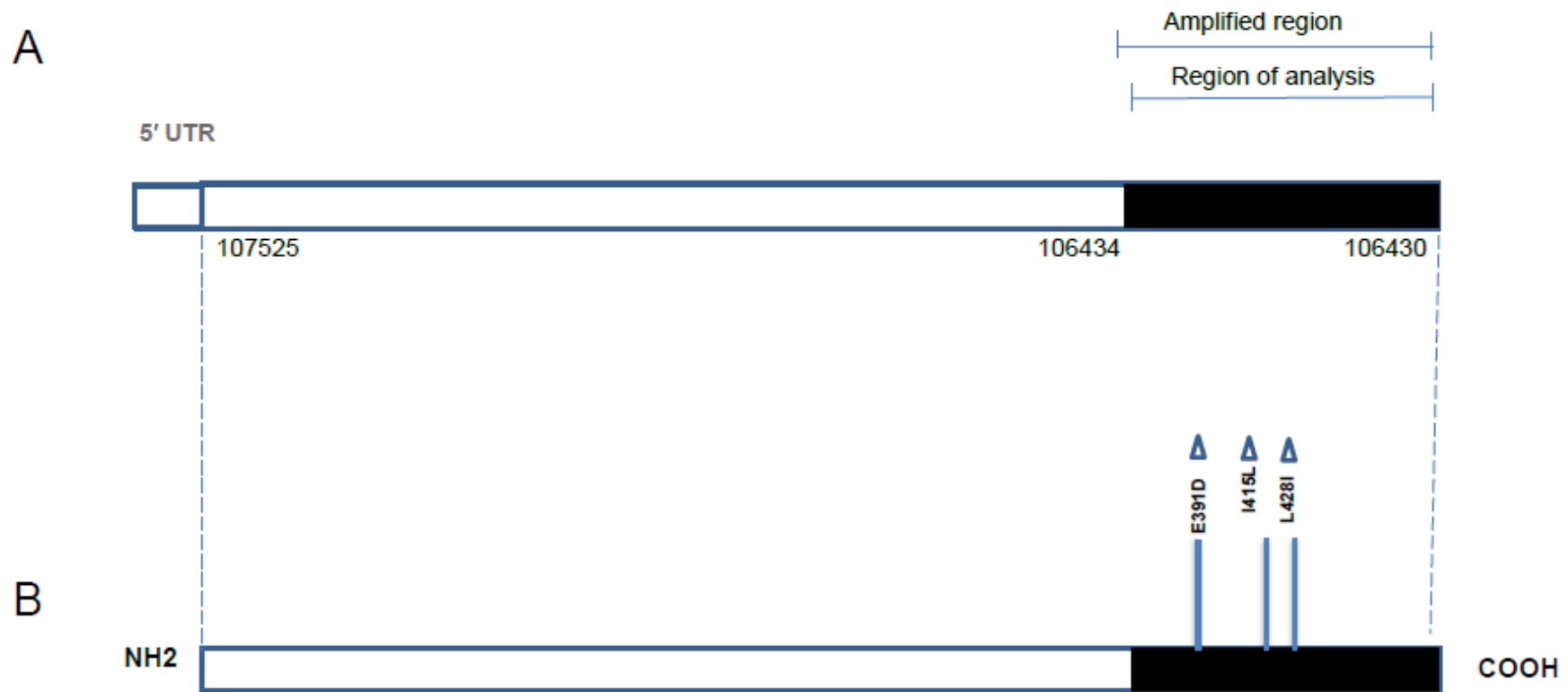


Figure 4.5: Distribution of the three identified mutations detected in the UL74 genes of HCMVUL54( $\Delta$ D413) within the chosen area of mutational analysis.

$\Delta$  refer to non synonymous mutations

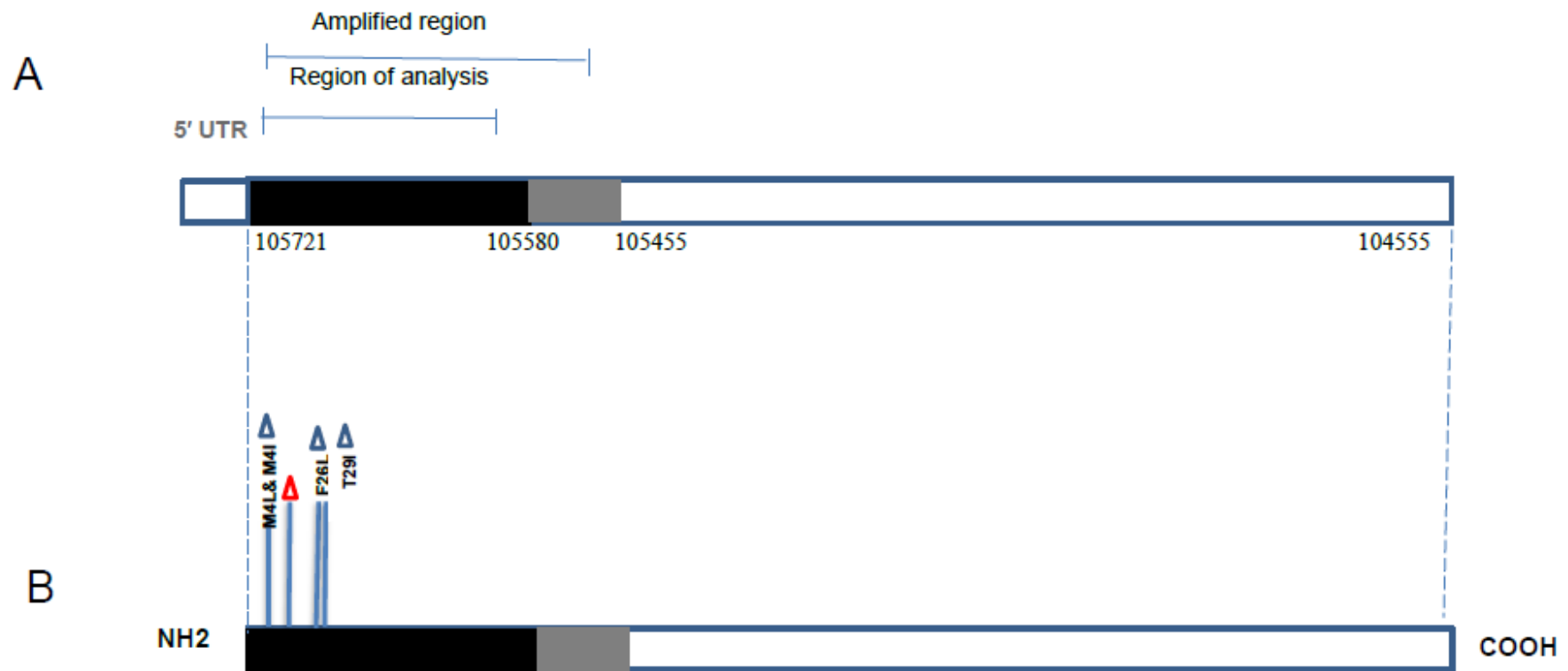


Figure 4.6: Distribution of five identified mutations detected in the UL72 genes of HCMVUL54( $\Delta$ D413) within the chosen area of mutational analysis.

$\Delta$  refer to non synonymous  $\Delta$  refer to synonymous mutatio



### 4.3 Discussion

The main findings in this chapter are first, that the mutation rate that achieved by HCMVUL54( $\Delta$ 413) is estimated as  $4.68 \times 10^{-6}$ . Measuring the mutation rate in two different regions within the HCMV genome showed that the mutation rate of HCMVUL54( $\Delta$ 413) is estimated as  $4.78 \times 10^{-6}$  for Region 1 and  $4.58 \times 10^{-6}$  for Region 2. The mutation rate for WT is below the detection threshold of our experiments (we detected no mutations in the WT). The mutation rate of the WT HCMV has been estimated as  $2 \times 10^{-7}$  (Renzette *et al.*, 2015; Sanjuán & Domingo-Calap, 2016) so we infer that the mutation rate of HCMVUL54( $\Delta$ 413) has been increased at least 20-fold. The absence of mutations in WT also indicates that the mutations observed in HCMVUL54( $\Delta$ 413) are not artefacts from our PCR polymerase – discussed further below. Second, analysis of Syn and non-Syn mutations showed that the two regions chosen for analysis were neutral. Third, as expected, the number of mutations that were detected in HCMVUL54( $\Delta$ 413) accumulate over passages. In low passage, we could not detect as many mutations in Region 1, while in Region 2 three mutations were detected. The number of mutations that were detected in HCMVUL54( $\Delta$ 413) high passage (p15) in Region 1 increased to six in each of 3 replicates. Eleven mutations were then found in HCMVUL54( $\Delta$ 413) high passage (p23). In Region 2 five mutations were detected in HCMVUL54( $\Delta$ 413) high passage (p15), followed by an increase to twelve mutations in HCMVUL54( $\Delta$ 413) high passage (p23). Importantly, we did not detect any mutations in the similar number of clones of HCMVUL54WT that we sequenced after high passage (p23). Our data show that mutations were accumulating during passaging HCMVUL54( $\Delta$ 413). These data suggests that the genetic deletion of codon 413 of domain II of the exonuclease region in UL54 affected the replication fidelity of the HCMV virus, resulting in accumulation of mutations over serial passage. The accumulation of mutations within viral genomes is known to affect viral fitness in RNA viruses in which their polymerase lack proof reading activity. This has been demonstrated empirically

in polio viruses, where it was shown that increasing the mutation frequency led to genomic catastrophe of the virus (Crotty, Cameron & Andino, 2001). We propose that the ability to cause the accumulation of mutations on virus viability could be exploited to intrinsically control the replication of the vaccine vector. Although we did not examine whether low fidelity of HCMVUL54( $\Delta$ 413) is associated with decreased virus fitness, the preliminary data from Dr. Chou's laboratory indicated that the infectivity of HCMVUL54( $\Delta$ 413) is lower than the HCMVUL54WT virus, which is consistent with a lower fitness of the virus.

The genetic reduction in the fidelity of CMV replication may introduce an unsustainable level of mutations during replication of the virus, thereby eventually resulting in 'genomic catastrophe' and demise of the virus. The long-term application of this project is to provide a means to control the replication of CMV for its use as a safe and effective live attenuated vaccine vector in a conventional or disseminating herpesvirus-based vaccine strategy. The present data are consistent with a mutation in domain II of the exonuclease region (deletion codon 413 that code for Asp) of HCMV DNA polymerase lowering the replication fidelity of the virus. In our study, the mutation rate of HCMVUL54( $\Delta$ 413) is similar to the Chou *et al.* (2008) report based on acquisition of Foscarnet resistance using a virus with a mutation in exonuclease domain II of DNA polymerase (aspartic acid being swapped for alanine). The authors reported that the mutation rate of HCMVUL54(D413A) had increased ~30-fold compared with the WT. Our results show that the mutation rate of HCMVUL54( $\Delta$ 413) increased to a comparable level of > 20-fold compared to the WT (which was not measurable in our experiments due to the lack of mutations).

The mutations in exonuclease domain III of HSV DNA polymerase (aspartic acid change to alanine at codon 581) is reported to affect the replication fidelity of HSV. The mutation frequency was also measured using two assays; in both of these assays, the virus replication was in the presence of the mutagen GCV. The mutation frequency was shown to have increased

20 to 80 fold compared to the WT based on a modified TK mutagenesis assay (Hwang *et al.*, 1997a). In contrast, the mutation frequency was increased 300 to 800 fold when using a recombinant virus assay (TK mutagenesis assay). The difference in magnitude for the increase in mutation frequency was explained as the first assay measuring the accumulation of mutations during one replication cycle, compared to measuring accumulation occur over many replication cycles in the second. In both of these methods the mutation frequency was increased more than ten-fold. However, our results are consist with the Hwang results as in both studies the mutation in exonucleases region affects the replication fidelity for these herpesviruses (HSV and HCMV) (Hwang *et al.*, 1997a). Our results are also consistent with the results of Uil *et al.* (2011), who found an increased mutation rate in an adenovirus polymerase mutant exonuclease. Their results showed that the mutation rates for the adenovirus polymerase mutant were  $10^{-4}$ , which was within the range of the mutation rate of RNA viruses (Uil *et al.*, 2011). In our results the mutation rate achieved by HCMVUL54( $\Delta$ 413) was  $\sim 5 \times 10^{-6}$ , which is lower than the mutation rate of adenovirus but still within the range of mutation rate of RNA viruses (Sanjuán *et al.*, 2010).

Several approaches have been used to detect mutation, some of these approaches based on direct sequencing (Eckerle *et al.*, 2007; Nobusawa & Sato, 2006) while others depend on screening for mutations conferring a specific phenotype and then sequencing. These latter assays include: selection for a mutation that confers resistance to a selective agent and then looking for revertants, screening for mutants that block an antibody or knock out a specific gene (Holland *et al.*, 1989), or by using a neutral reporter gene (typically a transgene) where null mutations (knock outs) lead to an observable phenotype (Mansky, 2000; Mansky & Temin, 1994; Pathak & Temin, 1990).

In our study, cloning combined with direct sequencing of genomic regions was chosen as the means by which to measure the frequency of mutation. This method was selected over these

other non-sequencing methods because it is a simple and accurate method (Beaucourt *et al.*, 2011).

Sanger sequencing platform was used for sequencing the selected two regions within HCMV genome, Region 1 that located within UL27 gene and Region 2 that contains UL73 gene and parts of UL72 and UL74 genes. This method of sequencing was used as the error rate is lower than for other platforms: capillary sequencing ( $1 \times 10^{-1}$ ), 454GS Junior ( $1 \times 10^{-2}$ ), PacBio RS ( $1 \times 10^{-2}$ ), Ion Torrent PGM ( $1 \times 10^{-2}$ ) SOLiD ( $2 \times 10^{-2}$ ) and Illumina ( $1 \times 10^{-3}$ ) next generation sequencing (NGS) platforms. In contrast the error rate that results from Sanger sequences is reported to range from  $1 \times 10^{-4}$  to  $1 \times 10^{-5}$  (Beerenwinkel *et al.*, 2012; Ewing *et al.*, 1998; Fox *et al.*, 2014). The read-length achieved by Sanger sequencing ( $\sim 800$ - $900$  bp) is also far longer than NGS platforms (Kircher & Kelso, 2010).

More recently developed NGS platforms (Tag-based methods and Duplex sequencing) have been developed to overcome the limitations related to the inherent high error rate of these current NGS platforms. With an estimated error rate of  $5 \times 10^{-8}$  (Fox *et al.*, 2014; Kennedy *et al.*, 2014), DNA duplexing was recently used for the estimation of the spontaneous mutation rate in another DNA virus, adenovirus 5 (Risso-Ballester, Cuevas & Sanjuán, 2016). However, construction of the necessary libraries are complex, costly and require specialized expertise (Ståhlberg *et al.*, 2016). To remove the possibility of errors from sequencing artefacts, we performed repeated sequencing using a M13 forward primer, which was then confirmed by resequencing of the same clones using both M13 forward and M13 reverse primers. These clones were also re-sequenced using an internal UL27 primer to ensure these mutations are real and it is not due to error during sequencing of the region. Finally, to minimise errors from PCR, the high fidelity Q5 enzyme was used for PCR amplification. As mentioned above, we did not observe any mutations in the WT virus, in which nearly 200,000nts (80,652nts of Region 1 and 100,300nts of Region 2) were examined. Thus we consider it unlikely that many of the

mutations observed in HCMVUL54 ( $\Delta$ 413) were artefactual and derived from PCR errors. Calculating an expected number of mutations caused by PCR errors is not straightforward. PCR is not 100% efficient so the number of doublings of the DNA is less than the number of PCR rounds. We did not measure the number of doublings of the DNA in our PCRs (each of 34 rounds), but McInerney *et al.* (2014) found on average an approximate 20-fold doubling of the DNA after 30 PCR rounds in experiments with six other polymerases (McInerney, Adams & Hadi, 2014). The Q5 enzyme amplifies the DNA with an error rate of  $5 \times 10^{-7}$  (Potapov & Ong, 2017) so assuming 20 doublings we might therefore expect one 'artefactual' mutation to appear in every 100,000 nucleotides examined ( $5 \times 10^{-7} \times 20 = 1 \times 10^{-5}$ ). Our results showed that the number of mutations that were detected in HCMVUL54 ( $\Delta$ 413) ranged between 5 and 10 in every 100,000 nucleotides examined (= the mutation frequency given in table 4.7).

Our sequencing data for UL27, suggests a trend towards clumping of mutations towards the right hand side of the sequenced region. Surprisingly, this apparent clumping of mutations is in a conserved region of the UL27 protein, based on sequence analysis of five reference strains (AD169, Towne, Toledo, Davis and Merlin) and 28 clinical HCMV samples (Hantz *et al.*, 2009). However, UL27 is not required for replication *in vitro* and therefore should not be under selective pressure (and our analysis of the non-synonymous/synonymous ratio is consistent with this). Several studies have indicated that such grouping of mutations might be related to specific DNA sequence patterns. For example, it has been shown that repetitive sequences such as direct and inverted repeats, microsatellite repeats and homonucleotide runs can lead to clumping of particular type of mutations (Rogozin & Pavlov, 2003). The presence of short direct repeats can also lead to slippage of the DNA polymerase causing deletions or duplications in DNA (Albertini *et al.*, 1982; Tran *et al.*, 1995). However, we did not detect any obvious motifs in this region. We therefore conclude that this observed trend is probably due

to chance. Consistent with this idea, the number of mutations in UL27 increased by passage 23 and only 4 of a total of 11 mutations were found in this region.

The mutations that were detected within UL73 in our study were within the variable region of that gene, which is reported in some studies as the region that could be under positive selection (Pignatelli, Dal Monte & Landini, 2001; Pignatelli *et al.*, 2003). In our study, three out of twelve mutations in this region were Syn, which represents the 25% expected under neutral evolution. It may be that there was no selective pressure in UL73, even though this is an essential gene *in vitro* (Dunn *et al.*, 2003; Yu, Silva & Shenk, 2003).

Pignatelli *et al.* (2001) also reported that the variants detected within UL73 variable region, which is under positive selection, did not change during *in vitro* passages (Pignatelli, Dal Monte & Landini, 2001). However, our result showed accumulation of the mutations over *in vitro* passages: at the low passage we detected two mutations, at passage 15 we detected three mutations (see table 4.6) and at passage 23 we detected 7 mutations (see table 4.7).

The ultimate aim for a low fidelity herpesvirus is to use the increased rate of mutational accumulation to decrease the fitness of the virus. Although fitness was not measured in these studies, the attenuated phenotype observed for HCMVUL54( $\Delta$ 413) by the Chou Laboratory suggests fitness of the virus may be lowered. In RNA viruses, the accumulation of mutations within the viral genome are known to cause a genomic catastrophe. The high mutation rate restrict RNA viruses to a small genome (small cage) (Belshaw *et al.*, 2008). Increasing the mutation within HCMV genome could presumably lead to genomic catastrophe. This is the ultimate aim of this project. It will be interesting in future work to measure the fitness of the HCMVUL54( $\Delta$ 413), and that could be performed by using an *in vitro* ‘plaque-to-plaque assay’ and using plaque diameter after each transfer as an indicator of fitness (Gnädig *et al.*, 2012). The genome-to- infectivity ratio for HCMVUL54 ( $\Delta$ 413) and HCMVUL54WT could be

determined after serial passages using quantitative real-time PCR or digital PCR and could compare to their relative parents (Gurczynski, Das & Pellett, 2014; Lock *et al.*, 2013; Womack & Shenk, 2010). Viral fitness could be measured also by counting virus particles with transmission electron microscopy and compared with genome copy number and infectivity of the virus by TCID<sub>50</sub> (Malenovska, 2013; Weidmann *et al.*, 2011).

In conclusion our result showed accumulation of the mutations over passages and this results could provid a proof of concept to use this strategy to control viral vaccine.

## Chapter 5 Flanking homology-based recombination as a strategy for intrinsic control of the herpesvirus-based vaccine platform

### 5.1 Introduction

This aspect of the thesis continues the focus on viral evolution by providing a method for intrinsic control of recombinant herpesviruses based on the use of regions of homology duplication to promote recombination within the virus genome. Specifically, we hypothesize that duplicated regions of sequence when positioned within the herpesvirus genome will target the region flanked by these repeat sequences for homologous recombination-based excision. We further hypothesize that the size of the duplicated region will kinetically determine the rate at which the intervening sequence will be excised. This hypothesis is based on earlier studies showing that the replication kinetics of herpesviruses containing heterologous genetic material within their genome is slowed compared to the wild type virus. In particular, insertion of a bacterial artificial chromosome (BAC) cassette within a number of different herpesviruses [MCMV(Wagner *et al.*, 1999), RhCMV (Chang & Barry, 2003) and MHV-68(Adler, Messerle & Koszinowski, 2001)] affects the replication of these viruses. These studies showed that excision of the BAC from viral genome, returning the viral genome to its normal size, resulted in a virus with WT properties. This led to the proposition that a size restriction for genome packaging within the herpesvirus capsid provides selective pressure for maintenance of a defined genome size (Wagner *et al.*, 1999). The study of Wagner *et al* (1999) further demonstrated that use of flanking regions of homology within an over-sized genome could effectively be used to target a defined region of the genome (in this case, the BAC cassette) for excision (Wagner *et al.*, 1999).

The impact of genome size on virus fitness due resulting from a presumed effect of genome capsid packaging constraints has been also observed for other herpesviruses (eg., Epstein Barr virus (Bloss & Sugden, 1994)) as well as for adenovirus, which similar to herpesviruses has an



encapsidated DNA genome (Bett, Prevec & Graham, 1993). Beyond the additional selective pressure resulting from size restrictions for encapsidation, the use of flanking homology regions to defined regions of the genome for excision is an approach that has been effectively used to excise *trans*-genes such as antibiotic resistance markers, during the development of the genetically modified plants (called intrachromosomal homologous recombination; IHR) (Yau & Stewart, 2013). The efficiency of the excision/recombination during IHR has been shown to be based on the length of the flanking repeated sequences (Fischer *et al.*, 1996; Puchta *et al.*, 1995). For a given repeat length, IHR was also shown to vary between different plant species. For example, in tobacco plants excision was observed when the length of the sequence tested was 174 bases or greater, but did not occur for an 137 base repeat (Zoubenko *et al.*, 1994) (Dufourmantel *et al.*, 2007; Iamtham & Day, 2000). In the single-celled green algae, *Chlamydomonas reinhardtii*, excision has been shown when the repeat length was 483 or 832 bp, but at a repeat below this size excision was not observed (Fischer *et al.*, 1996). These results suggest that flanking repetitive regions can specifically target genomic regions for excision even in large eukaryotic systems, and that the size of flanking region influences the excision process.

As a first step towards the adaptation of IHR as a strategy for intrinsic attenuation of herpesvirus-based vectors, I have engineered a panel of MCMV BACs in which the *trans*-genes are flanked by differing lengths of identical MCMV genomic regions. Given the propensity, both in CMV but also in transgenic plant genetic engineering technology, for such repeat regions to provide selective pressure towards the targeted removal of the intervening DNA sequence, we hypothesize that the presence of these flanking duplications will target the intervening heterologous *trans*-gene region for genetic deletion (See Figure 5.1). In contrast to the strategy based on fidelity and genomic catastrophic demise of the virus, this strategy aims to restore to recombinant virus to that of the WT CMV virus by excision of the transgene.

Rather than intrinsic control of the CMV virus itself, this strategy is therefore based on providing kinetic control over stability of the heterologous transgene within the recombinant virus. Although beyond the scope of the present thesis, we hypothesize that the presence of transgene between two homologues flanking region will lead to its excision from the recombinant virus and restoration of the WT (non-GMO) virus (these studies are ongoing through collaboration with Dr Redwood at the University of Western Australia). We further hypothesize that the rate of loss of the heterologous transgene can be regulated by length of homologous flanking region (Figure 5.1). The recent preliminary data from Dr Alec Redwood has demonstrated the proof of concept for this approach. PCR analysis for recombinant viral DNAs that were extracted over eight passages show i) flanking repeats regions can target the intervening region for excision, and ii) excision can be kinetically control based on the size of the flanking repeats sequences.

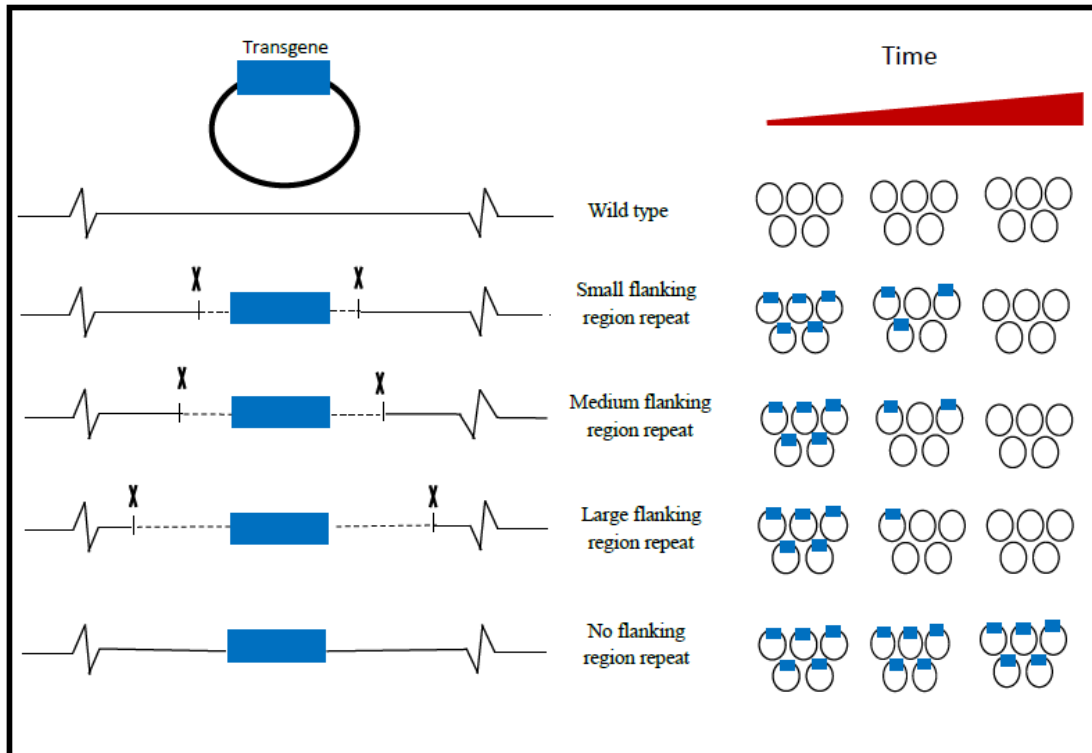


Figure 5.1: Schematic showing destabilization transgene strategy. (A) Schematic diagram of vector transgene regions, showing identical flanking regions of varying sizes. (B) Illustrates the expected relationship between the size of the flanking regions and the kinetic excision of the transgene.

## 5.2 Results

As a means to examine this intrinsic control strategy at a ‘proof-of-concept’ level, a panel of MCMV BAC constructs were designed in which a LacZ ( $\beta$ -galactosidase;  $\beta$ -gal)/Kan<sup>R</sup> expression cassette was inserted within the MCMV genome as a ‘heterologous gene marker’ to be targeted for deletion. Three constructs were designed with the LacZ/Kan<sup>R</sup> expression cassette being flanked by repeated MCMV sequence of either 250, 25 or 0nt. The LacZ/Kan<sup>R</sup> expression cassette was inserted to replace the M157 gene of MCMV (Figure 5.2). Deletion of M157 gene does not affect viral replication *in vitro* or *in vivo* (Bubić *et al.*, 2004), as the M157 gene product is not essential for viral replication and survival (Voigt *et al.*, 2003). Rather, the presence of this protein can negatively affect viral replication, with previous studies showing that M157 protein binds to the natural killer NK cell Ly49H receptor in MCMV susceptible

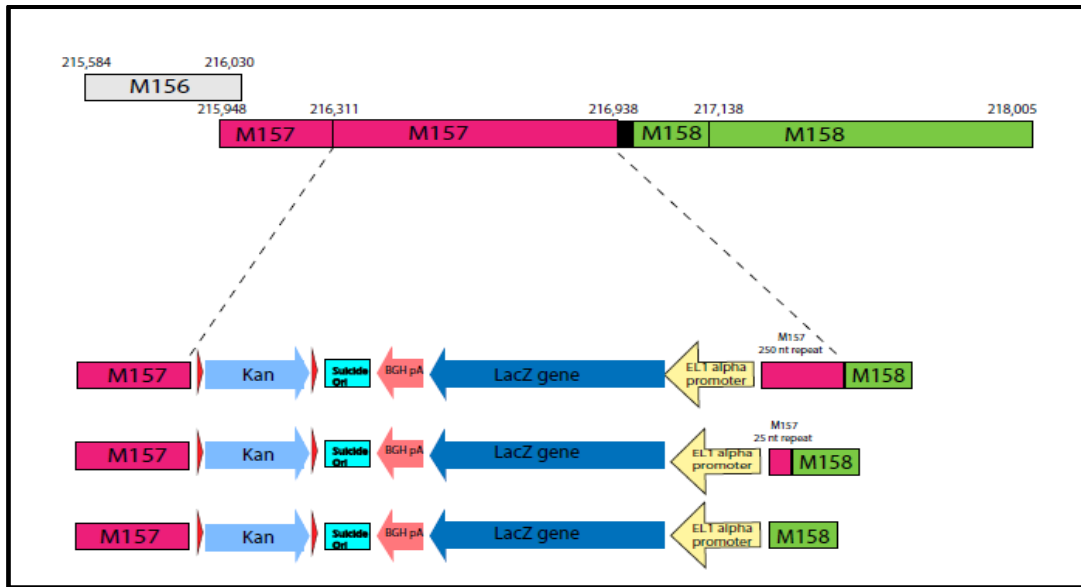
mice (strain C57B/6(B6)) leading to activation of NK cells and thereby elimination of viral replication (Arase *et al.*, 2002; Pyzik *et al.*, 2011). The M157 gene is also shown to be inactivated in recently isolated WT MCMV field strains (Redwood, Shellam & Smith, 2013). Multiple previous vaccine studies using the MCMV model have used MCMV vectors deleted for M157 gene from CMV (Klyushnenkova *et al.*, 2012; Tierney *et al.*, 2012; Tsuda *et al.*, 2011).

The MCMV LacZ/Kan<sup>R</sup> (0nt) without repeated sequence flanking the LacZ/Kan<sup>R</sup> cassette served as a critical control to assess the inherent rate of loss of the cassette from the MCMV genome in the absence of flanking regions. The LacZ gene was chosen as ‘heterologous gene marker’ for a number reasons. First, the presence of this gene could be monitored visually based on enzymatic activity. Second, the 3kb size of the LacZ gene was not expected to dramatically affect stability of the recombinant genome. Third, the insertion of this LacZ gene within MCMV genome has been shown not to affect replication of the virus either *in vitro* or *in vivo* during acute infection (Stoddart *et al.*, 1994). The Kan<sup>R</sup> gene was used for maintenance of the marker cassette both within bacteria (using kanamycin), as well as in eukaryotic cells (using G418). Specifically, the Kan<sup>R</sup> marker can be used both for initial selection of recombinant BACs following E/T-based recombination (using kanamycin), but also in murine embryonic cells during reconstitution of the virus to maintain the transgene cassette during stock production (using G418).

As detailed above (see Materials and Methods), the first step in construction of MCMV LacZ/Kan<sup>R</sup> flanking based vectors was generation of a plasmid containing the LacZ/Kan<sup>R</sup> expression cassette flanked by synthetic flanking regions for targeted insertion of LacZ/Kan<sup>R</sup> within the MCMV BAC genome. For these studies MCMV (strain K181) pARK14 was utilized. The MCMV K181 strain was originally isolated from salivary gland of infected mice following serial passage *in vivo* (Booth *et al.*, 1993; Redwood *et al.*, 2005). Importantly, within

pARK14, the BAC cassette was inserted with the concomitant excision of an equivalent region of the MCMV genome resulting in maintenance of a WT-sized genome. The BAC cassette is also fixed within the MCMV genome, not being flanked by repeat regions to enhance site-directed removal, as is the case for other existing MCMV BACs. Together, this will prevent results from these studies being confounded either by genome size issues or competition due to targeted excision of the BAC cassette. The pARK14 BAC was kindly provided by Alec Redwood (University of Western Australia).

A



B

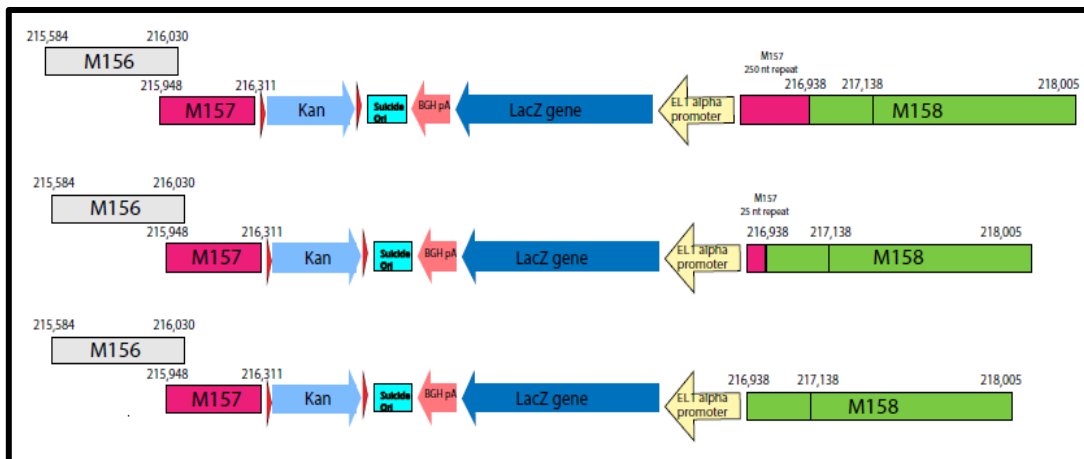


Figure 5.2: Schematic illustrating the construction of MCMV $\Delta$ M157 LacZ/Kan<sup>R</sup> flanked by repeats sequences. (A) LacZ/Kan<sup>R</sup> expression cassette flanked by 250nt repeated sequence, LacZ/Kan<sup>R</sup> expression cassette flanked by 25nt repeated sequence and LacZ/Kan<sup>R</sup> expression cassette were inserted within MCMV genome (K181) to partially replace the endogenous (M157 216,314-216,937). E/T recombination was used for construction of MCMV  $\Delta$ M157 LacZ/Kan<sup>R</sup> vectors. Linear vector carrying LacZ/Kan<sup>R</sup> expression cassette were electroporated into *E. coli* (EL250) where the recombinase proteins are expressed. M157 region (215,950-216,314) and M158 region (216,938-217,138) are flanking regions. The M157 250nt repeated region (216,064-216,313). The M157 25nt repeated region (216,288-216,313) are inserted at position (216,937) (B) MCMV $\Delta$ M157 LacZ/Kan<sup>R</sup> flanked by 250nt repeats, MCMV $\Delta$ M157 LacZ/Kan<sup>R</sup> flanked by 25nt repeats and MCMV $\Delta$ M157 LacZ/Kan<sup>R</sup> flanked by 0nt repeats.

### 5.2.1 Generation of LacZ/Kan<sup>R</sup> expression cassette that flank by repeated regions with flanking viral regions.

In order to construct MCMV BACs containing a LacZ/Kan<sup>R</sup> expression cassette flanked by different length of repeated sequences, three plasmids were generated to be inserted into the MCMV K181 genome. These plasmids contain the LacZ/Kan<sup>R</sup> expression cassette flanked by repeats of a region within the MCMV M157 gene of 0, 25 or 250nts. Construction of these plasmids was performed in two steps. The first step was cloning the synthetic repeated flanking viral regions into the pEF1 $\alpha$ /EBOV-GP (suicide-based vector), this vector contained the human elongation factor 1 alpha (EF1 $\alpha$  promoter), R6K $\gamma$  'suicide' origin of replication, bovine growth hormone polyadenylation signal (BGH pA) and Kan<sup>R</sup>/G418 selectable markers. The second step was cloning the lacZ gene into the constructed plasmid vectors. See Figure 5.3, Figure 5.4, Figure 5.5 and Figure 5.6.

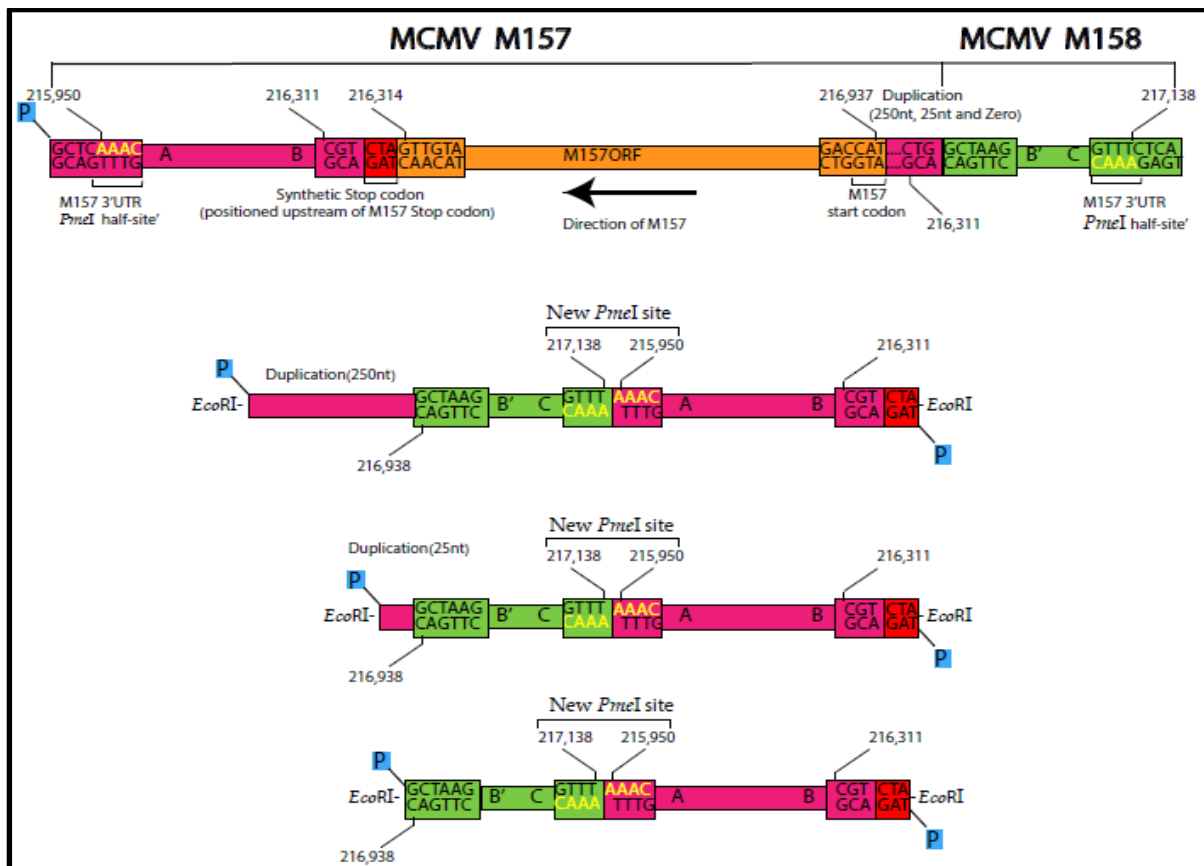


Figure 5.3: Schematic showing the design of the flanking regions with different length of repeated sequence. The flanking region cassette with new single *PmeI* site and necessary restriction sites (*EcoRI*) for insertion into the pEF1 $\alpha$ /EBOV-GP/Kan<sup>R</sup> (suicide-based vector) was synthesized (using an AmpR carrier vector from GeneArt from Invitrogen). The *PmeI* was selected based on its absence from the flanking regions and from within the suicide-based vector containing LacZ/Kan<sup>R</sup> for insertion. Three version of the flanking region were designed, the first version containing 250nt repeated sequence (216,064-216,313), the second version contain 25nt repeated sequence (216,289-216,313) and the third version was designed without repeated sequences.



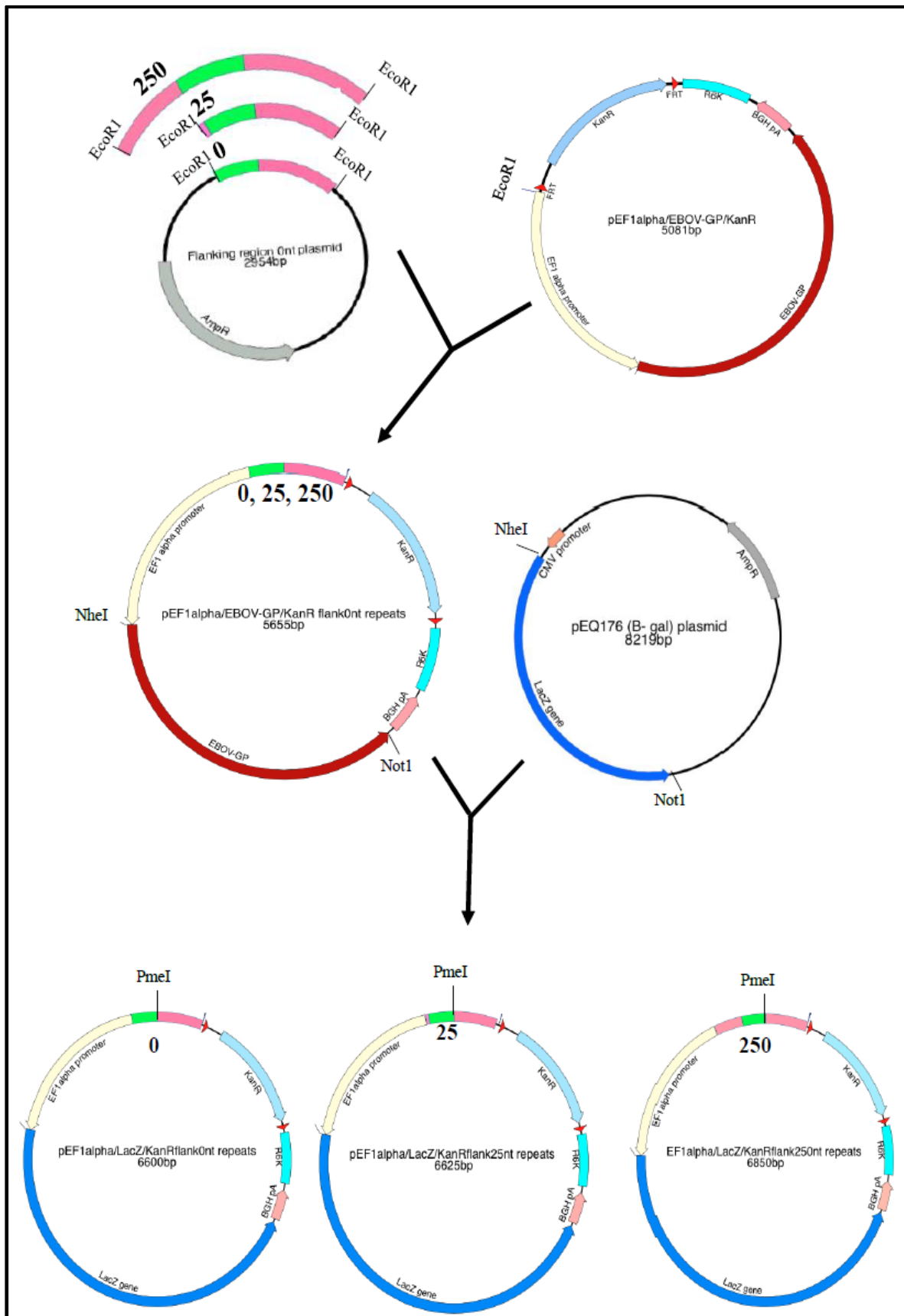


Figure 5.4: Schematic illustrating cloning strategy for generation of LacZ/Kan<sup>R</sup> expression cassette that flank by repeated regions with flanking viral regions. The first step in the construction was cloning the synthetic flanking regions with (0nt, 25nt and 250nt repeats) (GeneArt) into pEF1 $\alpha$ /EBOV-GP/Kan<sup>R</sup> (suicide-based vector) containing pEF1 $\alpha$  promoter and a /Kan<sup>R</sup> and *EcoRI* site (for insertion of the synthetic flanking region) were digested with *EcoRI* followed by ligation. In the second step, amplification of LacZ gene from pEQ176 plasmid by PCR, using synthetic oligonucleotide that contained restriction enzyme sites (*NheI* and *NotI*) was cloned into pEF1 $\alpha$ /EBOV-GP flank repeats sequences vectors (pEF1 $\alpha$ /EBOV-GP/Kan<sup>R</sup>flank0nt repeats, pEF1 $\alpha$ /EBOV-GP/Kan<sup>R</sup>flank25nt repeats and pEF1 $\alpha$ /EBOV-GP/Kan<sup>R</sup>flank250nt repeats). Indeed the LacZ gene was cloned into of EBOV-GP position. The resulting plasmids (pEF1 $\alpha$ /LacZ/Kan<sup>R</sup>flank0nt repeats, pEF1 $\alpha$ /LacZ/Kan<sup>R</sup>flank25nt repeats and pEF1 $\alpha$ /LacZ/Kan<sup>R</sup>flank250nt repeats containing the flanking homology to M157 for E/T based recombination within the MCMV K181 genome.

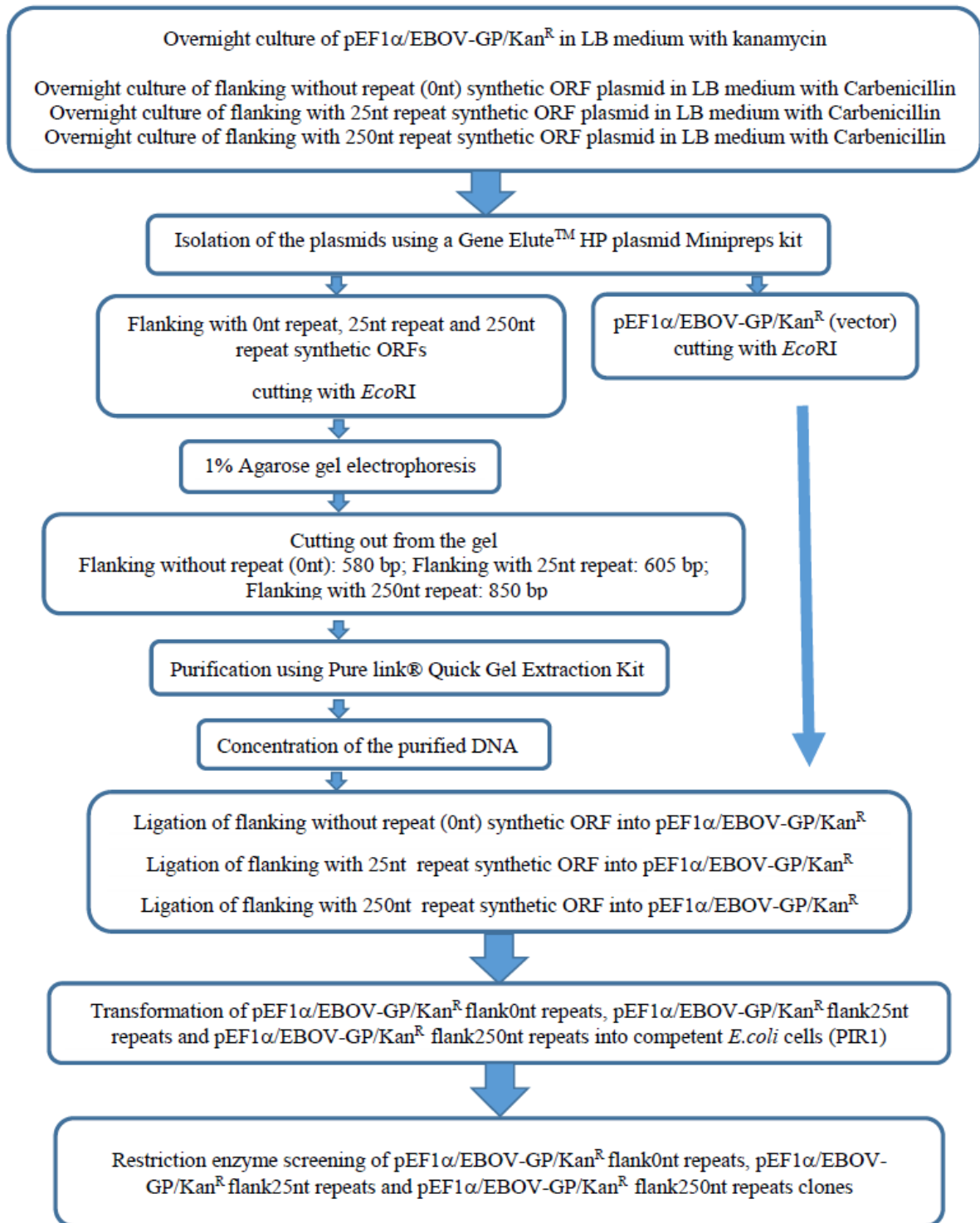


Figure 5.5: A flow-chart showing cloning flanking without repeat (0nt), flanking with 25nt repeat and flanking with 250nt repeat synthetic ORFs into pEF1α/EBOV-GP/Kan<sup>R</sup> vectors.

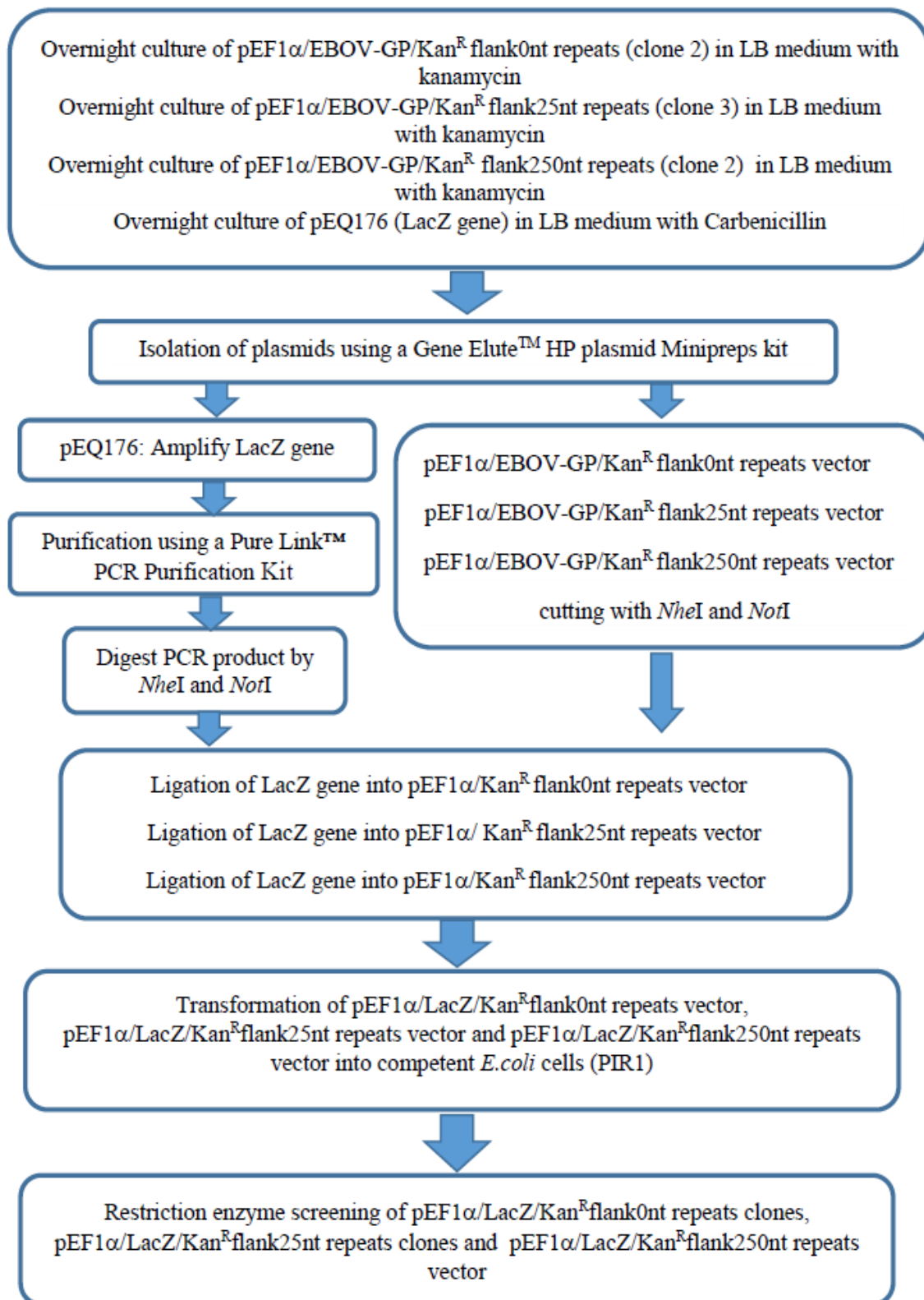


Figure 5.6: A flow-chart showing cloning LacZ gene into into pEF1α/Kan<sup>R</sup> flank0nt repeats, pEF1α/ Kan<sup>R</sup> flank25nt repeats and pEF1α/Kan<sup>R</sup> flank250nt repeats vectors.

### 5.2.1.1 Cloning flanking repeat sequences into the pEF1 $\alpha$ /EBOV-GP/Kan<sup>R</sup> plasmid

The aim was to insert the flanking regions that contain different length of repeats sequences into the suicide-based vector pEF1 $\alpha$ /EBOV-GP/Kan<sup>R</sup>. The flanking regions were generated synthetically as depicted in Figure 5.3. Endogenous *PmeI* half-sites within the MCMV genome flanking the site of insertion of the LacZ/Kan<sup>R</sup> cassette within the MCMV genome were identified. Orientation of these half-sites as shown within the final recombination plasmid enabled *PmeI* digestion to be used to linearize the LacZ/Kan<sup>R</sup> recombination fragment necessary for its insertion into the M157 locus. The plasmid called pEF1 $\alpha$ /EBOV-GP/Kan<sup>R</sup> was used as the background for assembly of the three LacZ/Kan<sup>R</sup> plasmids containing the different sized flanking regions.

The parental plasmid which contains the EBOV-GP gene under control of the human EF1 $\alpha$  promoter within a R6K-dependent ‘suicide vector’ required considerable re-engineering. First, the synthetic flanking repeat regions (GeneArt) were digested with *EcoRI* to release the flank region (Figure 5.7). *EcoRI* fragments were then ligated into a pEF1 $\alpha$ /EBOV-GP/Kan<sup>R</sup> that had been digested with *EcoRI* to linearize the vector. Digested plasmids were first separated on a 1% agarose DNA gel and then the bands of DNA representing the synthetic flanking repeat were cut from the gel. DNAs for the synthetic flank repeat were extracted from the gel using Pure link® Quick Gel Extraction Kit and concentrated using Zymo DNA Clean and Concentrator Kit. The results shown in (Figure 5.7 A, B and C) indicate that flank repeat sequence fragments were cut from their backbone plasmids, and in Figure 5.7 D, that pEF1 $\alpha$ /EBOV-GP/Kan<sup>R</sup> vector is linearized. The concentrated DNA for the synthetic flanking without repeat (0nt) and with repeats of 25nt and 250nt, were then ligated into the linearized pEF1 $\alpha$ /EBOV-GP/Kan<sup>R</sup> vector, generating pEF1 $\alpha$ /EBOV-GP/Kan<sup>R</sup>flank0nt repeat, pEF1 $\alpha$ /EBOV-GP/Kan<sup>R</sup>flank25nt repeat and pEF1 $\alpha$ /EBOV-GP/Kan<sup>R</sup>flank250nt repeat

plasmids, respectively. The ligated DNA was then transformed into competent PIR1 *E.coli* cells and plated onto LB agar containing 50 µg/ml Kan and incubated at 30°C. Ten colonies were picked from the LB plates and grown overnight with 50 µg/ml Kan. Plasmids were extracted from bacterial cells and purified using a Gene Elute™ HP plasmid Minipreps kit and then screened by restriction enzyme digest.

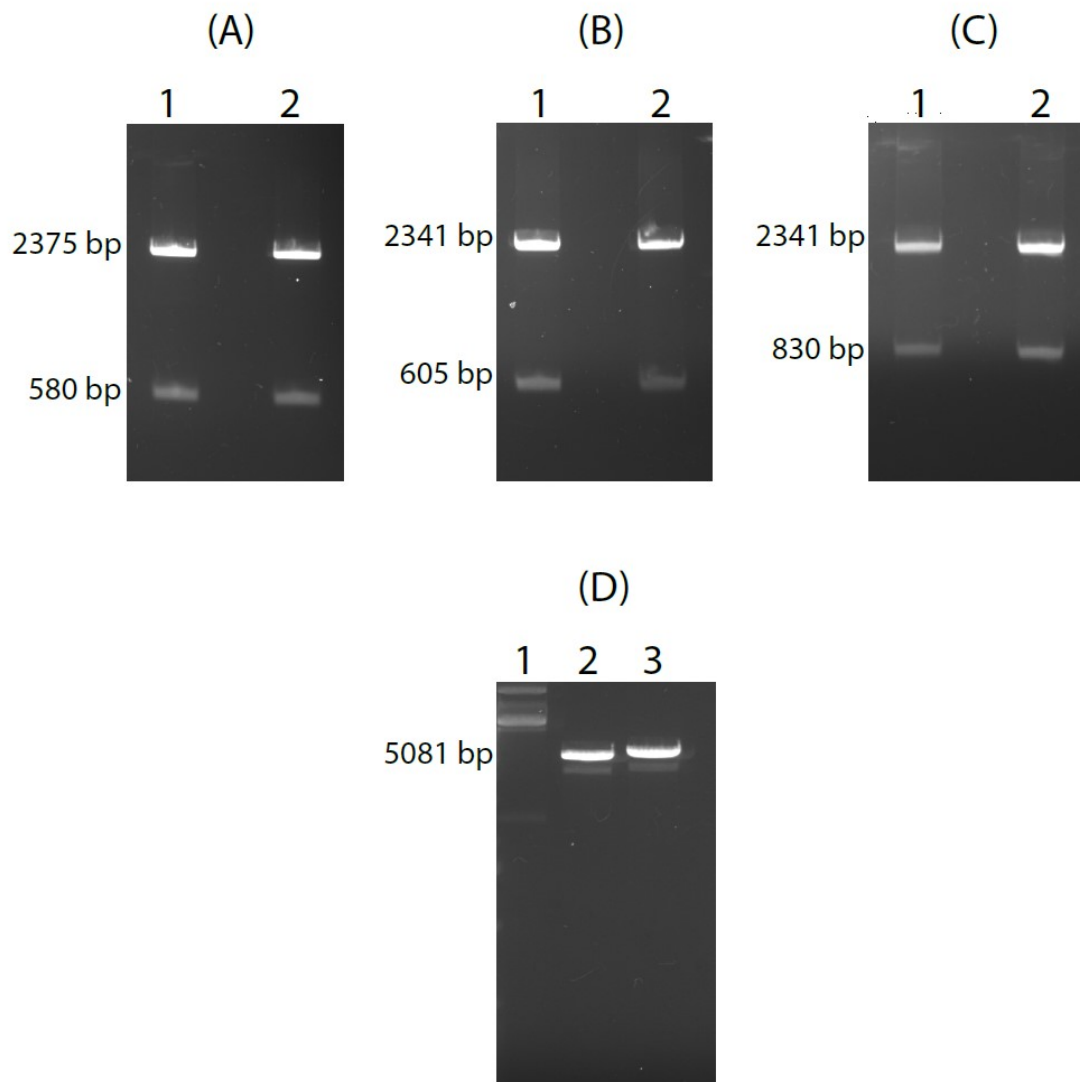


Figure 5.7: Gel electrophoresis showing *EcoRI* digestion of the synthetic flanking repeats sequences and pEF1 $\alpha$ /EBOV-GP/Kan<sup>R</sup> plasmid during cloning of pEF1 $\alpha$ /EBOV-GP/Kan<sup>R</sup>flank repeats (250nt/25nt/0nt). (A): restriction enzyme digestion of synthetic flanking 0nt repeat cut from its plasmid backbone by using *EcoRI* (508 bp). (B): restriction enzyme digestion of synthetic flanking 25nt repeat cut from its plasmid backbone by using *EcoRI* (605 bp). (C): restriction enzyme digestion of synthetic flanking 250nt repeat cut from its plasmid backbone by using *EcoRI* (830 bp). (D): uncut pEF1 $\alpha$ /EBOV-GP/Kan<sup>R</sup> vector (lane 1) was compared to *EcoRI* digested (lane 2 and 3) producing DNA fragment of approx. 5kb indicating digestion of the vector.

#### 5.2.1.1.1 Restriction digest analysis of pEF1 $\alpha$ /EBOV-GP/Kan<sup>R</sup> flank repeats sequences clones to confirm the insertion of the flanking repeats region

To determine whether the different synthetic flanking repeat sizes had been inserted into the pEF1 $\alpha$ /EBOV-GP/Kan<sup>R</sup>, plasmid DNA extracted from ten individual Kan-resistant clones for each construct (pEF1 $\alpha$ /EBOV-GP/Kan<sup>R</sup>flank0nt repeats, pEF1 $\alpha$ /EBOV-GP/Kan<sup>R</sup> flank25nt repeats and pEF1 $\alpha$ /EBOV-GP/Kan<sup>R</sup>flank250nt repeats) was analysed by *Eco*RI restriction. *Eco*RI digestion, which was used for insertion of the flanking regions into the pEF1 $\alpha$ /EBOV-GP/Kan<sup>R</sup>, was used for screening. As shown in Figure 5.9 and Figure 5.10, a subset of the clones gave the expected 605 bp and 830 bp (inserts) and 5081 bp (vector backbone) bands for pEF1 $\alpha$ /EBOV-GP/Kan<sup>R</sup>flank25nt repeats and pEF1 $\alpha$ /EBOV-GP/Kan<sup>R</sup> flank250nt repeats. These results show correct insert size in eight out of ten colonies from each construct. Two colonies from each construct were parental pEF1 $\alpha$ /EBOV-GP/Kan<sup>R</sup> (Figure 5.9 lane 8 (clone 2)) and lane 12 (clone 6) and Figure 5.10 lane 7 (clone 1) and lane 13 (clone 7)), and contained no inserts after digestion with *Eco*RI. The results (Figure 5.8) show correct insert size of synthetic flanking without repeat fragment (flanking repeat 0nt) (580 bp) and 5081 bp (vector backbone) in all colonies screened. Clones were also confirmed using *Pml*I and *Afl*III restriction digestion (data not shown).

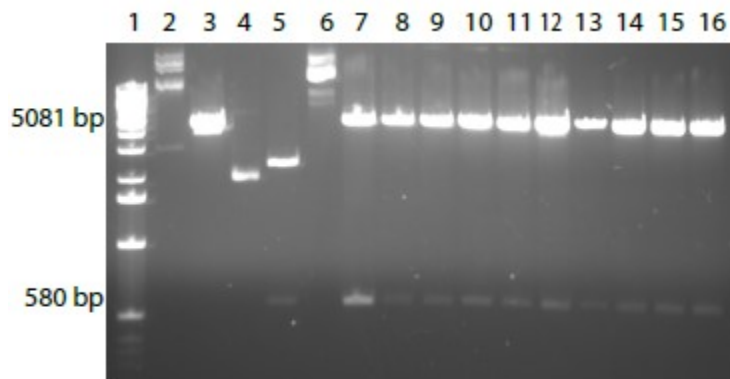


Figure 5.8: Restriction enzyme screening of pEF1 $\alpha$ /EBOV-GP/Kan<sup>R</sup>flank0nt repeat clones. Lane 1: DNA ladder (1 Kb DNA extension ladder). Lane 2: shows uncut pEF1 $\alpha$ /EBOV-GP/Kan<sup>R</sup>. Lane 3: restriction enzyme digestion of pEF1 $\alpha$ /EBOV-GP/Kan<sup>R</sup> parental plasmid by using *Eco*RI. Lane 4: shows uncut synthetic flanking without repeat (0nt) plasmid (GeneArt). Lane 5: restriction enzyme digestion of synthetic flanking without repeat (0nt) plasmid by using *Eco*RI. Lane 6: shows uncut pEF1 $\alpha$ /EBOV-GP/Kan<sup>R</sup>flank0nt repeat clone1. Lanes 7-16: restriction enzyme digestion of ten clones (clone 1-10) of pEF1 $\alpha$ /EBOV-GP/Kan<sup>R</sup>flank0nt repeat by using *Eco*RI. Two bands are observed in all of the ten clones that digested; upper band of approximately 5081bp indicated the vectors and the lower bands indicated the insert (synthetic flanking without repeat (0nt) fragment) of approximately 580 bp.

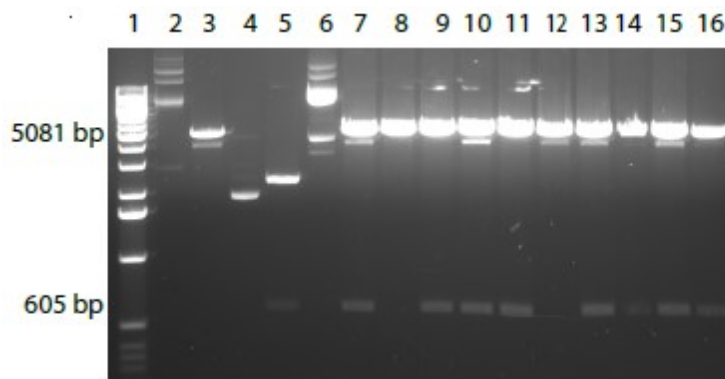


Figure 5.9: Restriction enzyme screening of pEF1 $\alpha$ /EBOV-GP/Kan<sup>R</sup>flank25nt repeats clones. Lane 1: DNA ladder (1 Kb DNA extension ladder). Lane 2: shows uncut pEF1 $\alpha$ /EBOV-GP/Kan<sup>R</sup>. Lane 3: restriction enzyme digestion of pEF1 $\alpha$ /EBOV-GP/Kan<sup>R</sup> parental plasmid by using *Eco*RI. Lane 4: shows uncut synthetic flanking with 25nt repeats plasmid (GeneArt). Lane 5: restriction enzyme digestion of synthetic flanking with 25nt repeats plasmid by using *Eco*RI. Lane 6: shows uncut pEF1 $\alpha$ /EBOV-GP/Kan<sup>R</sup>flank25nt repeats clone1. Lanes 7-16: restriction enzyme digestion of ten clones (clone 1-10) of pEF1 $\alpha$ /EBOV-GP/Kan<sup>R</sup>flank25nt repeats using *Eco*RI. Two bands are observed in eight clones (clone1, 3-5 and clones 7-10); upper band of approximately 5081bp indicated the vectors and the lower bands indicated the insert (synthetic flanking with 25nt repeats fragment) of approximately 605 bp.



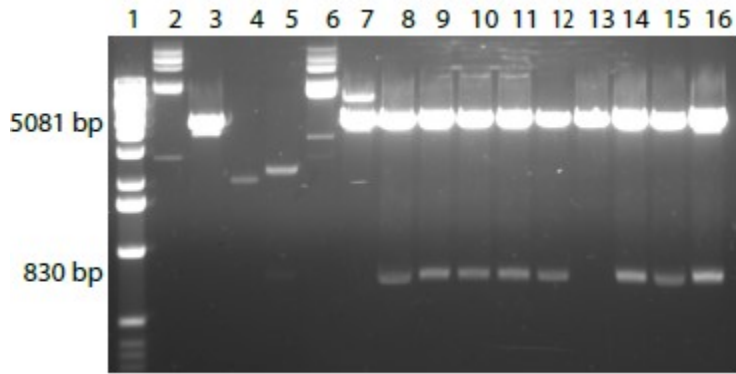


Figure 5.10: Restriction enzyme screening of pEF1 $\alpha$  /EBOV-GP/Kan<sup>R</sup>flank250nt repeats clones. Lane 1: DNA ladder (1 Kb DNA extension ladder). Lane 2: shows uncut pEF1 $\alpha$ /EBOV-GP/Kan<sup>R</sup>. Lane 3: restriction enzyme digestion of pEF1 $\alpha$ /EBOV-GP/Kan<sup>R</sup> parental plasmid without insert by using *Eco*RI. Lanes 4: shows uncut synthetic flanking with 250nt repeats plasmid (GeneArt). Lanes 5: restriction enzyme digestion of synthetic flanking with 250nt repeats by using *Eco*RI. Lanes 6: shows uncut pEF1 $\alpha$ /EBOV-GP/Kan<sup>R</sup>flank250nt repeats clone1. Lanes 7-16: restriction enzyme digestion of ten clones (clone 1-10) of pEF1 $\alpha$ /EBOV-GP/Kan<sup>R</sup>flank250nt by using *Eco*RI. Two bands are observed in eight clones (clone 2-6 and clones 8-10); upper band of approximately 5081bp indicated the vectors and the lower bands indicated the insert (synthetic flanking with 250nt repeat fragment) of approximately 830 bp.

#### 5.2.1.1.2 Restriction digest analysis of pEF1 $\alpha$ /EBOV-GP/Kan<sup>R</sup>flank repeats plasmids to determine the orientation of EBOV-GP expression cassette

Genes surrounding the site of heterologous gene insertion can affect the expression of the inserted antigens (Gillet *et al.*, 2005). Therefore, it was critical to determine the orientation of the EBOV-GP expression cassettes in the three constructed plasmids (section 5.2.1.1) prior to insertion of the LacZ in place of EBOV-GP. Orientation of the EBOV-GP expression cassette within the pEF1 $\alpha$ /EBOV-GP/Kan<sup>R</sup>flank repeat plasmids was determined based on the size of the digested plasmids following *Pme*I and *Nhe*I double digestion. Ten clones of each construct pEF1 $\alpha$ /EBOV-GP/Kan<sup>R</sup> flank0nt repeats clones, pEF1 $\alpha$ /EBOV-GP/Kan<sup>R</sup> flank25nt repeat clones and pEF1 $\alpha$ /EBOV-GP/Kan<sup>R</sup> flank250nt repeat (identified in section 5.2.1.1.1) were screened using *Pme*I and *Nhe*I restriction enzymes to determine the orientation of the expression cassette with the flanking regions. As shown in Figure 5.11 clone 6 and 7 of pEF1 $\alpha$ /EBOV-GP/Kan<sup>R</sup> flank0nt repeats had two bands of 4062 bp and 1593 bp, which confirmed forward orientation; clones 2, 3, 4, 5, 8, 9 and 10 had two bands 4226 bp and 1429

bp corresponding to a reverse orientation. Clone 1 was incorrect; in addition to the two expected bands, there was an extra band of approximately 600 bp in size. Figure 5.12 shows clones 1, 4, 7, 8, 9 and 10 of pEF1 $\alpha$ /EBOV-GP/Kan<sup>R</sup> flank25nt repeats had two bands 4087 bp and 1593 bp confirming forward orientation. Clones 3 and 5 had two bands 4226 bp and 1454 bp consistent with reverse orientation. Clones 2 and 6 were incorrect consistent with the results of *Eco*RI digestion (see Figure 5.9) showing that these clones contain only the pEF1 $\alpha$ /EBOV-GP/Kan<sup>R</sup> vector without insert. Figure 5.13 shows that clones 3, 5, 6, 9 and 10 of pEF1 $\alpha$ /EBOV-GP/Kan<sup>R</sup> flank250nt repeats had two bands of 4312 bp and 1593 bp confirming forward orientation. Clones 2, 4 and 8 had two bands 4226 bp and 1679 bp confirmed reverse orientation. Clone 1 and 7 were incorrect consistent with the *Eco*RI digestion (see Figure 5.10). Three clones in the same (reverse) orientation were selected to be used in the next step of cloning: clone 2 of pEF1 $\alpha$ /EBOV-GP/Kan<sup>R</sup> flank0nt repeats, clone 3 of pEF1 $\alpha$ /EBOV-GP/Kan<sup>R</sup> flank25nt repeats and clone 2 of pEF1 $\alpha$ /EBOV-GP/Kan<sup>R</sup> flank250nt repeats (see flanking maps in appendix Figure (A7-1))

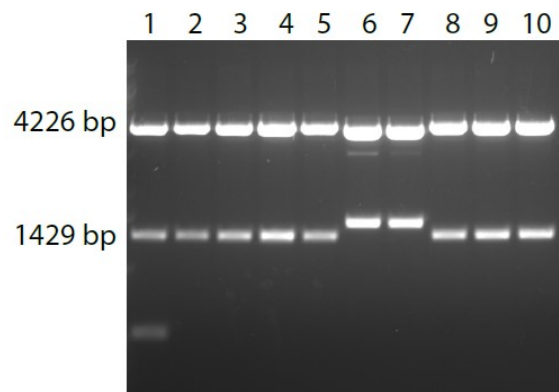


Figure 5.11: Restriction enzyme screening for the orientation of EBOV-GP expression cassette in the pEF1 $\alpha$ /EBOV-GP/Kan<sup>R</sup> flank0nt repeats clones. Lane 6 and 7 show confirmed forward orientation of EBOV-GP expression cassette in pEF1 $\alpha$ /EBOV-GP/Kan<sup>R</sup> flank0nt repeats clones following digestion with *Pme*I and *Nhe*I lanes (6 and 7). Two bands are observed; upper band of approximately 4062 bp and the lower bands of approximately 1593 bp. Lane 2, 3, 4, 5, 8, 9 and 10 show confirmed reverse orientation of EBOV-GP expression cassette in pEF1 $\alpha$ /EBOV-GP/Kan<sup>R</sup> flank0nt repeats clones following digestion with *Pme*I and *Nhe*I lanes (2, 3, 4, 5, 8, 9 and 10). Two bands are observed; upper band of approximately 4226 bp and the lower bands of approximately 1429 bp.

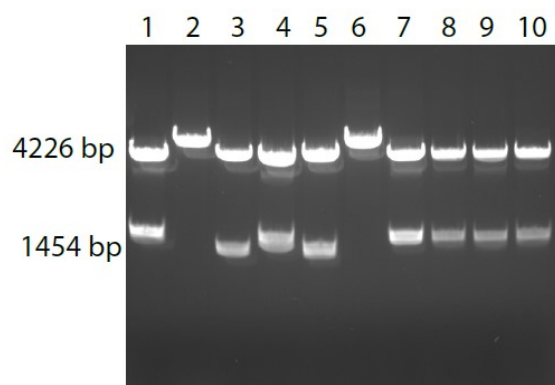


Figure 5.12: Restriction enzyme screening for the orientation of EBOV-GP expression cassette in the pEF1 $\alpha$ /EBOV-GP/Kan<sup>R</sup> flank25nt repeats clones. *PmeI* and *NheI* were used in digestion of ten clones. Lane 1, 4, 7, 8, 9 and 10 show confirmed forward orientation of EBOV-GP expression cassette in pEF1 $\alpha$ /EBOV-GP/Kan<sup>R</sup> flank25nt repeats clones following digestion with *PmeI* and *NheI* lanes (1, 4, 7, 8, 9 and 10). Two bands are observed; upper band of approximately 4087 bp and the lower bands of approximately 1593 bp. Lane 3 and 5 show confirmed reverse orientation of EBOV-GP expression cassette in pEF1 $\alpha$ /EBOV-GP/Kan<sup>R</sup> flank25nt repeats clones following digestion with *PmeI* and *NheI* lanes (3 and 5). Two bands are observed; upper band of approximately 4226 bp and the lower bands of approximately 1454 bp.

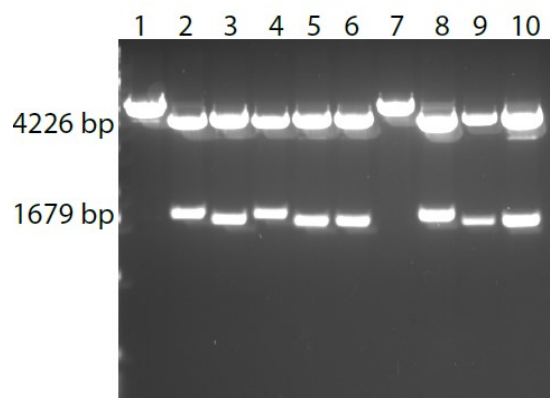


Figure 5.13: Restriction enzyme screening for the orientation of EBOV-GP expression cassette in the pEF1 $\alpha$ /EBOV-GP/Kan<sup>R</sup> flank250nt repeats clones. *PmeI* and *NheI* were used in digestion of ten clones. Lane 3, 5, 6, 9 and 10 show confirmed forward orientation of EBOV-GP expression cassette in pEF1 $\alpha$ /EBOV-GP/Kan<sup>R</sup> flank250nt repeats clones following digestion with *PmeI* and *NheI* lanes (3, 5, 6, 9 and 10). Two bands are observed; upper band of approximately 4312 bp and the lower bands of approximately 1593 bp. Lane 2, 4 and 8 show confirmed reverse orientation EBOV-GP expression cassette in pEF1 $\alpha$ /EBOV-GP/Kan<sup>R</sup> flank250nt repeats clones following digestion with *PmeI* and *NheI* lanes (2, 4 and 8). Two bands are observed; upper band of approximately 4226 bp and the lower bands of approximately 1679 bp.

#### 5.2.1.2 Cloning the LacZ gene into pEF1 $\alpha$ /Kan<sup>R</sup>flank repeats sequences vectors

LacZ( $\beta$ -gal)gene was cloned into pEF1 $\alpha$ /EBOV-GP flank repeat sequence vectors in order to generate LacZ/Kan<sup>R</sup> expression cassette flanked by different length of repeats sequences. To insert the LacZ reporter gene in place of the EBOV-GP gene between the synthetic flanking with repeated sequences, the LacZ gene was first amplified by PCR using as a template the pEQ176 plasmid (Addgene plasmid 83943) containing the LacZ gene. PCR amplification was carried out using synthetic  $\beta$ -gal primers. After a GCGC anchor region, the forward and reverse primers contained *NheI* and *NotI* restriction enzyme sites, respectively, to enable insertion of LacZ gene into the pEF1 $\alpha$ /flank repeats sequences. The PCR reaction was separated on a 1% agarose DNA gel in order to confirm PCR amplification of the LacZ gene. The results shown in (Figure 5.14) indicate that the LacZ gene had been amplified, resulting in production of a single band at the expected molecular size (3170 bp; Figure 5.14 lanes 3, 4 and 5).

The amplified LacZ-containing PCR product was purified using Pure Link<sup>TM</sup> PCR Purification Kit and then digested with *NheI* and *NotI* for two hours at 37°C. The pEF1 $\alpha$ /EBOV-GP flank repeat sequence vectors were similarly digested with *NheI* and *NotI*. The digested PCR product was then cloned into *NheI* and *NotI* sites of pEF1 $\alpha$ /EBOV-GP flank repeats sequences vectors. The LacZ *NheI/NotI* digested fragment was then ligated into the pEF1 $\alpha$ /flank repeat sequence vectors. The ligated DNA was transformed into competent PIR1 *E.coli* cells and then plated onto LB agar containing 50 $\mu$ g/ml Kan.

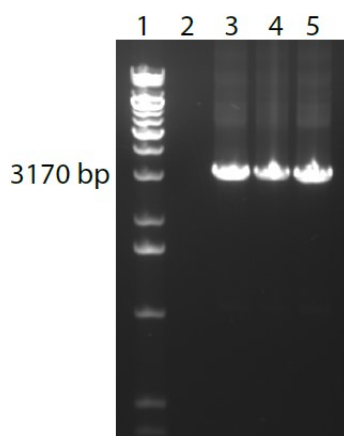


Figure 5.14: DNA gel showing PCR amplification of LacZ gene from pEQ176 plasmid. PCR was performed using Q5 hot start high-fidelity DNA polymerase. The product were separated on a 1% agarose DNA gel containing (0.5  $\mu\text{g/ml}$ ) EtBr to visualize the amplified DNA. The single DNA band at 3170 bp shows amplification of the desired LacZ gene (lane 3, 4 and 5). Lane 2 is a negative PCR control.

#### 5.2.1.2.1 Restriction digest analysis of pEF1 $\alpha$ /LacZ/Kan<sup>R</sup>flank repeats sequence clones to confirm the insertion of LacZ gene

Restriction enzyme digest analysis was used to confirm correct insertion of the LacZ gene into pEF1 $\alpha$ /flank repeats sequence vectors. Thirty clones (ten clones from each vector) were selected and analysed by restriction enzyme digestion using *NheI* and *NotI*. All clones of pEF1 $\alpha$ /LacZ flank0nt repeat and 250nt repeat had the correct insert size of 3170 bp (Figure 5.15 and Figure 5.17). For the pEF1 $\alpha$ /LacZ/Kan<sup>R</sup>flank25nt repeat, a subset of clones showed the expected 3170 bp (LacZ gene) insert and 3577 bp (vector backbone) Eight clones had the correct size of inserts from a total of ten clones that were screened. Clone 3 and 6 (Figure 5.16 lane 3 and 6) contained incorrect inserts.

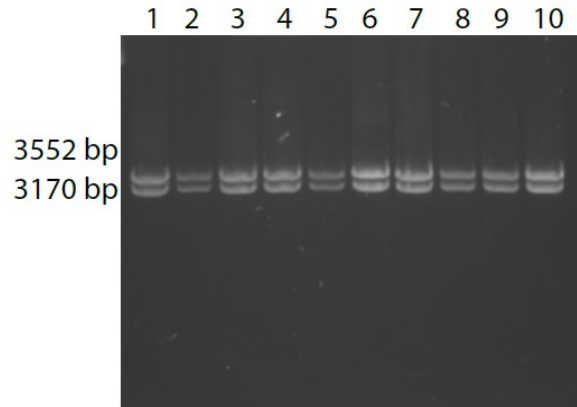


Figure 5.15: Restriction enzyme screening of pEF1 $\alpha$ /LacZ/Kan<sup>R</sup>flank0nt repeats sequence (Zero nt) clones. Ten clones of pEF1 $\alpha$ /LacZ/Kan<sup>R</sup>flank0nt repeat sequence were digested with *NheI* and *NotI*. Lanes 1-10 show digestion of ten clones (clone 1-10). Lanes 1-10 show confirmed pEF1 $\alpha$ /LacZ/Kan<sup>R</sup>flank0nt repeats sequence (Zero nt) clones following digestion with *NheI* and *NotI*. Two bands are observed following digestion with *NheI* and *NotI*; upper band of approximately 3552 bp indicated that the vector without EBOV-GP and the lower bands indicated the insert (LacZ gene) of approximately 3170 bp.

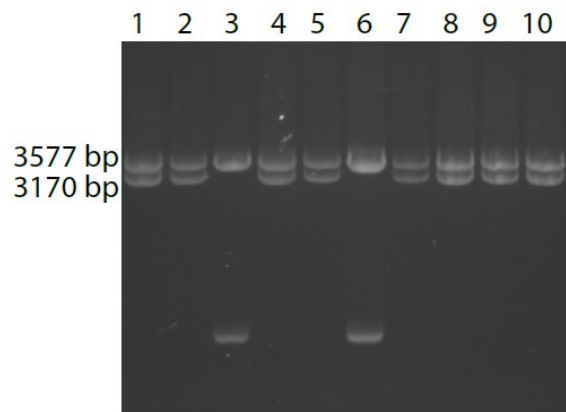


Figure 5.16: Restriction enzyme screening of pEF1 $\alpha$ /LacZ/Kan<sup>R</sup>flank25nt repeats sequence clones. Ten clones of pEF1 $\alpha$ /LacZ/Kan<sup>R</sup>flank25nt repeats sequence were digested with *NheI* and *NotI*. Lanes 1-10 show digestion of ten clones (clone 1-10). Lanes 1, 2, 4, 5, 7, 8, 9 and 10 show confirmed pEF1 $\alpha$ /LacZ/Kan<sup>R</sup>flank25nt repeats sequence clones following digestion with *NheI* and *NotI*. Two bands are observed following digestion with *NheI* and *NotI*; upper band of approximately 3577 bp indicated that the vector without EBOV-GP and the lower bands indicated the insert (LacZ gene) of approximately 3170 bp.

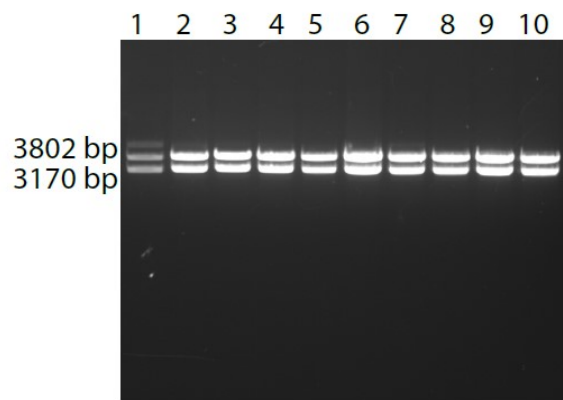


Figure 5.17: Restriction enzyme screening of pEF1 $\alpha$ /LacZ/Kan<sup>R</sup>flank250nt repeats sequence clones. Ten clones of pEF1 $\alpha$ /LacZ/Kan<sup>R</sup>flank250nt repeats sequence were digested with *NheI* and *NotI*. Lanes 1-10 show digestion of ten clones (clone 1-10). Two bands are observed following digestion with *NheI* and *NotI*; upper band of approximately 3802 bp indicated that the vector without EBOV-GP and the lower bands indicated the insert (LacZ) of approximately 3170 bp.

#### 5.2.1.2.2 Restriction enzyme screening of pEF1 $\alpha$ /LacZ/Kan<sup>R</sup>flank repeats sequence clones to confirmation the orientation of the LacZ expression cassette

To confirm orientation of the LacZ expression cassette, restriction digest analysis of positive clones was performed using *PmeI*, *NheI* and *AfeI*. Clones 1 and 2 from pEF1 $\alpha$ /LacZ/Kan<sup>R</sup>flank0nt, 25nt and 250nt repeat sequence (identified above in section 5.2.1.2.1 above) were analysed. The results in Figures 5.18 confirmed the reverse orientation of LacZ expression cassette, with three expected bands being observed following triple digestion with *PmeI*, *NheI* and *AfeI* (see flanking map in Appendix Figure (A7-1)). As shown in Figure 5.18 lanes 17 and 18, three bands are observed of 3354 bp, 1939 bp and 1429 bp following digestion pEF1 $\alpha$ /LacZ/Kan<sup>R</sup>flank0nt repeats sequence clones 1 and 2. For pEF1 $\alpha$ /LacZ/Kan<sup>R</sup>flank25nt repeats sequence clone 1 and clone 2, lanes 14 and 15 show the expected 3354 bp and 1939 bp bands, and a new band of 1454 bp. In addition to the 3354 bp and 1939 bp bands shown in lanes 11 and 12, the new 1679 bp band confirms the reverse orientation of pEF1 $\alpha$ /LacZ/Kan<sup>R</sup>flank250nt repeat clones 1 and 2.

Restriction digestion analysis using *Hin*DIII and *Eco*RI were also performed for further confirmation of the selected plasmids. As shown in Figure 5.19, three bands were again observed following digestion the extracted plasmids from clones 1 and 2 of the pEF1 $\alpha$ /LacZ/Kan<sup>R</sup>flanking repeat constructs. In lanes 17 and 18, and lanes 14 and 15 bands were observed 3577 bp, 2553 bp and 617 bp for clones 1 and 2 of 0nt and 25nt repeat plasmids, respectively. In lane 11 and lane 12, a new middle band size of 2778 bp in addition to the 3577 bp and 617 bp bands confirmed identity of pEF1 $\alpha$ /LacZ/Kan<sup>R</sup>flank250nt repeat sequence clones.

Figure 5.20 shows a final *Eco*RI restriction enzyme digestion analysis of clones 1 and 2. Three bands of 4333 bp, 1815 bp and 574 bp (lanes 17 and 18), 4333 bp, 1815 bp and 599 bp (lanes 14 and 15) confirmed identity of pEF1 $\alpha$ /LacZ/Kan<sup>R</sup>flank0nt repeat and pEF1 $\alpha$ /LacZ/Kan<sup>R</sup>flank25nt repeat plasmids, respectively. In contrast, *Eco*RI restriction digest analysis for the two clones of pEF1 $\alpha$ /LacZ/Kan<sup>R</sup>flank250nt repeats showed an incorrect digestion pattern for clone 1 (lane 11), with four bands being observed (including an extra band of 2500 bp). Restriction digest analysis for clone 2 showed the right size of the extracted plasmid (three bands of 4333 bp, 1815 bp and 824 bp (lane12)). Clone 2 from 0, 25 and 250nt repeat flanking constructs were selected for use in construction of corresponding recombinant MCMV vectors.



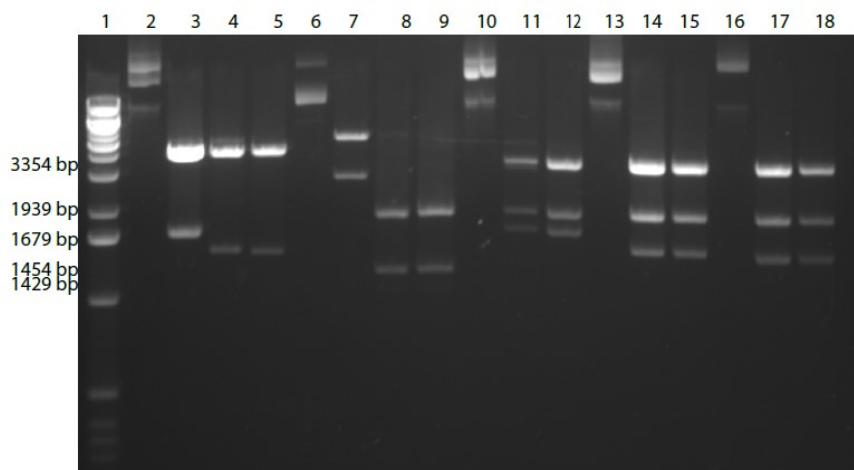


Figure 5.18: Restriction enzyme screening of pEF1 $\alpha$ /LacZ/Kan<sup>R</sup>flank250nt repeats sequences clones using *Pme*I, *Nhe*I and *Afe*I. Lane 1 DNA ladder (1 Kb DNA extension ladder). Lane 2 shows uncut pEF1 $\alpha$ /EBOV-GP/Kan<sup>R</sup>flank250nt repeats clone 2. Lane 3, 4, 5 show pEF1 $\alpha$ /EBOV-GP/Kan<sup>R</sup>flank250nt repeats clone 2, 25nt clone 3, Zero nt clone 2 digested vectors (parental). Lane 6 shows uncut pEQ176 plasmid. Lane 7 shows pEQ176 digested plasmid. Lane 8, 9 show undigested and digested PCR product of LacZ amplified from pEQ176 plasmid. Lane 10 shows uncut pEF1 $\alpha$ /LacZ/Kan<sup>R</sup>flank250nt repeats clone1. Lane 11, 12 show digestion of two clones of pEF1 $\alpha$ /LacZ/Kan<sup>R</sup>flank250nt repeats (clone 1-2). Three bands are observed; upper band of approximately 3354 bp, middle band of approximately 1939 bp and the lower bands of approximately 1679 bp. Lane 13 shows uncut of pEF1 $\alpha$ /LacZ/Kan<sup>R</sup>flank25nt repeats clone1. Lane 14, 15 show digestion of two clones of pEF1 $\alpha$ /LacZ/Kan<sup>R</sup>flank25nt repeats (clone 1-2). Three bands are observed; upper band of approximately 3354 bp, middle band of approximately 1939 bp and the lower bands of approximately 1454 bp. Lane 16 shows uncut of pEF1 $\alpha$ /LacZ/Kan<sup>R</sup>flank0nt repeats clone1. Lane 17, 18 show digestion of two clones of Lane 11, 12 show digestion of two clones of pEF1 $\alpha$ /LacZ/Kan<sup>R</sup>flank0nt repeats (clone 1-2). Three bands are observed; upper band of approximately 3354 bp, middle band of approximately 1939 bp and the lower bands of approximately 1429 bp.

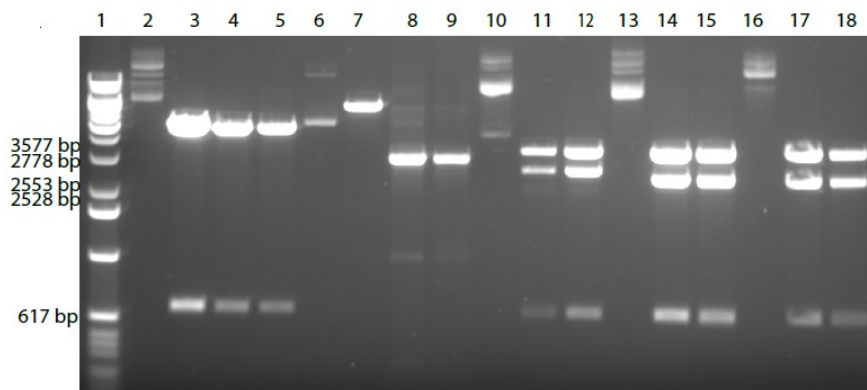


Figure 5.19: Restriction enzyme screening of pEF1 $\alpha$ /LacZ/Kan<sup>R</sup>flank repeats sequences clones using *Hind*III. Lane 1 DNA ladder (1 Kb DNA extension ladder). Lane 2 shows uncut pEF1 $\alpha$ /EBOV-GP/Kan<sup>R</sup>flank250nt repeats clone 2. Lane 3, 4, 5 show pEF1 $\alpha$ /EBOV-GP/Kan<sup>R</sup>flank250nt repeats clone 2, 25nt clone 3, Zero nt clone 2 digested vectors (parental). Lane 6 shows uncut pEQ176 plasmid. Lane 7 shows pEQ176 digested plasmid. Lane 8, 9 show undigested and digested PCR product of LacZ amplified from pEQ176 plasmid. Lane 10 shows uncut pEF1 $\alpha$ /LacZ/Kan<sup>R</sup>flank250nt repeats clone1. Lane 11, 12 shows digestion of two clones of pEF1 $\alpha$ /LacZ/Kan<sup>R</sup>flank250nt repeats (clone 1-2). Three bands are observed; upper band of approximately 3577 bp, middle band of approximately 2778 bp and the lower bands of approximately 617 bp. Lane 13 shows uncut pEF1 $\alpha$ /LacZ/Kan<sup>R</sup>flank25nt repeats clone1. Lane 14, 15 shows digestion of two clones of pEF1 $\alpha$ /LacZ/Kan<sup>R</sup>flank25nt repeats (clone 1-2). Three bands are observed; upper band of approximately 3577 bp, middle band of approximately 2553 bp and the lower bands of approximately 617 bp. Lane 16 shows uncut pEF1 $\alpha$ /LacZ/Kan<sup>R</sup>flank0nt repeats clone1. Lane 17, 18 show digestion of two clones of pEF1 $\alpha$ /LacZ/Kan<sup>R</sup>flank0nt repeats (clone 1-2). Three bands are observed; upper band of approximately 3577 bp, middle band of approximately 2553 bp and the lower bands of approximately 617 bp.

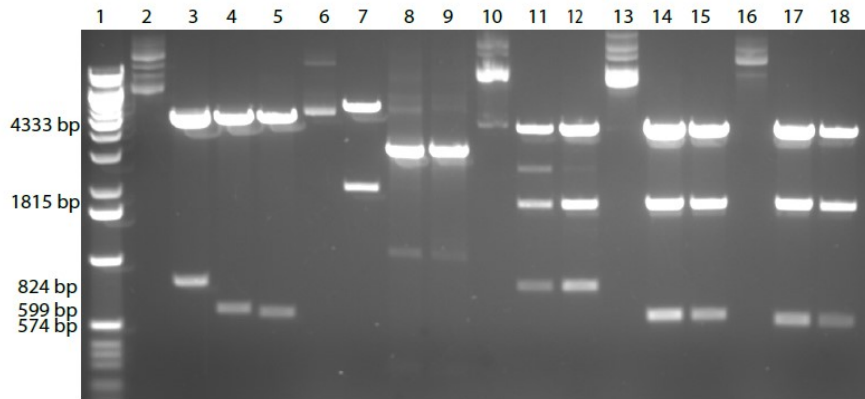


Figure 5.20: Restriction enzyme screening of pEF1 $\alpha$ /LacZ/Kan<sup>R</sup>flank repeats sequences clones using *Eco*RI. Lane 1 DNA ladder (1 Kb DNA extension ladder). Lane 2 shows uncut pEF1 $\alpha$ /EBOV-GP/Kan<sup>R</sup>flank250nt repeats clone 2. Lane 3, 4, 5 show pEF1 $\alpha$ /EBOV-GP/Kan<sup>R</sup>flank250nt repeats clone 2, 25nt clone 3, Zero nt clone2 digested vectors (parental). Lane 6 shows uncut pEQ176 plasmid. Lane 7 shows pEQ176 digested plasmid. Lane 8, 9 show undigested and digested PCR product of LacZ gene amplified from pEQ176 plasmid. Lane 10 shows uncut pEF1 $\alpha$ /LacZ/Kan<sup>R</sup>flank250nt repeats clone1. Lane 11, 12 show digestion of two clones of pEF1 $\alpha$ /LacZ/Kan<sup>R</sup>flank250nt repeats (clone 1-2). Three bands are observed in lane 12; upper band of approximately 4333 bp, middle band of approximately 1815 bp and the lower bands of approximately 824 bp. Lane 13 shows uncut pEF1 $\alpha$ /LacZ/Kan<sup>R</sup>flank25nt repeats clone 1. Lane 14, 15 show digestion of two clones of pEF1 $\alpha$ /LacZ/Kan<sup>R</sup>flank25nt repeats (clone 1-2). Three bands are observed; upper band of approximately 4333 bp, middle band of approximately 1815 bp and the lower bands of approximately 599 bp. Lane 16 shows uncut pEF1 $\alpha$ /LacZ/Kan<sup>R</sup>flank0nt repeats clone1. Lane 17, 18 show digestion of two clones of pEF1 $\alpha$ /LacZ/Kan<sup>R</sup>flank0nt repeats (clone 1-2). Three bands are observed; upper band of approximately 4333 bp, middle band of approximately 1815 bp and the lower bands of approximately 574 bp.

5.2.2 Construction of recombinant MCMV BAC clones carrying LacZ/Kan<sup>R</sup> expression cassette flanked by different length of repeats sequences (MCMVΔM157 LacZ/Kan<sup>R</sup> flanked by repeats sequences vectors) by using E/T- based linear recombination

The pEF1α/LacZ/Kan<sup>R</sup>flank0nt, pEF1α/LacZ/Kan<sup>R</sup>flank25nt and pEF1α/LacZ/Kan<sup>R</sup>flank250nt repeat vectors constructed in section 5.2.1 were used to make corresponding MCMV flanked attenuation vectors: MCMVΔM157 LacZ/Kan<sup>R</sup> flanked by 0nt repeats, MCMVΔM157 LacZ/Kan<sup>R</sup> flanked by 25nt repeats and MCMVΔM157 LacZ/Kan<sup>R</sup> flanked by 250nt repeats. Flanking cassettes were inserted to replace the M157 gene of MCMV K181 cloned as BAC (pARK14) by using E/T homologous recombination. The BAC DNA was extracted using alkaline lysis method (see section 2.2.1.2) and then electroporated into EL250 that expresses E/T recombinases required for recombination (see section 2.1.3). Thirteen Cam-resistant clones were selected and then recombinant BAC DNA was extracted using alkaline lysis method, followed with analysis by *EcoRI* restriction digest to confirm the integrity of the genome (Figure 5.21). Clone 1 was selected to be used for construction of the LacZ flanking MCMV recombinants.

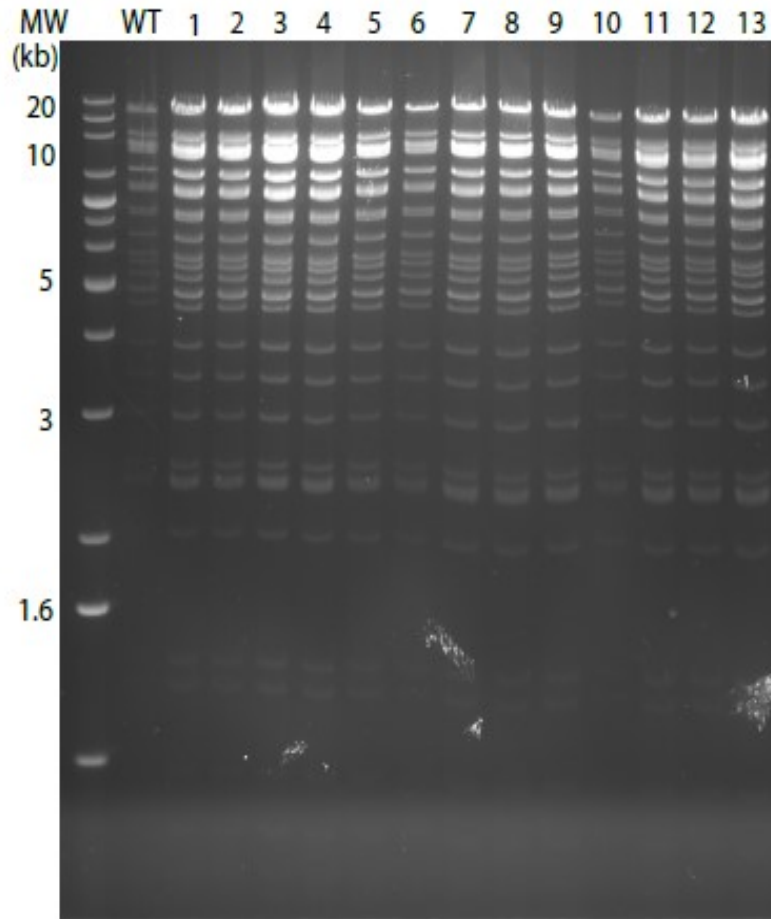


Figure 5.21: Characterization of WT MCMV (pARK 14) BAC, by using *EcoRI* restriction enzyme digestion. WT MCMV (pARK 14) and thirteen independent clones that selected after electroporation in to EL250, they were digested with *EcoRI* restriction enzyme. *EcoRI* digested fragments were then separated according their molecular weight by electrophoresis at 45 V for 18-20 hours and compared with the original wild type. Comparable restriction digestion pattern between the electroporated WT MCMV (pARK 14) BAC and WT MCMV (pARK 14) BAC showed the absence of any gross genomic rearrangements.

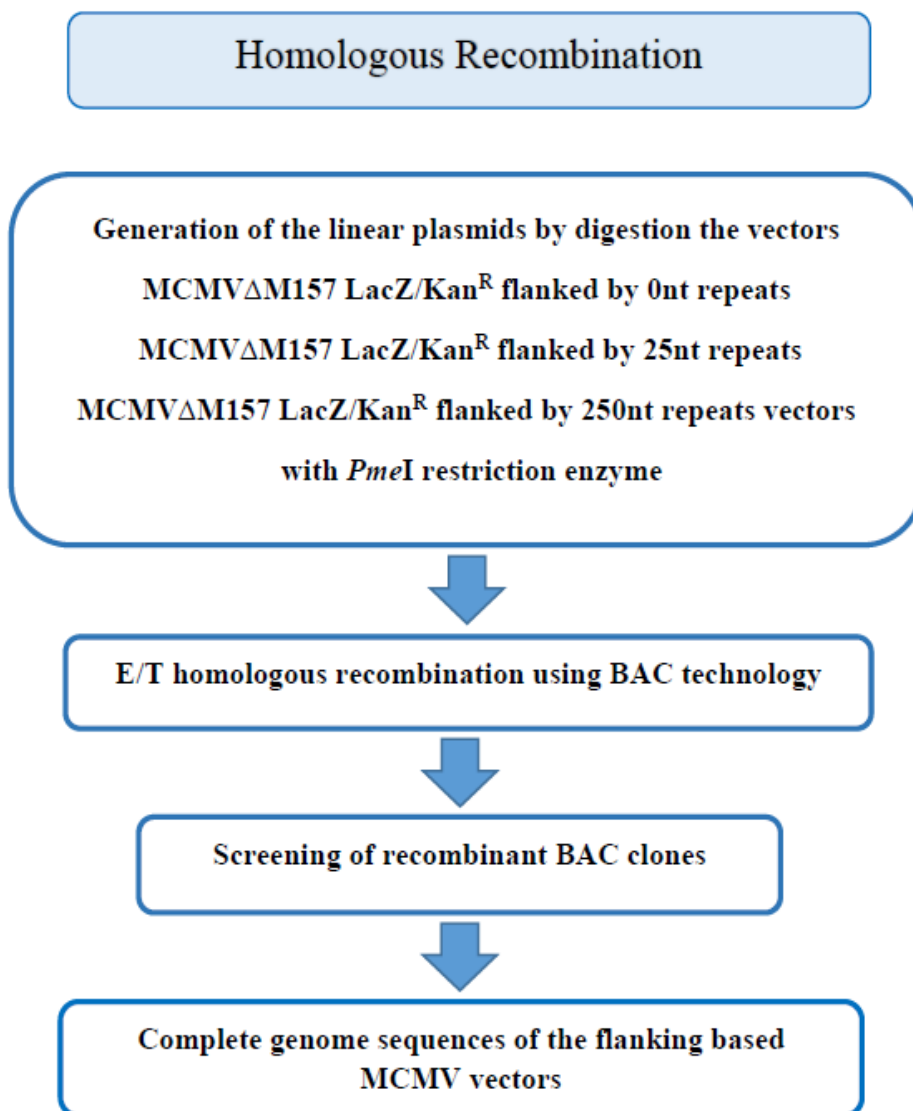


Figure 5.22: A flow-chart of construction of the flanking based MCMV vectors.

The initial step in construction of the MCMV $\Delta$ M157 LacZ/Kan<sup>R</sup> flank vectors was generation of linear recombination fragments. The pEF1 $\alpha$ /LacZ/Kan<sup>R</sup>flank0nt repeat (clone 2), pEF1 $\alpha$ /LacZ/Kan<sup>R</sup>flank25nt repeat (clone 2) and pEF1 $\alpha$ /LacZ/Kan<sup>R</sup>flank250nt repeat (clone 2) plasmids were digested with *PmeI* at 37°C for 2 hours (Figure 5.22). Digested plasmids were then purified using a Pure Link™ PCR Purification Kit (see section 2.2.6). Digestion of vectors resulting in production of linearized recombination fragments was confirmed by

electrophoresis on a 1% agarose DNA gels (Figure 5.23). Recombination fragment were then employed to generate MCMV $\Delta$ M157 LacZ/Kan<sup>R</sup> repeat by E/T homologous recombination in recombinase-induced EL250 containing WT MCMV (pARK 14) clone 1. These linear plasmids were transformed by electroporation into electrocompetent recombinase-induced EL250 cells (see section 2.2.8). Recombinant clones were then selected based on resistance to both Cam and Kan.

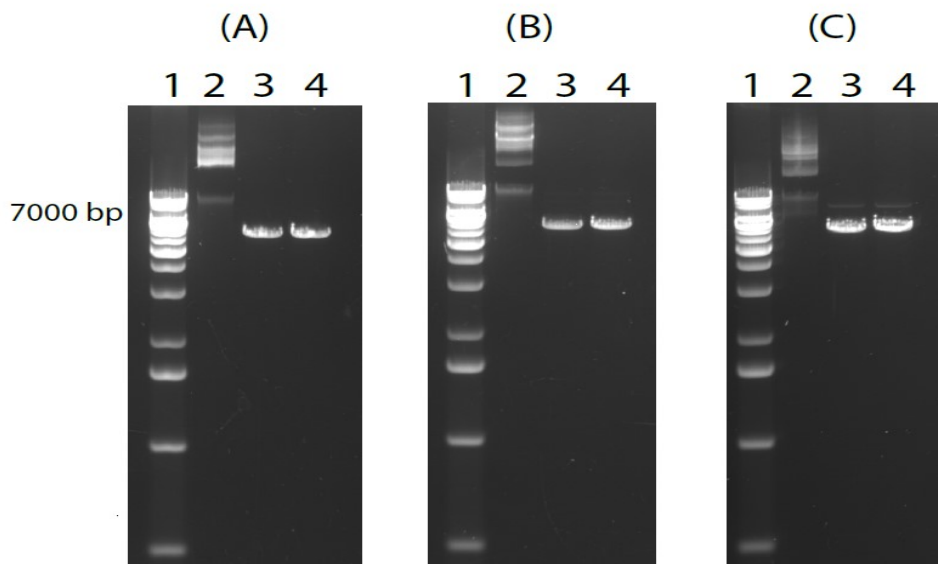


Figure 5.23: DNA gel showing recombinant fragments generated by digestion pEF1 $\alpha$ /LacZ/Kan<sup>R</sup> flank repeats sequence plasmid with *Pme*I. (A): lane 2 shows uncut show uncut pEF1 $\alpha$ /LacZ/Kan<sup>R</sup>flank0nt repeats sequence (clone 2). Lane 3 and 4 show pEF1 $\alpha$ /LacZ/Kan<sup>R</sup>flank0nt repeats sequence (clone 2) digested plasmids (6722 bp in size). (B): lane 2 shows uncut pEF1 $\alpha$ /LacZ/Kan<sup>R</sup>flank25nt repeats sequence (clone 2). Lane 3 and 4 show pEF1 $\alpha$ /LacZ/Kan<sup>R</sup>flank25nt repeats sequence (clone 2) digested plasmids (6747 bp in size). (C): Lane 2 shows uncut pEF1 $\alpha$ /LacZ/Kan<sup>R</sup>flank250nt repeats sequence (clone 2). Lane 3 and 4 show pEF1 $\alpha$ /LacZ/Kan<sup>R</sup>flank 250nt repeats sequence (clone 2) digested plasmids (6972 bp in size). The digested plasmids containing LacZ gene under EF1  $\alpha$ -promoter and Kan<sup>R</sup> gene flanked with different size of repeated sequences.

#### 5.2.2.1 Restriction digestion of recombinant MCMV LacZ/Kan<sup>R</sup> flank repeated sequences BAC DNA.

During E/T homologous recombination, it is possible that the components of the BAC genome undergoes rearrangement or deletion. Restriction digest analysis was performed to confirm genomic stability of the recombinant BACs clones as well as to ensure that the LacZ/Kan expression cassette flanked by repeated sequences had been inserted into pARK14. pARK14 (WT control) and five independent recombinant clones from each construct were selected and analysed by using *EcoRI* restriction enzyme followed by visualization after electrophoresis. As shown in Figure 5.24, this analysis showed the absence of gross genomic rearrangements. Complete genome sequencing was also performed on the following selected clones: MCMV BAC clones (MCMV $\Delta$ M157 LacZ/Kan<sup>R</sup> flanked by 0nt repeat (clone 5), MCMV $\Delta$ M157 LacZ/Kan<sup>R</sup> flanked by 25nt repeat (clone 3) and MCMV $\Delta$ M157 LacZ/Kan<sup>R</sup> flanked by 250nt repeat (clone 4). The results of complete genome sequencing confirmed the genetic integrity of the clones and the absence of undesired mutations. These three constructs were then selected for further analysis.



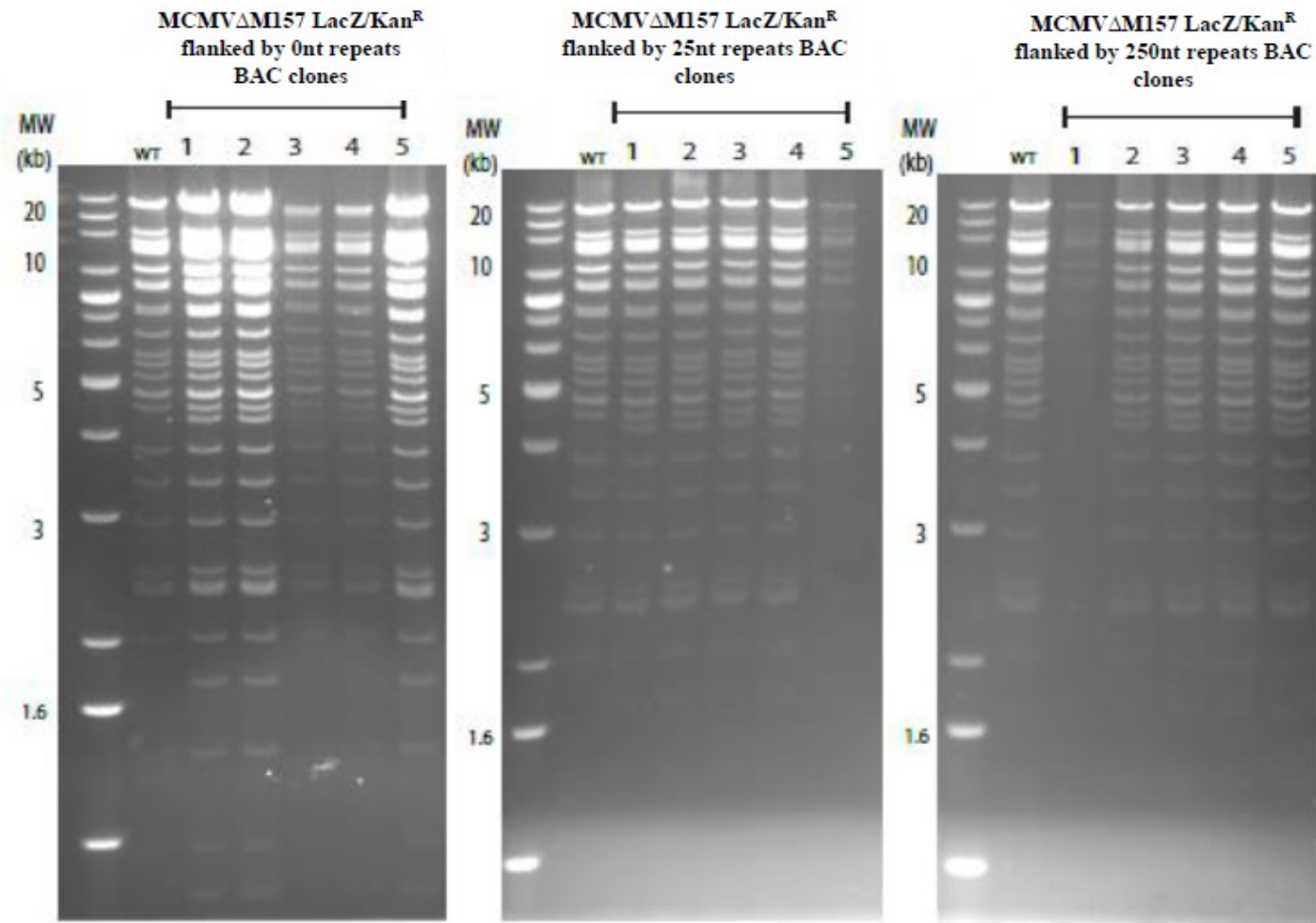


Figure 5.24: Characterization MCMV $\Delta$ M157 LacZ/Kan<sup>R</sup> flanked by 0nt repeats, MCMV $\Delta$ M157 LacZ/Kan<sup>R</sup> flanked by 25nt repeats. MCMV -WT and five independent clones from each construct were digested with *Eco*RI (restriction enzyme). *Eco*RI digested fragments were then separated according their molecular weight by electrophoresis at 45 V for 18-20 hours and compared with the wild type.

### 5.2.3 Expression of LacZ gene (reporter gene) in MCMV $\Delta$ M157 LacZ/Kan<sup>R</sup> flanked by repeats sequences vectors in bacterial cells

In order to determine whether the LacZ gene (reporter gene) was stable within the selected MCMV BACs in bacterial cells (EL250), the three recombinant MCMV BACs MCMV $\Delta$ M157 [LacZ/Kan<sup>R</sup> flanked by 0nt repeat (clone 5), MCMV $\Delta$ M157 LacZ/Kan<sup>R</sup> flanked by 25nt repeat (clone 3) and MCMV $\Delta$ M157 LacZ/Kan<sup>R</sup> flanked by 250nt repeat (clone 4)] were grown in LB agar containing Cam and X-gal substrate in the presence of Kan (the selectable marker). The parental MCMV pARK4 BAC (clone 1) was cultured in an identical fashion except for the absence of Kan. As shown in Figure 5.25 and Table 5.1, all MCMV recombinant BACs metabolized X-gal (resulting in blue colonies) demonstrating the stable maintenance and expression of functional  $\beta$ -gal in all recombinants (Figure 5.25 (A, B and C)). As expected, the parental pARK4 BAC resulted in only white colonies as expected consistent with the absence of LacZ gene.

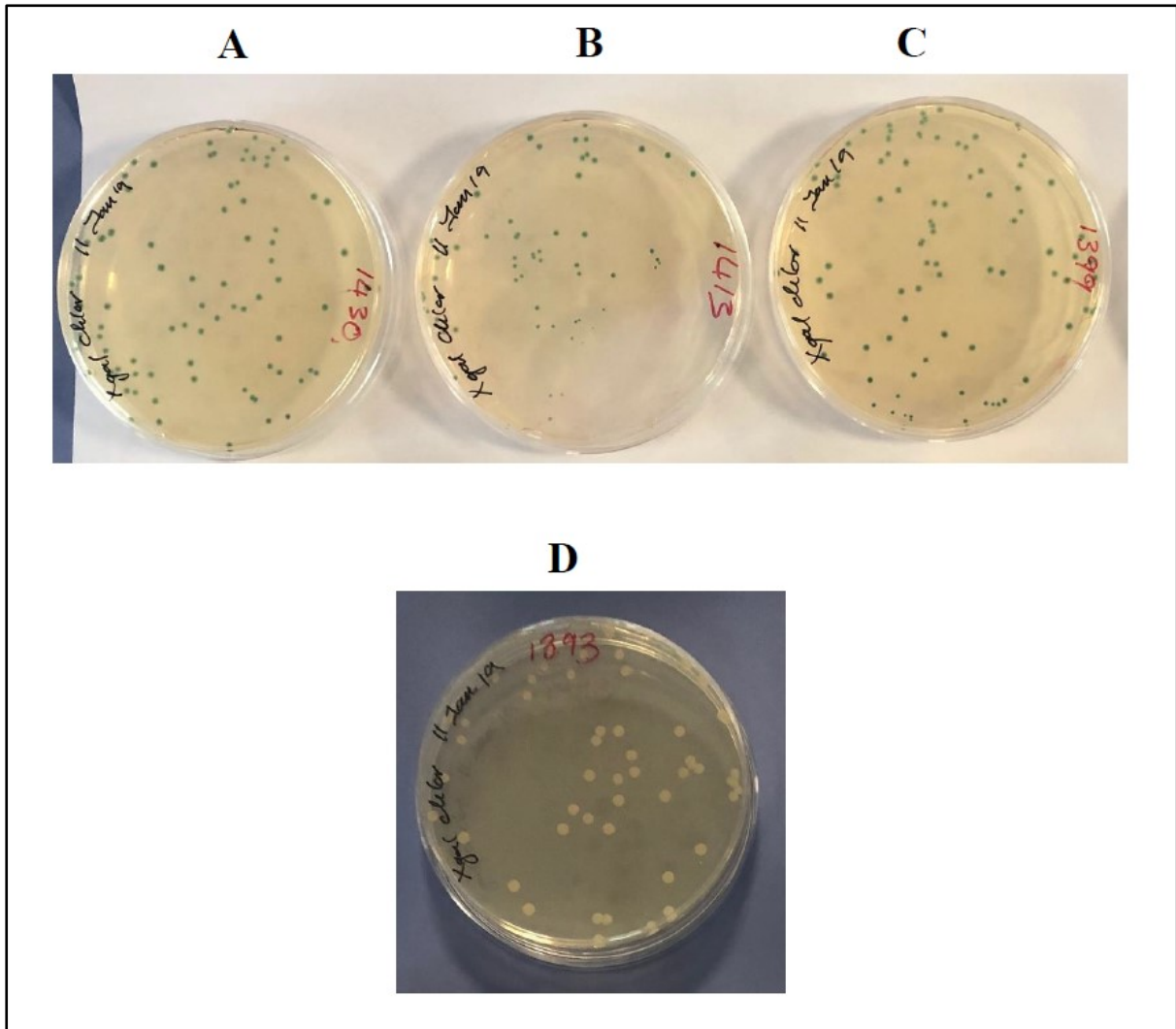


Figure 5.25: Growth of recombinant MCMV BAC clones in presence of X-gal reporter shows stable maintenance of  $\beta$ -gal marker in bacteria under non-restrictive conditions. (A): MCMV $\Delta$ M157 LacZ/Kan<sup>R</sup> flanked by 0nt repeats BAC clones. (B): MCMV $\Delta$ M157 LacZ/Kan<sup>R</sup> flanked by 25nt repeats BAC clones. (C): MCMV $\Delta$ M157 LacZ/Kan<sup>R</sup> flanked by 250nt repeats BAC clones and (C): Parental MCMV BAC clones (MCMV pARK4). The recombinant MCMV BAC clones were constructed by E/T recombination. The different recombinant MCMV BACs contain the LacZ/Kan<sup>R</sup> cassette flanked by indicated region of homology inserted within the non-essential M157 MCMV ORF BACs were selected on the basis of Kan-resistance. The table shows results of BAC clones grown on LB plates under non-restrictive (Cam only) conditions in the presence of X-gal as a reporter of  $\beta$ -Gal function. All BACs which contained the LacZ ( $\beta$ -gal) gene metabolized X-gal (resulting in blue colonies) demonstrating the stable expression of functional  $\beta$ -gal. MCMV pARK14 does not contain the LacZ gene and all colonies were white in appearance.

Table 5.1: Number of the recombinant MCMVBAC colonies expressing functional  $\beta$ -gal under non-restrictive condition (X-gal and Cam only)

Recombinant MCMV BAC clones (cultured in EL250 bacterial strains )	Total number of colonies [grown under non-restrictive conditions (X-gal + Cam only)]	Number of colonies expressing $\beta$ -gal (%)
MCMV pARK4 [Parental $\beta$ -gal negative] (EL250 clone 1)	54	0
MCMV $\Delta$ M157 LacZ/Kan <sup>R</sup> flanked by 0nt repeats (EL250 clone 5)	86	100
MCMV $\Delta$ M157 LacZ/Kan <sup>R</sup> flanked by 25nt repeats (EL250 clone 3)	60	100
MCMV $\Delta$ M157 LacZ/Kan <sup>R</sup> flanked by 250nt repeats (EL250 clone 4)	80	100

#### 5.2.4 *In vitro* testing of the flanking intrinsic attenuation system

A panel of fully characterised MCMV BAC constructs expressing a functional LacZ/Kan<sup>R</sup> cassette flanked by differing lengths of repeated regions was the goal of the present thesis. These constructs were sent to the laboratory of Alec Redwood (University of Western Australia), where the BACs have been reconstituted as MCMV viruses. The following data is presented from ongoing studies to test the proof-of-concept (kindly provided by Alec Redwood). Virus stocks of parental (ARK14) and the panel of three BAC flanking constructs were reconstituted by electroporation of BAC DNA into the M2-10B4 mouse fibroblast cell line. These viruses were then tested to their loss of the LacZ/Kan<sup>R</sup> expression cassette by PCR analysis of the viruses over increasing serial passage.

PCR analysis of WT MCMV(ARK14) (virus designated AR) and three recombinants MCMV viruses[MCMV $\Delta$ M157 LacZ/Kan<sup>R</sup> flanked by 0nt repeats (virus designated R0), MCMV $\Delta$ M157 LacZ/Kan<sup>R</sup> flanked by 25nt repeats (virus designated R25) and MCMV $\Delta$ M157 LacZ/Kan<sup>R</sup> flanked by 250nt repeats vectors (virus designated R250) that contain LacZ/Kan<sup>R</sup>

flanked by different length of repeat sequences] was performed by using two set of primers. The first set was designed to bind within the remaining section of M157 and within M158, at either side of LacZ/Kan<sup>R</sup> transgene. The expected size of the amplified region for WT MCMV(ARK14) was 1326 bp, and for recombinant viruses R0, R25 and R250, 6859 bp, 6884 bp and 7109 bp, respectively. The size of the amplified region following LacZ/Kan<sup>R</sup> transgene excision was 702 bp. Due anticipated inefficient amplification of the large (approx. 7kb) size of the region containing the transgene, a second set of primers were designed to amplify a region of 3024 bp within LacZ gene. AR and the three (R0, R25 and R250) recombinant viruses were passaged eight times and viral DNA was extracted from the infected cells at each passage followed by amplification using the two sets of primers. The locations of PCR primers are shown in Figure 5.26, with amplification being carried out with M157 primers and β-gal internal primers. Transgene excision revealed by the presence of 702 bp band amplified by M157 primers was detected in PCR products from viruses contain transgenes flanked by repeated sequences. The band was not detected in PCR products from viruses that contain the transgene flanked by zero repeat sequences (see Figure 5.26 (B)). A 3 kb β-gal band was visible in R0, R25 and R250 lanes. This data showed that (1) there is excision of the transgene when it is flanked by 25 bp and 250 bp of repeat sequences (2) the transgene is stable when it is not flanked by repeat sequences (0 bp).

The data presented in Figure 5.26 indicates that homologous flanking regions can target a heterologous target gene (in this case, LacZ/Kan<sup>R</sup>) for excision, which is consistent with homologous recombination being specifically targeted by the flanking regions as hypothesized. The impact of size of flanking region was determined by PCR over extended passage (P3 to P8) to ascertain whether flanking size would affect rate of excision. As shown in Figure 5.27, 702 bp bands for R25 and R250 differ in intensity, the intensity of the bands are increased over passage, with the P3 band for R25 being faint while the band for R250 was intense. The P8 bands for both R25 and R250 were intense. This data shows that transgene excision between

flanking repeats sequences detected at passage 1 to passage 8 increases more rapidly in R250 compared to R25 consistent with the hypothesis that the rate of excision can be kinetically controlled by the size of the region flanking the targeted transgene. The PCR was non-quantitative and provided a relative rate of LacZ/Kan<sup>R</sup> excision between R0, R25 and R250. The presence of a functional LacZ in the transgene cassette was also exploited as an additional measure by using the number of blue (LacZ/Kan<sup>R</sup>-containing) versus white (LacZ/Kan<sup>R</sup>-excised) to measure the rate of excision. Although preliminary, the data shown in Figure 5.28 shows the excision rate depended on the size of the flanking repeats, with the rate of the excision approaching 10% in R250 versus approximately 1% in R25, and being completely absent from the R0 virus lacking a repeat region. In summary, these initial *in vitro* studies strongly support our hypothesis that the presence of flanking repeat regions can target a heterologous target antigen for excision, and further indicate that the relative rate of this process can be influenced by the size of the flanking region.

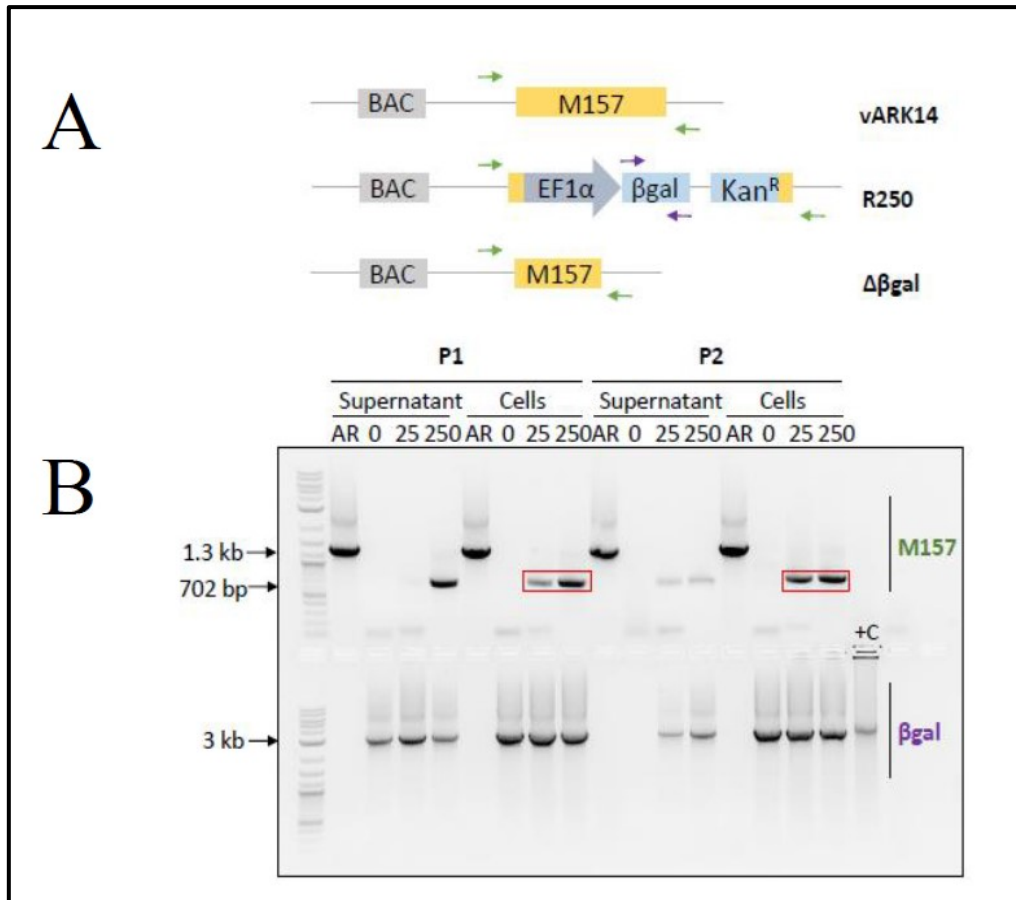


Figure 5.26: PCR analysis for transgene excision between flanking repeats sequences. (A): Schematic-showing locations of PCR primers in the viral genome that used in PCR analysis. The first set of primer (green arrows) are located at M157 and M158 which can bind in either side of transgene, the expected size for the amplified region in R0 is 6859 bp, R25 is 6884 bp and R250 is 7109 bp while in the AR the expected size is 1326 bp. In the case of transgene excision  $\Delta$  LacZ ( $\beta$ -gal) the size is 702 bp. The second set of primer LacZ ( $\beta$ -gal) internal primer (purple arrows) amplified a region within LacZ( $\beta$ -gal) gene about 3024 bp in length. (B): PCR analysis of the AR and the three recombinant viruses R0, R25 and R250 was performed using DNAs extracted from passage1 (P1) and passage2 (P2). The two sets of primers were used in amplification. Top panels: amplification with the first set of primers (M157). The bottom panels: amplification with LacZ ( $\beta$ -gal) internal primer. Bands identified at size 702 bp in the top panels indicate excision of the transgene from viral genome when it flanked by 25nt and 250nt repeat sequences. Bands at size 1.3 kb indicate that AR (WT) band. The presence of 702 bp bands that represent transgene excision in both R25 and R250 and the absence of the band in R0 support this idea. These results indicate that the transgene excision is consistent with homologous recombination (Data from Alec Redwood Laboratory).

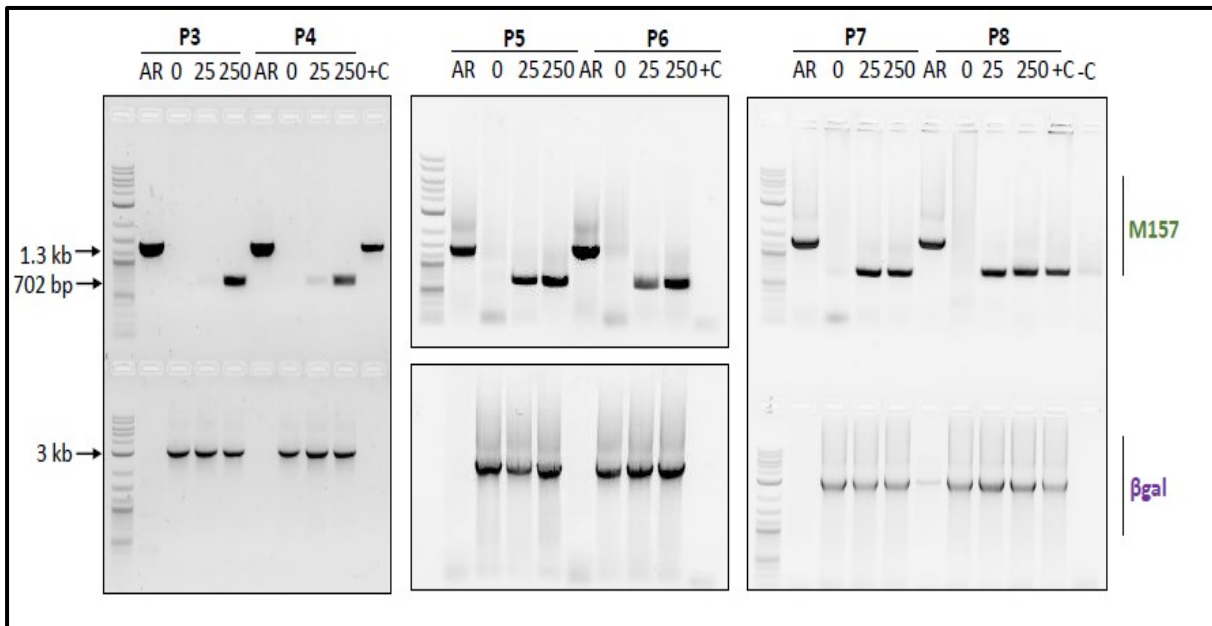


Figure 5.27: PCR analysis showing the impact of the size of the flanking repeats sequences on the excision of transgene from the recombinant viruses over passages. PCR was performed using viral DNAs extracted from six passages (P3 to P8). Top panels: amplification with the first set of primers (M157). The bottom panels: amplification with LacZ ( $\beta$ -gal) internal primer. Bands identified at size 702 bp in the top panels indicate excision of the transgene from viral genome when it flanked by 25nt and 250nt repeat sequences. Bands at size 1.3 kb indicate that AR (WT) band. 702 bp bands for R25 and R250 R250 differ in intensity. Comparing the intensity of the 702 bps bands in P3 through P8 this is showing that the R250 is accumulating at a greater rate than the R25. This results show that the size of the flanking sequences are kinetically effects excision (Data from Alec Redwood Laboratory) .

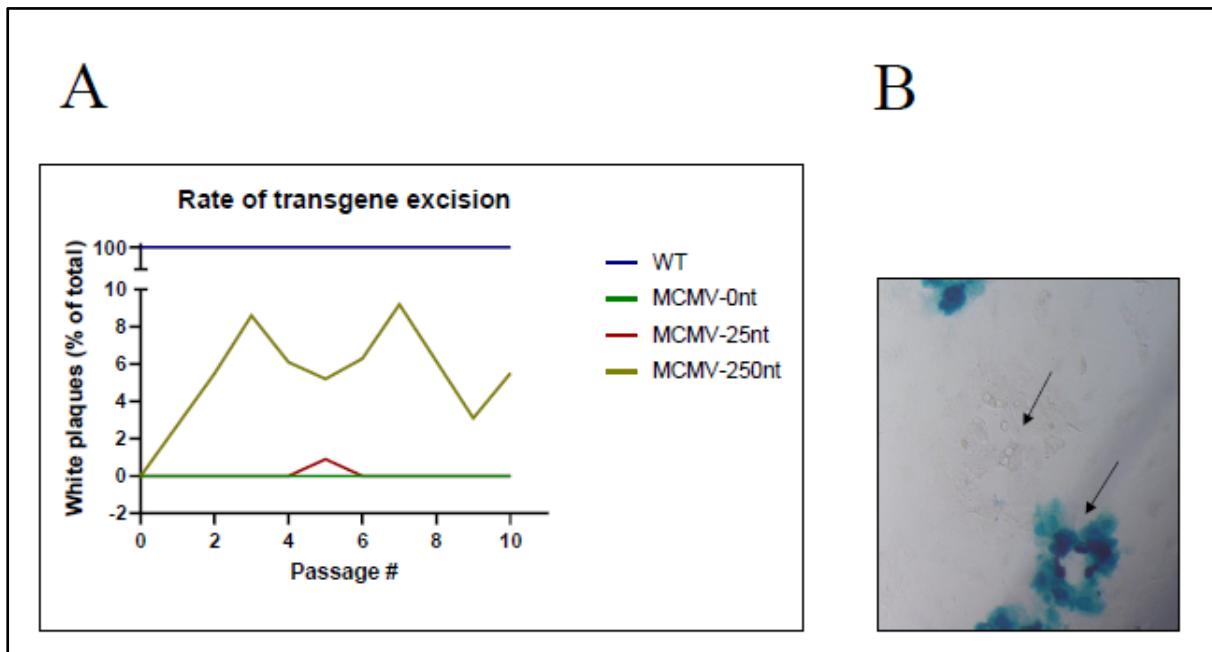


Figure 5.28: Rate of transgene excision in the flanking based attenuated viruses. (A): M2-10B4 infected with virus from indicated passages were stained with X-gal. White and blue plaques were counted and rate of transgene excision expressed as percentage of white plaques. (B): Representative blue and white plaques (Data from Alec Redwood Laboratory).



### 5.3 Discussion

The unpredictable risk for using recombinant viral vaccine vector (GMO) in regard to humans, animals and environment represent a major concern (Chan, 2006; Myhr & Traavik, 2011).

So far intrinsic control for recombinant virus vectors are not well developed. In Chapter 4, a strategy for intrinsic control based on modulating the genomic fidelity to alter the biological half-life of the virus vector itself was presented. In this present chapter, instead of controlling the viral vector, the aim was to control the transgene. This study set out with the aim of engineering MCMV vectors in which the transgene (LacZ) is flanked by different length of repeats sequences in order to develop a new strategy to control the genetically modified viruses by removing the transgene and restoration the WT virus. The hypothesis was that the presence of the transgene between two identical flanking sequences would lead to excision of transgene from the recombinant viral vector and restoration of the WT (non-GMO) virus.

The present study was designed to construct the genetically modified recombinant MCMV vector (LacZ-MCMV), which contains foreign DNA molecules (LacZ as transgene) that are flanked by two identical flanking regions. A panel of MCMV viruses expressing a measurable transgene flanked by different sized flanking regions were designed and constructed to test two hypotheses. First, whether the flanked region would be targeted for excision; and second whether the size of flanking region would affect the relative rate of excision. Within the constraints of the thesis the goal was to construct and fully characterize this panel of BACs, which would then be subsequently reconstituted as viruses and tested in future studies beyond the thesis in both *in vitro* and *in vivo*. The results of this study indicate that three BAC vectors (MCMV $\Delta$ M157 LacZ/Kan<sup>R</sup> flanked by 250nt repeats, MCMV $\Delta$ M157 LacZ/Kan<sup>R</sup> flanked by 25nt repeats and MCMV $\Delta$ M157 LacZ/Kan<sup>R</sup> flanked by 0nt repeats) were successfully engineered. Analysis of the complete nucleotide sequence of these vectors confirmed their successful design and construction, and the LacZ gene was shown to be functional and stable within the engineered MCMV BACs in the bacterial cells.

The LacZ gene was selected for use as transgene in this study for a number of reasons. First, it is well-characterized. Second, it does not impact fitness (previous studies that used this gene as a reporter gene in MCMV recombinants showed that expression of this gene does not affect the virus replication *in vitro* nor *in vivo* (Farrell *et al.*, 1994)). The  $\beta$ -gal protein also contains characterized T cell epitopes that can be used to measure impact on T cell responses. The LacZ expression cassette was designed to contain Kan<sup>R</sup> that can be used to maintain transgene in both bacteria and eukaryotic cells (in the latter by using G418). Two flanking regions with different size of repeats sequences were designed. The first contained a flanking regions with 250nt repeats sequences, which was chosen based on a previous study that showed rapid excision of a BAC cassette from MCMV was facilitated by using a 249nt repeat sequence size (Redwood *et al.*, 2005) (see below). The second flanking region was designed with 25nt repeats sequence, with the expected result of this repeat sequence causing a slower loss of transgene if size was inversely proportional to rate of excision as we hypothesized. A MCMV recombinant without repeats sequences was also used as a key control in the study. The M157 gene product is known to attenuate the virus replication in C57BL/6 mice. Consequently, we chose this site for the insertion of LacZ/Kan<sup>R</sup> expression cassette flanked by repeats sequences, with insertion leading to deletion of 623 bp of M157 gene (see below).

The hypothesis up which our study was based was derived from on insights gained from studies in molecular engineering of plants. These studies showed that removal of the selectable marker genes (antibiotic or herbicide resistance genes) from the genetically modified plant can be performed by flanking the selectable marker by two identical repeats sequences (Chong-Pérez & Angenon, 2013). For example, excision of streptomycin resistant gene from *Chlamydomonas reinhardtii* (Fischer *et al.*, 1996) and excision herbicide resistance gene from tobacco chloroplasts (Iamtham & Day, 2000) have both been achieved through this strategy. In both of these studies in plants, the rate of excision was shown to be affected by size of the flanking region. *Chlamydomonas reinhardtii*, excision was not observed when the size of

flanking region 230 bp but at size 483 or 832 bp excision was observed (Fischer *et al.*, 1996). Similarly, in tobacco excision was observed when the size of the flanking region was 418 bp while the excision was not observed when the size of the flanking region 137 bp (Zoubenko *et al.*, 1994).

Through collaboration with the Redwood Laboratory we present preliminary data testing whether flanking regions within a herpesvirus-based vector will similarly target a transgene for excision. Prior to the present study, the work of Wagner *et al* (1999) suggested the potential utility of this approach (Wagner *et al.*, 1999). However, the effect of size for controlling rate had never been examined. As shown in Figure 5.26 PCR, analysis showed the ability of the flanking repeats to remove transgene. The 702 bp band that represents transgene excision was detected in R25 and R250 while the band was absence in R0. The impact of size of flanking region on transgene excision was examined using two methods. The first method was based on the intensity of the 702 bp band, comparing the intensity of the excision bands over passages. The second method was based on calculating the blue and white plaques to measure the rate of the excision over passages (Figures 5.27 and 5.28). Consistent with size of the flanking region being positively correlated to the rate of excision, the increase in intensity of the 702 bps bands in P3 through P8 support that the R250 is accumulating at a greater rate than the R25. Although preliminary, this is the first study to show that within a virus vector the excision of a targeted transgene can be kinetically regulated.

In addition to size of the flanking region, there are many other factors that may affect the rate of transgene excision such as the size of the transgene, its biological nature, the sequence of the flanking regions, and position of insertion of the flanking regions in the virus. Prior studies have noted the effect of the size of genetic material inserted within the viral genome, resulting in 'over-length' viral genomes on viral replication *in vivo*, with multiple studies showing that removal of the inserted genome restored the virus to WT levels of replication (Adler, Messerle & Koszinowski, 2001; Chang & Barry, 2003; Wagner *et al.*, 1999). For adenovirus, insertion

genetic material more than 5% of the total genome size affected the efficiency of viral replication due to instability of the genome of the virus (Bett, Prevec & Graham, 1993). Together, these studies indicate that over length genomes are unstable and poorly packed in the capsid.

To avoid over-length instability of the transgene in our studies, MCMV K181-BAC (pARK14) was used for construction of the MCMV recombinants. The use of pARK14 BAC MCMV avoids the over-sized genome concern as it was constructed with deletion of 5.966 bp from MCMVK181 genome (genes m07 to m12 (6522 to 12488)), which is comparable to the size inserted. These deleted genes (genes m07 to m12) are not essential for replication of the virus *in vitro* and *in vivo*, it has been shown that the replication of pARK 14 was similar to the WT MCMV K181 virus. (Redwood *et al.*, 2005). In this study, we do not expect any impact of insertion LacZ/Kan<sup>R</sup> that flanked by repeats sequences into pARK 14 since we deleted about 623 bp of M157 in addition to 5.966 bp that already deleted from the wild type MCMV K181-BAC and the size of inserted fragments are LacZ/Kan<sup>R</sup>expression cassette with 0 repeats sequences, LacZ/Kan<sup>R</sup>expression cassette with 25 repeats sequences, LacZ/Kan<sup>R</sup>expression cassette with 250 repeats sequences are 6142 bp, 6167 bp and 6392 bp respectively.

It has been shown that the nature of the transgene could affect the rate of replication. Previous studies showed that insertion of heterologous antigen (EBOV- GP) within the RhCMV genome drops replication *in vitro* by approximately 1-log (Marzi *et al.*, 2016) while the insertion of SIV Gag, Rev-Tat-Nef fusion protein and Env within RhCMV genome did not affect the replication of the recombinants vectors that expressing these antigens (Hansen *et al.*, 2009). As indicated above, the LacZ was chosen for this initial proof-of-concept as it has been shown not to affect MCMV replication (Farrell *et al.*, 1994). However, the effect of the antigen both positively and negatively could potentially be used to alter kinetics of antigen loss, for example by altering the biological half life of the target antigen or by retargeting the antigen to prevent toxic accumulation.

It has been shown also that the expression of particular viral gene such as MCMV M157 gene could affect the replication of the virus in some animals (C57BL/6 mouse background) via activation natural killer (NK) cells. The activation of NK cells is controlled by cell surface receptor Ly49H(Arase *et al.*, 2002). The M157 binds to host NK receptors (Ly49H) and this binding delivers a signal to activate NK cells and consequently limiting viral replication (Pyzik *et al.*, 2011). Similar to previous studies, the NK activating M157 gene was inactivated to avoid NK control (Klyushnenkova *et al.*, 2012; Tierney *et al.*, 2012; Tsuda *et al.*, 2011). It is expected that the M157 deletion would give the recombinant virus an advantage over WT virus by targeting the NK activator. This could perhaps increase potential survival over the M157 expressing version. Moreover, it has been shown that co-infection with multiple strain with prolong co-replication could lead to increase the possibility of recombination between strains (McWhorter *et al.*, 2013; McWhorter *et al.*, 2015). In this study the MCMV was engineered in a way that M157 cannot restore after the transgene excision. If the viruses were engineered to restore the WT M157 following transgene excision, then this could potentially be used as a means to slow the rate of transgene loss by preferential selection based on preferred competition of the non-NK activating, M157-negative antigen containing virus. However, this NK-based selective pressure would only play a role *in vivo* not *in vitro*.

There are additional parameters that may affect stability of a flanked region. It has been shown that the excision of the BAC from MCMV (Smith strain) that flanked by a 527 bp repeats regions happened gradually, with excision of the BAC from the genome of all virus progeny by passage five (Wagner *et al.*, 1999). In contrast, excision of the BAC from MCMV (strain K181) that flanked by two 249 bp repeats regions from the genome happened rapidly, with 99% of infected cells at passage 2 showing evidence of transgene loss (on the basis of GFP expression) (Redwood *et al.*, 2005). The reason for there being no clear relationship between the efficiency of the recombination/excision and the size of repeated sequences in these two studies could be related to many different factors, including differences in the position of BAC

insertion/excision within viral genome, the particular sequence of the repeat regions, and the use of different viral strains. This may also indicate that as for plants, differences even between strains of the same virus may affect the rate of transgene loss.

A number of studies suggested that the nature of the flanking repeated DNA sequences (endogenous DNA sequences) could affect the accuracy of excision. Therefore flanking heterologous DNA sequences were used to excise the gene of interest in many plant studies to avoid deletions or inversion in the gene that could be result from the recombination between the repeated sequences, since the heterologous sequences would be unique and hence could not recombine with the endogenous DNA (Fischer *et al.*, 1996; Svab & Maliga, 1993). Until contributing variables that impact rate of transgene excision are known, this would indicate that a particular vaccine construct may need to be constructed and then characterized within the target host species in a 'case by case' fashion. However, high accuracy of excision of the transgene (in this case the BAC cassette) that flanked by two identical endogenous viral DNA repeats sequences was reported in both MCMV strains Smith and K181 (Redwood *et al.*, 2005; Wagner *et al.*, 1999).

The impact of transgene excision on the immunogenicity is unknown, but it can be predicted that losing the transgene will lead to an immune response more akin to a replication-defective or killed vaccine. This model is based on the idea that persistence of antigen is a main characteristic that sets herpesviruses apart from other vaccines, in terms of driving durable effector T cell-biased responses. Although boosting of animals may be required to enhance the immune response for vectors attenuated in such a fashion, given that these vectors can be reused, this reduced durability will need to be weighed against the high potential safety of such a modified vaccine. The actual impact of this control on these parameters of immunogenicity will be dependent, in large part, on the rate of transgene loss, which will only be able to be determined empirically. It is expected that the size of the flanking region could then be used to

fine-tune the vector to achieve the optimal rate of transgene loss for maximal durability and desired quality for a given threshold of safety.

## Chapter 6 Conclusion and future direction

Rapid increase in the use of GMOs, including recombinant viral vaccine vectors, has increased the importance of providing mechanisms for their biological control. Several strategies have been used towards the attenuation of these organisms in order to reduce the possible impact of these organisms to both the individual and the environment. For bacterial systems, a number of intrinsic strategies based on deletion or reengineering of essential genes in order to construct an auxotrophic organism have been shown to afford a high level of control. The most promising of these strategies depend on a growth requirement for synthetic amino acids which are unavailable in the environment. For eukaryotic systems, strategies based on deletion of essential genes have been used for attenuation. However, intrinsic control of eukaryotic systems, including eukaryotic viruses has proved more difficult and is considerably less developed.

Developing new strategies to intrinsically control genetically engineered viral vectors is clearly required for constructing safe vaccines. The aim of this thesis is to empirically examine a number of innovative intrinsic systems for the control of herpesvirus-based vaccine vectors. Studies described in Chapter 3 are concerned with the development of conditionally attenuated BoHV-4 deleted for ORF73 (BoHV-4  $\Delta$ ORF73) vaccine to target zoonotic disease. Chapter 4 develops a new strategy for intrinsic control based on modulation of genetic fidelity of the virus. In Chapter 5, we examine an intrinsic control strategy based on control of the transgene instead of the virus vector itself by using engineered MCMV to examine the ability of repeat sequences flanking the transgene to kinetically control excision of the transgene and thereby restore the WT virus.

In Chapter 3, we assessed the ability of conditionally attenuated BoHV-4 to induce the immune response against a heterologous antigens (*Mb* fusion protein) in the BoHV-4 permissive rabbit model. The aim was to test the hypothesis that the conditionally attenuated (latency defective) BoHV-4  $\Delta$ ORF73 can induce immune responses against its encoded heterologous antigens. We



constructed latency defective BoHV-4 expressing *Mb* fusion antigen (BoHV-4 $\Delta$ ORF73*Mb*) and tested the ability of the attenuated virus to induce immune response against *Mb*. The results of this investigation showed that conditionally attenuated version can induce the immune response against heterologous antigen, with antibodies being detected in three out of eight animals (38%). The transient nature of the vector resulting from the absence of ORF73 was also confirmed.

Studies detailed in Chapter 4 were designed to determine whether the mutation at exonuclease region domain II in UL54(UL54( $\Delta$ 413)) would affect the replication fidelity of HCMV UL54 (D413) virus. To test this hypothesis we used a molecular cloning technique to clone the amplified DNA that extracted from the HCMVUL54( $\Delta$ 413) and HCMVUL54WT from the low and high passages. Two regions within the viral genome were sequenced for detection of mutations and quantitation of the mutation rate. Our results showed that UL54( $\Delta$ 413) decreased the fidelity of replication of the virus corresponding to an accumulation of mutations over serial passage. Importantly, this study contributed additional evidence that suggested mutation at codon 413 affect the replication fidelity of the virus. The affect of this accumulation of the mutations on viral fitness will be an aim of future work.

Studies detailed in Chapter 5 were designed to test the hypothesis that flanking repeated sequences are able to destabilize the transgene leading to its excision from the recombinant virus. To test this hypothesis a panel of MCMV BAC vectors in which the LacZ/Kan<sup>R</sup> expression cassettes flanked by two identical repeats sequences (MCMV $\Delta$ M157 LacZ/Kan<sup>R</sup> flanked by repeats sequences) were constructed and characterized *in vitro*. The preliminary data showed the ability of the flanking repeats sequences to excise the LacZ/Kan<sup>R</sup> expression cassettes (the transgene). This finding supports our hypothesis that repeated sequences can target the transgene for excision by recombination between these sequences. These results also showed that the rate of excision is influenced by the size of flanking region regulates in a positive fashion. For future studies, will assess the effect of flanking repeats sequences on the

transgene *in vivo*. Importantly, these studies will also enable the impact of transgene excision on the quantity and quality of the resultant immune response against the target antigen to be assessed. The three different modes of control investigated in this thesis are expected to result in vaccines that differ in their level of safety, as well as the quality and quantity of the immunity they induce against the target antigen. The first strategy based on deletion of ORF73, a gene required for persistence of the virus and latency, is expected to result in an attenuated virus that can replicate for a short period of time but cannot become latent or reactivate (latency defective vector). In contrast, the vaccine attenuated by modulation of replication fidelity will be able to persist for a period of time, with its ability to establish latency and then reactivate being gradually lost (low replication fidelity vector). In the third model of control, based on targeting of the transgene rather than the virus, the vaccine will be fully replicative, and can be latent and then reactivate but in this case the antigen will be gradually lost after certain period of time (flanking based vector). We expect that the first and third models of control will result in viruses with a higher level of safety than viruses attenuated through modification of genetic fidelity. Decrease of replication fidelity does not always result in attenuation. A recent study has shown that the modulation of genetic fidelity for one of the herpesviruses (Marek's Disease Virus) results in production of a genetically diverse population of progeny viruses. These viruses when used to infect animals were more virulent than their attenuated parental viruses (Trimpert *et al.*, 2019). Higher mutation frequencies in RNA viruses are thought to aid the virus in rapid evolution and adaptation. The strain of Sabin vaccine, the live attenuated polioviruses vaccine has been shown that it could mutate or revert to more neurotropic form than that caused by wild type poliovirus (Chumakov *et al.*, 1992; Kew *et al.*, 1998; Pavlov *et al.*, 2006). The reversion of HCMVUL54(D413A) to the wild type was reported by Chou lab since this virus contained a substitution mutation in UL54 codon D413A rather than the codon deletion of the later  $\Delta$ 413 used in our studies (Chou & Marousek, 2008). Although the deleted codon virus version shows that the attenuated virus stable through passage 23, the possibility of reversion

to the wild type in the environment should not be excluded. However, in contrast to the above examples for RNA viruses, reversion of CMV to its WT form results in a virus that is non-pathogenic except in circumstances of severe immune suppression of the host.

Latency and reactivation are one of feature for herpesviruses (Sinclair & Sissons, 2006). The three different control strategies are also expected to result in viruses that also differ in their ability to reactivation. Whereas we expected that the latency defective BoHV-4 vector cannot reactivate due to deletion of the ORF73 region (since the virus genome is eventually lost from the host cell), we expected that low replication fidelity vector and the flanking based vector can be latent and then reactivate. In support of the inability of the ORF73-deleted virus to persist, Thirion *et al* (2010) showed in their study the inability of the latency defective BoHV-4(BoHV-4  $\Delta$ ORF73) to persist and to stay latent. Real-time PCR results for viral DNA extracted from PBMCs and spleen (the main two places for BoHV-4 latency) from the infected animals showed that the latency defective cannot be detected at day 12 (after an eclipse phase) until day 113 where the experiment was finished, while the WT BoHV-4 could be detected at these times. The inability of the latency defective BoHV-4 to reactivate was also confirmed by coculture the PBMCs and spleen cells with the permissive cells (Thirion *et al.*, 2010).

We predicted that the ability of the vaccine modified by the flanking-based strategy to establish latency and reactivation should not be affected by this mode of control. Attenuation based on modulation of the genomic fidelity could affect the ability of the low replication fidelity for latency and reactivation, but only gradually. Presumably increasing the number of mutations within the genes that their products required for latency and reactivation will lead to loss of the ability to become latent and reactivate. The three modified vaccine vectors also differ in their ability to recombine with WT virus or revert to WT. In the latency defective virus, the ORF73 is completely lost, whereas the low-fidelity vector will be eventually lost, but will carry the target antigen for the entire time. In contrast, the antigen flanked by repeat regions will still replicate essentially like WT virus, but will no longer carry the antigen. Recombination

between virus and vaccine strains has been reported *in vitro* and *in vivo*. Coinfection of vaccinia virus strain Ankara (MVA) vectored influenza vaccine with a cowpox virus isolate from Norway resulted in recombinant progeny (Hansen *et al.*, 2004). Similarly, recombination between oral poliovirus vaccine and related indigenous human enterovirus strains led to circulating vaccine-derived polioviruses (cVDPV), which caused paralytic disease (Kew *et al.*, 2004). In our studies, the design of the ORF73 deleted virus was critical to avoid the possibility for recombination with a WT virus being able to restore a functional ORF73 to the antigen carrying vector. Specifically, it was necessary to insert the different expression cassettes (*Mb* fusion protein expression cassette, RVFV-Gn expression cassette, *CB- Com1* and *CB- Mip* expression cassette) in place of ORF73 within BoHV-4 BAC vector. Finally, recombination between live attenuated laryngotracheitis herpesvirus vaccine strains was also reported in Australia and that resulted virulent field viruses (Lee *et al.*, 2012), with shedding and transmission of the vaccine strains reported between humans and within animals population (Fisher, 2014). However, herpesviruses are generally highly species-specific therefore such a concern of transmission of the wild type vaccine to human populations or another non-targeted animal species would not be expected.

We also expect that the mode of control may affect immunogenicity, in large part, due to the differential persistence of antigen expression expected for these different strategies. Several studies indicate that there is a direct correlation between persistence of antigens and the quantity and quality of the immune response. The yellow fever vaccine (YFV-17D) is a live attenuated vaccine that is known to provide durable protective immunity, which corresponds to persistent neutralizing antibodies and CD8 T cells. In a recent study there was shown to be a relationship between antigen load and the magnitude of CD8 T cell response, wherein the magnitude of CD8 T cell response was proportional to the amount of antigen when the vaccine load was below a key threshold; the CD8 T response was shown to become saturated when the viral load was above this threshold level. This study also showed that the presence of persistent antigen also

biased the immune response towards effector rather than central memory T cells (Akondy *et al.*, 2015; Miller *et al.*, 2008). While non-persistent antigen induced memory responses biased toward the central memory.

The ability of herpesvirus-based vectors to induce durable, effector-biased responses is consistent with their longterm antigen expression (Torti *et al.*, 2011). The impact of control on the immune response induced by the vaccine vector can be predicted based on persistence of the antigen. In the low replication fidelity vector, the antigen is persistent while in the latency defective and the flanking based vectors the antigen does not persist. Consistent with a role of antigen persistence also in maintaining antibody responses, we would predict antibody response induced by latency defective and flanking based vector is lower than that induced by low replication fidelity virus. In support of this model, it has been shown that the immune response induced by non persistent spread deficient MCMV virus (M94 gene deleted) was lower than that induced by wild type virus (Mohr *et al.*, 2010). Similar findings were observed in animals immunized with a single cycle simian immunodeficiency virus (SIV) (Jia *et al.*, 2009). In another study the antibody titer in the sera of animals infected with latency defective murine gammaherpesviruses 68 (non persistent) was lower than the that induced by persistent virus (Jia *et al.*, 2010).

Although T cells were not analysed, the different modes of control would also be expected to affect these responses. It is predicted that T cells responses induced by the low fidelity vector would be higher than those induced by latency defective vector or the flanking based vector, both latter vectors being onse in which antigen does not persist. The three different modes of control could also have an impact on the quality of the immune response. Based on the persistence of antigen, we predict that the memory responses induced by low fidelity vector will be biased towards effector memory responses, while in the latency defective vector and the flanking based vector central memory responses will predominate. Due to the limited time

of this study, we could not determine the impact of the persistence on the quality of the immune response induced by heterologous antigen.

The predicted impact on the durability of the immune response through losing persistent antigen expression as a consequence of the different modes of control is unknown. Losing the ability of the virus to persist could affect the durability of the immune response. In our study, the immune response induced by the attenuated BoHV-4 vector expressing *Mb* fusion antigen as recorded till the end of the study (three months) but it is unknown whether the immune response induced by the attenuated virus was durable. The impact of attenuation by modulation replication fidelity on the durability of the immune response presumably more less than the impact of other modes of attenuation. It has been shown that the spread defective CMV vector could induce a sustainable level of the immune response comparable to the wild type (Snyder *et al.*, 2011). It would be interesting in future to assess the impact of the three different modes of control on the durability of the immune responses.

In summary, the three modes of control investigated in this thesis predicated to have a different level of safety and immunogenicity. Future studies combining multiple layers of attenuation by using a different approach of attenuation would be very interesting to generate a safe vaccine. Such a combination approach based on multiple tiers of modulation of genetic fidelity with flanking base attenuation approach would possibly decrease the possibility of resulting wild type vaccine. Also, it would be interesting to combine the approach based on genetic deletion of the gene required for viral persistence and latency with flanking based methods.

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## Appendixes

### Appendix 1- Culture Media

#### **LB Broth**

Five gram of LB broth powder was added to 200 ml distilled H<sub>2</sub>O and sterilized by autoclaving at 15 lbs/in<sup>2</sup> for 15 minutes.

#### **LB Agar Plates**

Eight gram of LB agar powder was added to 200 ml distilled H<sub>2</sub>O and heated by microwave to completely dissolve then sterilized by autoclaving at 15 lbs/in<sup>2</sup> for 15 minutes. LB agar was used for bacterial culture.

### Appendix 2- BAC Preparation Buffers and Reagents

#### **P1 buffer**

Ingredients	Amount
Tris base	6.055 g
EDTA	3.722 g
H <sub>2</sub> O	800 ml

The pH was adjusted to 8.0 with HCl and then RNase was added.

#### **P2 buffer**

Ingredients	Amount
5M NaOH	4 ml
10% SDS	10 ml
H <sub>2</sub> O	86 ml

#### **P3 buffer**

Ingredients	Amount
KAc	294.45 g
H <sub>2</sub> O	500 ml

The pH was adjusted to 5.5 with glacial acetic acid.

### **3M NaOAc**

Ingredients	Amount
NaOAc	204.15 g
H <sub>2</sub> O	500 ml

The pH was adjusted to 5.5 with glacial acetic acid

### Appendix 3- PCR and Agarose Gel Electrophoresis

1- PCR (reaction and condition) that was performed using Q5 hot start high- fidelity DNA polymerase to amplify the two regions (Region 1 and Region 2) within HCMV DNA and amplify Lac Z gene from pEQ176 plasmid.

#### **PCR Reaction**

Ingredients	Amount
Forward primer (50 pmol/ $\mu$ l)	0.25 $\mu$ l
Reverse primer (50 pmol/ $\mu$ l)	0.25 $\mu$ l
10 mM deoxynucleotide triphosphate (dNTPs; New England Biolabs, USA)	0.5 $\mu$ l
Q5 hot start high- fidelity DNA polymerase (New England Biolabs, USA)	0.25 $\mu$ l
5X Q5 reaction (New England Biolabs, USA)	5 $\mu$ l
Distilled H <sub>2</sub> O	17.25 $\mu$ l
DNA template	1.5 $\mu$ l
Total	25 $\mu$ l

#### **PCR Conditions**

	Temperature	Duration	Number of cycles
Initial denaturation	98 °C	3 minutes	1 cycle
Denaturation	98 °C	10 seconds	34 × cycles
Annealing	66 °C	30 seconds	
Extension	72 °C	30 seconds	
Final extension	72 °C	2 minutes	1 cycle

- The annealing temperature depends on the melting temperatures <sup>TM</sup> of the primers.
- Extension time depends on the length of sequence to be amplified.

2- PCR (reaction and condition) that was performed using AccuPrime™ Taq DNA polymerase to amplify the DNA of BoHV-4 BACs and BoHV-4 viruses.

### **PCR Reaction**

Ingredients	Amount
Forward primer (50 pmol/μl)	0.5 μl
Reverse primer (50 pmol/μl)	0.5 μl
AccuPrime™ Taq DNA polymerase, high fidelity (Thermo Fisher Scientific, UK)	0.2 μl
10X AccuPrime™ PCR Buffer II (Thermo Fisher Scientific, UK)	2 μl
Distilled H <sub>2</sub> O	15.8 μl
DNA template	1 μl
Total	20 μl

### **PCR Conditions**

	Temperature	Duration	Number of cycles
Initial denaturation	94 °C	5 minutes	1 cycle
Denaturation	94 °C	30 seconds	35 × cycles
Annealing	58 °C	30 seconds	
Extension	68 °C	5 minutes	
Final extension	68 °C	10 minutes	1 cycle

- The annealing temperature depends on the melting temperatures <sup>TM</sup> of the primers.
- Extension time depends on the length of sequence to be amplified.

### **Molecular Ladder (1Kb)**

80 μl of Kb DNA Extension Ladder (Invitrogene) and 133.2 μl of Loading Dye (5 ×) were mixed with 586.41 μl of distilled H<sub>2</sub>O.

## Appendix 4- Virology

### **1.2% Seaplaque agar**

Ingredients	Amount
Sea Plaque agar	0.6 g
Sterile H <sub>2</sub> O	50 ml

Microwave for two minutes until boiled and then sterilized by autoclaving.

### **20 % Sorbitol Cushion**

Ingredients	Amount
D- sorbitol	100g
1M Tris pH 7.4	2.5 ml
1M MgCl <sub>2</sub>	1 ml
Distilled H <sub>2</sub> O to 500 ml	

Sterilized by filtration.

### **1M MgCl<sub>2</sub>**

Ingredients	Amount
MgCl <sub>2</sub> *6H <sub>2</sub> O	101.65 g
Distilled H <sub>2</sub> O to 500 ml	

### **2xDMEM**

Ingredients	Amount
DMEM powder	13.38 g
NaHCO <sub>3</sub>	49.3 ml
Distilled H <sub>2</sub> O to 500 ml.	
PSG	10 ml
FBS	100 ml

## Appendix 5- Western Blotting

### **10 × Tris Buffered Saline (TBS)**

Ingredients	Amount
Tris Base	24.2 g
NaCl	80 g
Distilled H <sub>2</sub> O to 1 L	

The pH was adjusted to 7.6 with HCl.

### **1× Tris Buffered Saline (TBS)**

Ingredients	Amount
10 × TBS	100 ml
Distilled H <sub>2</sub> O	900 ml
Tween 20	100 µl

Tween was added while on a magnetic stirrer at a medium setting

### **1× Tris Buffered Saline- Tween (TBS-T)**

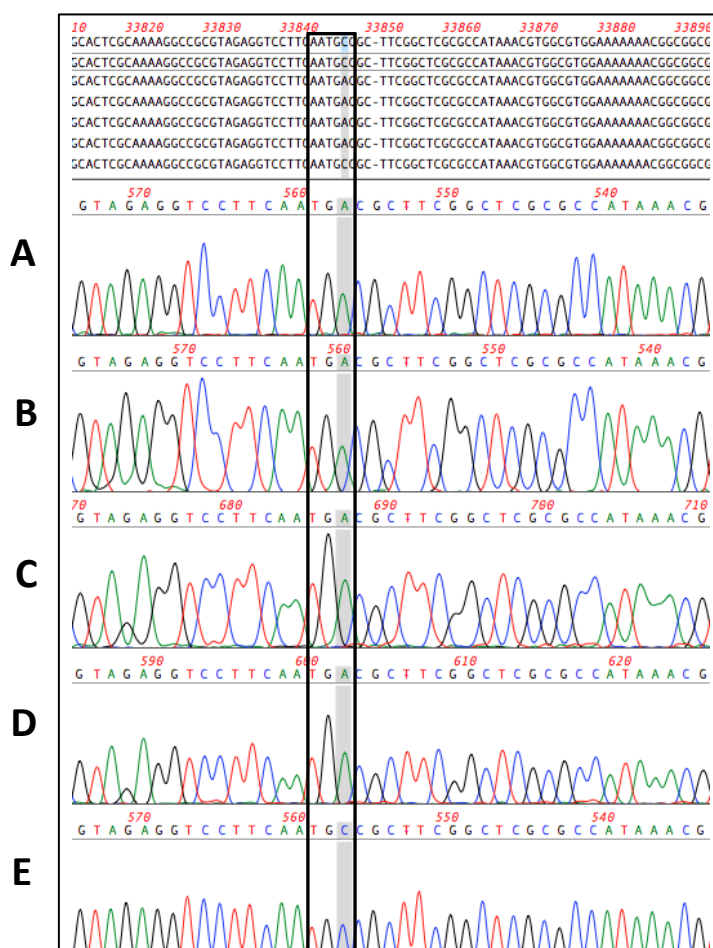
100 ml of 10 × TBS was added to 900 ml distilled H<sub>2</sub>O and then 100 µl of Tween 20 was added while on a magnetic stirrer at a medium setting.

### **Blocking Solution**

Ingredients	Amount
Skimmed Milk Powder	2.5 g
1 × PBS-T or 1 × TBS	50 ml



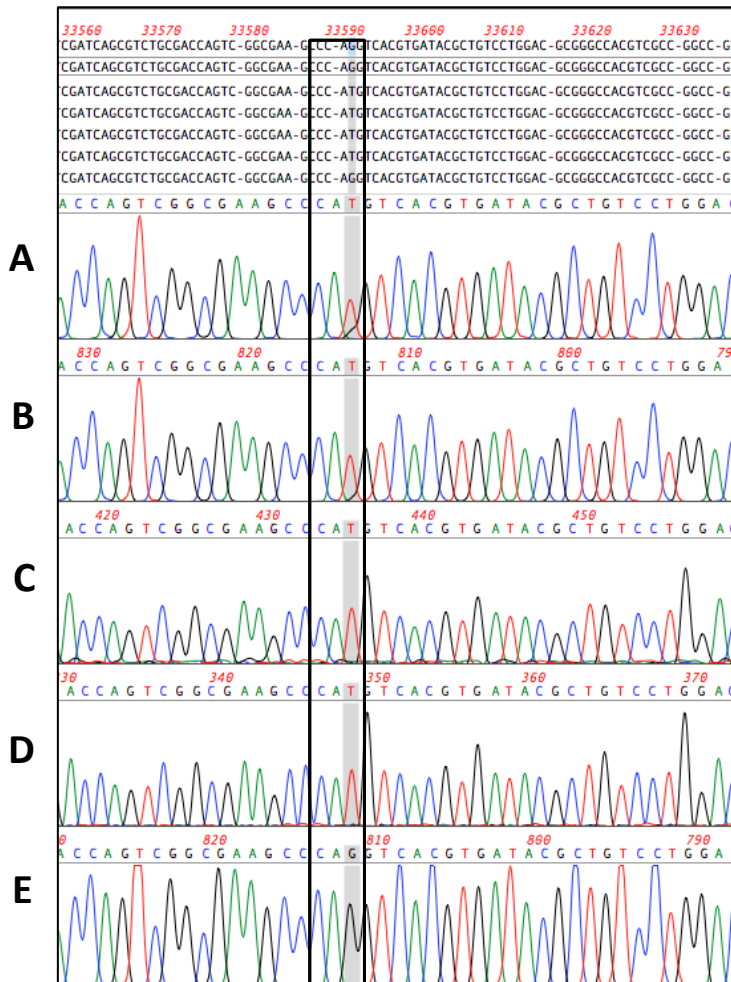
Appendix 6- Appendix Mutations



**Figure 1** Sequence diagram showing A272S mutation identified in sequence obtained from cloned PCR product of a region within UL27 gene of HCMV UL54 ( $\Delta$  D413) passage 15, C change to A at position 33844 (to the HCMVAD169 reference strain) or G change to T at position 814 (to the UL27 references strain) (non-synonymous mutation). (A) sequence using M13 forward primer, (B&C) re-sequence using forward and reverse M13 primer, (D) sequence using UL27 primer (internal reverse primer) and (E) WT sequence

Note: the UL27 gene in reverse orientation.

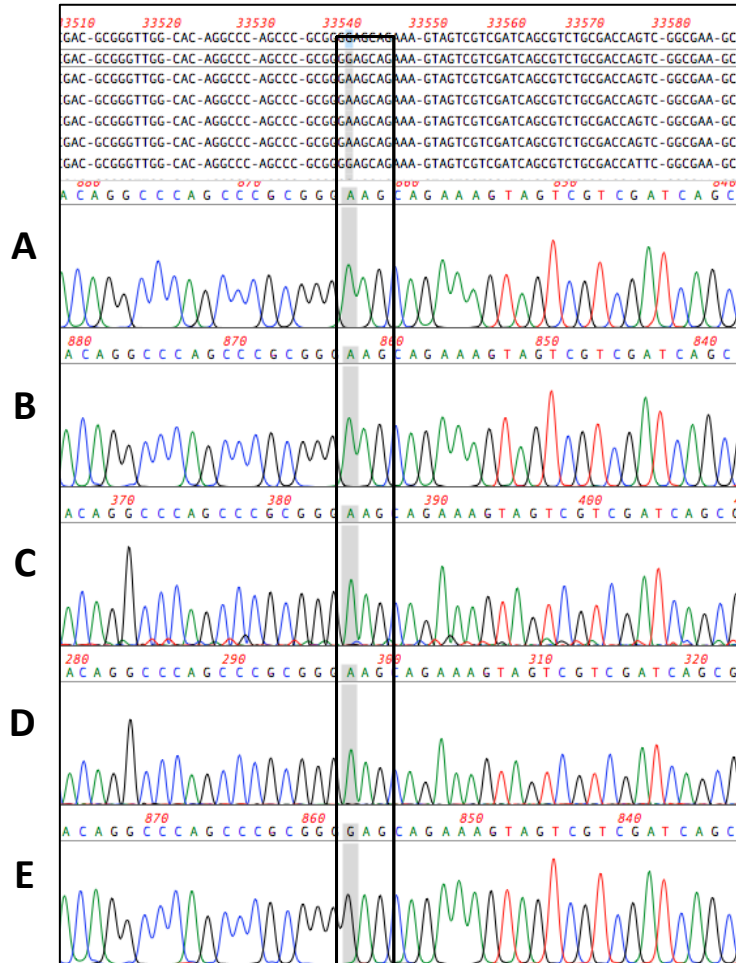
GCA change to TCA (Transversion)



**Figure 2** Sequence diagram showing L357M mutation identified in sequence obtained from cloned PCR product of a region within UL27 gene of HCMV UL54 ( $\Delta$  D413) passage 15, G change to T at position 33589 (to the HCMVAD169 reference strain) or C change to A at position 1069 (to the UL27 references strain) (non-synonymous mutation). (A) sequence using M13 forward primer, (B&C) re-sequencing using forward and reverse M13 primer, (D) sequence using UL27 primer (internal reverse primer) and (E) WT sequence.

Note: the UL27 gene in reverse orientation.

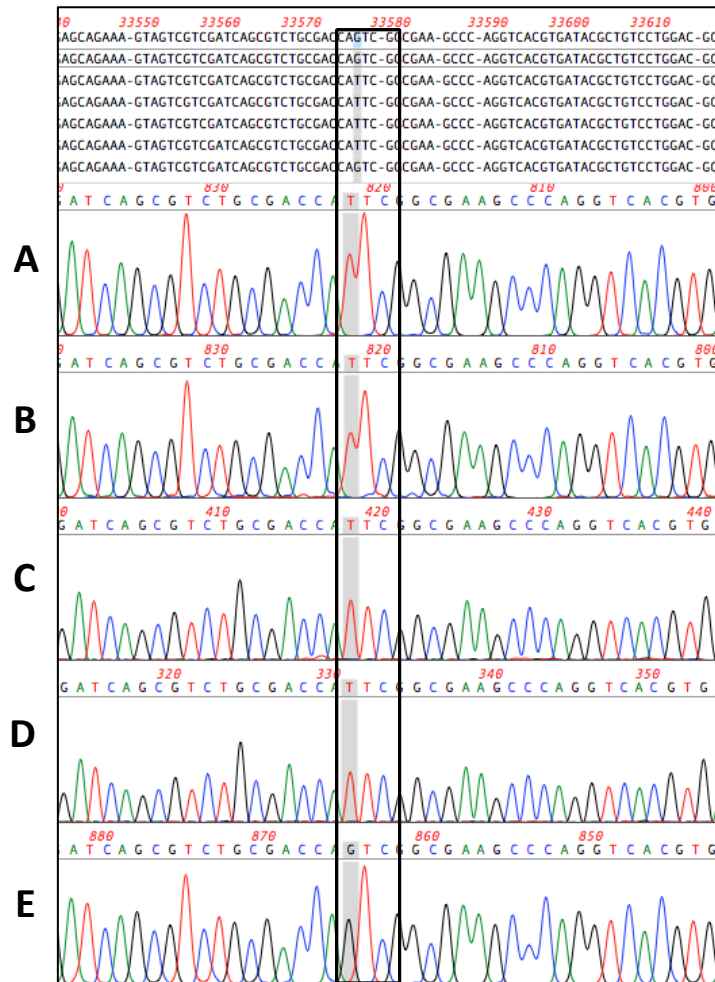
CTG change to ATG (Transversion)



**Figure 3** Sequence diagram showing a synonymous mutation identified in sequence obtained from cloned PCR product of a region within UL27 gene of HCMV UL54 ( $\Delta$  D413) passage 15, G change to A at position 33539 (to the HCMVAD169 reference strain) or C change to T at position 1119 (to the UL27 reference strain). (A) sequence using M13 forward primer, (B&C) re-sequencing using forward and reverse M13 primers, (D) sequence using UL27 primer (internal reverse primer) and (E) WT sequence.

Note: the UL27 gene is in reverse orientation.

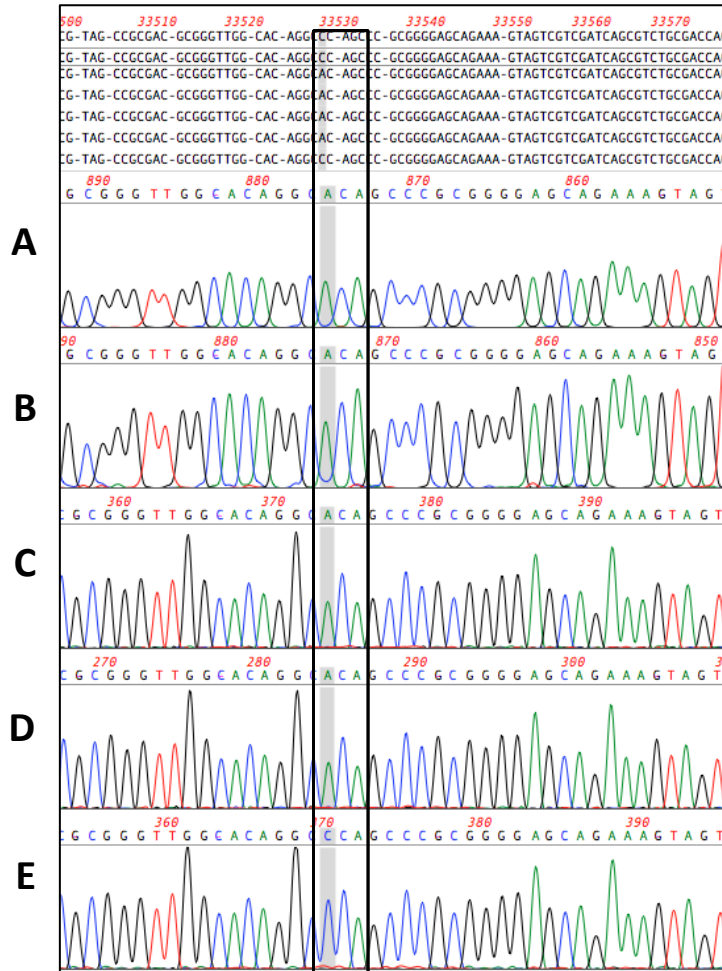
Codon (373) CTC change to CTT (Transitions)



**Figure 4:** Sequence diagram showing D361E mutation identified in sequence obtained from cloned PCR product of a region within UL27 gene of HCMV UL54 ( $\Delta$  D413) passage 15, G change to T at position 33575 (to the HCMVAD169 reference strain) or C change to A at position 1083 (to the UL27 references strain) (non-synonymous mutation). (A) sequence using M13 forward primer, (B&C) re-sequencing using forward and reverse using M13 primer, (D) sequence using UL27 primer (internal reverse primer) and (E) WT sequence.

Note: the UL27 gene in reverse orientation.

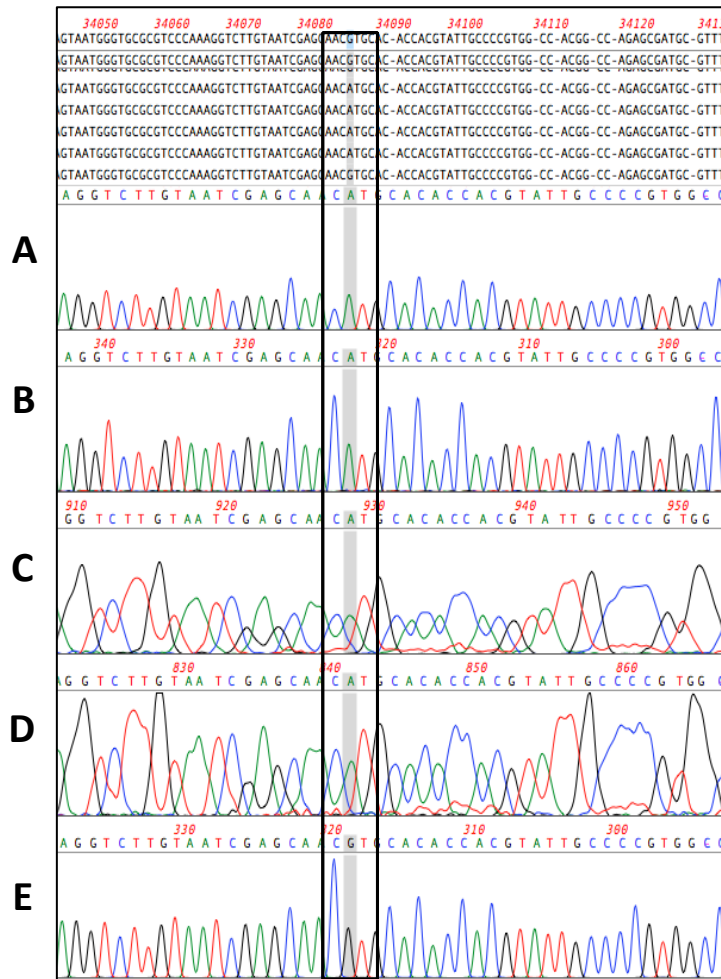
GAC change to GAA (Transversion)



**Figure 5:** Sequence diagram showing W 377C mutation identified in sequence obtained from cloned PCR product of a region within UL27 gene of HCMV UL54 ( $\Delta$  D413) passage 15, C change to A at position 33527 (to the HCMVAD169 reference strain) or G change to T at position 1131 (to the UL27 references strain) (non-synonymous mutation). (A) sequence using M13 forward primer, (B&C) re-sequence using forward and reverse using M13 primer, (D) sequence using UL27 primer (internal reverse primer) and (E) WT sequence.

Note: the UL27 gene in reverse orientation.

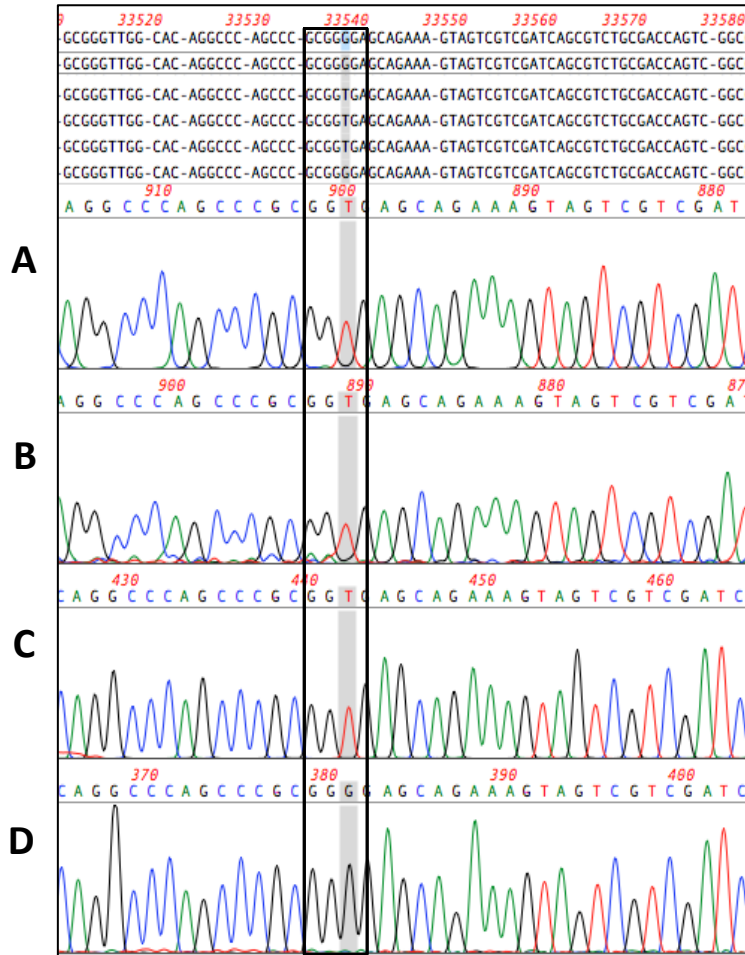
TGG change to TGT (Transversion)



**Figure 6** Sequence diagram showing T192M mutation identified in sequence obtained from cloned PCR product of a region within UL27 gene of HCMV UL54 ( $\Delta$  D413) passage 15, G change to A at position 34083(to the HCMVAD169 reference strain) or C change to T at position 575 (to the UL27 references strain) (non-synonymous mutation). (A) sequence using M13 forward primer, (B&C) re-sequence using forward and reverse using M13 primer, (D) sequence using UL27 primer (internal reverse primer) and (E) WT sequence.

Note: the UL27 gene in reverse orientation.

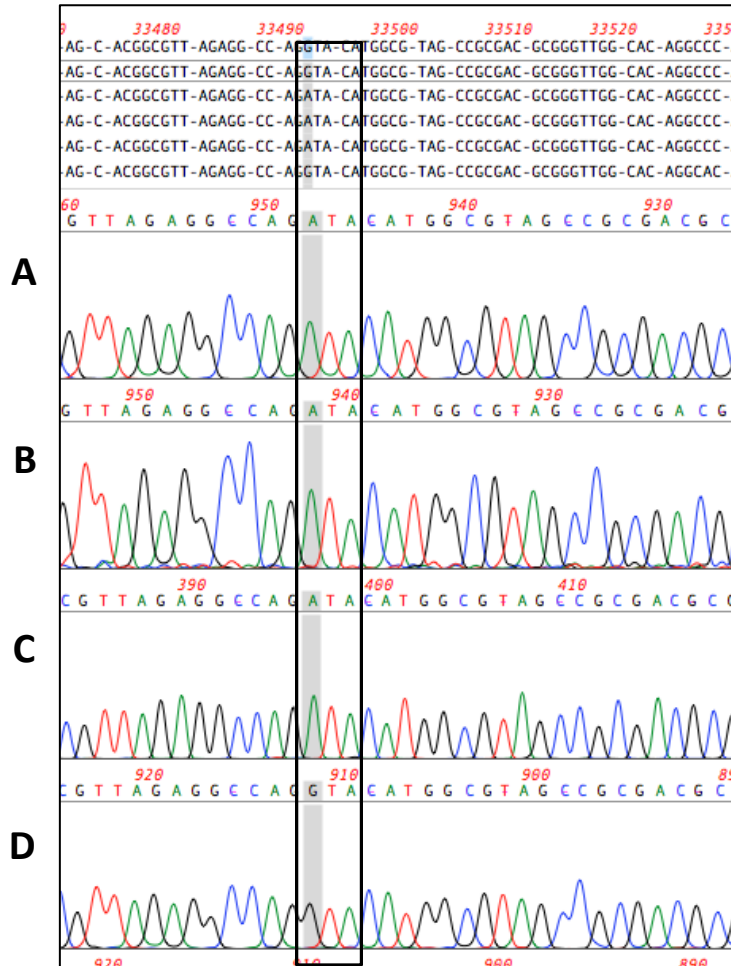
ACG to ATG (Transitions)



**Figure 7** Sequence diagram showing P374T mutation identified in sequence obtained from cloned PCR product of a region within UL27 gene of HCMV UL54 ( $\Delta$  D413) passage 15, G change to T in position 33538 (to the HCMVAD169 reference strain) or C change to A at position 1120 (to the UL27 references strain) (non-synonymous mutation). (A) sequence using M13 forward primer, (B&C) re-sequenced using forward and reverse using M13 primer and (D) WT sequence.

Note: the UL27 gene in reverse orientation.

CCC change to ACC (Transversion)

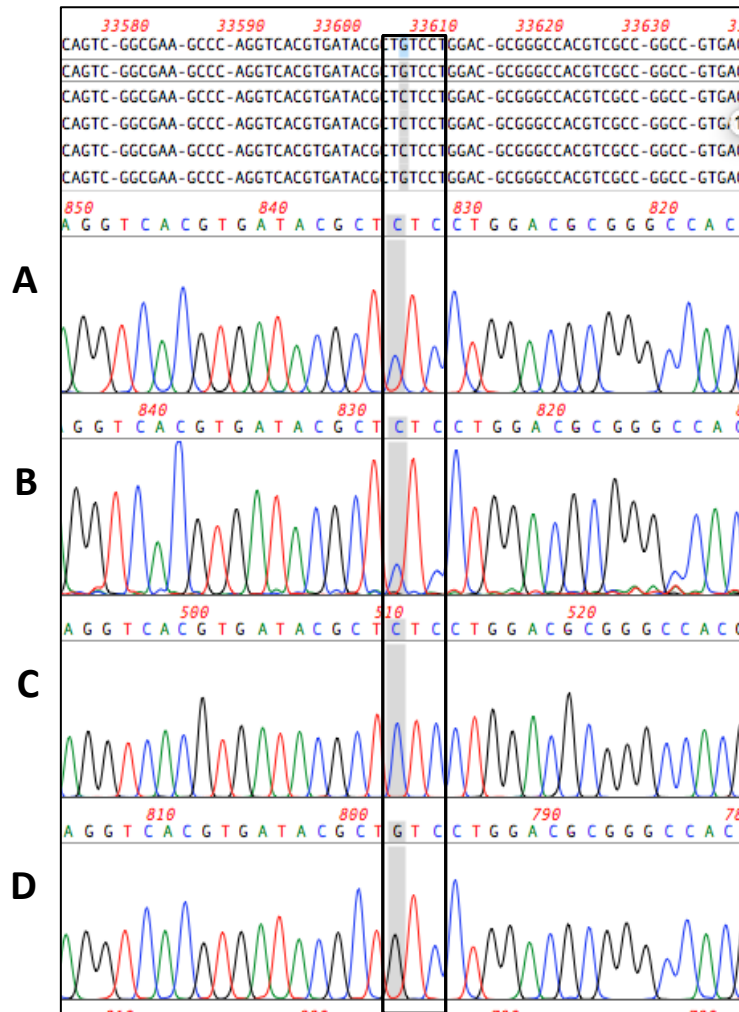


**Figure 8** Sequence diagram showing a synonymous mutation identified in sequence obtained from cloned PCR product of a region within UL27 gene of HCMV UL54 ( $\Delta$  D413) passage 15, G change to A in position 33491(to the HCMVAD169 reference strain) or C change to T at position 1167 (to the UL27 references strain). (A) sequence using M13 forward primer, (B&C) re-sequence using forward and reverse using M13 primer and (D) WT sequence.

Note: the UL27 gene in reverse orientation.

Codon 389 TAC change to TAT (Transitions)

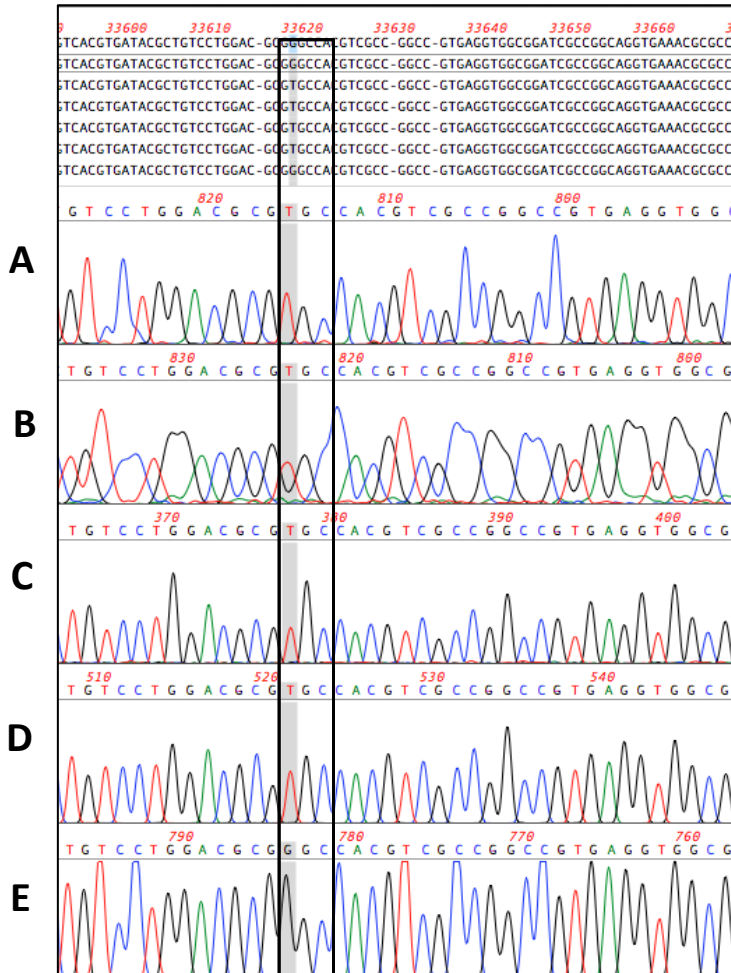




**Figure 9** Sequence diagram showing D351E mutation identified in sequence obtained from cloned PCR product of a region within UL27 gene of HCMV UL54 ( $\Delta$  D413) passage 15, G change to C at position 33605 (to the HCMVAD169 reference strain) or C change to G at position 1053 (to the UL27 references strain) (non-synonymous mutation). (A) sequence using M13 forward primer, (B&C) re-sequence using forward and reverse using M13 primer and (D) WT sequence.

Note: the UL27 gene in reverse orientation.

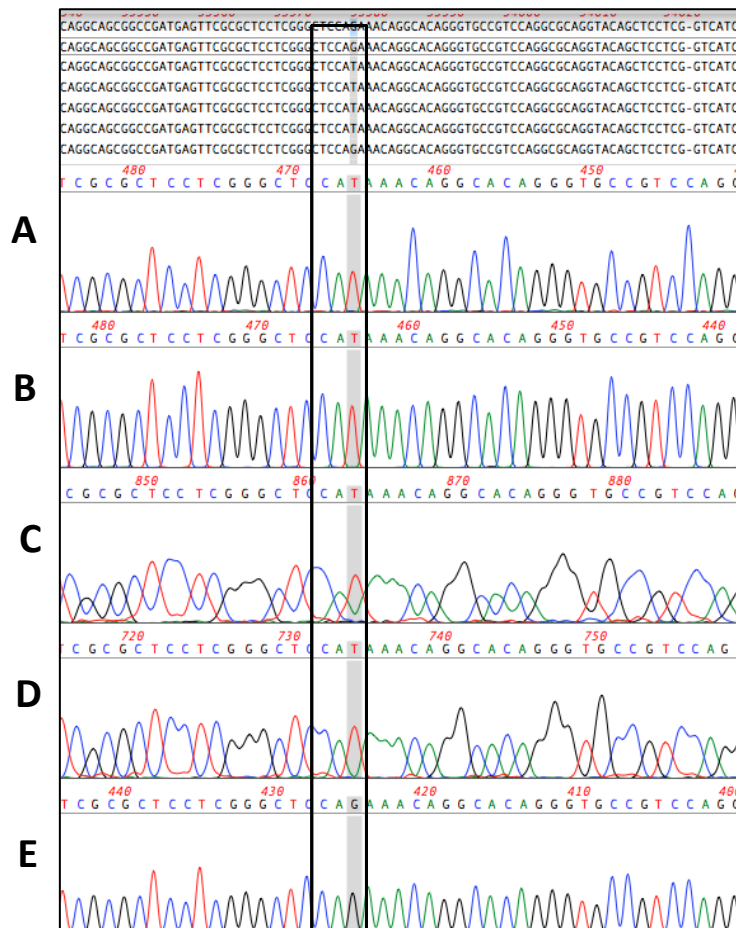
GAC change to GAG (Transversion)



**Figure 10** Sequence diagram showing a synonymous mutation identified in sequence obtained from cloned PCR product of a region within UL27 gene of HCMV UL54 ( $\Delta$  D413) passage 15, G change to T at position 33617(to the HCMVAD169 reference strain) or C to A at position 1041 (to the UL27 references strain) (synonymous mutation). (A) sequence using M13 forward primer, (B&C) re-sequence using forward and reverse using M13 primer, (D) sequence using UL27 primer (internal reverse primer) and (E) WT sequence.

Note: the UL27 gene in reverse orientation.

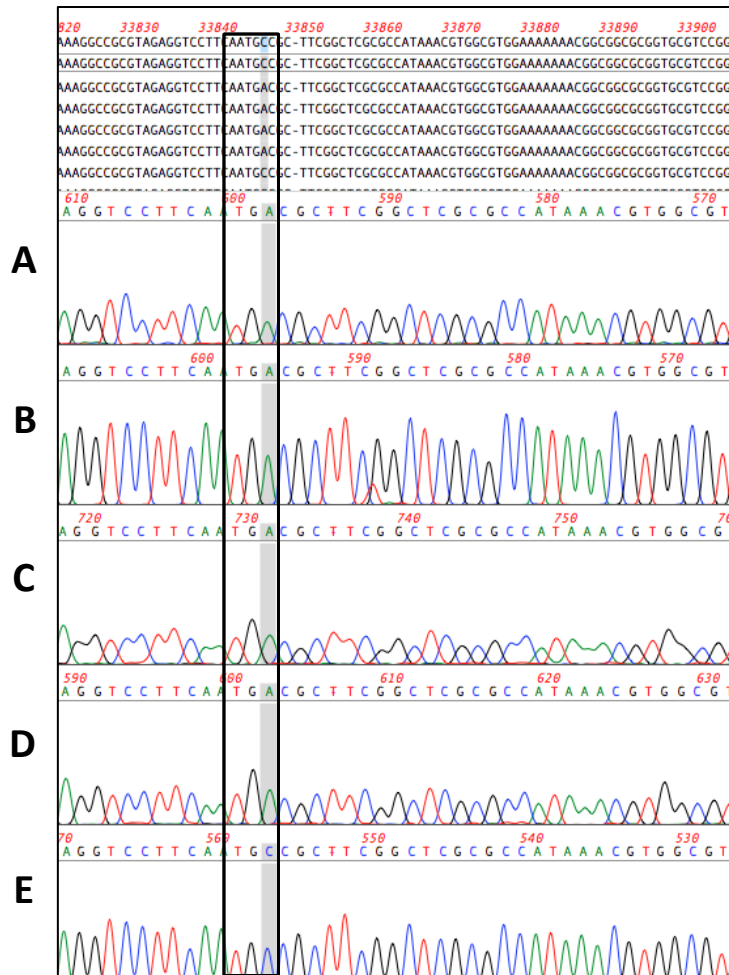
Codon (347) GCC change to GCA (Transversion)



**Figure 11** Sequence diagram showing L228M mutation identified in sequence obtained from cloned PCR product of a region within UL27 gene of HCMV UL54 ( $\Delta$  D413) passage 15, G change to T at position 33976(to the HCMVAD169 reference strain) or C change to A at position 682 (to the UL27 references strain) (non-synonymous mutation). (A) sequence using M13 forward primer, (B&C) re-sequencing using forward and reverse using M13 primer, (D) sequence using UL27 primer (internal reverse primer) and (E) WT sequence.

Note: the UL27 gene in reverse orientation.

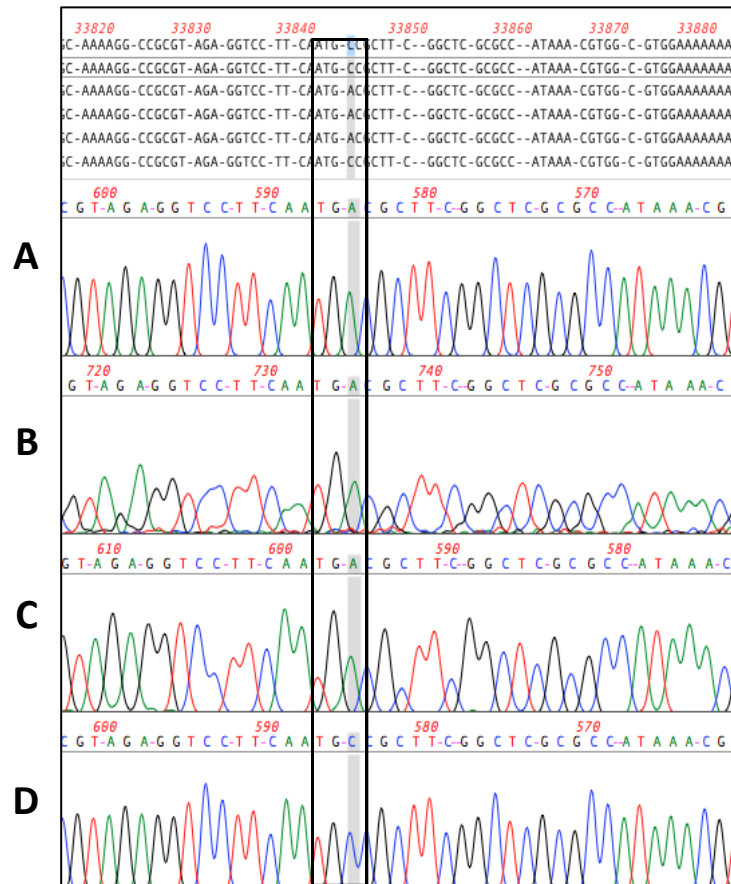
CTG change to ATG (Transversion)



**Figure 12** Sequence diagram showing A272S mutation identified in sequence obtained from cloned PCR product of a region within UL27 gene of HCMV UL54 (Δ D413) passage 15, C change to A at position 33844 (to the HCMVAD169 reference strain) or G change to T at position 814 (to the UL27 references strain) (non-synonymous mutation). (A) sequence using M13 forward primer, (B&C) re-sequencing using forward and reverse using M13 primer, (D) sequence using UL27 primer (internal reverse primer) and (E) WT sequence.

Note: the UL27 gene in reverse orientation.

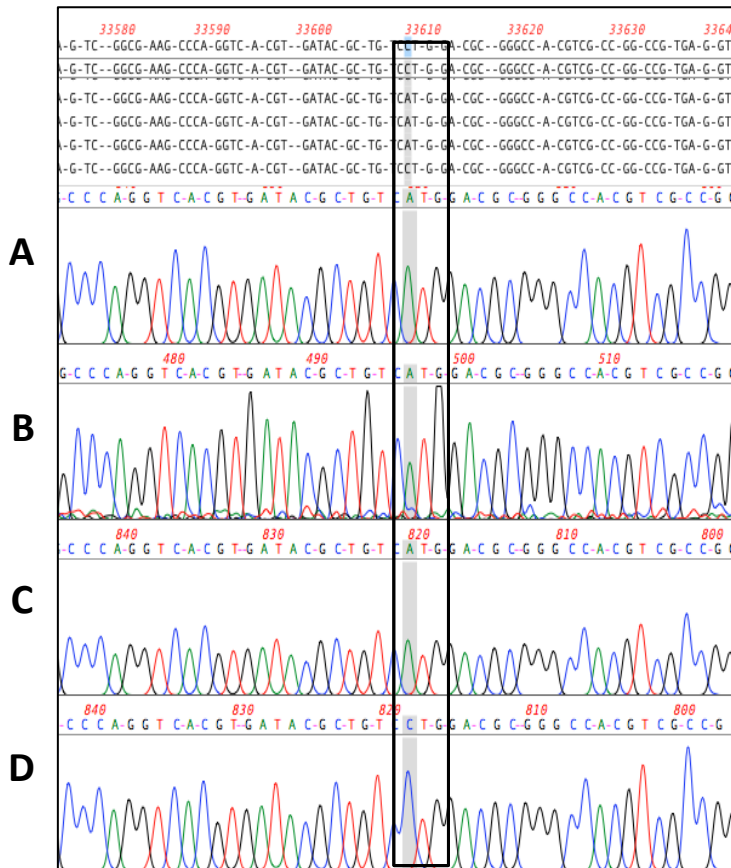
GCA change to TCA (Transversion)



**Figure 13** Sequence diagram showing A272S mutation identified in sequence obtained from cloned PCR product of a region within UL27 gene of HCMV UL54 ( $\Delta$  D413) passage 15, C change to A at position 33844 (to the HCMVAD169 reference strain) or G change to T at position 814 (to the UL27 references strain) (non-synonymous mutation). (A) sequence using M13 forward primer, (B&C) re-sequencing using forward and reverse using M13 primer and (D) WT sequence.

Note: the UL27 gene in reverse orientation.

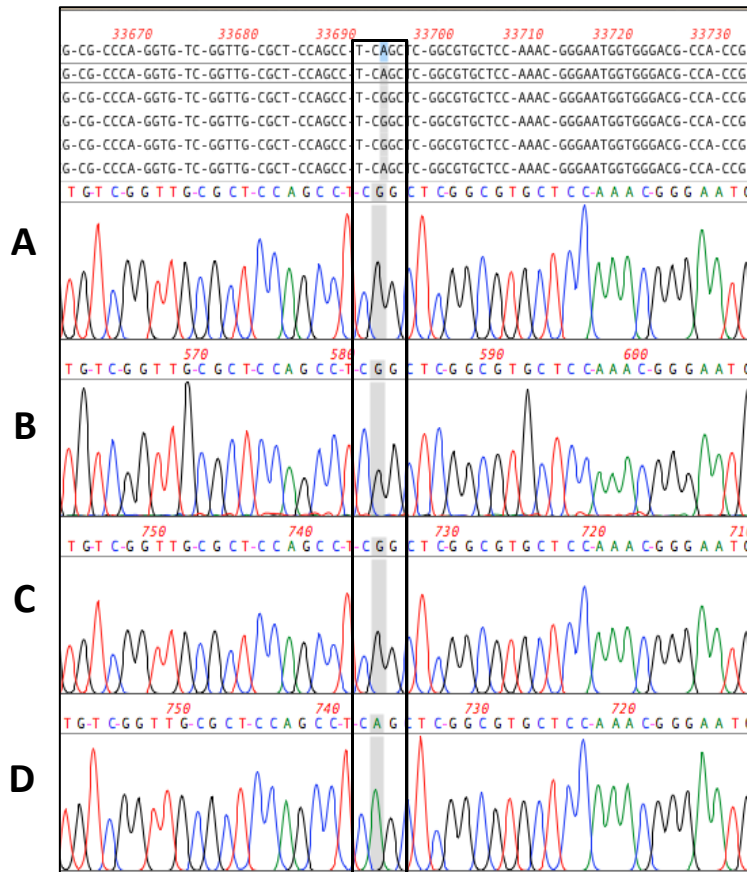
GCA change to TCA (Transversion)



**Figure 14** Sequence diagram showing Q352H mutation identified in sequence obtained from cloned PCR product of a region within UL27 gene of HCMV UL54 ( $\Delta$  D413) passage 15, C change to A at position 33608(to the HCMVAD169 reference strain) or G change to T at position 1050 (to the UL27 references strain) (non-synonymous mutation). (A) sequence using M13 forward primer, (B&C) re-sequence using forward and reverse using M13 primer and (D) WT sequence.

Note: the UL27 gene in reverse orientation.

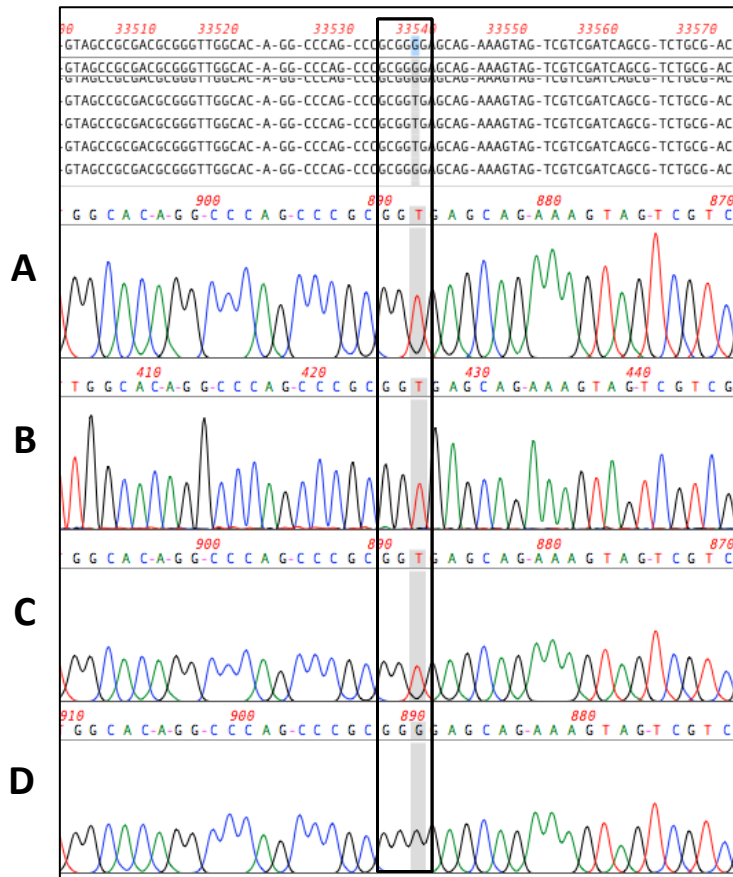
CAG change to CAT (Transversion)



**Figure 15** Sequence diagram showing L322P mutation identified in sequence obtained from cloned PCR product of a region within UL27 gene of HCMV UL54 ( $\Delta$  D413) passage 15, A change to G at position 33693 (to the HCMVAD169 reference strain) or T change to C at position 965 (to the UL27 references strain) (non-synonymous mutation). (A) sequence using M13 forward primer, (B&C) re-sequence using forward and reverse using M13 primer and (D) WT sequence.

Note: the UL27 gene in reverse orientation.

CTG change to CCG (Transitions)

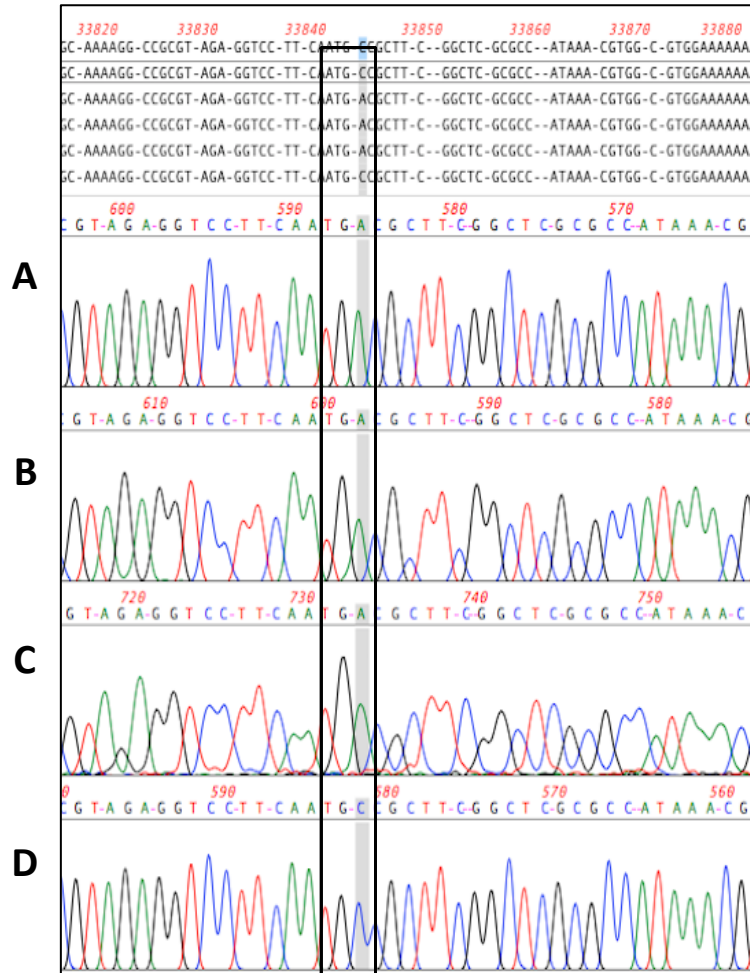


**Figure 16** Sequence diagram showing P374T mutation identified in sequence obtained from cloned PCR product of a region within UL27 gene of HCMV UL54 ( $\Delta$  D413) passage 15, G change to T in position 33538 (to the HCMVAD169 reference strain) or C change to A at position 1120 (to the UL27 references strain) (non-synonymous mutation). (A) sequence using M13 forward primer, (B&C) re-sequencing using forward and reverse using M13 primer and (D) WT sequence.

Note: the UL27 gene in reverse orientation.

CCC change to ACC (Transversion)

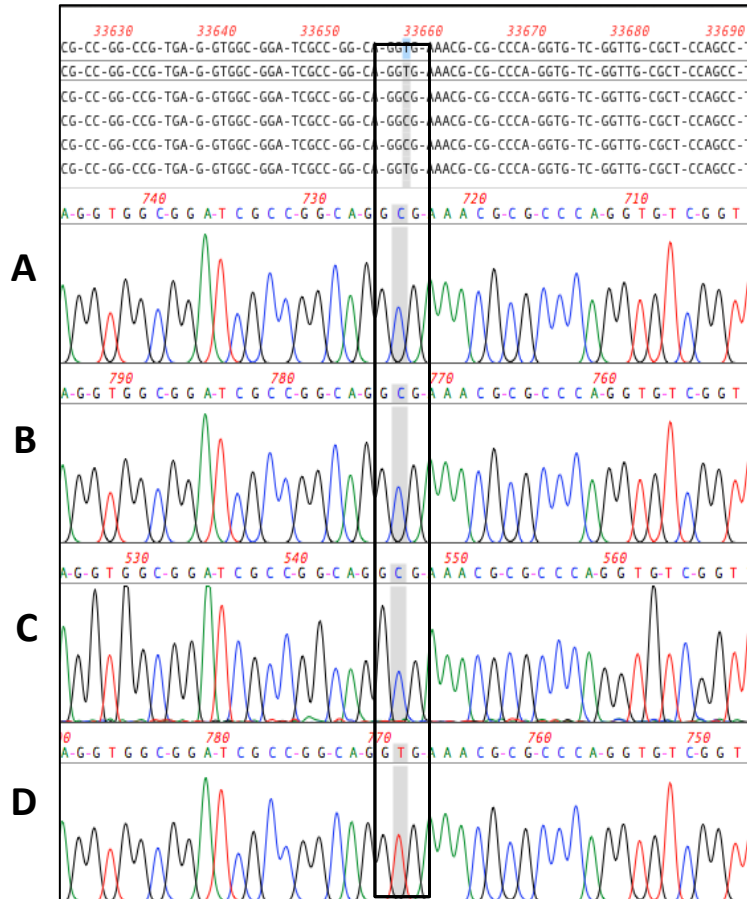




**Figure 17** Sequence diagram showing A272S mutation identified in sequence obtained from cloned PCR product of a region within UL27 gene of HCMV UL54 ( $\Delta$  D413) passage 15, C change to A at position 33844 (to the HCMVAD169 reference strain) or G change to T at position 814 (to the UL27 reference strain) (non-synonymous mutation). (A) sequence using M13 forward primer, (B&C) re-sequence using forward and reverse using M13 primer and (D) WT sequence.

Note: the UL27 gene in reverse orientation.

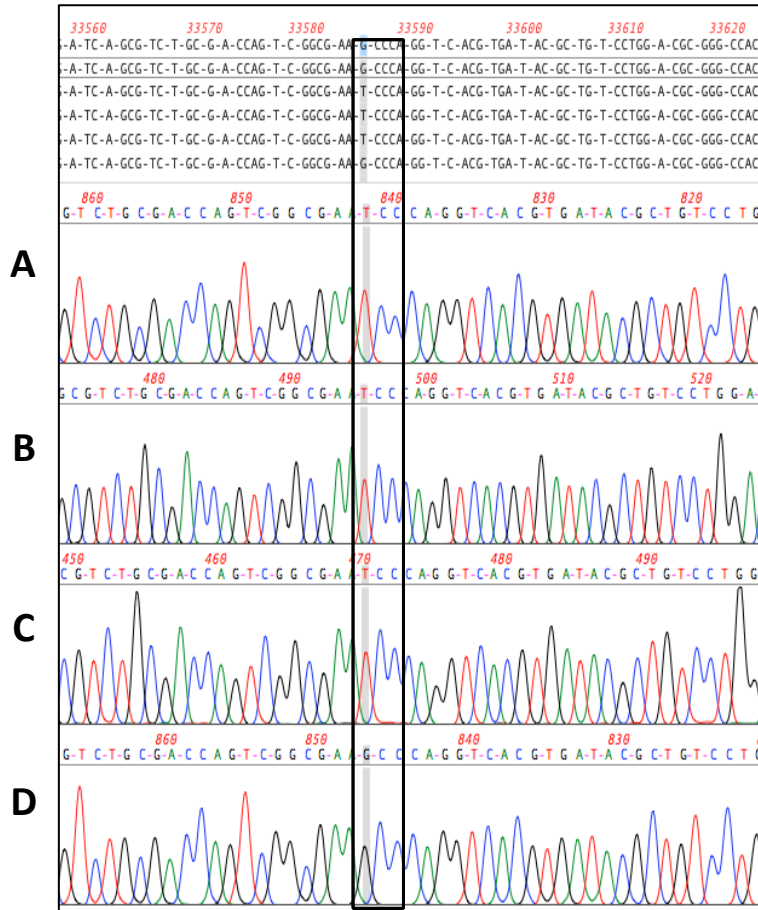
GCA change to TCA (Transversion)



**Figure 18** Sequence diagram showing H334R mutation identified in sequence obtained from cloned PCR product of a region within UL27 gene of HCMV UL54 ( $\Delta$  D413) passage 15, T change to C in position 33657(to the HCMVAD169 reference strain) or A change to G at position 1001(to the UL27 references strain) (non-synonymous mutation). (A) sequence using M13 forward primer, (B&C) re-sequence using forward and reverse using M13 primer and (D) WT sequence.

Note: the UL27 gene in reverse orientation.

CAC change to CGC (Transitions)

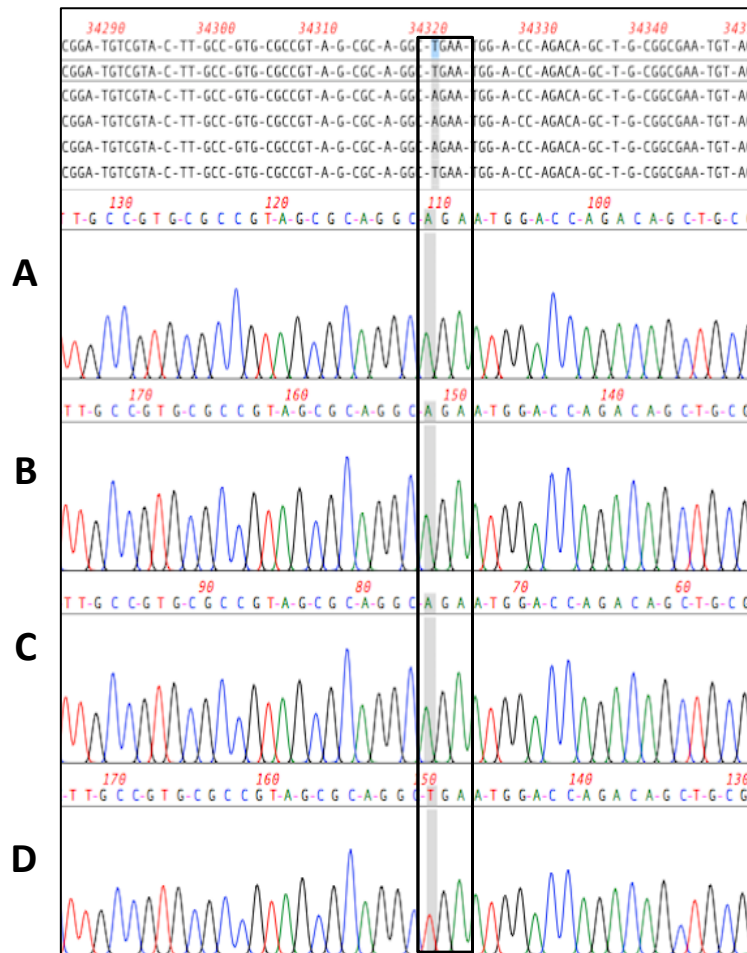


**Figure 19** Sequence diagram showing a synonymous mutation identified in sequence obtained from cloned PCR product of a region within UL27 gene of HCMV UL54 ( $\Delta$  D413) passage 23, G change to T at position 33584 (to the HCMVAD169 reference strain) or C to A at position 1074 (to the UL27 references strain). (A) sequence using M13 forward primer, (B&C) re-sequence using forward and reverse M13 primer and (D) WT sequence.

Note: the UL27 gene in reverse orientation.

Codon 358

GGC change to GGA (Transversion)

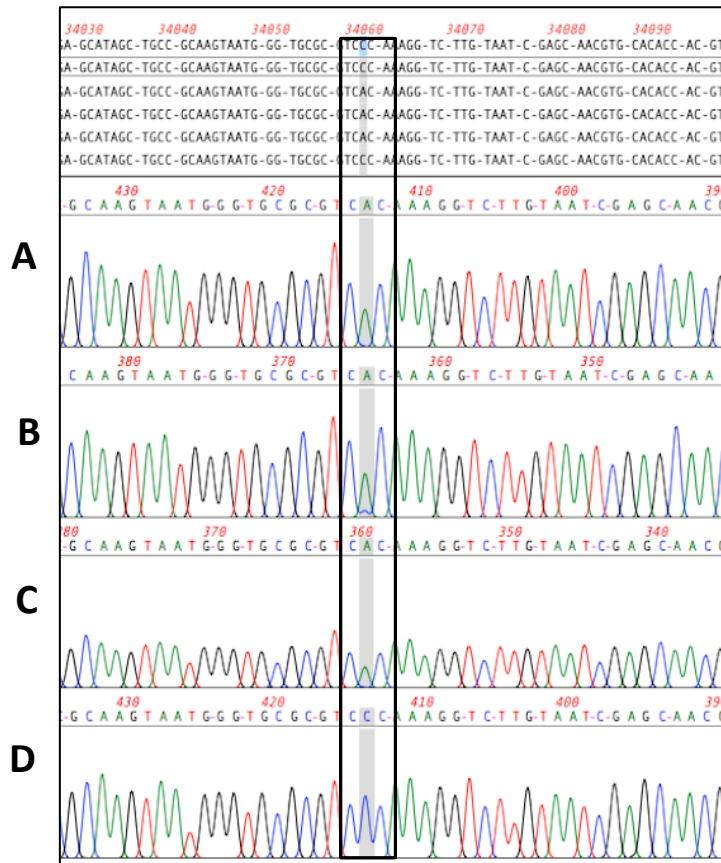


**Figure 20** Sequence diagram showing a synonymous mutation identified in sequence obtained from cloned PCR product of a region within UL27 gene of HCMV UL54 ( $\Delta$  D413) passage 23, T change to A at position 34319 (to the HCMVAD169 reference strain) or A change to T at position 339 (to the UL27 references strain). (A) sequence using M13 forward primer, (B&C) re-sequencing using forward and reverse M13 primer and (D) WT sequence.

Note: the UL27 gene in reverse orientation.

Codon 113

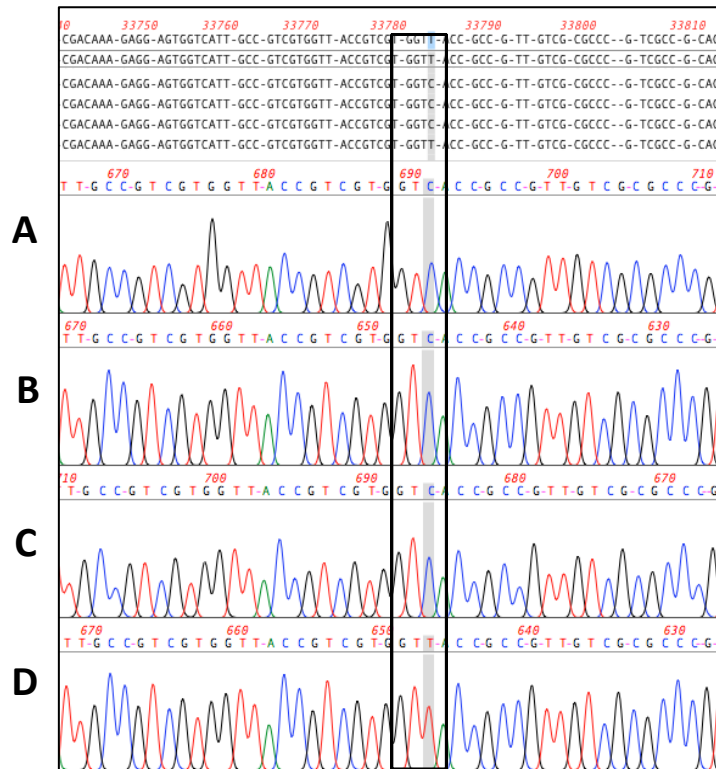
TCA change to TCT (Transversion)



**Figure 21** Sequence diagram showing G200V mutation identified in sequence obtained from cloned PCR product of a region within UL27 gene of HCMV UL54 ( $\Delta$  D413) passage 23, C change to A at position 34059 (to the HCMVAD169 reference strain) or G change to T at position 599 (to the UL27 references strain) (non-synonymous mutation). (A) sequence using M13 forward primer, (B&C) re-sequencing using forward and reverse using M13 primer and (D) WT sequence.

Note: the UL27 gene in reverse orientation.

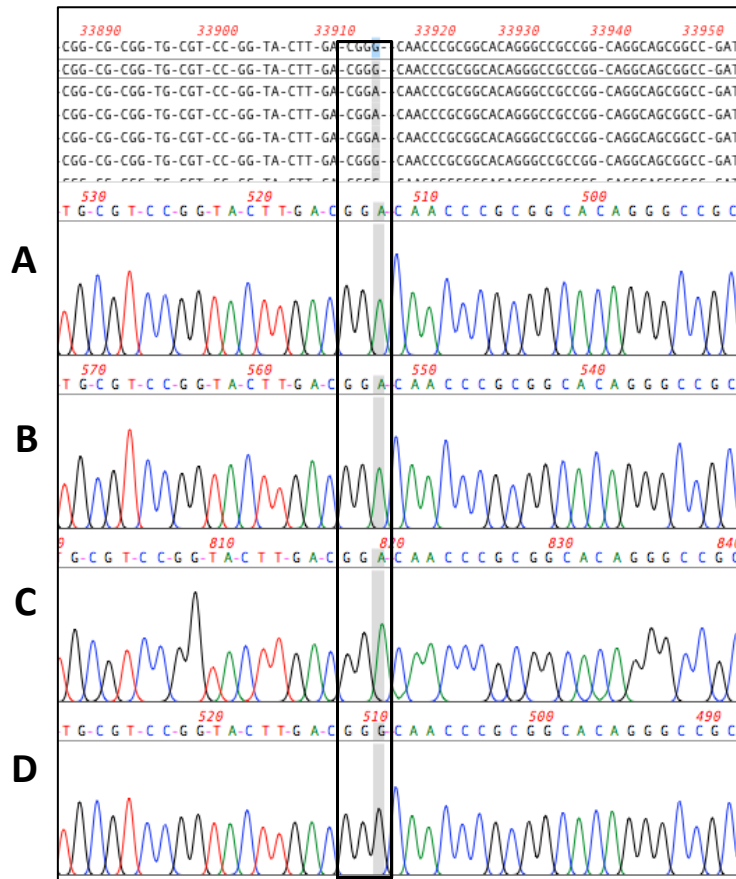
GGG change to GTG (Transversion)



**Figure 22** Sequence diagram showing N92D mutation identified in sequence obtained from cloned PCR product of a region within UL27 gene of HCMV UL54 ( $\Delta$  D413) passage 23, T change to C at position 33784 (to the HCMVAD169 reference strain) or A change to G at position 874 (to the UL27 references strain) (non-synonymous mutation). (A) sequence using M13 forward primer, (B&C) re-sequence using forward and reverse using M13 primer and (D) WT sequence.

Note: the UL27 gene in reverse orientation.

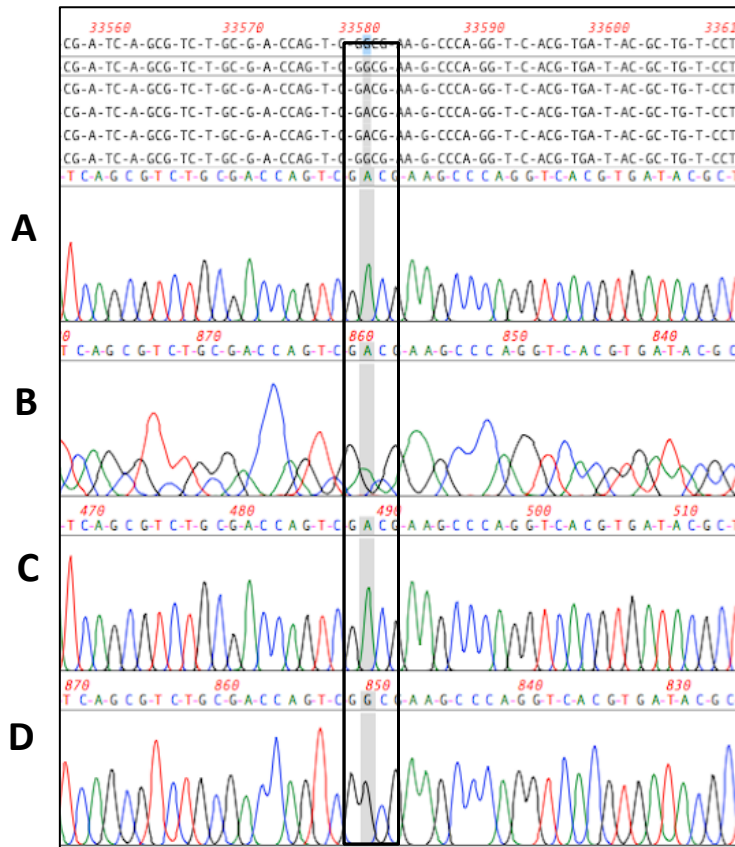
AAC change to GAC (Transitions)



**Figure 23** Sequence diagram showing P249S mutation identified in sequence obtained from cloned PCR product of a region within UL27 gene of HCMV UL54 ( $\Delta$  D413) passage 23, G change to A at position 33913(to the HCMVAD169 reference strain) or C change to G at position 744 (to the UL27 references strain) (non-synonymous mutation). (A) sequence using M13 forward primer, (B&C) re-sequence using forward and reverse using M13 primer and (D) WT sequence.

Note: the UL27 gene in reverse orientation.

CCC change to TCC (Transitions)

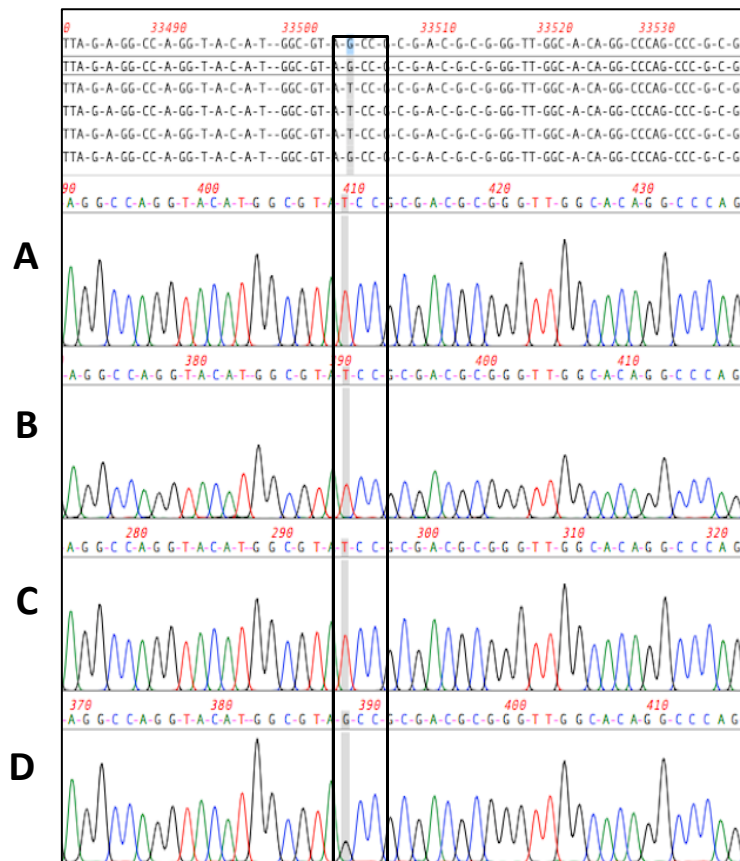


**Figure 24** Sequence diagram showing A360V mutation identified in sequence obtained from cloned PCR product of a region within UL27 gene of HCMV UL54 (Δ D413) passage 23, G change to A at position 33579 (to the HCMVAD169 reference strain) or C change to T at position 1079 (to the UL27 references strain) (non-synonymous mutation). (A) sequence using M13 forward primer, (B&C) re-sequencing using forward and reverse using M13 primer and (D) WT sequence.

Note: the UL27 gene in reverse orientation.

GCC change to GTC (Transitions)

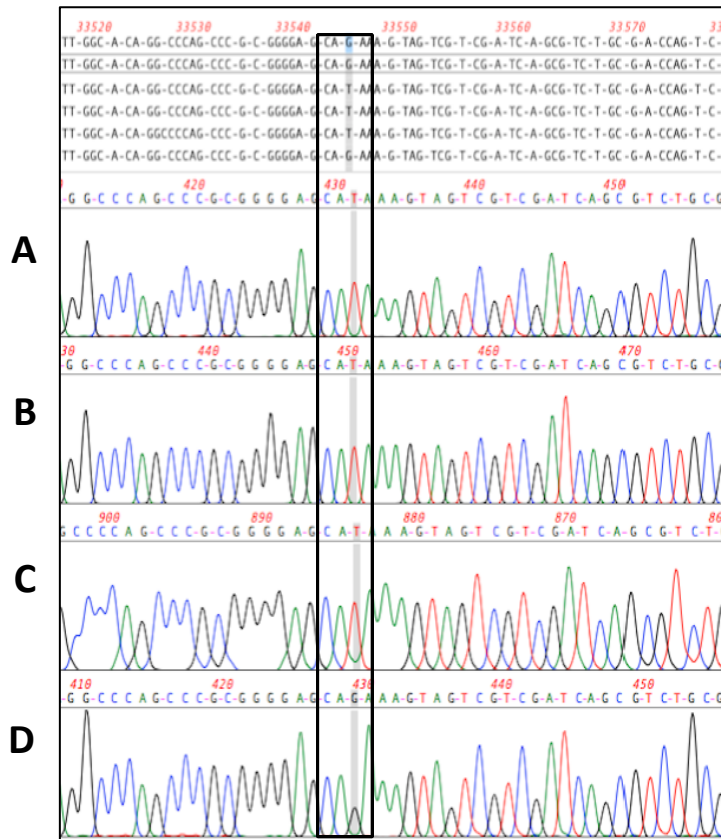




**Figure 25** Sequence diagram showing a synonymous mutation identified in sequence obtained from cloned PCR product of a region within UL27 gene of HCMV UL54 (Δ D413) passage 23, G change to T at position 33503(to the HCMVAD169 reference strain) or C to A at position 1155 (to the UL27 references strain). (A) sequence using M13 forward primer, (B&C) re-sequence using forward and reverse M13 primer and (D) WT sequence.

Note: the UL27 gene in reverse orientation.

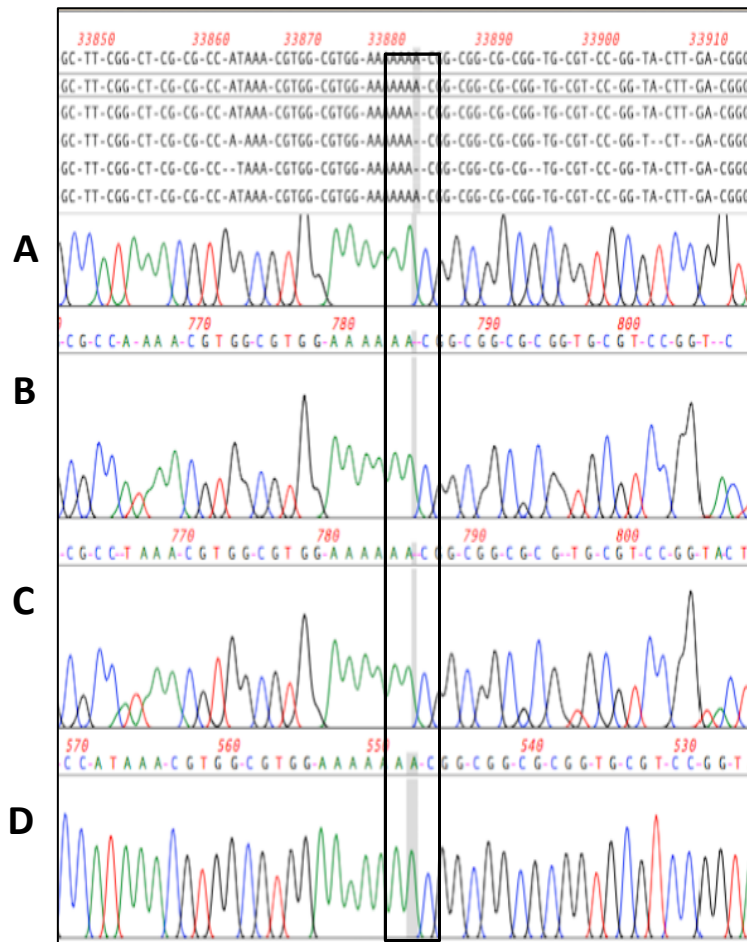
GGC change to GGA (Transversion)



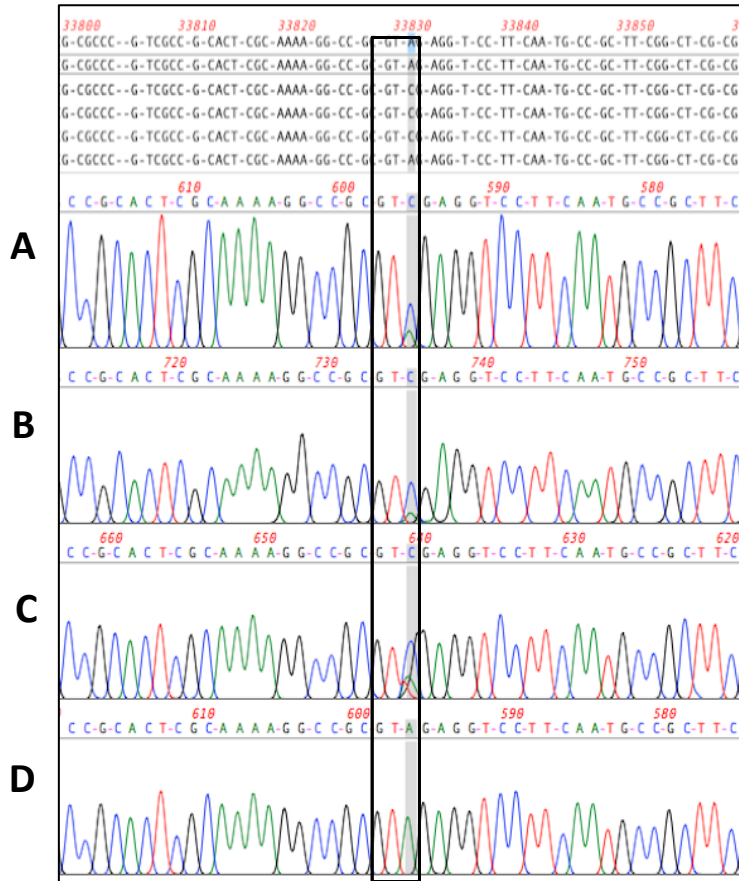
**Figure 26** Sequence diagram showing L372M mutation identified in sequence obtained from cloned PCR product of a region within UL27 gene of HCMV UL54 ( $\Delta$  D413) passage 23, G change to T at position 33544 (to the HCMVAD169 reference strain) or C change to A at position 1114 (to the UL27 reference strain) (non-synonymous mutation). (A) sequence using M13 forward primer, (B&C) re-sequence using forward and reverse using M13 primer and (D) WT sequence.

Note: the UL27 gene in reverse orientation.

CTG change to ATG (Transversion)



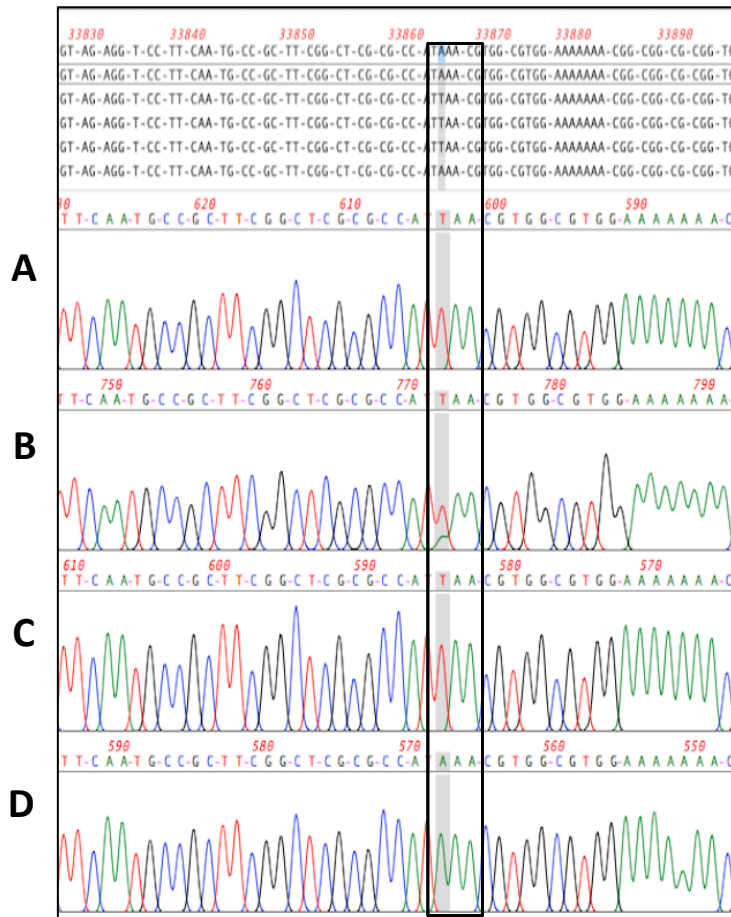
**Figure 27** Sequence diagram showing **deletion** mutation identified in sequence obtained from cloned PCR product of a region within UL27 gene of HCMV UL54 ( $\Delta$  D413) passage 23, A deleted at position 33882 (to the HCMVAD169 reference strain) or T deleted at position 776 (to the UL27 references strain) (synonymous mutation). (A) sequence using M13 forward primer, (B&C) re-sequence using forward and reverse using M13 primer and (D) WT sequence.



**Figure 28** Sequence diagram showing Y277D mutation identified in sequence obtained from cloned PCR product of a region within UL27 gene of HCMV UL54 ( $\Delta$  D413) passage 23, A change to C at position 33829 (to the HCMVAD169 reference strain) or T change to G at position 829 (to the UL27 references strain) (non-synonymous mutation). (A) sequence using M13 forward primer, (B&C) re-sequence using forward and reverse using M13 primer and (D) WT sequence.

Note: the UL27 gene in reverse orientation.

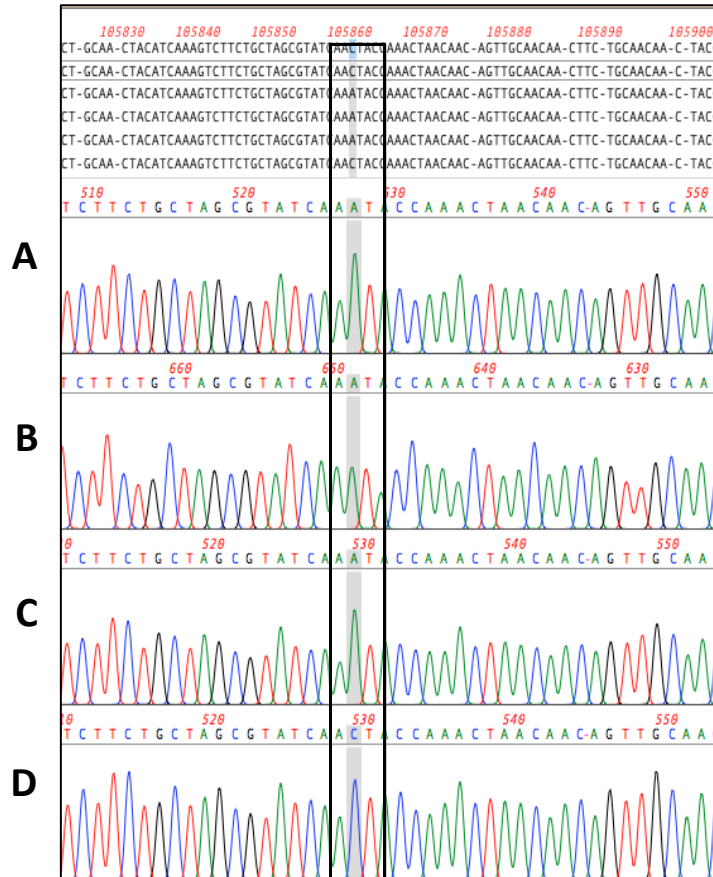
TAC change to GAC (Transversion)



**Figure 29** Sequence diagram showing F265L mutation identified in sequence obtained from cloned PCR product of a region within UL27 gene of HCMV UL54 ( $\Delta$  D413) passage 23, A change to T at position 33863 (to the HCMVAD169 reference strain) or T change to A at position 795 (to the UL27 references strain) (non-synonymous mutation). (A) sequence using M13 forward primer, (B&C) re-sequence using forward and reverse using M13 primer and (D) WT sequence.

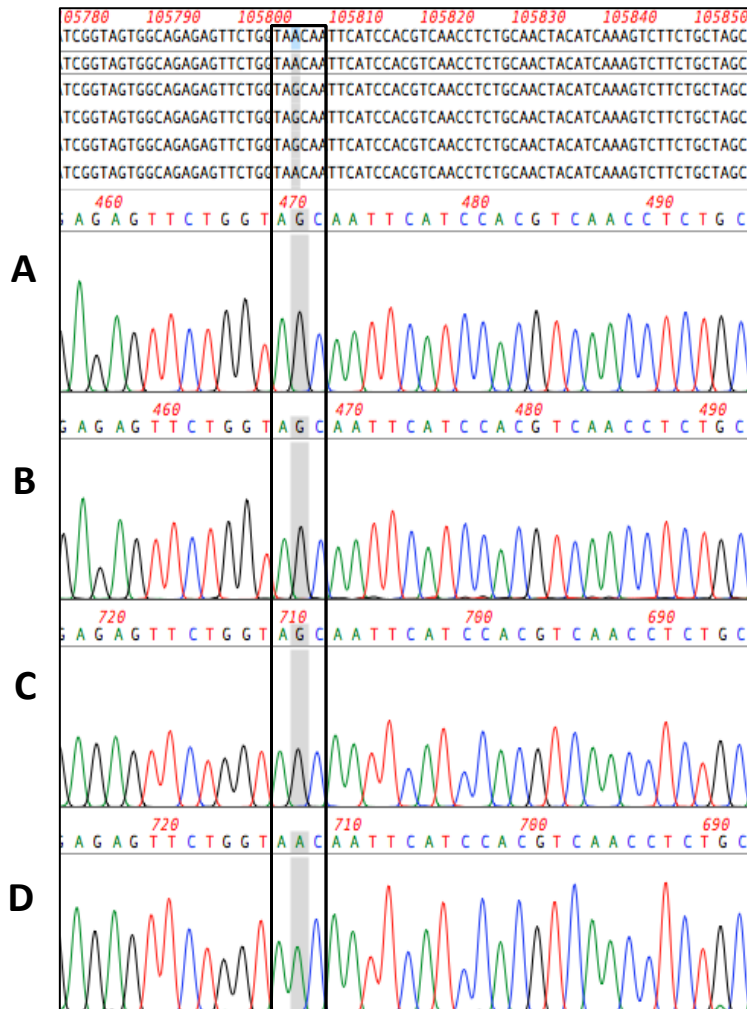
Note: the UL27 gene in reverse orientation.

TTT change to TTA (Transversion)



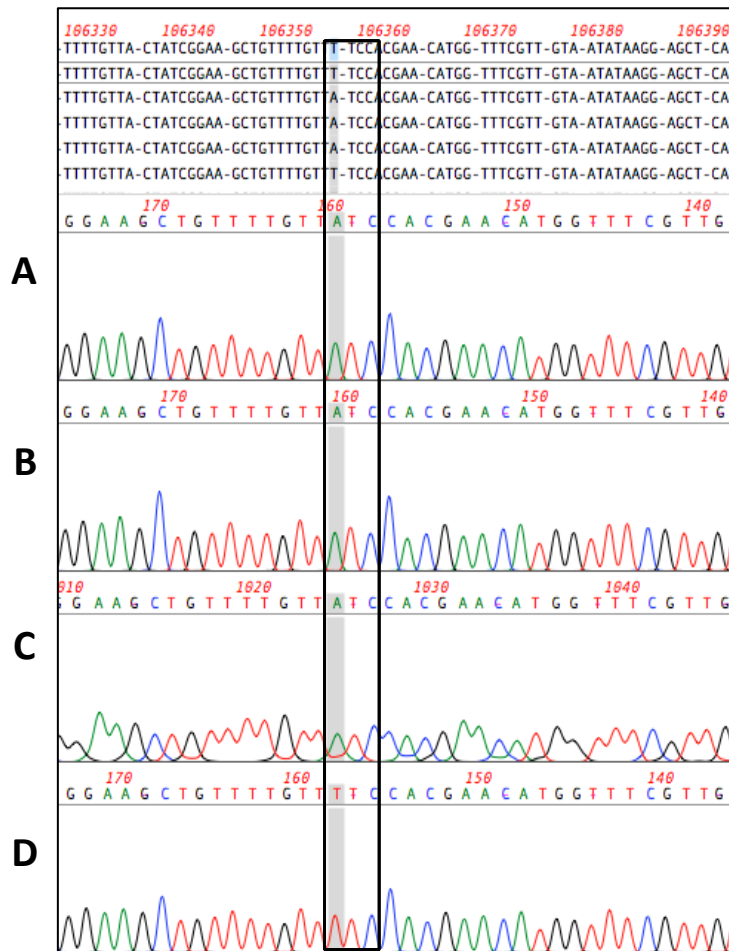
**Figure 30** Sequence diagram showing T41N mutation identified in sequence obtained from cloned PCR product of a UL72-UL74 spanned region of HCMV UL54 ( $\Delta$  D413) mutant early passage, C change to A at position 105858 (to the HCMVAD169 reference strain) or at position 122 (to the UL73 reference strain) (non-synonymous mutation). (A) sequence using M13 forward primer, (B&C) re-sequence using forward and reverse using M13 primer and (D) WT sequence.

ACT change to AAT (Transversion).



**Figure 31** Sequence diagram showing N22S mutation identified in sequence obtained from cloned PCR product of a UL72-UL74 spanned region of HCMV UL54 ( $\Delta$  D413) mutant early passage, A change to G at position 105801 to the HCMVAD169 reference strain) or at position 65 (to the UL73 references strain) (non-synonymous mutation). (A) sequence using M13 forward primer, (B&C) re-sequence using forward and reverse using M13 primer and (D) WT sequence .

AAC change to AGC (Transitions)

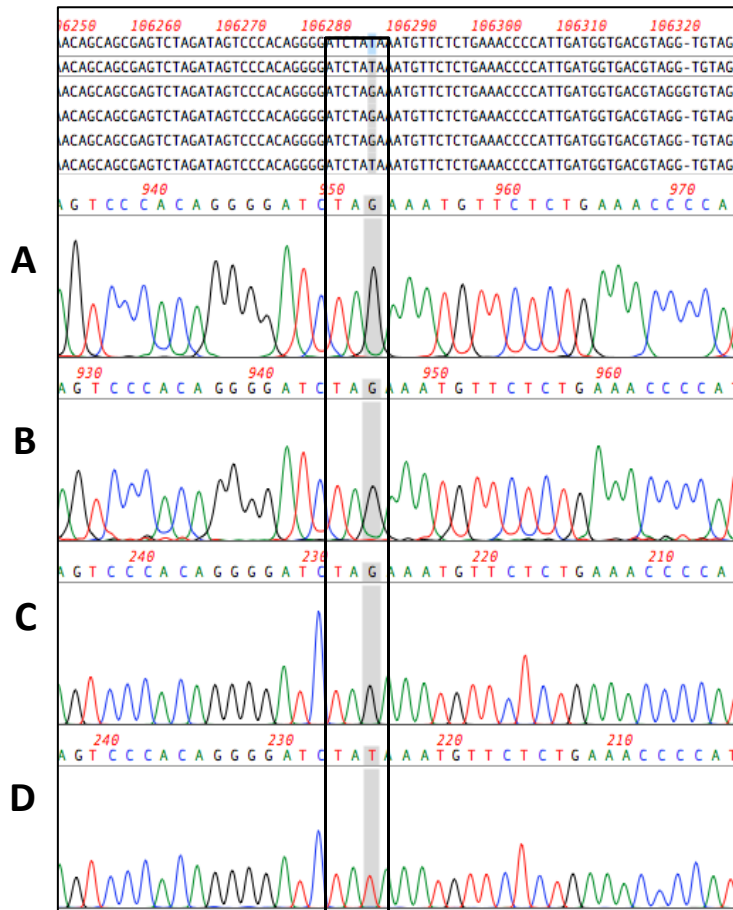


**Figure 32** Sequence diagram showing E391D mutation identified in sequence obtained from cloned PCR product of a UL72-UL74 spanned region of HCMV UL54 ( $\Delta$  D413) mutant early passage, T change to A at position 106353 to the HCMVAD169 reference strain) or A change to T at position 1173(to the UL74 references strain). (A) sequence using M13 forward primer, (B&C) re-sequence using forward and reverse using M13 primer and (D) WT sequence.

Note: the UL74 gene in reverse orientation.

GAA change to GAT (Transversion)

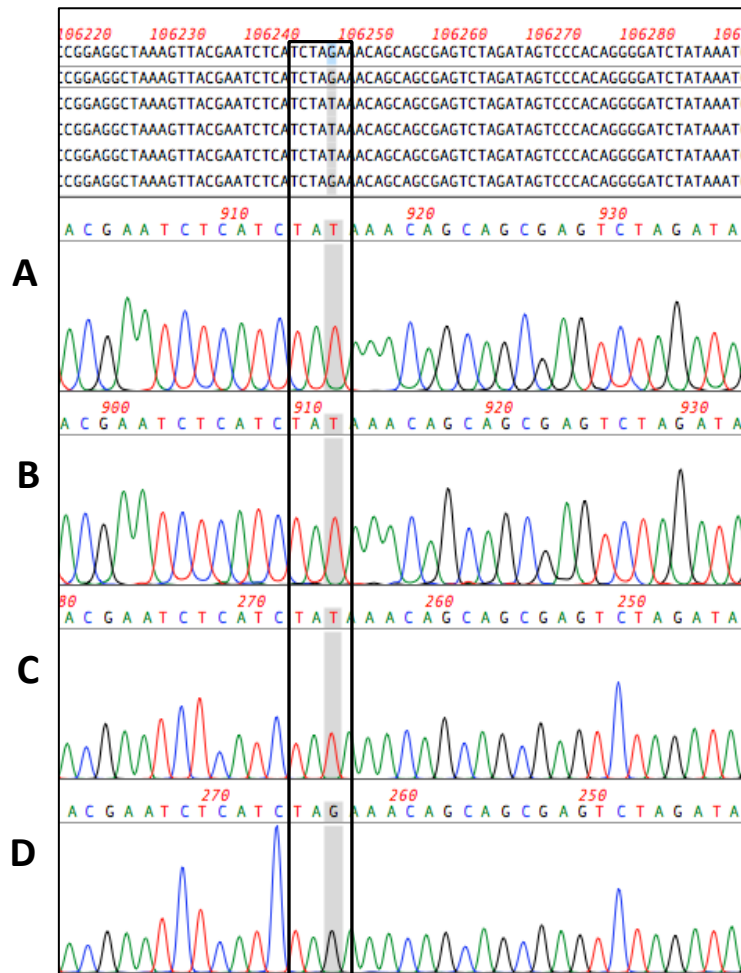




**Figure 33** Sequence diagram showing I415L mutation identified in sequence obtained from cloned PCR product of a UL72-UL74 spanned region of HCMV UL54 ( $\Delta$  D413) mutant late passage (P15), T change to G at position 106283 to the HCMVAD169 reference strain) or A change to C at position 1243 (to the UL74 references strain) (non-synonymous mutation). (A) sequence using M13 forward primer, (B&C) re-sequence using forward and reverse using M13 primer and (D) WT sequence.

Note: the UL74 gene in reverse orientation.

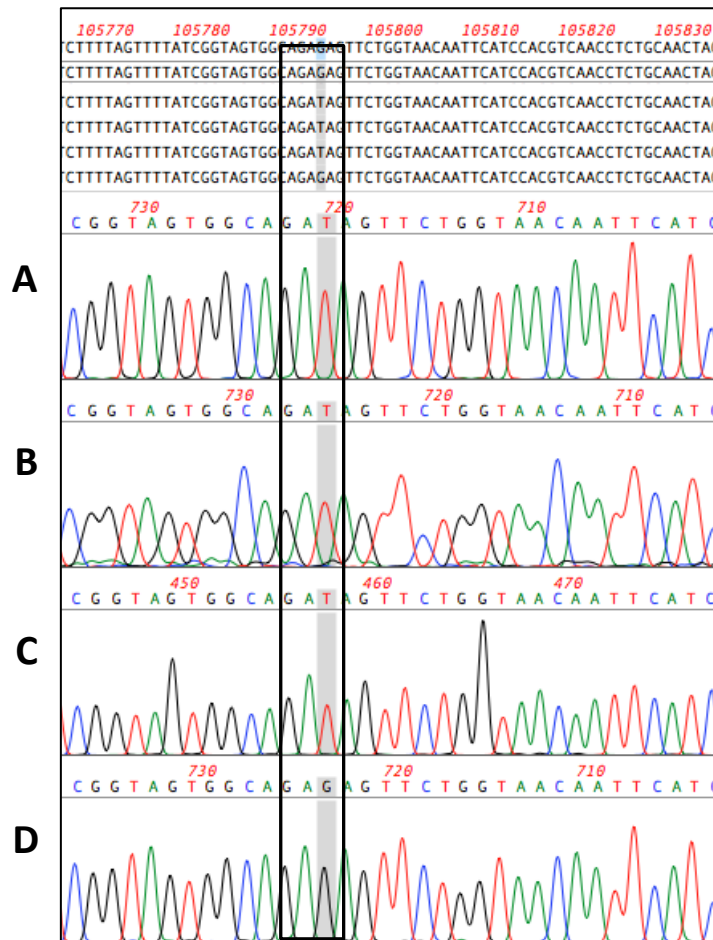
ATA change to CTA (Transversion)



**Figure 34** Sequence diagram showing L428I mutation identified in sequence obtained from cloned PCR product of a UL72-UL74 spanned region of HCMV UL54 ( $\Delta$  D413) mutant late passage (P15), G change to T at position 106244 to the HCMVAD169 reference strain) or C change to A at position 1282 (to the UL74 reference strain) (non-synonymous mutation). (A) sequence using M13 forward primer, (B&C) re-sequencing using forward and reverse using M13 primer and (D) WT sequence.

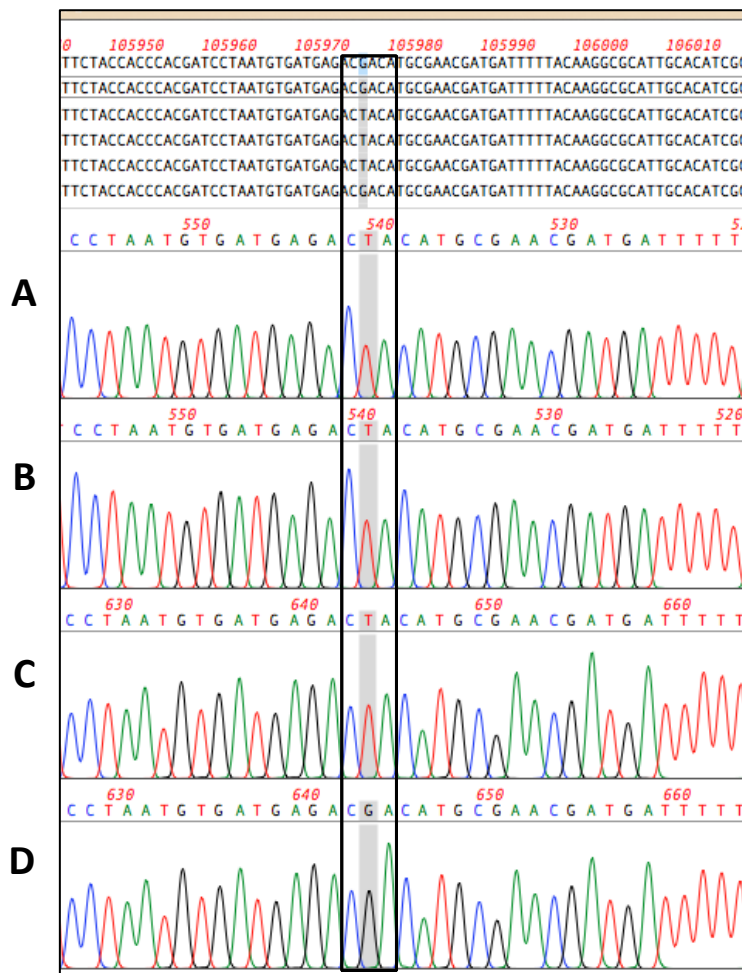
Note: the UL74 gene in reverse orientation.

CTA change to ATA (Transversion)



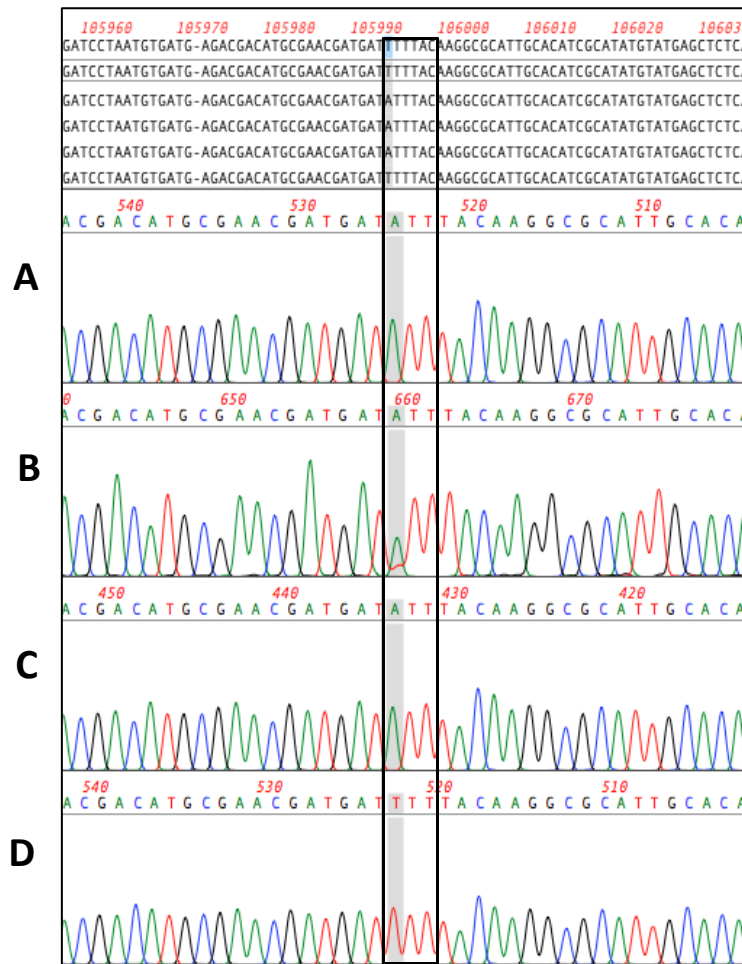
**Figure 35** Sequence diagram showing E18D mutation identified in sequence obtained from cloned PCR product of a UL72-UL74 spanned region of HCMV UL54 ( $\Delta$  D413) mutant late passage (P15), G change to T at position 105790 to the HCMVAD169 reference strain) or at position 54 (to the UL73 references strain) (non-synonymous mutation). (A) sequence using M13 forward primer, (B&C) re-sequence using forward and reverse using M13 primer and (D) WT sequence.

GAG change to GAT (Transversion)



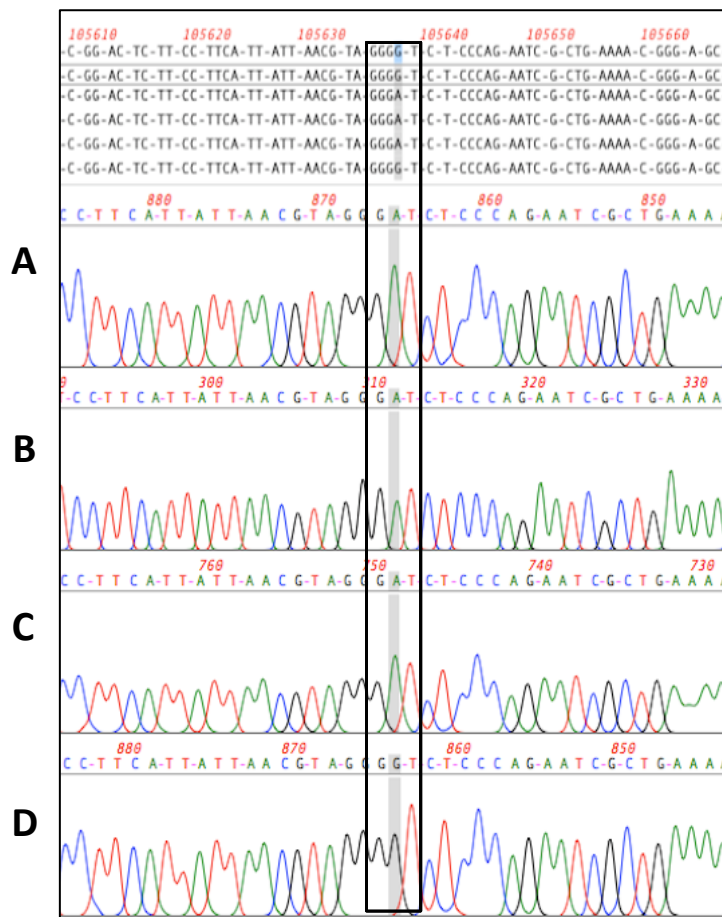
**Figure 36** Sequence diagram showing R79L mutation identified in sequence obtained from cloned PCR product of a UL72-UL74 spanned region of HCMV UL54 ( $\Delta$  D413) mutant late passage (P15), G change to T at position 105972 to the HCMVAD169 reference strain) or at position 236 (to the UL73 references strain) (non-synonymous mutation). (A) sequence using M13 forward primer, (B&C) re-sequencing using forward and reverse using M13 primer and (D) WT sequence.

CGA change to CTA (Transversion)



**Figure 37** Sequence diagram showing F85I mutation identified in sequence obtained from cloned PCR product of a UL72-UL74 spanned region of HCMV UL54 ( $\Delta$  D413) mutant late passage (P15), T change to A at position 105989 to the HCMVAD169 reference strain) or at position 253 (to the UL73 references strain) (non-synonymous mutation). (A) sequence using M13 forward primer, (B&C) re-sequencing using forward and reverse using M13 primer and (D) WT sequence.

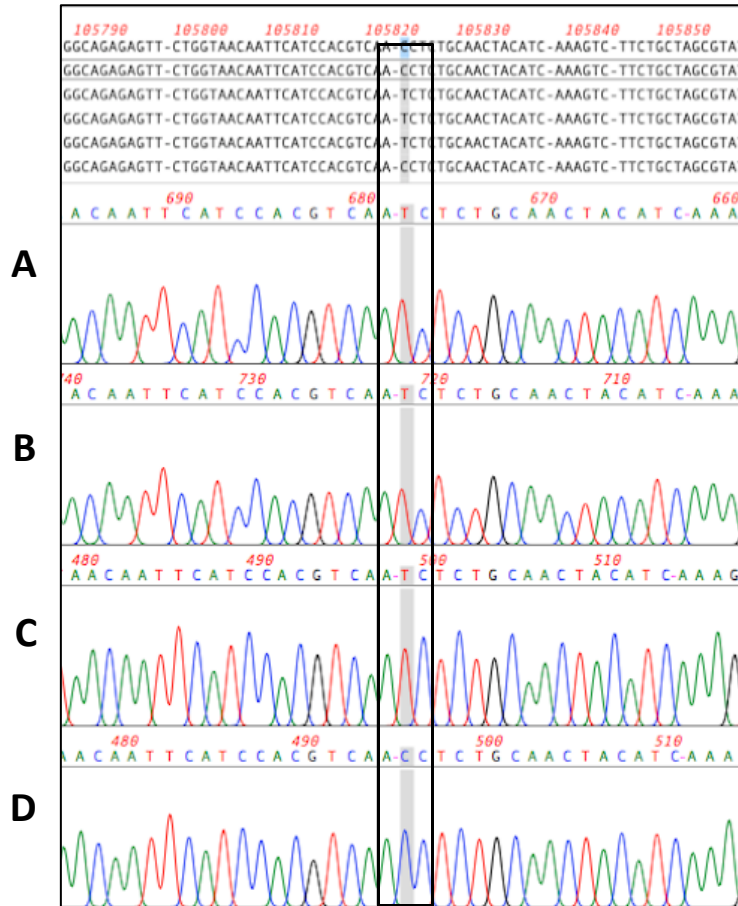
TTT change to ATT (Transversion)



**Figure 38** Sequence diagram showing T29I mutation identified in sequence obtained from cloned PCR product of a UL72-UL74 spanned region of HCMV UL54 ( $\Delta$  D413) mutant late passage (P23), G change to A at position 105636 to the HCMVAD169 reference strain) or C change to T at position 86 (to the UL72 references strain) (non-synonymous mutation). (A) sequence using M13 forward primer, (B&C) re-sequence using forward and reverse using M13 primer and (D) WT sequence.

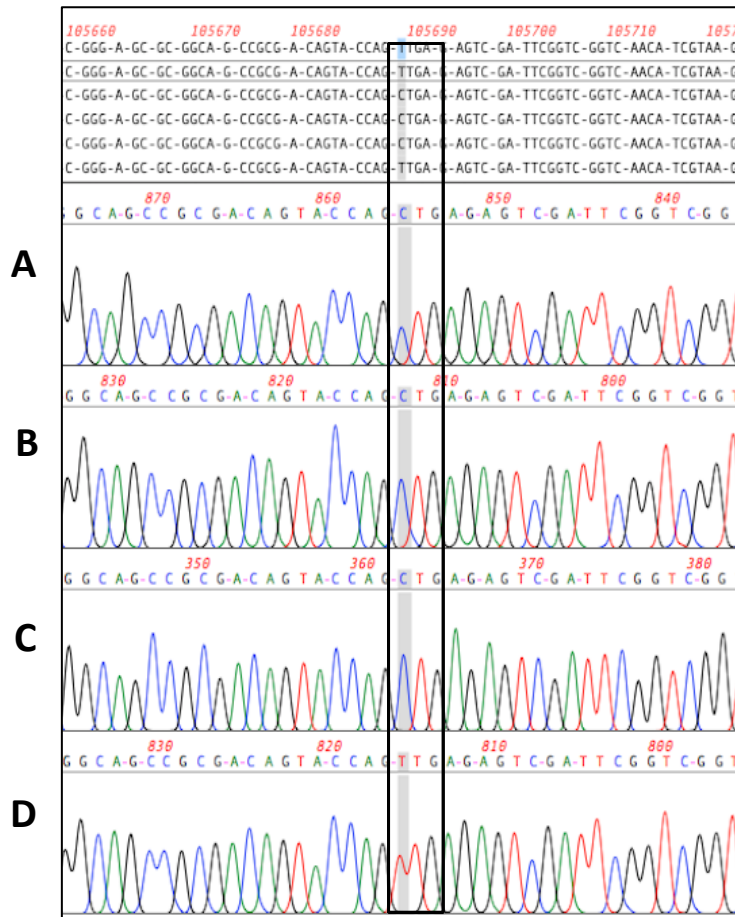
Note: the UL72 gene in reverse orientation.

ACC change to ATC (Transitions)



**Figure 39** Sequence diagram showing T28I mutation identified in sequence obtained from cloned PCR product of a UL72-UL74 spanned region of HCMV UL54 ( $\Delta$  D413) mutant late passage (P23), C change to T at position 105819 to the HCMVAD169 reference strain) or at position 83 (to the UL73 references strain) (non-synonymous mutation). (A) sequence using M13 forward primer, (B&C) re-sequence using forward and reverse using M13 primer and (D) WT sequence.

ACC change to ATC (Transitions)



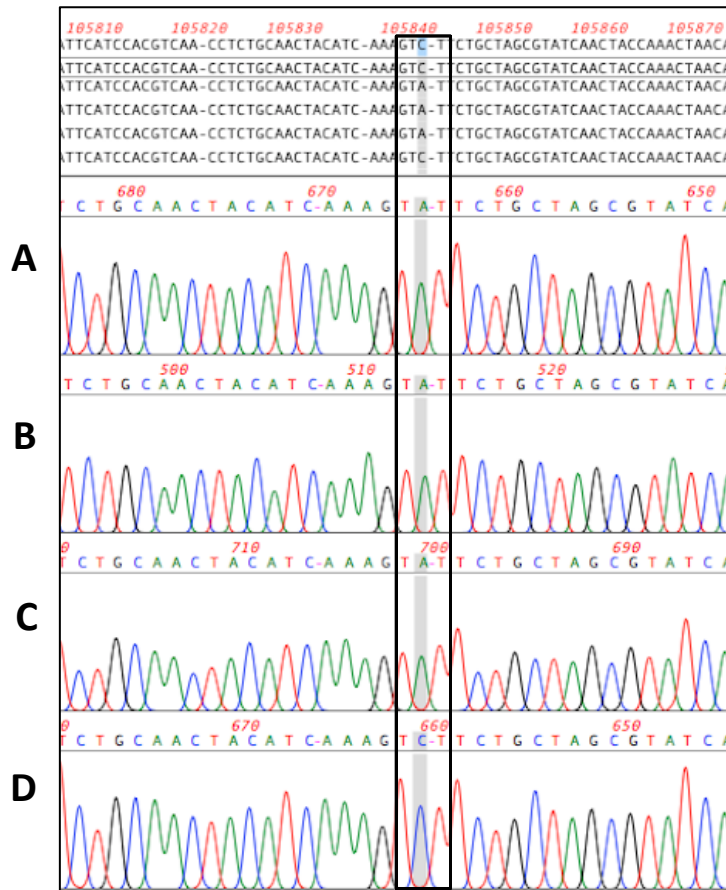
**Figure 40** Sequence diagram showing a synonymous mutation identified in sequence obtained from cloned PCR product of a UL72-UL74 spanned region of HCMV UL54 ( $\Delta$  D413) passage 23, T change to C at position 105686 (to the HCMVAD169 reference strain) or A to G at position 36 (to the UL72 reference strain). (A) sequence using M13 forward primer, (B&C) re-sequence using forward and reverse M13 primer and (D) WT sequence.

Codon 12

Note: the UL72 gene in reverse orientation.

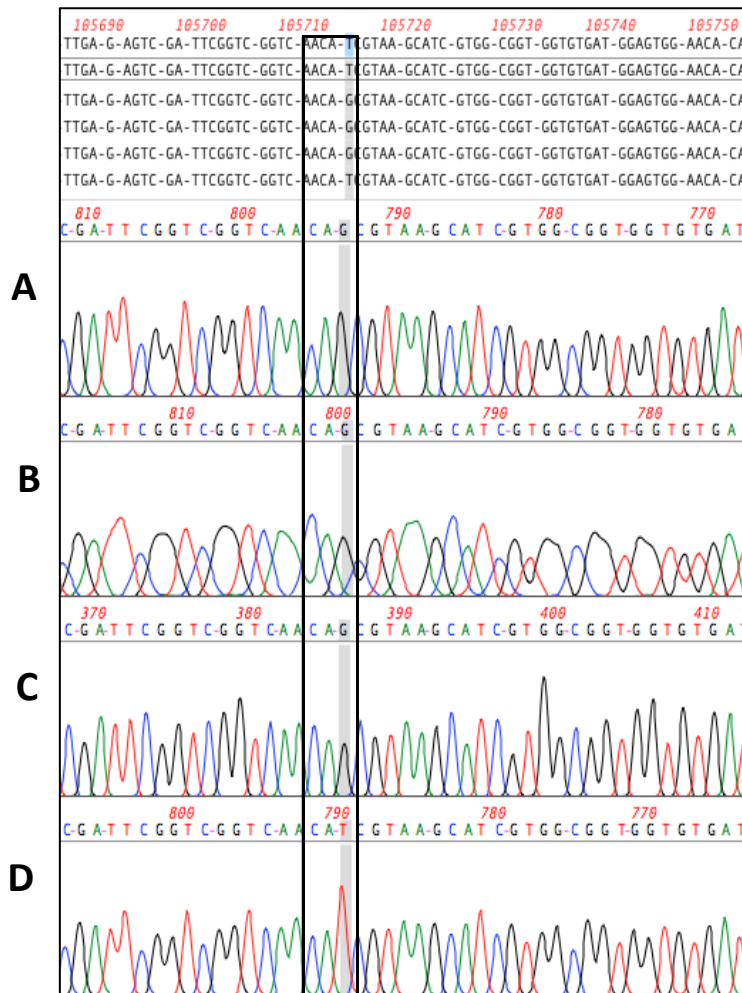
CAA change to CAG (Transitions)





**Figure 41** Sequence diagram showing S35Y mutation identified in sequence obtained from cloned PCR product of a UL72-UL74 spanned region of HCMV UL54 ( $\Delta$  D413) mutant late passage (P23), C change to A at position 105840 to the HCMVAD169 reference strain) or at position 104 (to the UL73 references strain) (non-synonymous mutation). (A) sequence using M13 forward primer, (B&C) re-sequence using forward and reverse using M13 primer and (D) WT sequence.

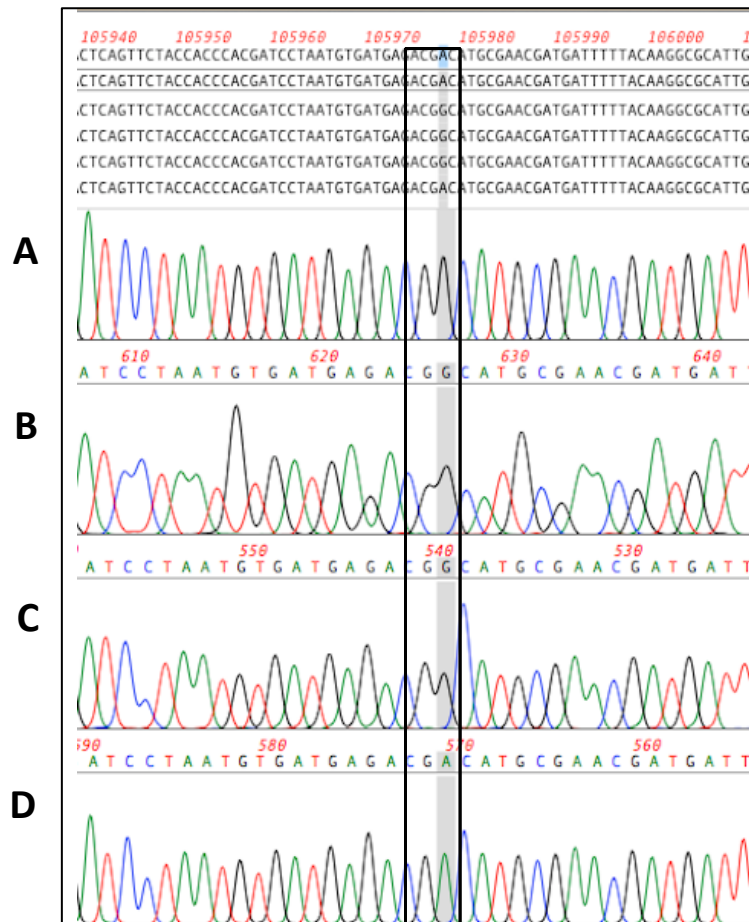
TCT change to TAT (Transversion)



**Figure 42** Sequence diagram showing M4L mutation identified in sequence obtained from cloned PCR product of a UL72-UL74 spanned region of HCMV UL54 ( $\Delta$  D413) mutant late passage (P23), T change to G at position 105712 to the HCMVAD169 reference strain) or A change to C at position 10 (to the UL72 references strain) (non-synonymous mutation). (A) sequence using M13 forward primer, (B&C) re-sequencing using forward and reverse using M13 primer and (D) WT sequence.

Note: the UL72 gene in reverse orientation.

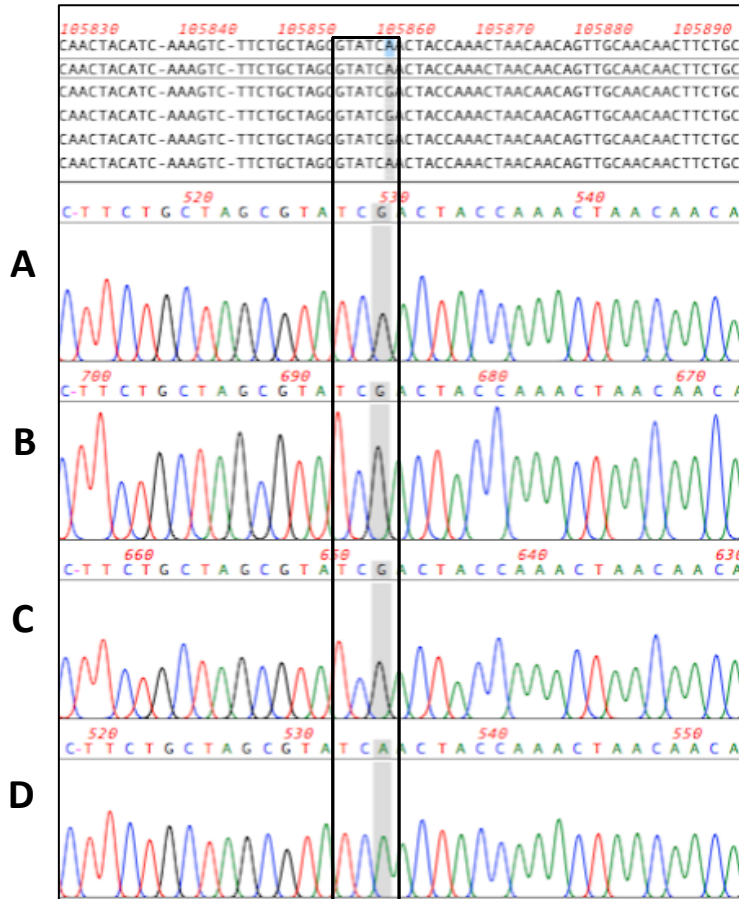
ATG change to CTG (Transversion)



**Figure 43** Sequence diagram showing a synonymous mutation identified in sequence obtained from cloned PCR product of a UL72-UL74 spanned region of HCMV UL54 ( $\Delta$  D413) passage 23, A change to G at position 105973 (to the HCMVAD169 reference strain) or at position 237 (to the UL73 reference strain). (A) sequence using M13 forward primer, (B&C) re-sequencing using forward and reverse M13 primer and (D) WT sequence.

Codon 79

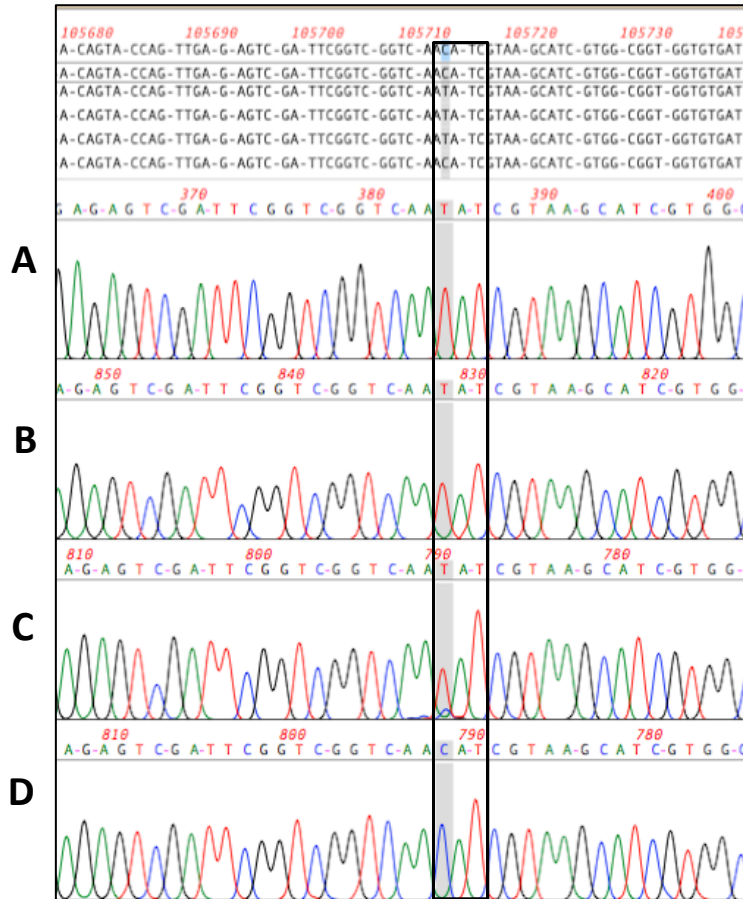
CGA change to CGG (Transitions)



**Figure 44** Sequence diagram showing a synonymous mutation identified in sequence obtained from cloned PCR product of a UL72-UL74 spanned region of HCMV UL54 ( $\Delta$  D413) passage 23, A change to G at position 105856 (to the HCMVAD169 reference strain) or at position 120 (to the UL73 reference strain). (A) sequence using M13 forward primer, (B&C) re-sequenced using forward and reverse M13 primer and (D) WT sequence.

Codon 40

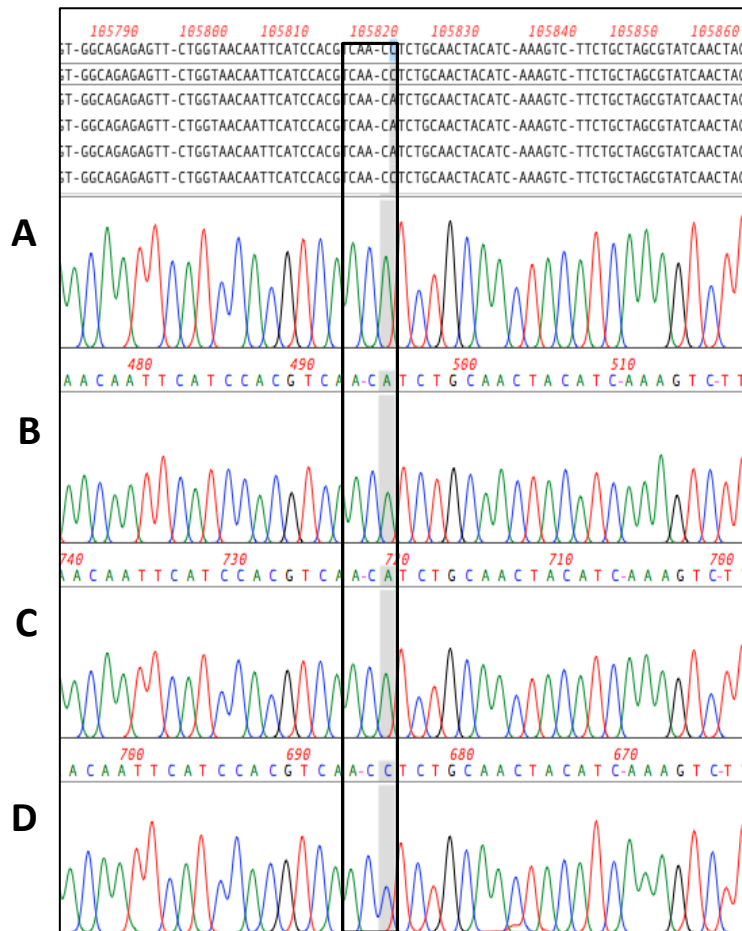
TCA change to TCG (Transitions)



**Figure 45** Sequence diagram showing M4I mutation identified in sequence obtained from cloned PCR product of a UL72-UL74 spanned region of HCMV UL54 ( $\Delta$  D413) mutant late passage (P23), T change to G at position 105710 to the HCMVAD169 reference strain) or G to A at position 12 (to the UL72 reference strain) (non-synonymous mutation). (A) sequence using M13 forward primer, (B&C) re-sequencing using forward and reverse using M13 primer and (D) WT sequence.

Note: the UL72 gene in reverse orientation.

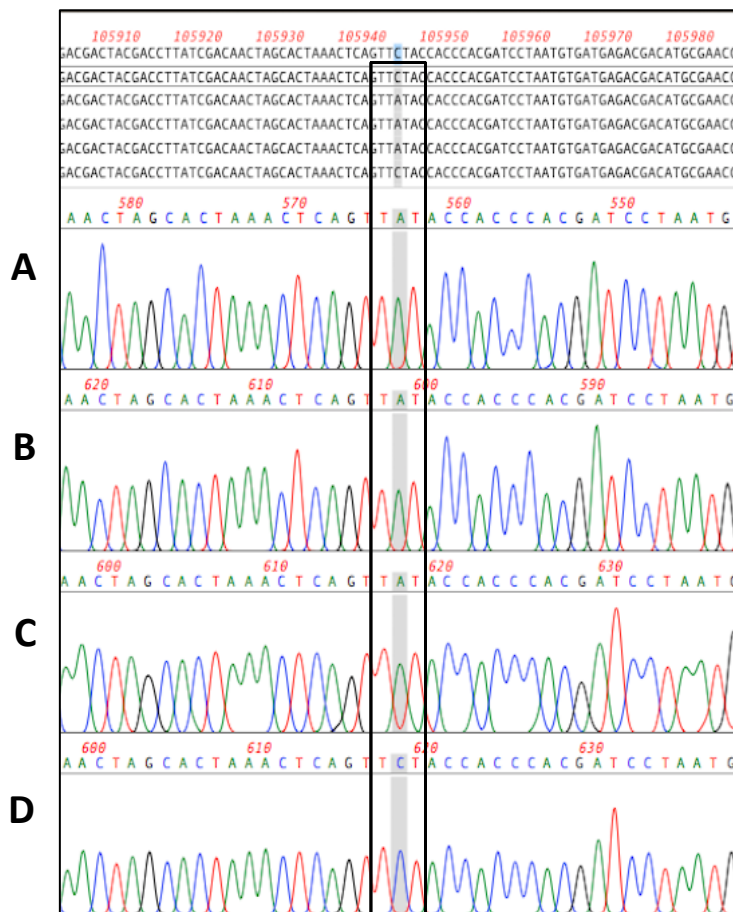
ATG change to ATA (Transitions)



**Figure 46** Sequence diagram showing a synonymous mutation identified in sequence obtained from cloned PCR product of a UL72-UL74 spanned region of HCMV UL54 ( $\Delta$  D413) passage 23, C change to A at position 105820 (to the HCMVAD169 reference strain) or at position 84 (to the UL73 references strain). (A) sequence using M13 forward primer, (B&C) re-sequencing using forward and reverse M13 primer and (D) WT sequence.

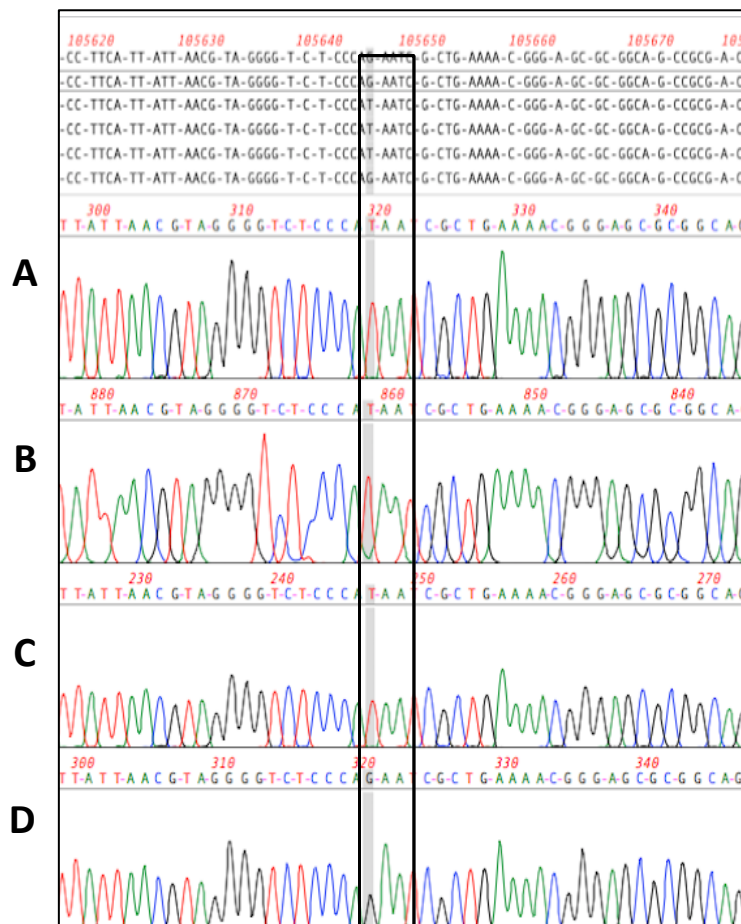
Codon 28

ACC change to ACA (Transversion)



**Figure 47** Sequence diagram showing S69Y mutation identified in sequence obtained from cloned PCR product of a UL72-UL74 spanned region of HCMV UL54 ( $\Delta$  D413) mutant late passage (P23), C change to A at position 105942 to the HCMVAD169 reference strain) or at position 206 (to the UL73 reference strain) (non-synonymous mutation). (A) sequence using M13 forward primer, (B&C) re-sequencing using forward and reverse using M13 primer and (D) WT sequence.

TCT change to TAT (Transversion)

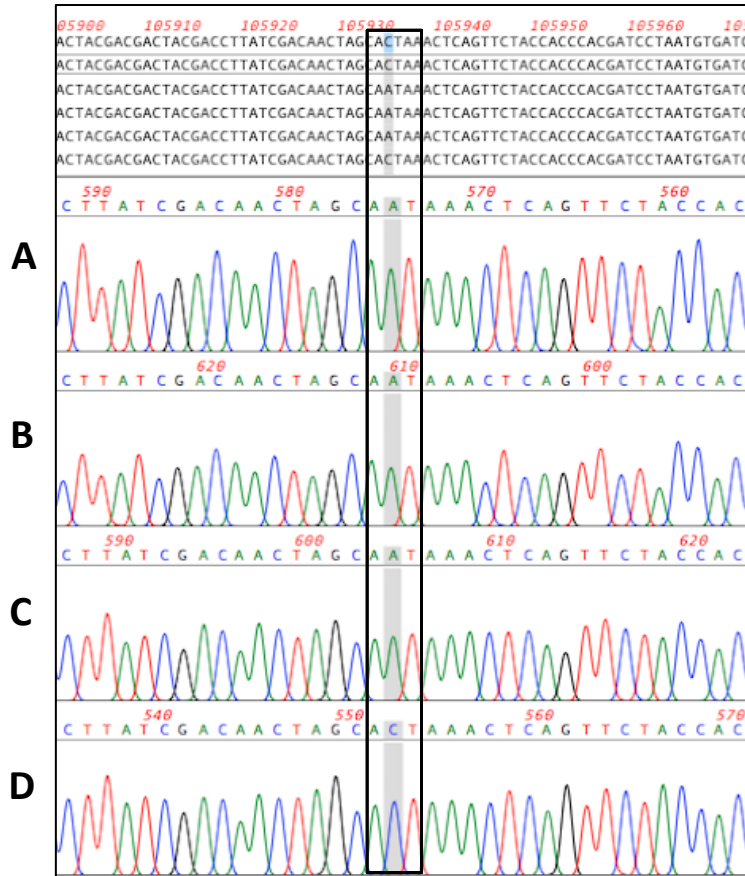


**Figure 48** Sequence diagram showing F26L mutation identified in sequence obtained from cloned PCR product of a UL72-UL74 spanned region of HCMV UL54 ( $\Delta$  D413) mutant late passage (P23), G change to T at position 105644 to the HCMVAD169 reference strain) or C change to A at position 78 (to the UL72 references strain) (non-synonymous mutation). (A) sequence using M13 forward primer, (B&C) re-sequence using forward and reverse using M13 primer and (D) WT sequence.

Note: the UL72 gene in reverse orientation.

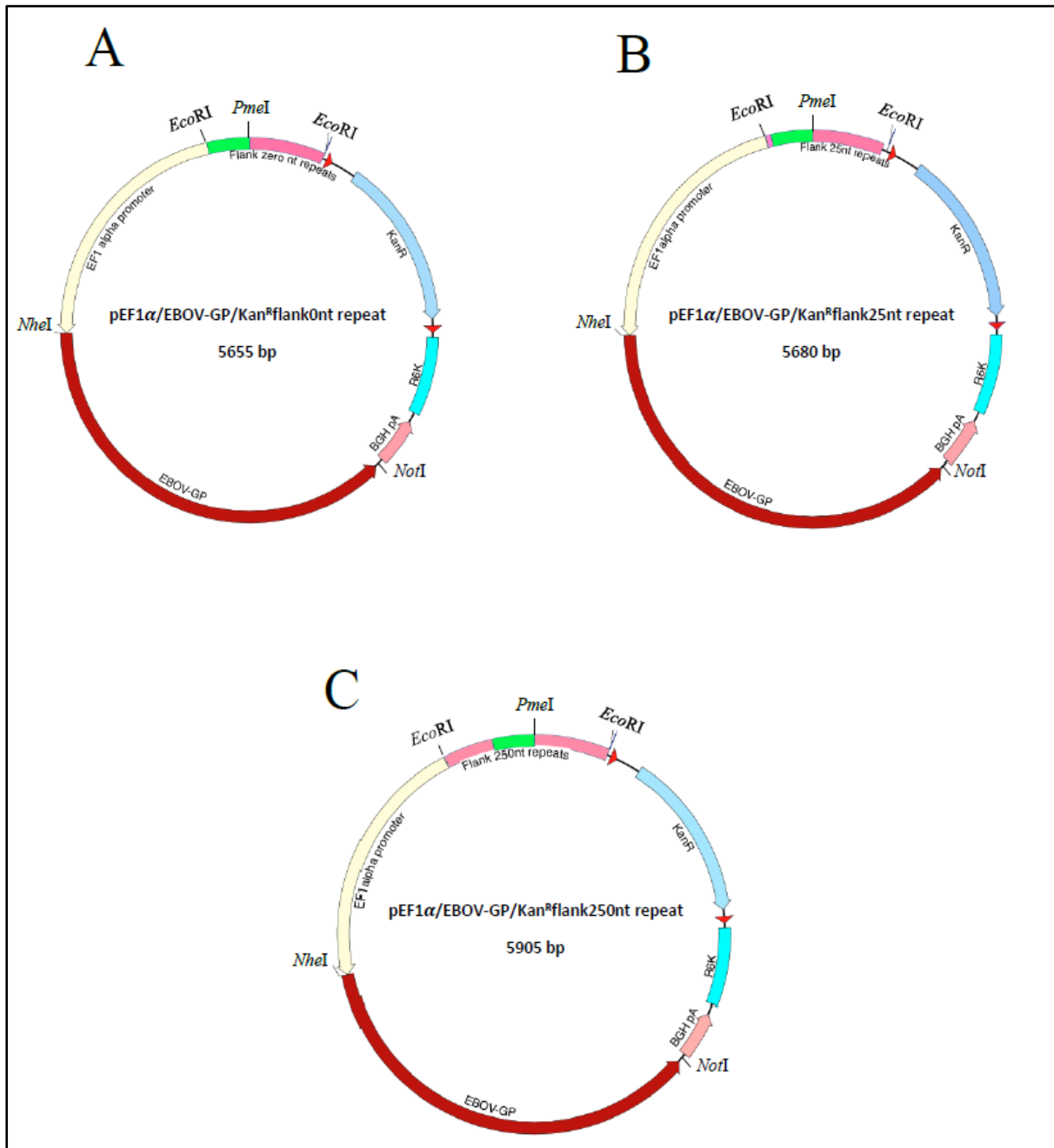
TTC change to TTA (Transversion)



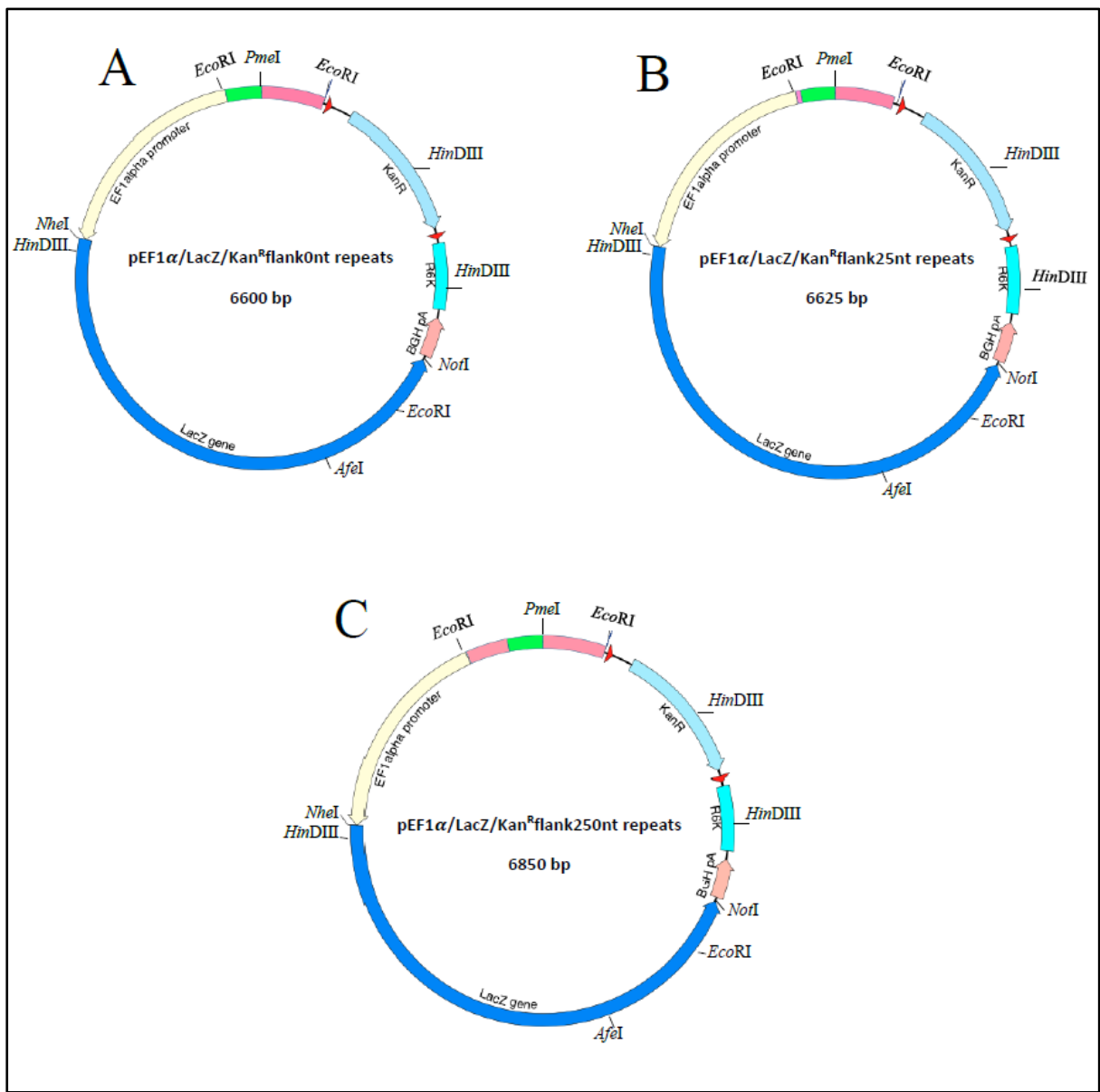


**Figure 49** Sequence diagram showing T65N mutation identified in sequence obtained from cloned PCR product of a UL72-UL74 spanned region of HCMV UL54 ( $\Delta$  D413) mutant late passage (P23), C change to A at position 105930 to the HCMVAD169 reference strain) or at position 194 (to the UL73 references strain) (non-synonymous mutation). (A) sequence using M13 forward primer, (B&C) re-sequencing using forward and reverse using M13 primer and (D) WT sequence.

ACT change to AAT (Transversion)



**Figure (A7-1):** Schematic showing *PmeI* and *NheI* restriction sites of pEF1α/EBOV-GP/Kan<sup>R</sup> flank repeats sequences vectors. (A) pEF1α/EBOV-GP/Kan<sup>R</sup>flank0nt repeat vector, (B) pEF1α/EBOV-GP/Kan<sup>R</sup>flank25nt repeats vector and (C) pEF1α /EBOV-GP/Kan<sup>R</sup>flank250nt repeats.



**Figure (A7-2):** Schematic showing *PmeI*, *NheI*, *AfeI*, *HindIII* and *EcoRI* restriction sites of pEF1α/EBOV-GP/Kan<sup>R</sup> flank repeats sequences vectors (A)pEF1α/LacZ/Kan<sup>R</sup>flank0nt repeats vector, (B)pEF1α/LacZ/Kan<sup>R</sup>flank25nt repeats vector and (C) pEF1α/LacZ/ Kan<sup>R</sup> flank250nt repeats vector.