# DEVELOPMENT AND CHARACTERISATION OF RECOMBINANT LSDV-VECTORED DUAL VACCINES AGAINST BOVINE LEUKAEMIA VIRUS AND LUMPY SKIN DISEASE VIRUS

**Akiko Suzuki**

**December 2019**

University of Cape Town


**Supervisor: Professor Anna-Lise Williamson**
**Co-supervisor: Dr. Rosamund Chapman**

**Division of Medical Virology**

**Department of Pathology**

**Faculty of Health Sciences**

**University of Cape Town**

The copyright of this thesis vests in the author. No quotation from it or information derived from it is to be published without full acknowledgement of the source. The thesis is to be used for private study or non-commercial research purposes only.

Published by the University of Cape Town (UCT) in terms of the non-exclusive license granted to UCT by the author.

University of Cape Town

## GENERAL TABLE OF CONTENTS

## ABSTRACT

Bovine leukaemia virus (BLV) and lumpy skin disease virus (LSDV) are endemic to Africa and cause significant economic losses to the beef and dairy industries. Vaccines are the most cost-effective and efficient way to prevent infection and outbreaks. Currently, there is no commercially available vaccine against BLV. In contrast, there are several live attenuated vaccines against LSDV. A recombinant LSDV which could protect cattle against both LSDV and BLV would be of great benefit to the African continent.

This Master's degree project involved three objectives. Firstly, the genetic variabilities and phylogenetic relationships of eight South African BLV isolates with other BLV strains from different geographical regions worldwide with known genotypes were determined. The BLV full-length *envelope* (*env*) and *gag* genes were successfully sequenced from total DNA extracted from the blood of BLV-infected cattle from a single herd. The analyses indicated that the seven of the South African isolates characterised in this study belonged to genotype 4 and the eighth to genotype 1. Furthermore, amino acid substitutions in the BLV Env and Gag sequences unique to the South African isolates were identified.

Secondly, the activity of five selected poxvirus promoters in cells infected with LSDV was assessed by the detection of transient expression of an enhanced green fluorescent protein (eGFP) reporter gene driven by the poxvirus promoters. The promoters tested were a modified early fowlpox virus promoter (pmFP), an early-late promoter of a 7.5 kilodalton polypeptide gene of vaccinia virus (VACV) (p7.5), a synthetic early-late promoter of VACV (pS), a modified early-late promoter of the H5 gene of VACV (pmH5) and a synthetic early-late optimised promoter of VACV (pLEO). The results showed that all the poxvirus promoters were functional in the LSDV-infected cells and the eGFP expression was stable over the 72-hour study period.

Lastly, two LSDV-vectored dual vaccines containing BLV immunogen(s) were developed and characterised. The first recombinant LSDV-vectored vaccine contained the BLV Env and Gag immunogens and the second recombinant LSDV-vectored vaccine contained the BLV Env immunogen alone. The presence of the BLV *env* gene in the recombinant LSDV vaccine was confirmed by polymerase chain reaction (PCR) and the BLV *env* sequence was confirmed by Sanger sequencing. Furthermore, BLV Env and Gag protein expression were confirmed by immunofluorescent staining and Western blotting, respectively.

Future work will involve further purification of the recombinant viruses, confirmation of the production of BLV Gag virus-like particles and the preparation of high titre stocks of the vaccines to test in cattle.

## DECLARATION

I, Akiko Suzuki, hereby declare that the work on which this dissertation/thesis is based is my original work (except where acknowledgements indicate otherwise) and that neither the whole work nor any part of it has been, is being, or is to be submitted for another degree in this or any other university.

I empower the university to reproduce for the purpose of research either the whole or any portion of the contents in any manner whatsoever.

Signature: Signed by candidate

Date: 27.9.2019

**ACKNOWLEDGMENTS**

I'd like to thank the following people and organisations:

My supervisor Prof. Anna-Lise Williamson and co-supervisor Dr. Ros Chapman for giving me an opportunity to conduct this project and for their assistance and guidance.

Dr. Niki Douglass for her support, guidance and insightful advice.

My colleagues at Anna-Lise lab for their technical advice and assistance. I'd like to extend my deepest gratitude to Dr. Henry Munyanduki who always made time for me, who has been patient with me and who has been an inspiring scientist. I'm also grateful to Ruzaiq Omar for his moral support.

Ms. Susan Lanfear and Ms. Zainonesa Hartley for their assistance with my application for funding and other administrative processes.

CPS staff for keeping me safe and for friendly conversations.

Enkosi, Mama Monica for her amazing job, Mr. Lunga Sayo and other DAs for their assistance whenever I needed something for my experiments and also for fun conversations.

Ms. Nakedi Ribane and Ms. Eleanor Kunene for their encouragement, trust and unwavering support over the years. Thank you so much for being my mentors.

Ms. Susan Komori for her moral support and friendship.

South African Research Initiative and Poliomyelitis Research Foundation for financial support.

## LIST OF ACRONYMS AND ABBREVIATIONS

| | |
|---|---|
| 49 flank | 3’ end of LSDV *049* ORF |
| 50 flank | 3’ end of LSDV *050* ORF |
| Ab | Antibody |
| AEFI | Adverse effects following immunisation |
| AGID | Agar gel immunodiffusion |
| Approx. | Approximately |
| ATP | Adenosine triphosphate |
| BCIP/NTB | 5-bromo-4-chloro-3-indolyl phosphate / nitro blue tetrazolium |
| BHK | Baby hamster kidney |
| BLAST | Basic Local Alignment Search Tool |
| BLV | Bovine leukaemia virus |
| BoLA | Bovine major histocompatibility complex |
| bp | Base pairs |
| Ca | Capsid |
| CaPV | *Capripoxvirus* or capripoxvirus |
| cDMEM | Complete Dulbecco's Modified Eagle Medium |
| CPE | Cytopathic effect |
| CRE | cyclic-AMP responsive |
| C-terminus | Caboxyl-terminus |
| CTL | Cytotoxic T cell |
| Cyclic-AMP | Cyclic adenosine monophosphate |
| $ddH_2O$ | Double-distilled water |
| DMEM | Dulbecco's Modified Eagle Medium |
| DMSO | Dimethyl sulfoxide |
| DNA | Deoxyribonucleic acid |
| dNTP | Deoxynucleotide |
| *E. coli* | *Escherichia coli* |
| e.g., | For example |
| EBL | Enzootic bovine leukosis |
| Ecogpt | *Escherichia coli* xanthine guanine phosphoribosyltransferase |
| EDTA | Ethylenediaminetetraacetic acid |
| EEV | Extracellular enveloped virus |
| eGFP | Enhanced green fluorescent protein |
| ELISA | Enzyme-linked immunosorbent assay |

| | |
|---|---|
| Env | Envelope |
| FBT | Foetal bovine rete testes |
| FCS | Fetal calf serum |
| FP | Fluorescent protein |
| Gag | Group-specific antigen |
| Gp | Glycoprotein |
| GPT | Xanthine guanine phosphoribosyltransferase |
| HIV | Human immunodeficiency virus |
| HKY | Hasegawa-Kishino-Yano |
| HPL | High proviral load |
| HPLC | High-performance liquid chromatography |
| H-ras | Harvey rat sarcoma virus |
| HTLV | Human T-lymphocyte leukaemia virus |
| i.e., | Namely |
| IEV | Intracellular enveloped virus |
| IMV | Intracellular mature virus |
| IN | Integrase |
| ISD | Immunosuppressive domain |
| ITAM | Immunoreceptor tyrosine-based activation motif |
| JTT | Jones-Taylor-Thornton |
| K2 | Kimura-2-parameter |
| kb | Kilobase |
| kDa | Kilodalton |
| KSGP | Kenyan sheep and goat poxvirus |
| LB | Luria-Bertani |
| LEO | Early-late optimised promoter |
| LPL | Low proviral load |
| LSD | Lumpy skin disease |
| LSDV | Lumpy skin disease virus |
| LTR | Long terminal repeat |
| MA | Matrix |
| MDBK | Madin-Darby bovine kidney |
| mFP | Modified early fowlpox virus promoter |
| $Mg_2Cl$ | Magnesium chloride |
| mH5 | Modified early-late promoter of the H5 gene of VACV |

| | |
|---|---|
| miRNA | microRNA |
| ML | Maximum-likelihood |
| MOI | Multiplicity of infection |
| mRNA | Messenger ribonucleic acid |
| NAb | Neutralising antibody |
| NC | Nucleocapsid |
| NCBI | National Centre for Biotechnology Information |
| NJ | Neighbour-joining |
| nLSDV | Neethling lumpy skin disease virus |
| nPCR | Nested polymerase chain reaction |
| N-terminus | Amino-terminus |
| ORF | Open reading frame |
| p7.5 | Promoter of 7.5 kDa polypeptide gene of vaccinia virus |
| PBMC | Peripheral blood mononuclear cells |
| PBS | Phosphate buffered saline |
| PCR | Polymerase chain reaction |
| Pen-Strep | Penicillin and streptomycin |
| PFE/L | Fowlpox virus early/late |
| pHyb | Hybrid early-late promoter |
| PL | Persistent lymphocytosis |
| Pol | Polymerase |
| Poly(A) | Polyadenylation |
| PPRV | Peste des petits ruminant virus |
| Pr | Precursor |
| Pro | Protease |
| pS | Synthetic early-late promoter |
| REF | Rat embryo fibroblast |
| RFLP | Restriction fragment length polymorphism |
| RT | Reverse transcriptase |
| RVFV | Rift Valley fever virus |
| RVV | Recombinant vaccinia virus |
| sa | Splice acceptor |
| SBL | Sporadic bovine leukosis |
| sd | Splice donor |
| SIV | Simian immunodeficiency virus |

| | |
|---|---|
| SNV | Spleen necrosis virus |
| SOD | Superoxide dismutase |
| SPPV | Sheep poxvirus |
| SU | Surface protein |
| TBE | Tris-brate-EDTA |
| TF | Transcription factor |
| Th | T helper |
| TM | Transmembrane protein |
| TN83 | Tamura-Nei |
| tPA | Tissue-type plasminogen activator |
| TxRE | Tax responsive element |
| UCT | University of Cape Town |
| UPGMA | Unweighted pair group method with arithmetic mean |
| VACV or VV | *Vaccinia virus* |
| VLP | Virus-like particle |

## LIST OF FIGURES

## LIST OF TABLES

# CHAPTER 1

## LITERATURE REVIEW

## 1.1. LUMPY SKIN DISEASE VIRUS (LSDV)

Lumpy skin disease (LSD) is a notifiable disease of cattle in South Africa [1] and is endemic in Africa and the Middle East [2]. LSD is caused by lumpy skin disease virus (LSDV), which is a DNA virus from the *Capripoxvirus* (CaPV) genus of the *Poxviridae* family. LSDV naturally infects cattle and Asian water buffalo and the transmission of LSDV is predominantly mediated by arthropod vectors [3, 4]. LSDV infection causes acute or subclinical diseases including high fever (> 41°C), lymphadenopathy, salivation, nasal discharge, lachrymation and skin nodules that are characteristic of poxvirus infection [5-7]. The morbidity rate varies depending on age, breeds and immune status of animals but it is usually less than 20% [8, 9]. The mortality rate is less than 10% but it can reach more than 75% during outbreaks [8-11].

### 1.1.1. Epizootiology of lumpy skin disease (LSD)

LSD has never been eradicated from the endemic countries. Rather, endemic countries have been plagued with recurrent outbreaks and LSDV geographic distribution has been rapidly expanding from Africa to the Middle East and further into Europe (Figure 1.1) [2]. Furthermore, there has been a concern about the incursion of LSD into Asia if LSDV continues expanding its geographic distribution at this alarming rate. The financial cost to control outbreaks is generally enormous and the economic damages of an LSD outbreak can be severe particularly in resource-limited countries.

Since LSDV virions are stable in cutaneous lesions and body fluids such as blood, saliva, and semen for several days or even more than a month, extensive transmission of LSDV is possible in the presence of efficient vectors such as blood-sucking insects and ticks that directly transfer the virus into the vascular system of naïve animals [5, 6, 12-15]. The transmission of LSDV by insect vectors and unrestricted movement of cattle have been shown to have contributed to recent spread into the Middle East and recurrent outbreaks in Africa and the Middle East [8, 16]. In general, eradication of arboviruses, such as LSDV and dengue virus, is extremely difficult as standing water that harbours insects exists everywhere throughout the year. Controlling movement of cattle is also challenging as nomadic pastoralists and refugees can mediate the spread of the disease [17]. Although the eradication of LSDV might be a daunting task, it remains important to prevent LSDV outbreaks.

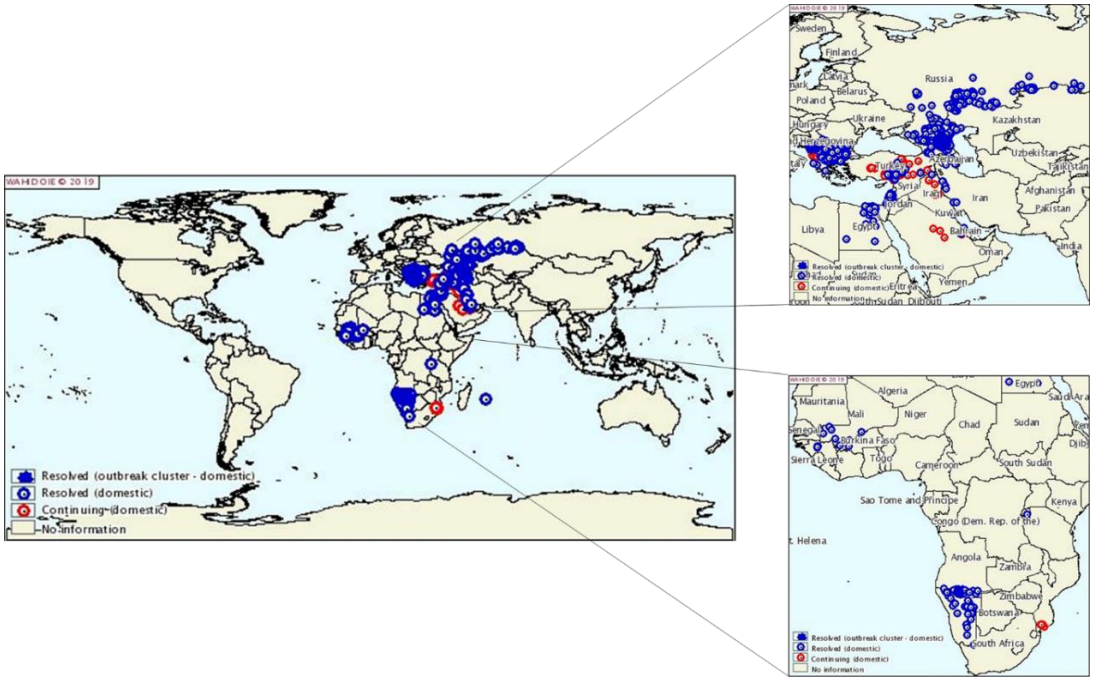



**Figure 1.1: Geographic distribution of lumpy skin disease outbreaks from January 2005 to July 2019.** Adapted from World Organisation for Animal Health (OIE) (2019)[2].

### 1.1.2. LSDV vaccines

There are several manufacturers marketing LSDV vaccines. All commercially available LSDV vaccines are live-attenuated vaccines based on the LSDV strains, Kenyan sheep and goat poxvirus (KSGP) strains or sheep poxvirus (SPPV) strains as there is cross-protection amongst CaPV strains. The use of SPPV- and KSGP-based vaccines against LSDV, however, is not allowed in sub-Saharan countries, where SPPV and KSGP are not co-circulating with LSDV. Therefore, vaccines based on LSDV strains are the only option in such a region. However, local reaction at the injection site, adverse effects following immunisation (AEFI), incomplete protection amongst cattle vaccinated with non-homologous LSDV strains, insufficient attenuation and residual virulence of vaccine viruses have been reported [18-23]. Together, these issues discourage cattle owners from using LSDV vaccines in fear of reduced milk and beef production and damage to skin and hides [8, 18, 22-24].

### 1.1.3. Recombinant LSDV-vectored dual vaccines

As a member of the poxvirus family, LSDV has been explored as a vaccine vector for several infectious diseases including HIV and rabies [25-28]. In addition to the use as a homologous vaccine, LSDV-vectored dual vaccines have been developed to protect animals against lumpy skin disease and other ruminant pathogens such as peste des petits ruminant virus (PPRV), Rift Valley fever virus (RVFV), rinderpest virus (RPV) and SPPV [29-34]. The use of the LSDV

vector is particularly useful for the protection against ruminant pathogens as the vaccine expresses LSDV antigens and only infects and replicates in goats, sheep and cattle, thereby preventing the transfer of vaccine viruses to non-target animals, including humans [35]. Some of the previous vaccine studies showed complete dual protection against homologous virus (LSDV or SPPV) and heterologous virus (RVFV, RPV or PPRV) [30-34]. In addition, long-term protection (~2 years after vaccination) against LSDV was demonstrated and recombinant LSDV vaccines did not transmit the vaccine virus to in-contact animals nor induce adverse effects [32, 33]. These studies motivated us to develop a recombinant LSDV-vectored dual vaccine expressing heterologous bovine leukaemia virus (BLV) envelope (Env) and group-specific antigen (Gag) proteins to protect animals from both LSDV and BLV infections.

## 1.2. BOVINE LEUKAEMIA VIRUS (BLV)

Bovine leukosis is the most common neoplastic disease in cattle and consists of two types of leukosis: enzootic bovine leukosis (EBL) and sporadic bovine leukosis (SBL). The occurrence of EBL is more frequent than that of SBL [36, 37]. SBL is not transmissible and a causative agent of SLB remains unknown. The aetiological agent of EBL is bovine leukaemia virus (BLV), which is a B-lymphotropic oncovirus, belonging to the *deltaretrovirus* genus of the *Retroviridae* family [38]. BLV naturally infects cattle, water buffalo and zebu [39]. BLV infection is characterised by chronic lymphoproliferative disorder, termed persistent lymphocytosis (PL), and development of multicentric lymphomas (or lymphosarcoma) in various organs [40, 41].

### 1.2.1. Host animals and transmission of BLV

#### 1.2.1.1. Host animals

As a natural reservoir, BLV persistently infects four domesticated bovine species: domestic cattle (*Bos taurus*), zebu (*Bos indicus*), water buffalo (*Bubalus bubalis*) and yak (*Bos grunniens*). There is no viral reservoir in wild animals. However, there is a concern about the establishment of the BLV reservoir in other bovine species, particularly indigenous cattle [42]. BLV infection in indigenous cattle has been reported in Asia and Africa (see Section 1.2.2.3) but the BLV seroprevalence appeared to vary amongst different breeds. For example, previous serosurveys in Asia found that individual BLV seroprevalence of Pakistan water buffaloes and Cambodian draught cattle was 0.8% [43] and 5.3% [44], respectively, whereas that of Philippine water buffaloes and Chinese yaks was 27.6% [45] and 21.09% [42], respectively.

It has been shown that dairy cattle, particularly highly productive European cattle raised in modern cattle farming systems are more susceptible to various diseases possibly due to negative energy balance [46-51] and nature of the housing system (crowded environment in tie-stall barns or increased physical contact under the loose housing system) [52-54]. In addition, host genetic backgrounds also influence disease progression. Studies on genotyping of the bovine major histocompatibility complex (BoLA) and its association with PL status of BLV-infected cattle have shown that polymorphism in BoLA class II *DRB3* gene exon 2 (*DRB3.2*) is highly associated with proviral load and resistance or susceptibility to the progression to PL or lymphosarcoma in BLV-infected animals [55-66]. Previous studies have demonstrated that *DRB3.2*0902* and *DRB3.2*0201* alleles are associated with low proviral load (LPL) and resistance to progression to PL and lymphosarcoma whereas *DRB3.2*1201* and *DRB3.2*1501* alleles are associated with high proviral load (HPL) and susceptibility to PL [57-63]. Higher allelic frequency of the susceptible alleles, compared to that of the resistant alleles, have been observed in Philippine, Chilean, Korean, Argentina and Japanese Holstein and Holstein crossbreeds [63, 64, 67-70]. High frequency of susceptible alleles in the Holstein cattle can partly explain why BLV is highly prevalent in dairy cattle and in Japan and Argentina (see Section 1.2.2.2).

In addition to its natural host, BLV can infect a wide variety of mammalian cells [71] and BLV can experimentally infect rats [72], sheep [73-78], goats [75, 79] and rabbits [80-82]. However, pathogenesis and disease symptoms in these BLV-infected animals differ. Sheep develop lymphosarcoma at a higher frequency (i.e., almost all infected sheep) and disease progress faster (i.e, a 1-4 year latency period) than cattle [75, 78, 83-87]. For these reasons, sheep were considered to be a useful animal model to monitor the disease progression and protective effects of vaccines [87-96]. However, it has been shown that BLV-infected cattle and sheep display different kinetics of proliferating B cells and protective effects on vaccines [87, 93, 97]. Therefore, cattle are a more suitable animal model to assess vaccine-induced immune responses and efficacy of vaccines. In contrast to sheep and cattle, experimental infection of BLV in rabbits results in immune dysfunction but do not develop lymphoma [80-82]. Therefore, rabbits are not an ideal animal model to study mechanisms of pathogenesis and disease progression but can be used to test the safety of vaccines and monitor the presence of anti-BLV immune responses [82].

Recently, anti-BLV Gag antibodies were detected from human sera [98] and a segment of the BLV genome was detected from human mammary epithelium [99] and breast tissue [100]. These observations indicate that BLV has a wider host tropism than previously thought and this has raised a concern about the zoonotic infection of BLV into humans through BLV-infected milk and possible association of BLV infection in women with breast cancer [99].

### 1.2.1.2. Pathogenesis – silent infection

EBL is a chronic infection, characterised by abnormal proliferation of non-neoplastic B cells (10,000 cells/µl blood) during the PL stage and culminating in the development of malignant neoplastic lymphosarcoma in various organs (Figure 1.2) [41]. All animals with lymphosarcoma result in death.

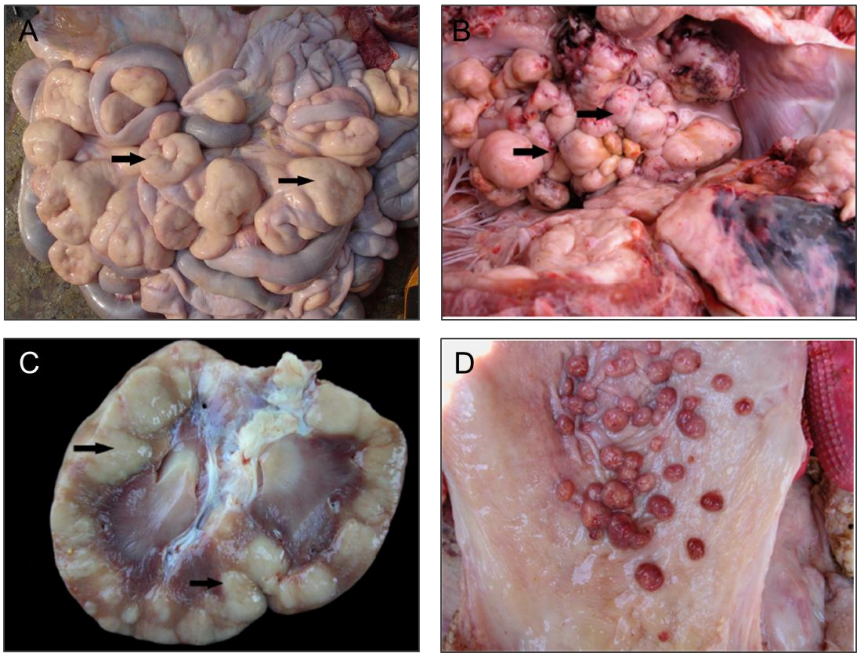



**Figure 1.2: Mass of neoplastic tissues formed in abdominal and thoracic organs. (A)** White enlarged mesenteric lymph nodes (arrows) in the intestine. **(B)** Nodular neoplastic masses bulging from the endocardial surface of the right atrium and ventricles. **(C)** White nodules of leukotic tissues on the surface of cortex and medulla of a kidney. **(D)** Pink neoplastic masses on the mucosal surface of the urinary bladder. Adapted from Yoon *et al.* (2005) [41].

Despite the devastating results following the infection, BLV infection is characterised by its silent nature. Firstly, infected animals do not show visible disease symptoms such as lymphadenopathy and nasal discharge and thus, BLV infection can be only confirmed by serological or molecular tests. Secondly, BLV infection does not cause PL or lymphosarcoma to all infected cattle (Figure 1.3) and the disease progresses slowly without apparent viremia during a 7 to 10 year latency period [83, 101]. Up to 30% of infected animals (PL animals) develop persistent lymphocytosis (PL) within 3 to 6 years after infection. The remaining 70% of the infected animals are aleukemic and asymptomatic without any haematological or clinical abnormalities. These aleukemic or non-PL animals harbour high (≥100,000 copies) or low (≤100 copies) proviruses in their infected lymphocytes (HPL and LPL, respectively) [102]. The non-PL animals with HPL are able to transmit the infection as efficiently as PL animals [102]. Of all the infected animals, irrespective of PL status, only 5 to 10% of the cows eventually

develop lymphoma after a long latency period. The lymphosarcoma stage is characterised by increased monoclonal populations of neoplastic B lymphocytes and eventually results in death [101].

Without appropriate diagnostic tests, it is difficult to accurately determine infectious status amongst herds as only less than 10% of the infected animals exhibit clinical symptoms. However, since both PL animals (30%) and non-PL animals with HPL (28%) are efficient transmitters of infection, actual infectious animals constitute approximately 60% of the infected animals. Thus, infectious status at the herd level is often underestimated and as a result, adherence to sterile methods during veterinary procedures tends to be neglected by veterinarians and cattle owners. Consequently, the infection can be spread extensively within and between herds by the time clinical symptoms are visible or animals die or are slaughtered.

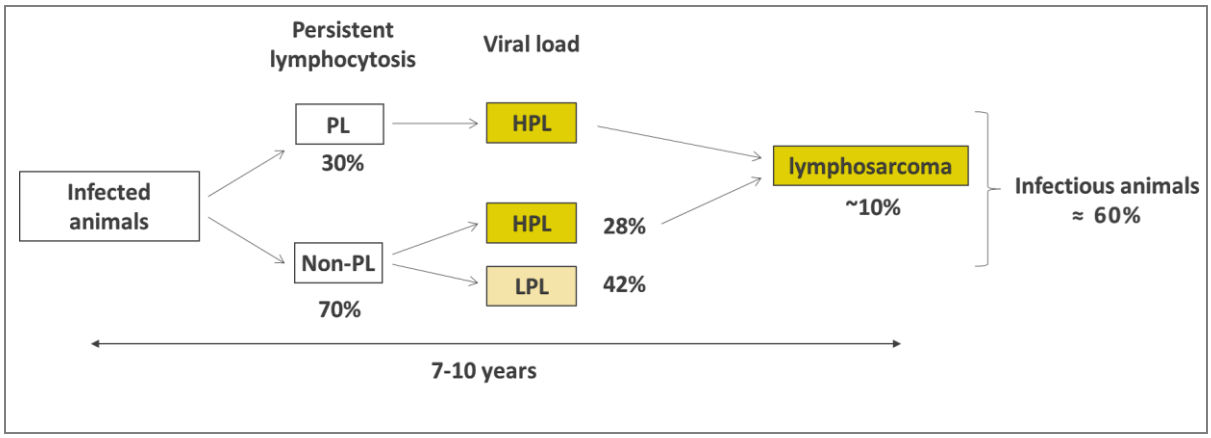



**Figure 1.3: Disease progression of BLV-infected cows.** BLV-infected animals with persistent lymphocytosis (PL) or without PL (non-PL) are determined by total lymphocyte count (TLC) (Bendixen (1965) [103]; Ferrer, *et al.* (1978) [83]). These animals are further classified into two groups, animals with high proviral load (HPL) or low proviral load (LPL), based on the number of proviral copies per microgram of DNA in infected peripheral blood lymphocytes (PBLs) and antibody titres against BLV p24 and gp51 proteins as specified by Juliarena *et al.* (2007) [102]. The dark yellow boxes indicate highly infectious animals whereas the light-yellow box indicates less infectious animals.

### 1.2.1.3. Within-herd transmission

BLV infection can spread in two ways, horizontal and vertical transmissions. Horizontal transmission occurs by physical contact with infected animals, BLV-carrying insect vectors and iatrogenic procedures. Since BLV virions are unstable, the transmission of BLV is considered to occur exclusively through the transfer of infected cells in blood or milk [104]. As a result, the majority of the transmission is mediated by direct transfer of infected bloodily fluids during iatrogenic procedures such as ear tattooing, dehorning, reuse of needles and gloves for veterinary procedures and plastic sleeves for rectal palpation for artificial insemination, detection of pregnancy and embryo transfer [105-109], which are common practices in the management of dairy and beef cattle. Ear tattooing, dehorning and vaccination

inevitably involve bleeding from an applied area, and reuse of instruments, gloves, needles permit transfer and spread of BLV-infected blood between animals. Due to a large number of animals that have to be processed at a time and lack of awareness of BLV infection, septic practices during veterinary examination have been frequently neglected [110, 111].

Vertical transmission from dam to calf involves utero/placental transmission, perinatal transmission during delivery or postnatal transmission through the consumption of infected colostrum and milk. BLV provirus and antibodies have been detected in colostrum [111-118] and a study examining 129 calves born to infected dams showed that overall, 18.6% of the calves were infected by vertical transmission, in which 10.8% and 7.7% of infected calves were due to transplacental and perinatal transmission, respectively. In another study examining 26 calves born to infected dams, no transplacental transmission was observed [119]. Although vertical transmission appears inefficient, the consequence of vertical transmission is not negligible. A study conducted [117] in Argentina showed that despite stringent management practices during iatrogenic procedures, BLV seroprevalence in a dairy farm increased with age: 11.4% amongst calves, 61.7% following first delivery and 86.5% in adult cows. Their follow-up study [112] showed that all calves born to infected dams reached high proviral load at 12 months of birth, which persisted during a 3-year study period. Since calves are immunosuppressed during the first 4 months after birth [120, 121], unlike infected adult cows [122, 123], infected calves could not efficiently control BLV infection. Therefore, infected calves can become a source of infection and facilitate the spread and persistence of BLV infection within-herd if dams are not tested regularly.

In addition to new-born calves, an Iranian study [110] revealed that imported animals and newly-brought heifers examined in this study did not undergo a serological test for BLV infection and these animals were subsequently placed in existing herds. These studies together highlight the significance of the compulsory serological tests for pregnant dams, newly-brought animals as well as semen and embryos for artificial insemination and fertilisation.

Once BLV-infected animals are detected, these animals need to be slaughtered or separated from uninfected animals to prevent further spread of infection. As a result, additional facilities and costs are required and these measures are indubitably expensive for small-scale and private farmers if governmental subsidiaries are not available. Therefore, a more cost-effective and less labour-intensive preventative measure should be implemented, and vaccines can live up to such requirements.

### 1.2.2. Epizootiology of enzootic bovine leukosis (EBL)

#### 1.2.2.1. History of BLV prevalence

The occurrence of leukosis in cattle was first reported by Leisering [124] in 1871 in Germany, who found yellowish nodules in the enlarged spleen of a cow. However, the causative agent of the leukosis in cattle remained unknown for the next 100 years. During the early to mid-1990s, various studies demonstrated that C-type retroviruses would cause leukaemia and lymphosarcoma in other animal species [125] such as chicken [126-128], mice [129-132] and cats [133-135]. It had long been suspected that a C-type virus was also associated with enzootic bovine leukosis (EBL) [136-139]. In 1969, bovine leukaemia virus (BLV) was isolated from cattle with lymphosarcoma and it was identified as the aetiological agent of EBL [140].

The origin of BLV is unknown but an initial epicentre of the EBL incidence is considered to be in Klaipeda in Lithuania [141]. Early studies in the late 1800s and early 1900s indicate that ELB was already endemic in Europe and spreading to BLV-free countries in Western Europe [142-144]. The importation of BLV-infected cattle into BLV-free countries appeared to facilitate the global spread of BLV [145]. BLV is thought to have been introduced into North America during the early 1900s and it is assumed that BLV was reintroduced from Canada into some BLV-free European countries through the importation of BLV-infected cattle [146, 147]. BLV was also thought to be introduced into other geographic regions worldwide, such as Ireland [148, 149], Bulgaria [150], Russia [151], Egypt [145] and Mongolia [152] through the importation of BLV-infected cattle.

#### 1.2.2.2 Current status of BLV prevalence

##### 1.2.2.2.1. Europe

Geo-economic situations in the European Community (now European Union) particularly facilitated the spread of BLV amongst European countries in the early and mid-1990s. For example, in the Netherlands, due to the strict national import policy, EBL incidence amongst imported and exported cattle was not reported prior to 1965 [153]. However, following the amendment of European Economic Community regulations in 1965, importation of cattle from partner countries became easier, resulting in the introduction of BLV in the Netherlands [153].

Despite the persistence of BLV for many decades, Europe became a role model as a BLV-free region. The European Community played a pivotal role in the successful eradication and elimination of EBL from the majority of the European countries (Figure 1.4). A framework to monitor, control and eradicate, animal diseases (e.g., bovine tuberculosis, brucellosis and EBL) was established in 1977 to protect livestock sectors from influences of animal disease

outbreaks [154]. The EU co-financed the EBL eradication program was started in 1993 and covers the cost of serological and milk tests as well as compensation for slaughtering animals [155, 156].

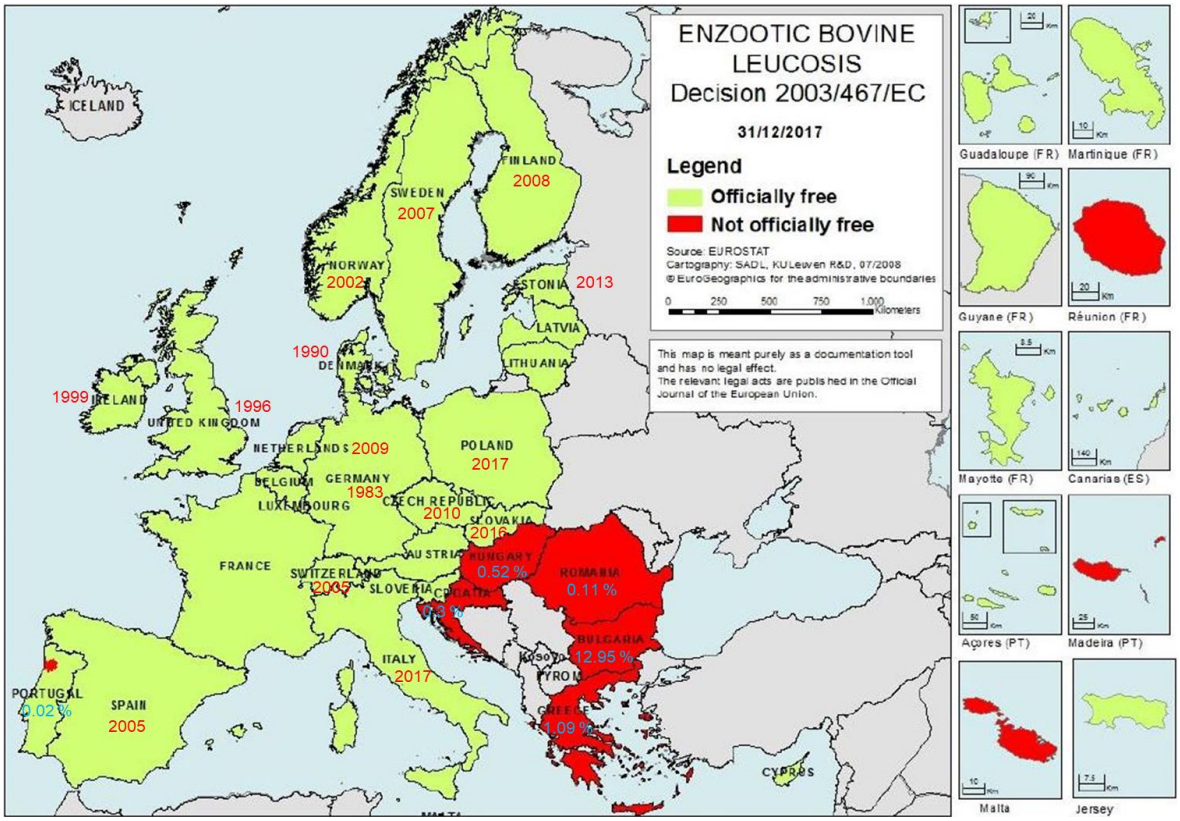



**Figure 1.4: Disease status of enzootic bovine leukosis in Europe in 2017.** A year BLV-free status achieved [157-162] and herd-prevalence (percent) in 2015 [160] are shown in the figure. Map adapted from Bovine and Swine Diseases Situation 2017 [160].

Since the implementation of this program, the EBL incidence has significantly decreased in the EU Member States. In 2006, another program [163] was started to co-finance several countries (Italy, Lithuania, Latvia, Poland and Portugal) to accelerate the eradication process where BLV still persisted. All these countries except Portugal achieved BLV-free status by 2017 and EBL is near eradication in Portugal, being present only in specific areas with low herd prevalence (0.02% in 2015) [164]. In 2010, the EU co-financed EBL eradication program was terminated following the successful eradication of EBL in the vast majority of the Member States [165]. As of 2017, 22 Member States have achieved EBL-free status and EBL remains present only in six countries (Figure 1.4). A 2015 survey showed low herd prevalence in five of these countries (Greece, Hungary, Croatia, Romania and Portugal), approximately 1% or less (Figure 1.4). Only in Bulgaria, was there a high herd prevalence of 12.95%.

#### 1.2.2.2.2. Global trend

Currently, BLV is endemic globally (Figure 1.5). Although it is possible to eradicate BLV, some countries have extremely high BLV seroprevalence. Table 1.1 summarises herd and individual BLV seroprevalence of dairy and beef cattle in Japan, Canada, USA and Argentina. Although it is difficult to compare seroprevalence between study years, different regions and countries, these countries show more than 65% of herd prevalence in dairy herds and varying degrees of individual seroprevalence (20.8% to 70.0%) in dairy cattle, which is generally higher than the individual seroprevalence of beef cattle. Furthermore, herd and individual seroprevalence of dairy cattle in all four countries remains high or has been increasing during 10- to 20-year study periods. Figure 1.6 is a graph showing higher individual BLV seroprevalence in dairy cattle compared to beef cattle and an increasing trend of individual BLV seroprevalence in dairy cattle in 1980, 1982 and 2007 in Japan [52].

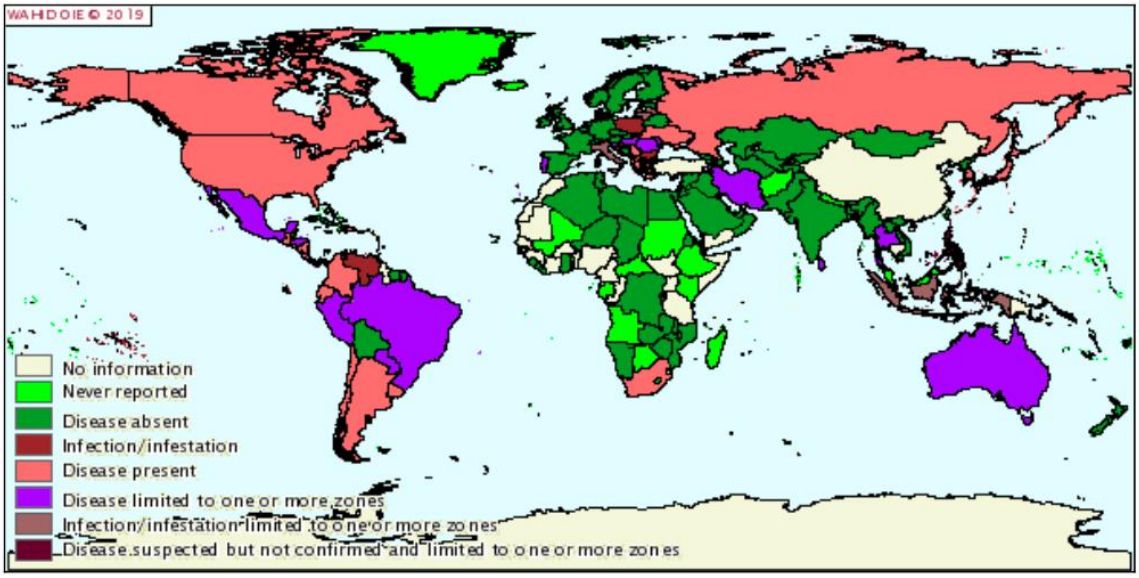



**Figure 1.5: Worldwide infectious status of enzootic bovine leukosis from January to June in 2016.** Taken from World Organisation for Animal Health (OIE) (2019) [166].

Persistence and high prevalence of BLV in some countries such as Bulgaria, Argentina, Japan, Canada and the USA appears to be mainly due to the delayed implementation of national eradication programmes in formerly non-EU countries (i.e., prior to the enlargement of the European Union in 2007), the absence of national compulsory disease control schemes [52, 167], modern housing system (tie-stall *vs* loose housing system; the latter increases physical contact between animals) [52-54, 168] and low awareness of BLV infection amongst veterinarians and cattle farmers [111, 169]. Currently, there is no national BLV eradication program in these countries including Japan [170], Canada [171, 172] and the USA [111, 173].

**Table 1.1: Nationwide and regional BLV seroprevalence for various years in Japan, Canada, USA and Argentina.**

| Country | Region | Study year | Test method | Herd prevalence | | Individual prevalence | | References |
|---|---|---|---|---|---|---|---|---|
| | | | | Dairy cattle | Beef cattle | Dairy cattle | Beef cattle | |
| **Japan** | 7 prefectures | 2007 | ELISA and AGID | 67.5% (overall)<br>79.1% | 22.0% | 28.6% (overall)<br>34.7% | 11.9% | [52, 174] |
| | Nationwide | 2009-2011 | ELISA | 78.0% | 69. 0% | 35.2% (overall)<br>40.9% | 28.7% | [170] |
| **Canada** | Maritime region | 1998-1999 | ELISA | 70.0% | | 20.8% | | [175] |
| | Alberta | 2002 | ELISA | 86.7% | | 26.9% | | [176] |
| | Manitoba | 2002 | ELISA | 97.4% | | 60.8% | 10.3% | [177] |
| | Saskatchewan | 2001 | ELISA | 89.1% | | 37.4% | | [178] |
| | Maritime region | 2013 | ELISA | 90.8% | | 30.4% | | [179] |
| **USA** | Nationwide | 1996 | AGID | 89.0% | | 43.5% | | [180, 181] |
| | Nationwide | 1997 | AGID | | 38.0% | | 10.3% | [181] |
| | Kansas | 2004 | AGID | | | | 8.5% | [182] |
| | 17 states | 2007 | ELISA | 83.9% | | | | [183] |
| | Nationwide | 2014-2015 | ELISA | | | 38.6% (overall)<br>47.6% | 33.6% | [184] |
| | 11 states | 2018 | ELISA | 94.2% | | | | [111] |
| **Argentina** | 4 provinces | 1998-1999 | ELISA, AGID and PCR | 84% | | 32.9% | | [167] |
| | 5 sites | 2004 | ELISA and PCR | 73.5% | | 70.0% | | [185] |
| | Rafaela (Santa Fe) | 2009 | ELISA and PCR | 86% | | | | [185] |
| | General Roca (Río Negro) | 2009 | ELISA and PCR | 90.5% | | | | [185] |
| | Susana (Santa Fe) | 2009 | ELISA and PCR | 92% | | | | [185] |
| | Ataliva (La Pampa) | 2009 | ELISA and PCR | 85% | | | | [185] |

ELISA, enzyme-linked immunosorbent assay; AGID, agar gel immunodiffusion.

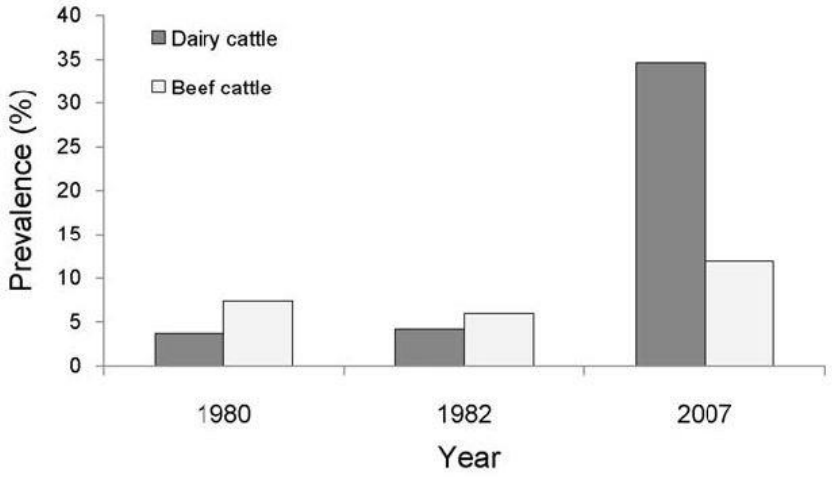



**Figure 1.6: Individual BLV seroprevalence in dairy and beef cattle in Japan in 1980, 1982 and 2007.** Taken from Murakami *et al*. (2011) [52].

Eradication of BLV is a lengthy process and achievement of BLV-free status following the implementation of eradication programmes usually takes more than 10 years. Therefore, implementation of proper disease control schemes and adherence to disease control programmes also play essential roles in accelerating eradication processes. For example, an increase in BLV seroprevalence from 8.49% to 33.38% in Bulgaria from 1997 to 2012 [150, 186] was due to the implementation of the serological tests alone (i.e., no culling of BLV-positive animals or herds) amongst underfinanced cattle farmers [150]. In Malta, EU co-financed serological testing was implemented in 2002 but it was interrupted until 2007 [187]. A national control scheme started only recently, in 2010 [187], and as of 2018, BLV is still present in Malta [188]. In contrast, Australia is an exemplary country for the successful and speedy elimination of BLV from dairy herds. Australia is officially declared BLV-free status in dairy herds in 2013 following the implementation of the National Dairy EBL Eradication Program in 2008 [189-191].

#### 1.2.2.2.3. Africa

Since serosurveys for the occurrence of BLV in Africa have not been conducted, the prevalence of BLV in Africa is largely unknown. Available data indicate relatively low seroprevalence amongst African dairy and beef cattle (Table 1.2). However, the individual seroprevalence of dairy and beef cattle in Tanzania was 41% and 21.4%, respectively during a study period between 1991 and 1994 [192]. Dairy cattle in Tanzania originated from Europe and Canada whereas beef cattle originated from an area where there was no contact with exotic European cattle [192]. Therefore, it was suspected that BLV was introduced into dairy cattle by the importation of cattle from BLV-present countries, and beef cattle were infected

through contact with infected dairy cattle in communal pastoral sites. To the best of our knowledge, there is no recent data of BLV seroprevalence in South Africa, but high EBL incidence has been reported in South Africa (Afrivet, 2018).

**Table 1.2: BLV seroprevalence for various years in six selected African countries.**

| Country | Study year | Herd prevalence | Individual prevalence | | References |
|---|---|---|---|---|---|
| | | | Dairy cattle | Beef cattle | |
| **Botswana** | 1990 | | 7.75% (overall) | | [193] |
| | | | 5.55% | 8.67% | |
| **Kenya** | 2016 | | 7.6% (overall) | | [194] |
| **Somalia** | 2008-2009 | 30.0% for native Somali breed | 1.5% for native Somali breed | | [195] |
| **South Africa** | 1996 | | 12.6% for mixed herds (Mafikeng area of the North West province) | | [196] |
| | 2011 | | 4.54% for dairy cattle (Gauteng and Mpumalanga provinces) | | [169] |
| **Tanzania** | 1991-1994 | | 41% | 21.4% | [192] |
| **Zambia** | 2001 | | 5.0% for indigenous cattle | | [197] |

### 1.2.3. Virology of BLV

#### 1.2.3.1. BLV virion and genomic structures

BLV is a member of the *Deltaretrovirus* genus of the *Retroviridae* family. BLV is phylogenetically related to human T-lymphocyte leukaemia virus (HTLV), which is a T-lymphotropic oncovirus in humans [198, 199]. BLV is an enveloped virus and has pleomorphic shapes, with its diameter ranging from 80 to 130 nm [140, 200, 201]. The outer layer of the BLV virion is a lipid bilayer in which envelope glycoproteins (Env) are embedded (Figure 1.7). Two copies of a positive-sense single-stranded RNA, reverse transcriptase (RT) and integrase (IN) are packed in each BLV virion and they are surrounded by capsid protein (p24) and matrix protein (p15). Genomic RNAs are associated with nucleocapsid proteins (p12). These structural proteins have been poorly studied, and only the Env transmembrane protein [202] and capsid protein [203] have been resolved by crystallography. More studies on structural proteins will help identify major antibody target sites and immunodominant epitopes useful for the design of efficacious vaccines.

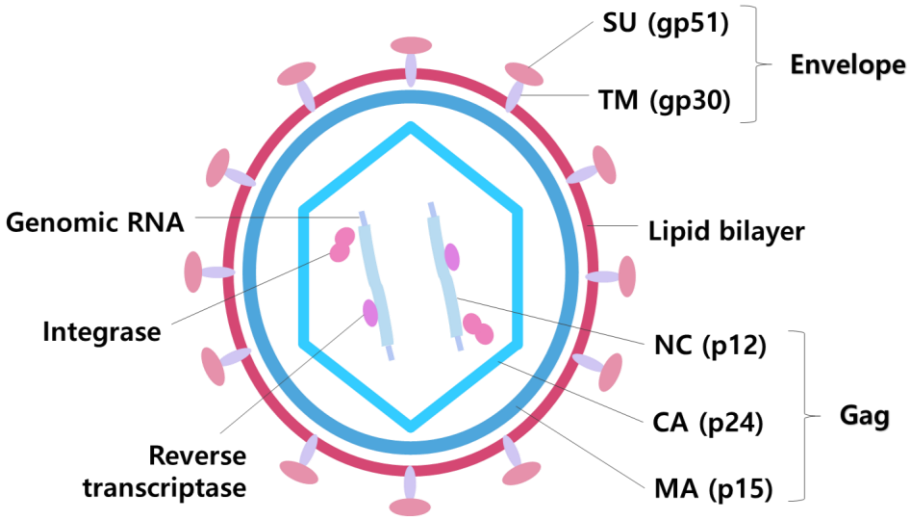



**Figure 1.7: Schematic representations of BLV virion. SU**, surface protein; **TM**, transmembrane protein; **NC**, nucleocapsid; **CA**, capsid; **MA**, matrix. Adapted from Barez *et al.* (2015) [204], Gillet *et al.* (2007) [200] and Polat *et al*. (2017) [205].

The complete BLV proviral DNA genome consists of 8,714bp [206]. As a complex retrovirus, BLV genome has a conserved modular genomic organisation with a set of structural genes (*gag*, *pro*, *pol* and *env*) at the 5' end of the proviral genome, followed by accessory genes (*G4, R3* and microRNAs) and regulatory genes (*tax, rex*) at the 3' end (Figure 1.8). The BLV proviral genome is flanked by two identical long terminal repeats (LTRs) at the 5' and 3' ends of the BLV genome. Both LTRs comprise three consecutive regions, U3, R and U5 regions, and contain a number of *cis*-acting regulatory sequences such as an enhancer sequence called Tax responsive element (TxRE), E box regulatory sequences and NF-κB binding site.

The structural genes encode proteins essential for the viral infection and replication whereas the regulatory gene products modulate viral gene expression and are involved in leukemogenesis. Functions of the accessory genes remain largely unknown but they are likely to be involved in the posttranslational regulation of the viral gene expression and pathogenesis.

The genomic organisation of a 1.8 kb region between the *env* gene and 3' LTR was long unknown (thereby called pX region). Subsequently, it was discovered that the pX region contains a microRNA (miRNA) ORF, regulatory [207, 208] and accessory genes [209]. Later studies [73, 74, 210] demonstrated that deletion in the *R3*/*G4* genes or *G4* gene resulted in reduced proviral load. Viral propagation and pathogenesis in rabbits and sheep inoculated with BLV deletion mutant showed that the deletion mutants were infectious. These studies led to the development of live-attenuated vaccines using deletion mutants (see Section 1.2.4.2).

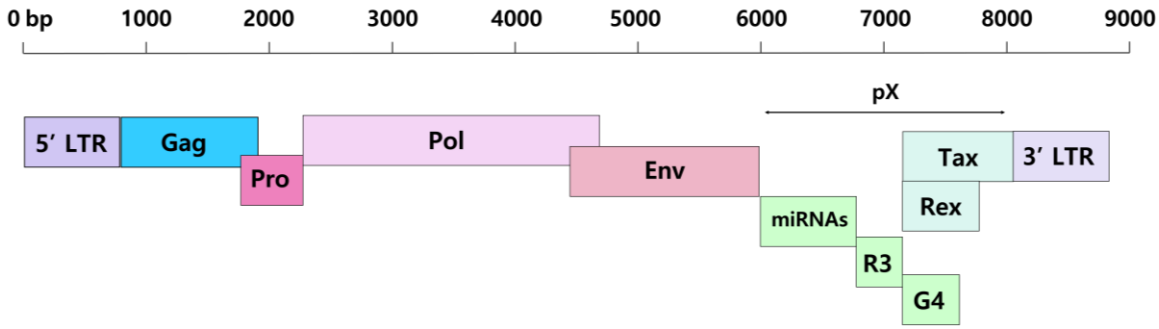



**Figure 1.8: Modular organisation of BLV proviral genome. LTR**, long terminal repeat; **Pro**, protease; **Pol**, DNA-dependent RNA polymerase; **Env**, envelope; **miRNA**, microRNA. **pX,** pX region. Adapted from Barez *et al*. (2015) [204], Gillet *et al*. (2007) [200], Polat *et al*. (2017) [205] and Rice *et al*. (1985) [211].

### 1.2.3.2. Replication, transcription and translation of BLV

The mechanism of BLV replication is identical to that of the human immunodeficiency virus (HIV), using the viral reverse transcriptase and strand transfer events to synthesise double-stranded viral DNA, which is subsequently integrated into the host genome [212, 213]. It has been shown that only 25-30% of circulating lymphocytes in leukemic animals harbour the provirus whereas approximately 70% of the tumour cells harbour the provirus [214, 215]. It has been also demonstrated that 1 to 4 copies of the provirus can be integrated as an intact or truncated viral genome [216]. The proviral integration appears to be random and not site-specific and thus, it is unlikely that insertional mutation contributes to BLV leukemogenesis [101, 216, 217].

All promoters except for those driving the expression of viral miRNAs are located in the 5’ LTR [218]. Transcription initiates from the CAP site at the 3’ end of the U3 region and terminates at the transcription terminal signal at the 3’ end of the R region in the 3’ LTR (Figure 1.9). The viral mRNAs are then polyadenylated [219] and exported into the cytoplasm [220], where the synthesis of viral proteins and posttranslational processing of polyproteins takes place [221].

The genomic RNA also serves as a template to transcribe the monocistronic Gag-Pro-Pol mRNA containing three different ORFs with the termination signal at the 3’ end of each ORF [211]. The translation of the Gag-Pro-Pol mRNA produces Gag precursor (pr45) (see Section 1.2.3.4), Gag-Pro precursor (pr66) or Gag-Pro-Pol precursor (pr145) proteins (Figure 1.10). Normal translational termination in the *gag* ORF results in the production of the Gag precursor whereas the frameshift translation produces the Gag-Pro or Gag-Pro-Pol precursors [211]. The posttranslational cleavage of the Gag-Pro precursor by the viral protease [221] results in the production of the protease whereas that of the Gag-Pro-Pol precursor protein (pr145) produces the reverse transcriptase (RT) [211]. The BLV RT has RNA-dependent DNA polymerase, DNA-dependent DNA polymerase, RNase H and integrase activities. The BLV RT has the mutation rate of $1.2 \times 10^{-5}$ mutation per bp per replication cycle [222], which is lower

than that of the HIV RT, ranging from $1.4x10^{-5}$ to $3.4x10^{-5}$ mutation per bp per replication cycle [223, 224].

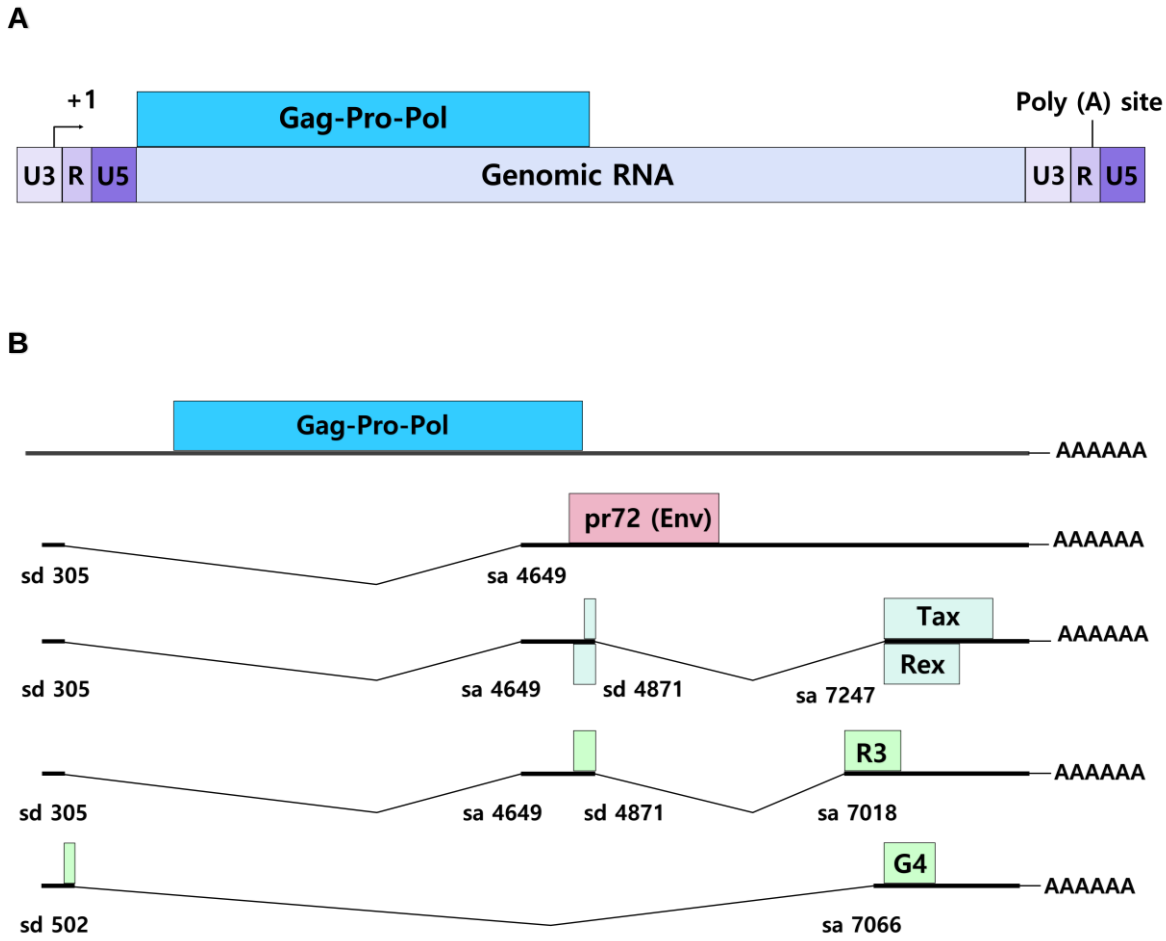



**Figure 1.9: Schematic representations of BLV proviral genomic RNA and transcript organisation. (A)** Genomic RNA and a template for transcription of a Gag-Pro-Pol precursor. **(B)** Messenger RNAs of a Gag-Pro-Pol precursor, Env precursor (pr72), Tax, Rex, R3 and G4 proteins. Nucleotide positions of splicing donor and acceptor sites are indicated by numbers. **LTR**, long terminal repeat; **Pro**, protease; **Pol**, polymerase; **Env**, envelope; **miRNA**, microRNA. **+1**, transcription start site; **Poly(A) site**, polyadenylation site; **pr**, precursor; **sd**, splice donor; **sa**, splice acceptor. **AAAAAA**, polyadenylation. Adapted from Alexandersen *et al*. (1993) [209], Barez *et al*. (2015) [204], Gillet *et al*. (2007) [200], Polat *et al*. (2017) [205] and Rice *et al*. (1985) [211].

The Env, R3 and G4 mRNAs are also monocistronic and single spliced Env and G4 mRNAs encode Env precursor (pr72) (see Section 1.2.3.3) and G4 protein (p11), respectively whereas the double spliced R3 mRNA encodes R3 protein (p5) [209]. The G4 expression appears to have oncogenic potential. Alexandersen *et al*. (1993) [209] demonstrated that the G4 mRNA was expressed at the beginning of the PL stage and the abundance of the G4 protein correlated with high lymphocyte counts in PL cows. Furthermore, Kerkhofs *et al*. (1997) [210] showed that the co-transfection of primary rat embryo fibroblast (REF) cells with plasmids expressing the *G4* gene and Harvey rat sarcoma virus (H-ras) oncogene transformed the REF

cells and induced tumours in mice. The function of the R3 protein remains unknown but is likely to be involved in the post-translational regulation of viral gene products [209].

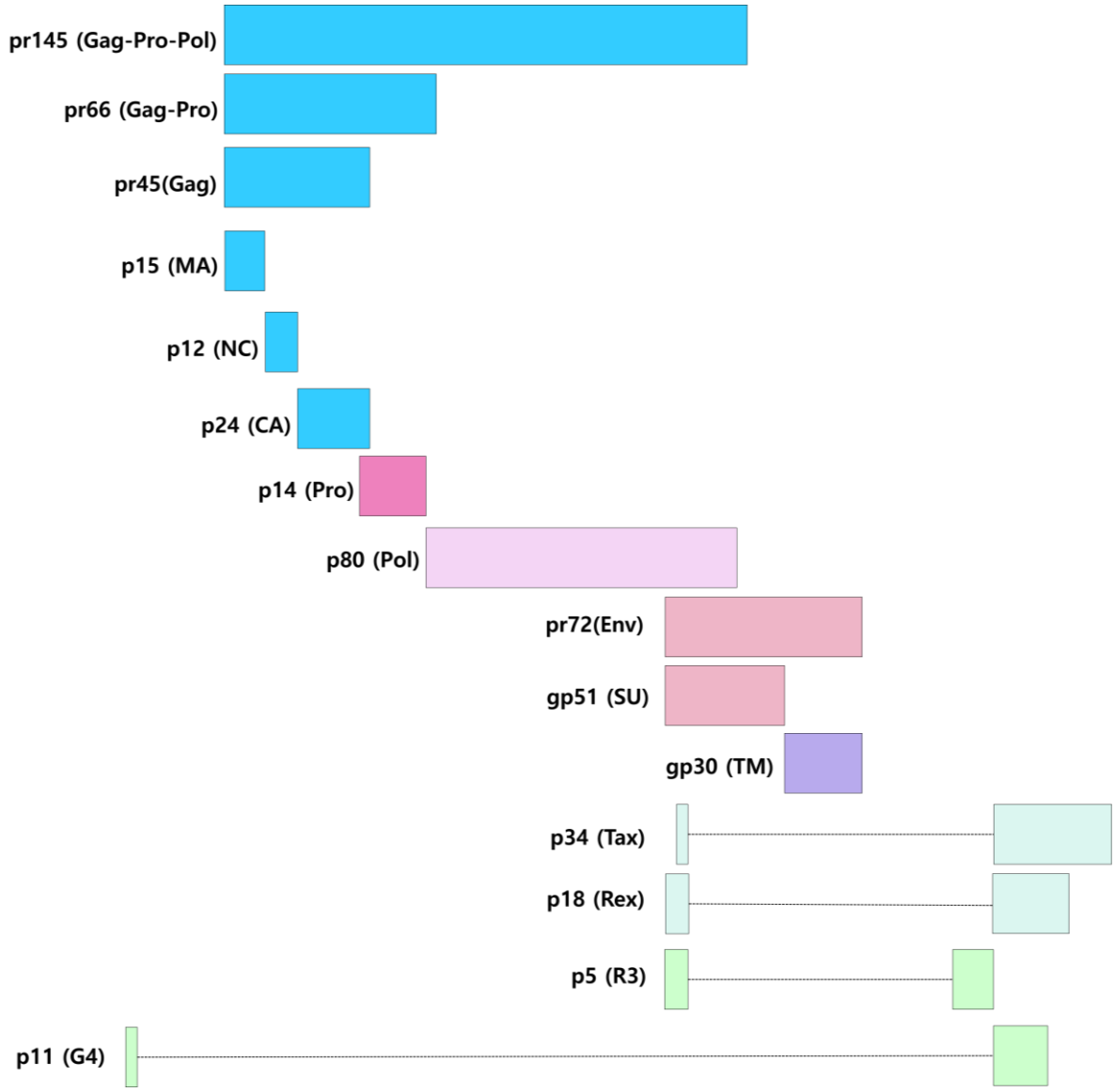



**Figure 1.10: Schematic representation of precursor polyproteins and posttranslationally processed proteins**. **Gag-Pro-Pol,** precursor polyprotein of Gag, protease and polymerase; **Gag-pro**, precursor polyprotein of Gag and protease; **MA**, matrix; **NC**, nucleocapsid; **CA**, capsid; **Pro**, protease; **Pol**, DNA-dependent RNA polymerase; **Env**, envelope; **SU**, surface protein; **TM**, transmembrane protein. Adapted from Barez *et al*. (2015) [204], Gillet *et al.* (2007) [200] and Polat *et al.* (2017) [205].

Transcription of *tax* and *rex* genes also initiates in the U3 region of the 5' LTR, producing a bicistronic mRNA [225]. The double spliced Tax-Rex mRNA encodes Tax and Rex proteins from two different but overlapping open reading frames (ORFs). The Tax protein appears to remain in the nucleus of the infected cells, where like the HIV Tat protein, the Tax protein functions as a transcriptional activator (transactivator) [207, 226-228] through its binding on

the cyclic-AMP responsive (CRE) site in the 5' LTR [227]. Like G4 protein, Tax protein also appears to be involved in pathogenesis and leukemogenesis. Co-transfection of primary REF cells with expression vectors containing the BLV *tax* gene and the H-ras oncogene immortalised and transformed the REF cells [229].

The Rex protein, a nuclear phosphoprotein, was also found to act as a *trans*-activating protein in the nucleus [220]. The Rex protein is likely to play a role in the nuclear export of full-length genomic mRNA (also Gag-Pro-Pol mRNA) and single spliced mRNA (Env mRNA) through the nuclear export signal in the R region of the 3' LTR [220]. This leads to increased production of structural proteins and decreased production of a double spliced *tax-rex* mRNA.

In 2012, *Kincaid et al.* [230] identified five precursor hairpins of viral miRNAs (named B1 to B5) in a persistently infected bovine B -cell line, which were transcribed from a region between *env* and *R3* genes. Subsequent studies have indicated that the BLV miRNAs are likely to be oncomiR [230-232] and they are abundantly expressed (i.e., up to 40% of the total miRNA in BLV-infected cells) in pre-leukemic cells, BLV-infected plasma, PBMC and tumours, where the structural and regulatory gene expression is suppressed [232, 233]. It was also shown that the level of the BLV miRNA expression appears to correlate with proviral load [232]. Furthermore, a functional reporter assay identified host transcripts that are targeted by the BLV miRNAs and demonstrated that the BLV miRNAs are likely to be involved in the suppression of apoptosis, tumorigenesis and viral persistence in BLV-infected cells [232].

#### 1.2.3.3. Envelope (Env)

The BLV envelope (Env) proteins are glycoproteins presented on the surface of the virion as a trimer of surface (gp51) and transmembrane protein (gp30) heterodimers. The BLV *env* gene is 1,548bp in size with the 55bp 5' end of the *env* ORF overlapping with the 3' end of the *pol* ORF [189]. The *env* gene encodes the Env precursor (pr72), which is cleaved at the RVRR cleavage site by subtilisin/kexin-like convertases, such as furin, into the gp51 and gp30 glycoproteins [234] (also see Section 4.2.2.1). Disulphide bonds between the gp51 and gp30 glycoproteins link them together, conferring stability to the gp51-gp30 complex [235]. The Env glycoproteins play an important role in receptor binding [236] and cell fusion during the natural infection [237].

The gp51 surface protein is rich in binding domains and epitopes such as putative receptor-binding domain (RBD) [236, 237], antibody epitopes (conformational and linear epitopes) [238-243], T-cell epitopes [244, 245], neutralising domains [241, 246-248] and $Zn^{2+}$-binding site [237], which are important in cell fusion and entry during the natural infection we as well as immunogenicity (also see Chapter 2). The gp51 glycoprotein also contains a leader sequence,

which is important to target the Env proteins to the endoplasmic reticulum (ER) membrane [249] (also see Section 4.2.1.2). The gp30 transmembrane protein contains a fusion peptide at the N-terminus, which is important for cell fusion and entry [250-252], and a cytoplasmic tail at the C-terminus, which is essential for viral entry and immune evasion [250, 253]. Unlike the gp51, the gp30 is not rich in domains and epitopes and poorly immunogenic. Yet, the gp30 contains some important domains and epitopes such as highly immunogenic GD21 epitope, which is conserved in all primate T-lymphotropic viruses and BLV genus [247], immunosuppressive domain (ISD) [254, 255] and immunoreceptor tyrosine-based activation motif (ITAM) [250, 253]. The ITMA in the cytoplasmic tail appears to play a role in viral infectivity and propagation, packaging of Env glycoproteins in nascent virions, and downregulation of Env expression on the virion surface to evade host immune responses [250, 253]. Importantly, the Env protein is one of the major antibody targets and thus, has been used as an antigen in various BLV vaccines (see Section 1.2.4). The Env sequence is also utilised for phylogenetic analyses and genotyping of BLV natural isolates (see Chapter 2).

#### 1.2.3.4. Group-specific antigen (Gag)

The group-specific antigen (Gag) is another structural protein essential to form a BLV virion. The *gag* gene is 1,180bp in size and encodes the Gag-Pro precursor (pr66) from the Gag-Pro-Pol mRNA [256, 257]. The Gag-Pro precursor is then, proteolytically cleaved by the viral protease into the Gag precursor (pr45). The Gag pr45 is further processed by the viral protease into the matrix (p15), capsid (p24) and nucleocapsid (p12). The nucleocapsid, which is tightly interacted with the secondary structure of the genomic RNA at the 5' end, is necessary for morphogenesis and encapsidation [258-260]. The capsid contains T-cell epitopes [261] and a major homology region (MHR) [262], which is conserved amongst *Retroviridae* [263] and indispensable for viral infectivity. The capsid is another major target of host immune response but unlike the anti-gp51 antibody, which is persistently detectable in leukemic and aleukemic animals with low proviral load, the anti-p24 antibody is undetectable in aleukemic animals with low proviral load [102].

The matrix is myristoylated at the N terminus [264] and plays an essential role in encapsidation and localisation of nascent BLV virions to the host cellular membrane as well as subsequent budding from the infected cells [264-270]. It has been shown that the presence of the Gag-Pro or Gag-Pro-Pol sequences are sufficient for the formation of the virus-like particles (VLPs) and budding [260, 271]. In other words, the VLP formation and budding do not require other viral genes and genomic RNA. This minimum genetic requirement and self-assembly capacity are useful for the formation of VLPs from a simple Gag expression vector and VLPs derived

from other families of viruses have been widely used and tested in various vaccines [272, 273] such as human papillomavirus (HPV) [274] and hepatitis B virus (HBV) [275]. As a vaccine platform, VLPs serve as a scaffold to present an antigen(s) in a dense and repetitive manner [276]. For this reason, VLPs are highly immunogenic and known to induce strong and long-lasting humoral and cellular responses [274, 277-279], both of which are particularly important for the protection against virus infection [280]. Furthermore, since the VLPs are hollow virions and lack the viral genome inside them, they are non-infectious and safe as vaccines [276, 281].

### 1.2.4. Vaccines

Currently, there is no commercially available BLV vaccine. Although several BLV vaccine modalities have been explored in the last 30 years, research on the development of efficacious BLV vaccines has not progressed significantly. Furthermore, none of the vaccines developed so far were able to achieve complete protection in vaccinated animals. Most of these vaccines focused on the induction of anti-Env antibody. The development of this type of vaccine was motivated by early studies showing that the anti-gp51 antibody neutralised viral cell fusion *in vitro* [282] *and in vivo* [283, 284] and maternal antibody from BLV infected dam and anti-Env antibody isolated from BLV-infected animals protected vaccinated animals from BLV infection [285, 286]. However, recent studies demonstrated that BLV spreads its infection through lytic cycle (i.e., infection into new cells after cell lysis) immediately after the infection [287, 288] and then, these viruses stay inside infected cells and infection continues through mitotic infection (i.e., clonal expansion of infected cells) at later stage [204, 289-292]. This indicates that both humoral and cellular immunities are critical in controlling BLV infection.

#### 1.2.4.1. Inactivated vaccines and cell-derived vaccines

Early attempts to protect animals from BLV infection using inactivated viruses were unsatisfactory as the antibody (Ab) responses that were induced were weak and short-lived and complete protection was not achieved even against a low challenge dose of BLV [283, 293, 294]. However, these studies did show that high Ab titres prior to the challenge were essential for protection [293, 294]. Based on the lessons learnt from these inactivated vaccine trials, cell-derived vaccines were developed with the aim of inducing high Ab titres and long-lasting immune responses [295-297]. NP-2 cells derived from foetal lamb kidney cells infected with BLV were utilised. They contained a defective viral genome, which only expressed p24 capsid proteins of Gag and all Env protein derivatives (pr72 precursor, gp 51 and gp30 proteins). The results of vaccine studies appeared to be promising but not convincing. This

vaccine was shown to be safe and the vaccination induced the rapid development of binding Ab responses and high anti-Env neutralising antibody (NAb) titres which were retained for up to 1 year following repeated vaccination. In addition, all vaccinated cattle were protected over the 4-year study period [296, 297]. However, the absence of the challenge virus in these animals should be verified using PCR.

#### 1.2.4.2. Live-attenuated vaccines

Two research groups have extensively explored the use of modified BLV proviruses as a live attenuated vaccine [74, 82, 88, 96, 298-301]. Boris-Lawrie *et al.* constructed a hybrid virus consisting of BLV and spleen necrosis virus (SNV) that was replication-competent but non-pathogenic. This hybrid virus contained BLV structural genes and sequences essential for viral replication, integration and translation but the BLV regulatory genes, promoters and regulatory sequences were replaced with the corresponding sequences from SNV to reduce viral pathogenicity [82, 298-300]. Their experiments in rats and rabbits demonstrated persistent infection of the vector virus and the formation of the provirus as well as transcription of vector genes in these animals. Furthermore, the presence of anti-Gag and anti-Env Abs and complete protection from the challenge virus were confirmed in all three vaccinated rabbits [82, 299, 300]. However, the statistical significance of protection is questionable due to the small sample size and the vaccine efficacy in the rabbit animal model cannot be translated into cattle as rabbits do not develop the lymphoproliferative disease nor lymphoma [80, 81]. Furthermore, although the vector virus was shown to be non-pathogenic *in vivo*, there remains the possibility of the induction of insertional mutagenesis and oncogenesis as the integration of the vector BLV provirus was shown in the vaccinated animals [302, 303].

Willems *et al.* developed an attenuated BLV provirus in which the nucleotide sequences for accessory R3 and G4 proteins were deleted. These proteins are dispensable for viral infection *in vivo* and BLV lacking these genes has shown low levels of transcription and propagation in sheep and cows and is thus less pathogenic than the wild type virus [73, 74, 96, 210]. All vaccinated animals seroconverted prior to challenge and all vaccinated sheep and two out of three cows were protected from the viral challenge for at least 18 months following the challenge [88]. However, despite the lower challenge dose than the dose used by Boris-Lawrie *et al.*, complete protection was not achieved in vaccinated cows and the challenge virus in a vaccinated cow at 12 months after the challenge was detected. This incomplete protection amongst the vaccinated cows might have been attributed to low anti-Env Ab titres prior to and following the challenge It is also possible that the vaccine virus was too attenuated for the vaccinated cows to induce strong, sustained immune responses, which subsequently allowed

the challenge virus to propagate in these animals. In contrast, vaccinated sheep generated much higher anti-Env Ab titres than vaccinated cows before and after the challenge and thus, the level of the vaccine virus attenuation could be appropriate to induce protective immune responses in sheep.

Live attenuated vaccines generally have an intrinsic risk of reversion of the vaccine virus to the virulent virus through back mutation and compensatory mutation, which arise during manufacturing processes using serial passages in different cell lines and are driven by the persistent infection and replication of vaccine viruses in their hosts or in immunocompromised individuals [304-309]. Even though these reversions are rare, this possibility cannot be excluded if revertants exhibit wild-type persistent infection and if they propagate in BLV-infected animals with altered immunity. In addition, the transmission of vaccine viruses to non-vaccinated animals can also facilitate the reversion of vaccine viruses to virulent viruses [310-313]. Recombination between wild-type and vaccine viruses and between a vaccine virus and related species is a mechanism that results in an emergence of virulent viruses, which has been reported for some veterinary vaccines [314-319].

#### 1.2.4.3. Peptide vaccines

In addition to humoral immune responses, cellular immune responses have been shown to correlate with the protection from BLV infection [320-323]. One approach to induce cellular immune responses is to design and synthesise k-mer peptides *in silico* that span T-cell epitope sequences of the gp51 glycoprotein. However, previous studies demonstrated that vaccination of peptide vaccines in mice and sheep only induced weak cytotoxic T cell (CTL) and T helper (Th) 1-type immune responses [320-323]. Furthermore, not all vaccinated animals were protected or vaccination only resulted in the delayed emergence of BLV in challenged animals [321-323].

To solve issues relating to weak induction of immune responses, Aida *et al.* used more immunogenic carbonate apatite as a delivery system and optimised the binding affinity of peptides using *in silico* screening [324, 325]. Their preliminary results demonstrated the induction of cellar immune responses in mice and significant suppression of viral propagation in vaccinated cows harbouring BLV-susceptible BoLA alleles [324, 325]. However, the suppression of viral growth was only seen in three out of six cows. In addition, in another study, Hislop *et al*. demonstrated that the re-stimulation of peripheral blood mononuclear cells (PBMCs) from sheep vaccinated with BLV-infected cells, which reflects natural infection, did not induce sufficient CTL responses [322]. These results together suggest the difficulties in

inducing strong, protective cellular immune responses in animals vaccinated with peptide vaccines.

#### 1.2.4.4. Recombinant vaccinia virus vaccines

Another extensively explored vaccine modality is a recombinant vaccinia virus (RVV). This vaccine modality is different from traditional vaccines, such as inactivated vaccines and live-attenuated vaccines, in that the RVV encodes vaccine antigens *in situ* to prime immune responses. Despite its theoretically simple mode of action, it is not easy to induce solid immune responses in vaccinated animals. RVV vaccine studies have shown variable and inconsistent results depending on the experimental animals, vaccine dose, challenge dose, vaccine virus strains and promoters to drive the antigen expression. RVVs (Lister strain) expressing the whole Env protein under the control of the p7.5 promoter or a promoter of A-type inclusion bodies were tested in rabbits, sheep and cows and binding and neutralising Ab responses were detected only in vaccinated rabbits [90-92, 95, 326]. The vaccines failed to induce Ab responses in sheep and cows but induced cellular immune responses in these animals [90-92, 95]. However, none of the vaccinated sheep and cows was protected when challenged with BLV and the vaccination only suppressed BLV growth in these animals.

A study using the Copenhagen strain of vaccinia virus (VV) expressing gp51 and partial gp30 proteins or the complete Env protein under the control of the p7.5 or fowlpox virus early/late (PFE/L) promoter demonstrated that all sheep vaccinated with RVV expressing the full-length Env protein under the control of the p7.5 promoter were protected whereas only two out of six sheep vaccinated with RVV expressing the full-length Env protein under the control of the PFE/L promoter were protected [94, 327]. In another study using an RVV (Copenhagen strain) expressing gp51, the full-length Env protein or uncleaved precursor Env protein from the H6 or three different vaccinia virus promoters, all vaccinated rabbits and sheep induced NAbs and all except one sheep were protected [93]. Finally, cows vaccinated with RVV expressing BLV Gag-Pol-Env proteins or uncleaved precursor Env protein all induced binding and neutralising Abs with varying degrees [97]. Despite the induction of Ab responses, no cows were protected. Therefore, none of these vaccine studies were able to demonstrate sufficient efficacy and correlates of protection, whether humoral or cellular immune responses or both. These previous vaccine studies also highlight the need for reassessment of vaccine strains, promoters and vaccine doses for the development of efficacious vaccines.

## 1.3. RATIONALE AND AIMS

This Master's degree project involved three aims.

**Aim 1: To sequence the BLV *env* and *gag* genes of some South African BLV isolates and investigate phylogenetic relationships with other BLV strains from different geographical regions worldwide with known genotypes.**

Molecular characterisation of BLV strains from Africa is limited and a Zambian isolate is the only isolate that has been genotyped so far. More studies on genetic variations and genotypes of BLV strains currently circulating in Africa will be necessary and such knowledge will provide valuable insight into the development of efficacious BLV vaccines tailored to the African BLV strains.

**Aim 2: To assess the activity of five selected poxvirus promoters (a modified early fowlpox virus promoter, an early-late promoter of a 7.5 kilodalton polypeptide gene of vaccinia virus (VACV), a synthetic early-late promoter of VACV, a modified early-late promoter of the H5 gene of VACV and a synthetic early-late optimised promoter of VACV) in cells infected with lumpy skin disease virus.**

To the best of our knowledge, the activity of these poxvirus promoters that have been extensively used in various recombinant poxvirus vectors has not been assessed in recombinant LSDV. This study will fill the gap in the knowledge in the selection of the poxvirus promoters so that genes of interest can be expressed from recombinant LSDV vaccines**.**

**Aim 3: To construct and characterise two recombinant LSDV-vectored dual vaccines designed to protect cattle against BLV and LSDV.**

These vaccines are based on a recombinant LSDV which is the only CaPV strain that is allowed to be used in sub-Saharan Africa, where sheep poxvirus and/or goat poxvirus are absent. These dual vaccines were designed to express BLV envelope and Gag proteins so that virus-like particles (VLPs) consisting of arrays of Env proteins embedded into the Gag proteins elicit humoral and cellular responses against BLV antigens. The LSDV vector backbone is known to be immunogenic against LSDV.

# CHAPTER 2

# PHYLOGENETIC ANALYSIS OF SOUTH AFRICAN BLV ISOLATES

## 2.1. INTRODUCTION

Globally, ten genotypes of the BLV strains have been identified based on the phylogenetic analyses of the BLV *env* nucleotide sequences and BLV whole-genome sequences as well as the genotype-specific amino acid substitutions [249, 328-330] (Table 2.1). The use of the BLV *env* nucleotide sequence for phylogenetic analysis has been a gold standard owing to its critical functions during the viral life cycle. The Env protein is necessary for cell entry and viral infection and contains various motifs and domains that are important for receptor binding, cell fusion and syncytium [237, 239, 331, 332]. Due to its location on the surface of the virus, the Env protein is a target for neutralising antibodies as well as $CD4^+$ and $CD8^+$ T cells [244, 245]. As a result, Env protein is subject to strong selective pressure [185, 249, 333, 334], which drives the generation of BLV variants.

During the late 1990s and early 2000s, the *env* sequences of the BLV isolates from Belgium, France, Italy, America, Japan, Australia, Brazil and Argentina were utilised to assess the relationships between genetic variants of the *env* gene and the serological status of infected animals [335-337]. Identification of genetic variants of the *env* gene subsequently aided the classification of different BLV strains into distinct groups using the restriction fragment length polymorphism (RFLP) analysis (often in conjunction with sequencing) [185, 335-338]. Restriction digestion of PCR products from various BLV isolates with *Bam*HI, *Bcl*I, *Bgl*I, *Hae*III and *Pvu*II was thought to be a useful marker to differentiate BLV strains [335-337]. However, classification based on the RFLP was not comprehensive nor consistent [336-339]. This led to the misclassification of BLV genotypes in some studies [335-337]. In addition, BLV isolates initially used for genotyping were obtained from the limited number of countries, whereby only allowing classification of two to four genotypes [185, 338, 340].

Later, sequencing-based genotyping replaced the RFLP-based genotyping and sequencing of the BLV *env* gene or the BLV whole genome from a variety of countries have been providing more comprehensive insights into the genetic variabilities and global distribution of different BLV strains. Currently, BLV whole-genome sequences are available from genotypes 1, 2, 4, 6, 9 and 10. Whilst the BLV whole-genome sequences provide more phylogenetically informative sites and phylogenetic analyses based on the BLV whole-genome sequences are supported by high bootstrap values and posterior probabilities [330, 341, 342], Rola-Łuszczak *et al.* [151] and Yang *et al.* [343] amongst others [45, 329, 344-347] correctly grouped BLV global isolates using partial *env* sequences. In 2009, Rodríguez *et al*. [328] conducted a seminal study by comparing the BLV full-length *env* sequences of 28 Argentine isolates and 46 sequences that had been previously described in various studies but had different genotype classification amongst different studies.

**Table 2.1: Global distribution of ten BLV genotypes**

| Region | Country | Genotype | | | | | | | | | | Reference |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | | 1 | 2 | 3 | 4 | 5 | 6 | 7 | 8 | 9 | 10 | |
| Europe | Belarus | | | | ✓ | | | | | | | [151] |
| | Belgium | | | | ✓ | | | | | | | **[253, 301, 348]** |
| | Croatia | | | | | | | | ✓ | | | **[151, 349]** |
| | France | | | ✓ | ✓ | | | | | | | **[249, 348, 350]** |
| | Germany | ✓ | | | | | | | | | | [336, 350] |
| | Italy | | ✓ | | | | ✓ | ✓ | ✓ | | | [351-353] |
| | Moldova | | | | ✓ | | | ✓ | | | | [334] |
| | Poland | | | | ✓ | | | ✓ | | | | [151] |
| | Russia | ✓ | | | ✓ | | | ✓ | ✓ | | | [151, 354, 355] |
| | Ukraine | | | | ✓ | | | ✓ | ✓ | | | [151] |
| Africa | Zambia | | | | ✓ | | | | | | | [345] |
| Middle East | Egypt | ✓ | | | | | | | | | | [145] |
| | Iran | ✓ | | | | | | | | | | [53] |
| | Jordan | ✓ | | | | | ✓ | | | | | [356] |
| | Turkey | ✓ | | | | | | | | | | [357, 358] |
| Oceania | Australia | ✓ | | | | | | | | | | [189] |
| Asia | China | | | | | | ✓ | | | | ✓ | [344] |
| | India | | | | | | ✓ | | | | | [359] |
| | Japan | ✓ | ✓ | ✓ | | ✓ | | | | | | [206, 249, 329, 360] |
| | Korea | ✓ | | ✓ | | | | | | | | [346, 361] |
| | Mongolia | ✓ | | | ✓ | | | ✓ | | | | [152] |
| | Myanmar | | | | | | | | | | ✓ | [341] |
| | Philippines | ✓ | | | | | ✓ | | | | | [45] |
| | Thailand | ✓ | | | | | ✓ | | | | ✓ | [329] |
| | Vietnam | ✓ | | | | | ✓ | | | | | [342] |
| North America | Costa Rica | ✓ | | | | ✓ | | | | | | [249, 353] |
| | Dominica | | | | | | | | | | | [343] |
| | U.S.A. | ✓ | | ✓ | ✓ | | | | | | | **[249, 348]** |
| | Mexico | ✓ | | | | | | | | | | [362, 363] |
| | St. Kitts | ✓ | | | | | | | | | | [343] |
| South America | Argentina | ✓ | ✓ | | ✓ | | ✓ | | | | | [185, 247, 249, 340, 353, 364] |
| | Bolivia | ✓ | ✓ | | | | ✓ | | | ✓ | | [330] |
| | Brazil | ✓ | ✓ | | | ✓ | ✓ | ✓ | | | | [249, 339, 353, 365-367] |
| | Chile | | | | ✓ | | | ✓ | | | | [249, 338] |
| | Colombia | ✓ | | ✓ | | | | | | | | [368] |
| | Paraguay | ✓ | ✓ | | | | ✓ | | | | | [330] |
| | Peru | ✓ | ✓ | | | | ✓ | | | | | [330] |
| | Uruguay | ✓ | | | | | | | | | | [248, 353] |

Checkmark (✓) indicates the presence of BLV strains with relevant genotype. This table combines genotype data from RFLP-based and sequencing-based genotyping of the BLV full-length or partial *env* nucleotide sequences or whole genome. Genotypes of historical isolates in the currently BVL-free countries are also included.

They re-classified these isolates into seven genotypes (G1 to G7) using neighbour-joining, Bayesian, maximum-likelihood and parsimony methods. In 2012, genotype 8 (G8) was identified in Croatia [349] and subsequently in Ukraine and Russia in 2013 [151]. In 2016, using the BLV whole-genome sequences of genotype-1, 2, 4 and 6 strains as well as 25 newly identified isolates from Peru, Bolivia and Paraguay, Polat *et al*. [330] identified genotype 9

(G9) from Bolivian isolates [330]. Recently, genotype 10 (G10) was identified in three Asian counties, Thailand in 2016 [329], Myanmar in 2017 [341] and China in 2018 [344].

Genotype 1, 4 and 6 (G1, G4 and G6, respectively) are circulating in all continents. G1 was called a Japan-USA subgroup [348] or a Japanese group [340] whereas G4 was considered to be a European genotype (Belgium-France subgroup [348] or European cluster [249]). However, G1 and G4 are currently distributed worldwide as a result of international trades of cattle, semen and embryo, particularly from some European countries such as the Netherlands, Germany and Denmark that have been actively exporting cattle worldwide since the post-World War II [151, 369]. Previously, Licursi *et al*. [370] identified G2 from Argentine isolates and classified them as an Argentine group. Later studies conducted by Polat *et al.* [330] and Camargos *et al.* [339] revealed the greater geographical distribution of G2 strains in other South American countries such as Bolivia, Paraguay, Peru and Brazil. G3 was previously classified as a US-Californian cluster [249] but G3 isolates have been sporadically detected in Japan [360], Korea [346] and Colombia [371]. G6 had been identified in the Middle East, Asian, South America but it was recently identified in Italy in 2016 [352], which was the first case in Europe. In Europe, along with the classic European genotype G4, the presence of G7 and/or G8 isolates was recently confirmed in Poland, Ukraine and Russia [151]. A G7 isolate (GenBank accession number: S83530) was identified in Italy in 1996 [351] and genotyped in 2010 [353]. A recent study conducted between 2012 and 2016 in Italy showed that the newly identified G7 isolates were clustered with this old G7 isolate (S83530) [352]. Therefore, the G7 isolates appeared to persist since at least 1996. The same study also identified the presence of G2, G4, G6 and G8 isolates in Italy [352]. Italy was declared BLV-free in 2017 (seroprevalence < 0.2%) [372, 373]. The presence of multiple strains and the long persistence of the G7 isolates prior to the BLV-free status suggest multiple introductions into Italy and highlights the significance of continued efforts in conducting preventative measures such as restriction on the movement of infected animals and serological tests for the newly- acquired animals.

In addition to the worldwide spread of 10 genotypes, multiple genotypes are circulating in Western Europe, Asia and South America. Coincidently, some countries in these regions such as Italy, Japan and Argentina had/have a long history of BLV prevalence or high BLV prevalence rates (See Section 1.2.2.2), indicating that the prolonged or delayed eradication process not only results in the persistence of BLV but also may allow the introduction of new and multiple genotypic strains. This is a serious concern regarding the development of an efficacious BLV vaccine.

To date, only one Zambian isolate has been genotyped from Africa. Although high BLV prevalence has been reported in South Africa [169, 196], there has been no molecular

characterisation of South African BLV strains. Therefore, the aim of this chapter is to sequence the *env* and *gag* genes of some South African BLV isolates and investigate phylogenetic relationships with other BLV strains from different geographical regions worldwide with known genotypes. A Japanese strain (GenBank accession number, AP018021) that was used to design the recombinant LSDV-BLV-Env-Gag and LSDV-BLV-Env (See Chapter 4) was included in the phylogenetic analyses. Since this study was conducted in parallel with the development and characterisation of the recombinant LSDV-BLV-Env-Gag and LSDV-BLV-Env, the phylogenetic analyses between the South African isolates and the Japanese strain AP018021 were also conducted concurrently to assess whether the Japanese isolate AP018021 is closely related to the South African isolates.

## 2.2. MATERIALS AND METHODS

### 2.2.1. Collection of bovine whole blood samples

Whole blood samples were obtained from a single herd, where leukosis case was suspected, in Port Elizabeth, Eastern Cape province, South Africa in 2018. The blood samples were delivered to the University of Cape Town for further analyses.

### 2.2.2. Extraction of total bovine genomic DNA

The bovine genomic DNA was extracted from the eight whole blood samples using New MagNA Pure Compact Nucleic Acid Isolation Kits and MagNA Pure Compact Instrument (Roche, Switzerland). Following the thawing of the frozen blood samples at room temperature, the vials were vortexed briefly and 400µl of each blood sample were transferred into sample tubes. The blood samples, elution tubes and DNA extraction reagents were placed in MagNA Pure Compact Instrument and the whole bovine genomic DNA was extracted in 100µl of an elution volume as per manufacturer's instructions. The extracted DNA was stored at -20°C until needed.

### 2.2.3. Amplification of the BLV full-length *env* and *gag* sequences by nested polymerase chain reaction (nPCR) and Sanger sequencing of the complete *env* and *gag* genes

The BLV full-length *env* and *gag* sequences were amplified from the eight samples by nested polymerase chain reaction (nPCR) using primers outlined in Table 2.2. To amplify the full-length BLV *env* sequence, both rounds of nPCR were performed in a 20µl reaction mixture

containing 1µl of the total genomic DNA or PCR product from the first round of nPCR, 1µl of 2x KAPA 2G Robust HotStart ReadyMix (KAPA BIOSYSTEMS, USA), 1µl of each primer (10µM each) and high-performance liquid chromatography (HPLC) water (Sigma-Aldrich, USA) using a thermal cycler (Applied Biosystems, USA). A reaction mixture without template

DNA was included as a PCR control. The cycling parameters were as follows: 95°C for 3 minutes followed by 25 cycles (30 cycles for the second round) of 95°C for 15 seconds, 55°C (61°C for the second round) for 15 seconds, 72°C for 2 minutes and 72°C for 4 minutes.

**Table 2.2: Primer sets designed and used to amplify BLV full-length env and gag sequences**

| Amplified gene | | Orientation | Primers | Primer sequence |
|---|---|---|---|---|
| *env* | Outer | Forward | Env4594OF | 5'- CCTCCTACCAATTCTAAAGACC -3' |
| | | Reverse | Env6535OR | 5'- CACGCAGAAGCGACAATCTC-3' |
| | Inner | Forward | Env4660if | 5' - GGGCGGAGAAACACCYAAGG-3' |
| | | Reverse | Env6421ir | 5'- CACTGACTATTCCACTAAGCC -3' |
| *gag* | Outer | Forward | Gag551OF | 5'- GATTGATCACCCCGGAACCC -3' |
| | | Reverse | Gag1988OR | 5'- GTATTTTCAGCCCCGGTGTCC -3' |
| | Inner | Forward | Gag579if | 5'- CTCTGGACCCACCCCCTCG -3' |
| | | Reverse | Gag1866ir | 5'- CATTCTARTTCGGCCTCACTAAG-3' |

To amplify the full-length BLV *gag* sequence, both rounds of nPCR were conducted in a 20µl reaction mixture containing 5µl of the total genomic DNA or 1µl of the PCR product from the first round of nPCR, 1µl of 2x KAPA 2G Robust HotStart ReadyMix (KAPA BIOSYSTEMS, USA), 1µl of each primer (10µM each) and high-performance liquid chromatography (HPLC) water (Sigma-Aldrich, USA) using a thermal cycler (Applied Biosystems, USA). A reaction mixture without the template DNA was included as a PCR control. The cycling parameters were as follows: 95°C for 3 minutes followed by 40 cycles (35 cycles for the second round) of 95°C for 15 seconds, 56°C (61°C for the second round) for 15 seconds, 72°C for 2 minutes and 72°C for 4 minutes.

Both *gag* and *env* PCR products were separated on 0.8% agarose TBE gel stained with ethidium bromide (0.5µg/ml) at 100V. A 1,782bp fragment of the BLV *env* gene and a 1,307bp fragment of the BLV *gag* gene were gel purified using the Zymoclean Gel DNA Recovery Kit (Zymo Research, USA) as per the manufacturer's protocol. Sanger sequencing of the full-length *env* and *gag* genes was conducted using primers outlined in Table 2.3 and

Table 2.4 by Central Analytical Facilities at the University of Stellenbosch (South Africa). The eight *env* and *gag* genes sequenced were designated sample ID K1170, K1194, M1878, M2746, P591, P2152, P2677 and L3401.

**Table 2.3: Primers used to sequence the BLV full-length *env* sequence**

| Primers | Primer sequence |
|---|---|
| Env4763 fwd | 5'- AGGCGCTCTCCTGGCTAC -3' |
| Env5044 fwd | 5'- CARGTCTCCCAGATACAC -3' |
| Env5185 rev | 5'- ATCTGCCCCCACATAAGG -3' |
| Env5312 fwd | 5'- TGGGGATATGATCCCCTG -3' |
| Env5524 rev | 5'- GATGGTTTTGTTATATAC -3' |
| Env5636 fwd | 5'- AATGTTTCTCAAGGCAAC -3' |
| Env5820 rev | 5'- AGTCTCTGATGGCTAAGG -3' |
| Env 5919 fwd | 5'- CCCAGAACCGACGGGGGC -3' |
| Env6067 rev | 5'- GACTCTTTGCGAGAGAGG -3' |
| Env6198 fwd | 5'- AATGCTTGACCTCTCGCC - 3' |
| Env6404 rev | 5'- ACAGCCTGGGGGTGCGTG -3' |

Fwd, forward primer; rev, reverse primer.

**Table 2.4: Primers used to sequence the BLV full-length *gag* sequence**

| Primers | Primer sequence |
|---|---|
| Gag590 fwd | 5' - CCCTCGGCGGCRTTTTGG - 3' |
| Gag928 rev | 5'- GGGTTGTTCYTCKGGGGC-3' |
| Gag1229 rev | 5'- GRTTAAAACCCTGGAGGG -3' |
| Gag1300 fwd | 5' CCTYTGGCTTCAGGCCTG -3' |
| Gag1543 rev | 5' YCCCACCGGGGCGGCCAC -3' |
| Gag1665 fwd | 5'- GACCATGCTATCGATGCC -3' |
| Gag1870 rev | 5'- CCTCACTAAGRGRATCTG - 3' |

Fwd, forward primer; rev, reverse primer.

### 2.2.4. Phylogenetic analysis of the BLV full-length *env* and *gag* nucleotide sequences

The BLV full-length *env* (1,548bp) and *gag* (1,184bp) nucleotide sequences of the eight South African isolates were aligned with the BLV sequences obtained from the Basic Local Alignment Search Tool (BLAST) database [374] on CLC Main Workbench (QIAGEN Bioinformatics, Denmark) (Table S2.1). One hundred BLV nucleotide sequences from the BLAST dataset that shared the highest nucleotide sequence identity with the South African isolates were included in an initial sample pool. The BLV sequences with unknown genotypes were excluded whereas those with known genotypes that had not been included in the initial sample pool were manually included by obtaining the sequences from the GenBank nucleotide database. Pair-wise comparison of the sequences in this sample pool was performed to remove the sequences with the 100% nucleotide sequence identity from the same genotype and the same country.

Subsequently, 29 BLV full-length *env* sequences representing 9 genotypes, 56 partial *env* sequences representing 10 genotypes and 20 BLV full-length *gag* sequences representing 6

genotypes obtained from BLAST database [374] and GenBank nucleotide database were aligned with the eight South African *env* and *gag* nucleotide sequences, using MUSCLE on MEGA7 [375]. The 444bp fragment of the BLV *env* gene from the South African isolates were aligned with those obtained from the BLAST database [374] and the GenBank nucleotide database using MUSCLE on MEGA7 [375]. The BLV full-length *env*, partial *env* and full-length *gag* nucleotide sequences were translated into amino acid sequences and aligned using MUSCLE on MEGA7 [375]. Model testing was performed using MEGA7 [375] to select the best evolutionary model based on the Bayesian information criterion (BIC) [376].

Neighbour-joining (NJ) and Maximum-likelihood (ML) trees based on the BLV full-length and 444bp partial *env* nucleotide sequences were constructed using the Tamura-Nei substitution model with the Gamma distribution (TN83 + G) [377] and the Kimura-2-parameter model with the Gamma distribution (K2 +G) [378], respectively, on MEGA7 [375].

NJ and ML trees based on the BLV full-length *gag* nucleotide sequences were constructed using the TN83 + G [377] and the Hasegawa-Kishino-Yano substitution model with the Gamma distribution (HKY + G) [379], respectively on MEGA7 [375].

The nonparametric bootstrap analysis with 1000 iterations [380] was used to evaluate the robustness of evolutionary relationships.

### 2.2.5. Comparison of the BLV Env and Gag nucleotide and amino acid sequences of the South African and global isolates

To evaluate the nucleotide and amino acid sequence percent identities and differences, pair-wise comparison of the BLV *env* and *gag* sequences between the South African isolates and the global isolates selected in Section 2.2.4 was performed on CLC Main Workbench 7.9.3 (QIAGEN Bioinformatics, Denmark).

To estimate the mean intragenotype and intergenotype distances of the BLV Env and Gag nucleotide and amino acid sequences, 115 full-length Env and 56 full-length Gag sequences from the global isolates with 10 and 6 known genotypes, respectively were obtained from the BLAST database [374]. Five hundred full-length *env* nucleotide sequences and 250 full-length *gag* nucleotide sequences from the BLAST dataset that shared the highest nucleotide sequence identity with the South African isolates were included in the initial sample pool. The BLV sequences with unknown genotypes were excluded whereas those with known genotypes that had not been included in the initial sample pool were manually included by obtaining the sequences from the GenBank nucleotide database. The nucleotide sequences

were translated into amino acid sequences on MEGA7 [375] and their deduced amino acid sequences were confirmed with BLV sequences available on the NCBI [381].

The mean intragenotype and intergenotype distances of BLV Env nucleotide and amino acid sequences were estimated using the Tamura-Nei substitution model with the Gamma distribution (TN83 + G) [377] and the Jones-Taylor-Thornton model with the Gamma distribution (JTT + G) [382], respectively, on MEGA7 [375]. The mean intragenotype and intergenotype distances of the BLV Gag nucleotide and amino acid sequences were estimated using the HKY + G [379] model on IQ-TREE [383] and the JTT + G model [382] on MEGA7 [375], respectively. Nonparametric bootstrap analysis with 1000 iterations [380] was used to evaluate the robustness of evolutionary relationships.

To identify genotype-specific amino acid substitutions as well as those unique to the South African isolates, the full-length Env and Gag amino acid sequences of the South African isolates were aligned with full-length and partial Env and Gag amino acid sequences obtained from the Basic Local Alignment Search Tool (BLAST) database [374] using MUSCLE on MEGA7 [375].

## 2.3. RESULTS

### 2.3.1. Phylogenetic analyses of the BLV *env* and *gag* nucleotide sequences

Total bovine DNA was extracted from blood obtained from eight BLV-infected cattle and the full-length *env* and *gag* sequences were successfully amplified and sequenced from eight samples (designated as sample ID K1170, K1194, M1878, M2746, P591, P2152, P2677 and L3401). In the process of selecting BLV global sequences for the comparison with the South African sequences, as many BLV sequences with known genotypes from various countries as possible were included in the sample pool but the BLV sequences with the 100% sequence identity from the same genotype and the same country were excluded. Subsequently, the South African BLV full-length *env* (1,548bp), partial *env* (444bp) and full-length *gag* (1,184bp) nucleotide sequences were aligned with 29 full-length *env* sequences representing nine genotypes, 56 partial *env* sequences representing ten genotypes and 20 full-length *gag* sequences representing six genotypes, respectively, to perform phylogenetic analyses.

#### 2.3.1.1. Phylogenetic analysis of BLV *env* nucleotide sequences

##### 2.3.1.1.1. Phylogenetic analysis of the South African isolates based on the full-length *env* nucleotide sequences

To determine evolutionary relationships between the South African and global isolates, neighbour-joining (NJ) (Figure 2.1) and maximum-likelihood (ML) (Figure 2.2) trees based on the full-length *env* nucleotide sequences were constructed using the TN83 + G substitution model G [377]. Both phylogenetic trees showed congruent topologies with high bootstrap values. The NJ and ML trees showed that the seven South African isolates (K1170, K1194, M1878, P591, P2152, P2677 and L3401) are closely related to each other with 99% bootstrap value. These seven South African isolates appeared to belong to G4, clustering with the G4 isolates with 99% bootstrap value (NJ and ML). Furthermore, these seven South African isolates appear to be most closely related to the G4 Russian isolate (JN695878) with 79% (NJ) and 81% (ML) bootstrap values.

Interestingly, the NJ and ML trees also showed that one South African isolate (M2746) did not cluster with the seven other South African isolates but clustered within the G1 group with 94% (NJ and ML) bootstrap value. Furthermore, this M2746 isolate appeared to be most closely related to the G1 Saint Kitts and Nevis isolate (KX674369) with 90% (NJ) and 87% (ML) bootstrap values. Therefore, the eight South African isolates from a single herd appeared to contain two genotypes, G1 and G4.

This phylogenetic analysis also indicates that the G4-like South African isolates were more divergent from the other G4 isolates whereas the G1-like South African isolate was less divergent from some of the closest G1 global isolates. The genetic distance between the G1-like South African isolate and the Saint Kitts and Nevis isolate (KX674369) was estimated to be 0.003 nucleotide substitutions per site whereas the genetic distance between the G4-like South African isolates and the Russian isolate (JN695878) was estimated to be 0.013 nucleotide substitutions per site.

The Japanese AP018021 isolate which had been used to design the recombinant LSDV-BLV-Env-Gag and LSDV-BLV-Env clustered with the South African M2746 isolate with low (39% for both trees) bootstrap value but also appeared to belong to G1.

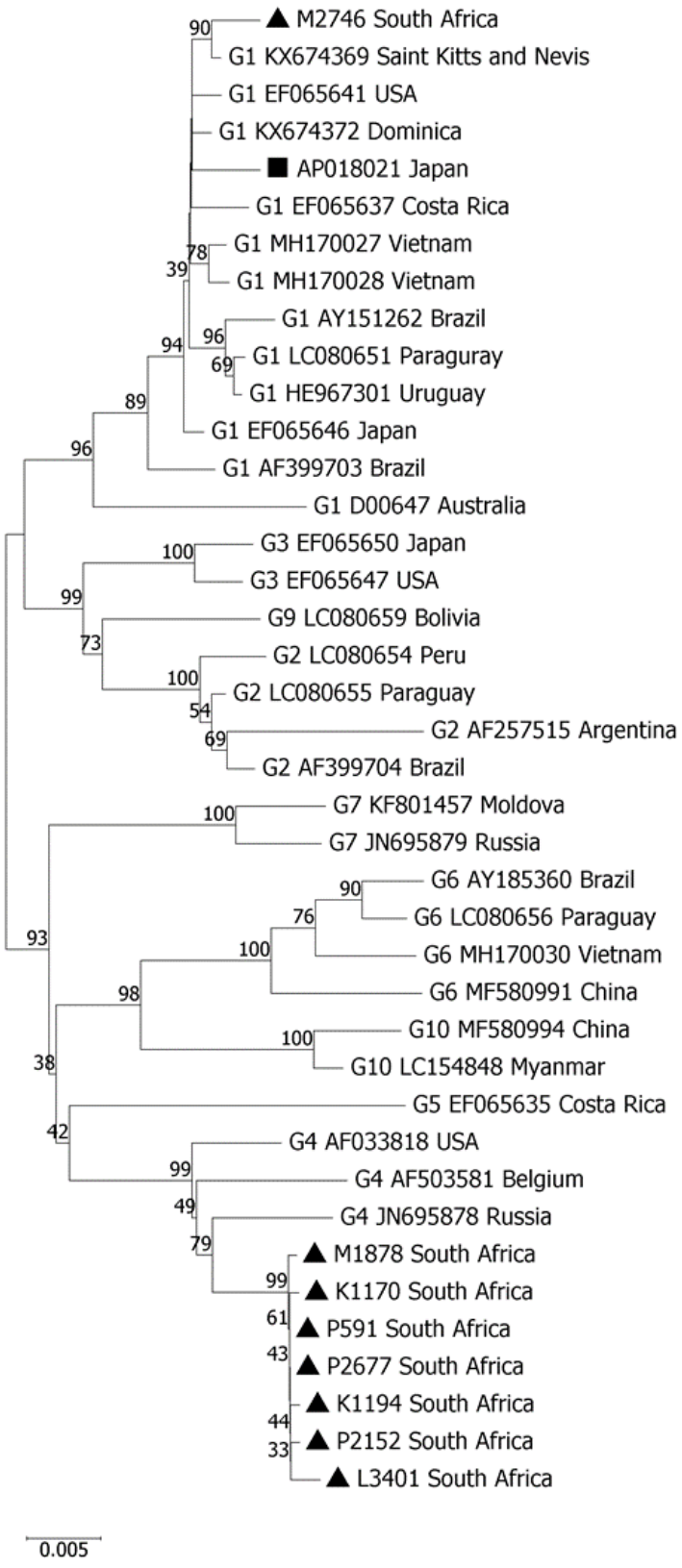



**Figure 2.1: Neighbour-joining phylogenetic tree based on the BLV full-length *env* nucleotide sequences from South Africa and other geographic regions worldwide.** BLV strains identified in this study are indicated by filled triangles (▲) followed by their sample ID. The BLV strain used to design recombinant LSDV-BLV-Env-Gag and LSDV-BLV-Env is indicated by a filled square (■). Other strains are shown by genotype followed by GenBank accession number and country of origin. Numbers at the branches denote bootstrap support (1000 iterations). The bar at the bottom of the figure denotes genetic distance.

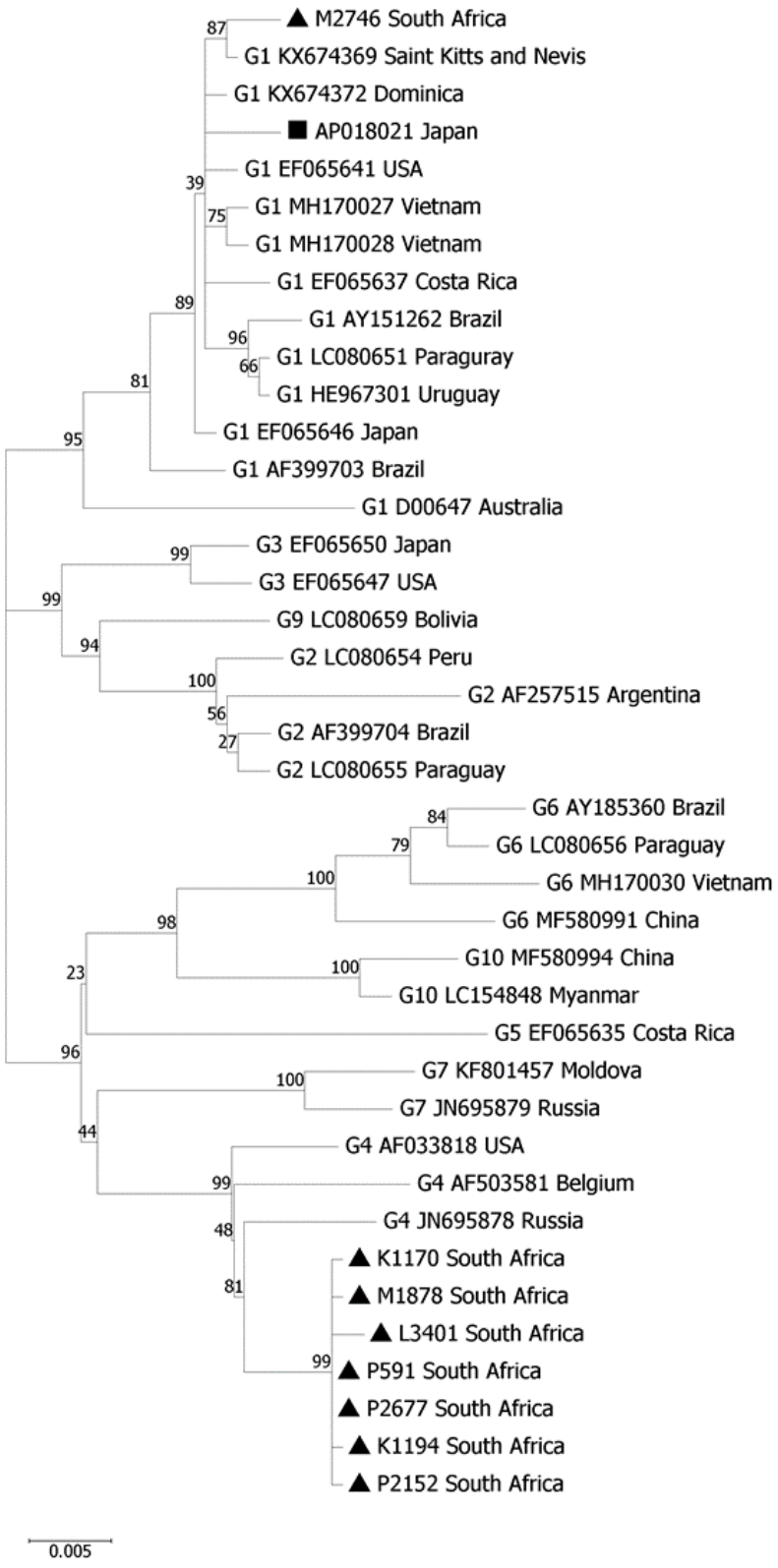



**Figure 2.2: Maximum-likelihood phylogenetic tree based on the BLV full-length *env* nucleotide sequences from South Africa and other geographic regions worldwide.** BLV strains identified in this study are indicated by filled triangles (▲) followed by their sample ID. The BLV strain used to design recombinant LSDV-BLV-Env-Gag and LSDV-BLV-Env is indicated by a filled square (■). Other strains are shown by genotype followed by GenBank accession number and country of origin. Numbers at the branches denote bootstrap support (1000 iterations). The bar at the bottom of the figure denotes genetic distance.

#### 2.3.1.1.2. Phylogenetic analysis of the South African isolates based on the 444bp partial *env* nucleotide sequences

The only African BLV sequence that had been sequence was a Zambian isolate with a partial *env* sequence [345]. Furthermore, since the 444bp partial *env* sequences have been used for the BLV genotyping in various studies [329, 336-338, 346, 356, 384], the 444bp partial *env* sequences are available from all 10 genotypes and from various geographic regions. To confirm the results obtained from the phylogenetic analyses based on the full-length *env* nucleotide sequences and to further investigate the phylogenetic relationships between the South African isolates with more diverse BLV isolates from different geographic regions, NJ (Figure S2.1) and ML (Figure 2.3) trees based on the 444bp partial *env* nucleotide sequences were constructed using the K2P + G model [378].

Despite the low to moderate bootstrap values, this analysis was consistent with the analysis using the full-length *env* nucleotide sequences, showing that the seven South African isolates belong to G4 and one South African isolate (M2746) belongs to G1. The seven South African isolates clustered within the G4 group with 48% bootstrap value (NJ and ML) whilst one South African isolate (M2746) clustered with fourteen phylogenetically closest G1 isolates as well as the Japanese AP018021 isolate with 40% (NJ) and 39% (ML) bootstrap values. None of the G1 isolates shared moderate to high bootstrap values with the South African isolate (M2746) and the Japanese isolate AP018021 but the South African (M2746) and Japanese (AP018021) isolates were clustered with all other G1 isolates with 73% (NJ) and 74% (ML) bootstrap values, confirming that these two isolates belong to G1. This second analysis also showed that despite the low bootstrap values, these seven South African isolates are more closely related to the Zambian isolate (LC193462; a black arrow in Figure 2.3) than other G4 isolates with 46% (NJ) and 50% (ML) bootstrap values. Furthermore, these sequences appeared significantly similar to each other, showing 0.002 nucleotide substitutions per site between the G4-like South African isolates and the Zambian isolate.

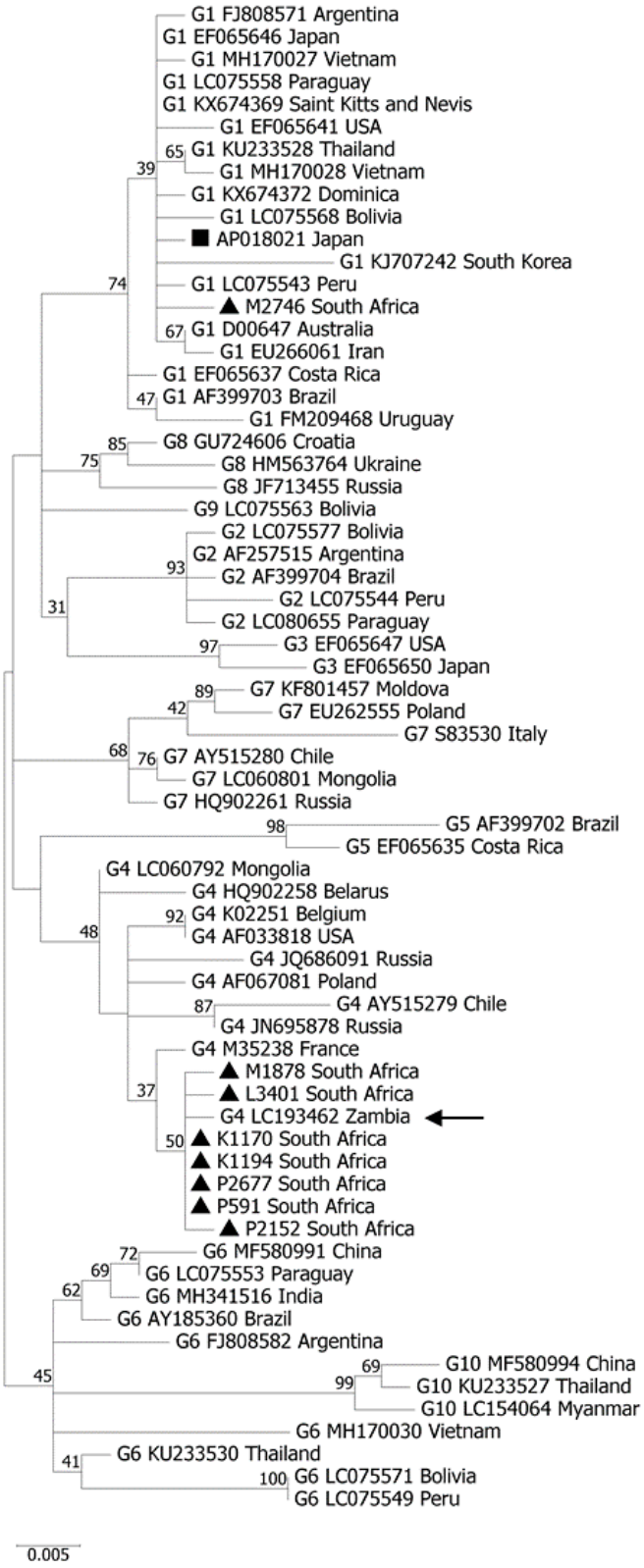



**Figure 2.3: Maximum-likelihood phylogenetic tree based on the BLV partial *env* nucleotide sequences from South Africa and other geographic regions worldwide.** BLV strains identified in this study are indicated by filled triangles (▲) followed by their sample ID. The BLV strain used to design recombinant LSDV-BLV-Env-Gag and LSDV-BLV-Env is indicated by a filled square (■). A black arrow indicates the Zambain isolate which is most closely related to the seven South African isolates. Other strains are indicated by their genotype, GenBank accession number and country of origin. Numbers at the branches denote bootstrap support (1000 iterations). The bar at the bottom of the figure denotes genetic distance.

### 2.3.1.2. Phylogenetic analysis of the BLV *gag* nucleotide sequences

Although the BLV *gag* sequence has been rarely used for phylogenetic analyses, the BLV *gag* sequence was included in this study to add more insight into the genetic variabilities of the BLV isolates and to compare the results with the phylogenetic analyses of the BLV *env* nucleotide sequences. NJ (Figure S2.2) and ML (Figure 2.4) trees based on the BLV full-length *gag* nucleotide sequences were constructed using the HKY + G [379] and the TN83 + G [377] models, respectively.

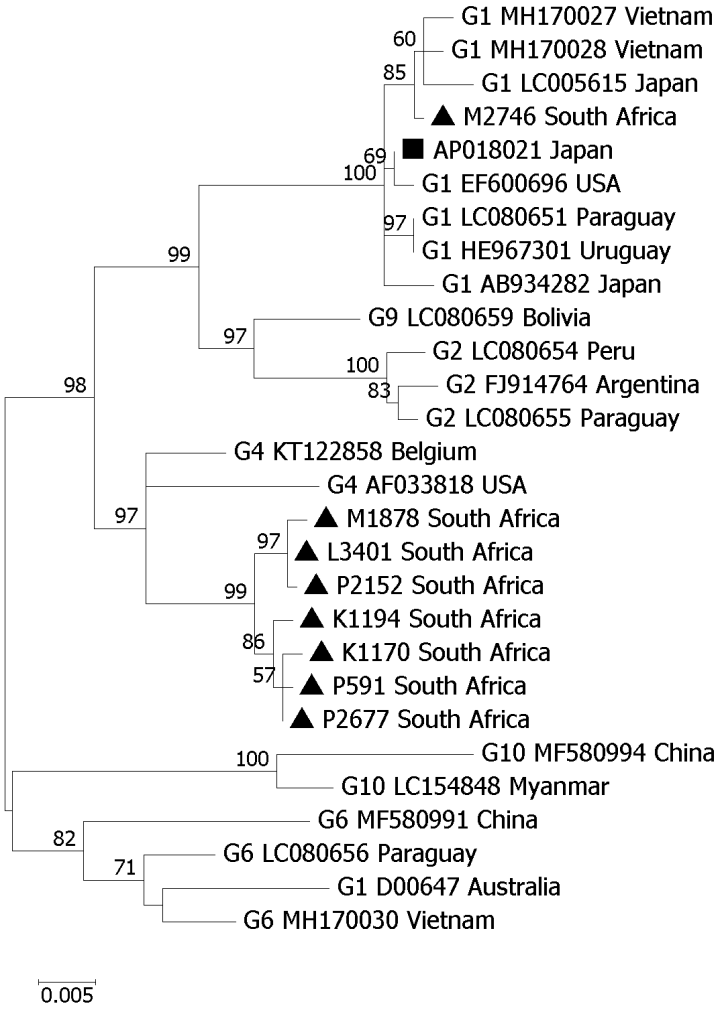



**Figure 2.4: Maximum-likelihood phylogenetic tree based on the BLV full-length *gag* nucleotide sequences from South Africa and other geographic regions worldwide.** BLV strains identified in this study are indicated by filled triangles (▲) followed by their sample ID. The BLV strain used to design recombinant LSDV-BLV-Env-Gag and LSDV-BLV-Env is indicated by a filled square (■). Other strains are shown by genotype followed by GenBank accession number and country of origin. Numbers at the branches denote bootstrap support (1000 iterations). The bar at the bottom of the figure denotes genetic distance.

Despite the limited number of the available BLV full-length *gag* nucleotide sequences, the NJ and ML trees showed congruent topologies with the phylogenetic analyses of the BLV full-

length and partial *env* nucleotide sequences. Furthermore, the phylogenetic analyses based on the full-length *gag* nucleotide sequences were supported by higher bootstrap values compared to those based on the *env* nucleotide sequences. Like the BLV *env* phylogenetic trees, the BLV *gag* trees showed that one South African isolate (M2746) belongs to G1 with 100% bootstrap value (NJ and ML) whereas the seven South African isolates belong to G4 with 93% (NJ) and 97% (ML) bootstrap values.

The result showed that the G1-like South African isolate (M2746) was clustered with two G1 Vietnamese isolates (MH170027 and MH170028) and a G1 Japanese isolate (LC005615) with 83% (NJ) and 85% (ML) bootstrap values. The result also showed that the Japanese isolate AP018021 belongs to G1 as consistent with the phylogenetic analyses based on the full-length and partial *env* nucleotide sequences.

Interestingly, there appeared to be more genetic variabilities in the BLV *gag* nucleotide sequences than the BLV *env* nucleotide sequences as evident from the tree topology. Consistent with the phylogenetic analyses based on the full-length and partial *env* sequences, the G4-like South African *gag* nucleotide sequences appeared more divergent from those of the other G4 isolates but the G1-like South African *gag* nucleotide sequence appeared less divergent from those of some of the closest G1 global isolates. The genetic distance between the seven G4-like South African and the G4 Belgian isolate (KT122858) was estimated to be 0.02 nucleotide substitution per site, which is approximately 1.5 times higher than the nucleotide substitution rate between the G4-like South African and the G4 Russian isolate (JN695878) and ten times higher than the nucleotide substitution rate between the G4-like South African and the G4 Zambian isolate (LC193462).

### 2.3.2. Pair-wise comparison of the BLV Env and Gag sequences

To confirm the results obtained from the phylogenetic trees based on the full-length and partial *env* as well as full-length *gag* nucleotide sequences and to investigate the genetic variabilities of the BLV strains, pair-wise comparison of the nucleotide and amino acid sequences between South African and global isolates was performed.

#### 2.3.2.1. Pair-wise comparison of the BLV full-length Env sequences

Pair-wise comparison of the BLV full-length Env nucleotide and amino acid sequences of the South African and global isolates was consistent with the phylogenetic analyses based on the NJ and ML trees. The results showed that one South African isolate (M2746) was most closely related to the G1 isolates and the seven remaining South African isolates were closely related

to each other and most closely related to the G4 isolates (Table 2.5, Figure 2.5 and Table S2.2 for the full data).

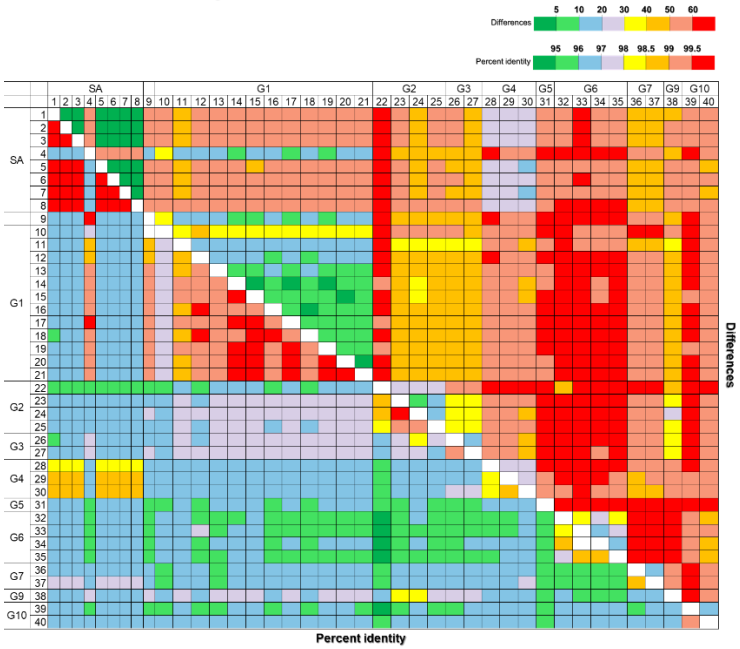
**Table 2.5: Nucleotide and amino acid differences in the BLV full-length Env sequences between South Africa isolates and global isolates representing nine genotypes**

| Genotype | GenBank accession number | Country of origin | South Africa | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|
| | | | K1170 | K1194 | M1878 | M2746 | P591 | P2152 | P2677 | L3401 |
| | K1170 | South Africa | | | | | | | | |
| | K1194 | South Africa | 2 (1) | | | | | | | |
| | M1878 | South Africa | 2 (1) | 2 (2) | | | | | | |
| | M2746 | South Africa | 56 (15) | 56 (15) | 56 (16) | | | | | |
| | P591 | South Africa | 1 (0) | 1 (1) | 1 (1) | 55 (15) | | | | |
| | P2152 | South Africa | 2 (0) | 2 (1) | 2 (1) | 56 (15) | 1 (0) | | | |
| | P2677 | South Africa | 1 (0) | 1 (1) | 1 (1) | 55 (15) | 0 (0) | 1 (0) | | |
| | L3401 | South Africa | 4 (0) | 4 (1) | 4 (1) | 58 (15) | 3 (0) | 4 (0) | 3 (0) | |
| | AP018021 | Japan | 56 (14) | 56 (15) | 56 (15) | 14 (1) | 55 (14) | 56 (14) | 55 (14) | 58 (14) |
| G1 | D00647 | Australia | 53 (14) | 53 (15) | 53 (15) | 38 (9) | 52 (14) | 53 (14) | 52 (14) | 55 (14) |
| | AF399703 | Brazil | 49 (14) | 49 (15) | 47 (13) | 19 (7) | 48 (14) | 49 (14) | 48 (14) | 51 (14) |
| | AY151262 | Brazil | 56 (16) | 56 (17) | 54 (15) | 16 (3) | 55 (16) | 56 (16) | 55 (16) | 58 (16) |
| | EF065637 | Costa Rica | 53 (16) | 53 (17) | 53 (17) | 13 (3) | 52 (16) | 53 (16) | 52 (16) | 55 (16) |
| | KX674372 | Dominica | 51 (15) | 51 (16) | 51 (16) | 9 (2) | 50 (15) | 51 (15) | 50 (15) | 53 (15) |
| | EF065646 | Japan | 50 (14) | 50 (15) | 50 (15) | 10 (3) | 49 (14) | 50 (14) | 49 (14) | 52 (14) |
| | LC080651 | Paraguay | 54 (16) | 54 (17) | 52 (15) | 13 (3) | 53 (16) | 54 (16) | 53 (16) | 56 (16) |
| | KX674369 | St. Kitts and Nevis | 52 (15) | 52 (16) | 52 (16) | 6 (2) | 51 (15) | 52 (15) | 51 (15) | 54 (15) |
| | HE967301 | Uruguay | 54 (15) | 54 (16) | 52 (14) | 13 (2) | 53 (15) | 54 (15) | 53 (15) | 56 (15) |
| | EF065641 | USA | 52 (14) | 52 (15) | 52 (15) | 10 (1) | 51 (14) | 52 (14) | 51 (14) | 54 (14) |
| | MH170027 | Vietnam | 53 (16) | 53 (17) | 53 (17) | 11 (3) | 52 (16) | 53 (16) | 52 (16) | 55 (16) |
| | MH170028 | Vietnam | 53 (15) | 53 (16) | 53 (16) | 11 (2) | 52 (15) | 53 (15) | 52 (15) | 55 (15) |
| G2 | AF257515 | Argentina | 68 (29) | 68 (30) | 68 (30) | 63 (25) | 67 (29) | 68 (29) | 67 (29) | 70 (29) |
| | AF399704 | Brazil | 52 (13) | 52 (14) | 52 (14) | 47 (11) | 51 (13) | 52 (13) | 51 (13) | 54 (13) |
| | LC080655 | Paraguay | 49 (12) | 49 (13) | 49 (13) | 44 (10) | 48 (12) | 49 (12) | 48 (12) | 51 (12) |
| | LC080654 | Peru | 53 (14) | 53 (15) | 53 (15) | 48 (13) | 52 (14) | 53 (14) | 52 (14) | 55 (14) |
| G3 | EF065650 | Japan | 50 (13) | 50 (14) | 50 (14) | 45 (11) | 49 (13) | 50 (13) | 49 (13) | 52 (13) |
| | EF065647 | USA | 49 (13) | 49 (14) | 49 (14) | 44 (11) | 48 (13) | 49 (13) | 48 (13) | 51 (13) |
| G4 | AF503581 | Belgium | 24 (7) | 26 (8) | 26 (8) | 60 (14) | 25 (7) | 26 (7) | 25 (7) | 28 (7) |
| | JN695878 | Russia | 21 (6) | 21 (7) | 21 (7) | 59 (13) | 20 (6) | 21 (6) | 20 (6) | 23 (6) |
| | AF033818 | USA | 20 (6) | 20 (7) | 20 (7) | 54 (13) | 19 (6) | 20 (6) | 19 (6) | 22 (6) |
| G5 | EF065635 | Costa Rica | 54 (17) | 54 (18) | 54 (18) | 64 (18) | 53 (17) | 54 (17) | 53 (17) | 56 (17) |
| G6 | MF580991 | China | 59 (13) | 59 (14) | 59 (14) | 65 (15) | 58 (13) | 59 (13) | 58 (13) | 61 (13) |
| | AY185360 | Brazil | 60 (15) | 60 (16) | 60 (16) | 64 (18) | 59 (15) | 60 (15) | 59 (15) | 62 (15) |
| | LC080656 | Paraguay | 59 (12) | 59 (13) | 59 (13) | 62 (15) | 58 (12) | 59 (12) | 58 (12) | 61 (12) |
| | MH170030 | Vietnam | 58 (13) | 58 (14) | 58 (14) | 64 (16) | 57 (13) | 58 (13) | 57 (13) | 60 (13) |
| G7 | KF801457 | Moldova | 48 (13) | 48 (14) | 48 (14) | 56 (12) | 47 (13) | 48 (13) | 47 (13) | 48 (13) |
| | JN695879 | Russia | 46 (13) | 46 (14) | 46 (14) | 56 (13) | 45 (13) | 46 (13) | 45 (13) | 46 (13) |
| G9 | LC080659 | Bolivia | 49 (14) | 51 (15) | 51 (15) | 46 (12) | 50 (14) | 51 (14) | 50 (14) | 53 (14) |
| G10 | MF580994 | China | 54 (14) | 54 (15) | 54 (15) | 65 (19) | 53 (14) | 54 (14) | 53 (14) | 54 (14) |
| | LC154848 | Myanmar | 50 (13) | 50 (14) | 50 (14) | 59 (16) | 49 (13) | 50 (13) | 49 (13) | 50 (13) |

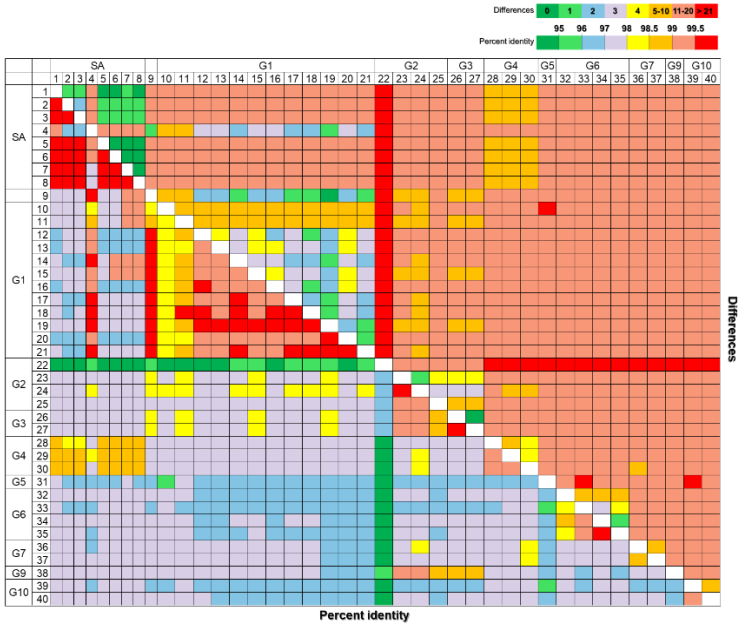
Amino acid sequence differences are shown in brackets. Each cell in the table is highlighted according to amino acid differences.

Differences: 0 | 1 | 2 | 3 | 4 | 5-10 | 11-20 | > 21

**A. Nucleotide sequence**




**B. Amino acid sequence**




**C**

| | GenBank accession number | Country of origin |
|---|---|---|
| 1. | K1170 | South Africa |
| 2. | K1194 | South Africa |
| 3. | M1878 | South Africa |
| 4. | M2746 | South Africa |
| 5. | P591 | South Africa |
| 6. | P2152 | South Africa |
| 7. | P2677 | South Africa |
| 8. | L3401 | South Africa |
| 9. | AP018021 | Japan |
| 10. | D00647 | Australia |
| 11. | AF399703 | Brazil |
| 12. | AY151262 | Brazil |
| 13. | EF065637 | Costa Rica |
| 14. | KX674372 | Dominica |
| 15. | EF065646 | Japan |
| 16. | LC080651 | Paraguay |
| 17. | KX674372 | St. Kitts and Nevis |
| 18. | HE967301 | Uruguay |
| 19. | EF065641 | USA |
| 20. | MH170027 | Vietnam |
| 21. | MH170028 | Vietnam |
| 22. | AF257515 | Argentina |
| 23. | AF399704 | Brazil |
| 24. | LC080655 | Paraguay |
| 25. | LC080654 | Peru |
| 26. | EF065650 | Japan |
| 27. | EF065647 | USA |
| 28. | AF503581 | Belgium |
| 29. | JN695878 | Russia |
| 30. | AF033818 | USA |
| 31. | EF065635 | Costa Rica |
| 32. | MF580991 | China |
| 33. | AY185360 | Brazil |
| 34. | LC080656 | Paraguay |
| 35. | MH170030 | Vietnam |
| 36. | KF801457 | Moldova |
| 37. | JN695879 | Russia |
| 38. | LC080659 | Bolivia |
| 39. | MF580994 | China |
| 40. | LC154848 | Myanmar |

**Figure 2.5*:* Heat map showing percent identities and differences of nucleotide and amino acid sequences in the BLV full-length Env sequences from South Africa and other geographic regions worldwide.** Percent identities (lower matrix) and differences (upper matrix) of BLV full-length Env (A) nucleotide and (B) amino acid sequences were compared between eight South African isolates (No. 1-8) and 32 sequences representing nine genotypes (G1-G7, G9 and G10) from other geographic regions worldwide (No. 9-40). (C) A table summarises sequence number, GenBank accession number and country of origin used in Figures A and B. SA, South Africa.

Furthermore, the full-length Env nucleotide and amino acid sequences of the G1-like South African isolate (M2746) shared high percent identities (>98.50%) and less sequence differences (< 20 nucleotides, < 5 amino acids) with the G1 sequences whereas those of the G-4 like South African isolates showed moderately high percent identities (98.00%-98.99%) and more sequence differences (<30 nucleotides, 5-29 amino acids) with the G4 sequences. However, intragenotype comparison showed that the full-length Env sequences of the G4-like South African isolates are more conserved than other genotypes. The pair-wise comparison of the full-length Env sequences amongst the seven G4-like South African isolates showed that they shared 99.74-100.00% nucleotide sequence identities and 99.61-100.00% amino acid sequence identities. In particular, the P591 and P2677 isolates had identical nucleotide sequences whereas despite some nucleotide sequence variabilities (99.74%- 99.94% or 1-4 nucleotide differences) amongst the other five South African isolates (K1170, P591, P2152, P2677 and L3401), their amino acid sequences shared 100.00% identity, indicating that their nucleotide substitutions were synonymous mutations.

Consistent with the phylogenetic analysis, the G-1like South African M2746 isolate was most closely related to the Saint Kitts and Nevis isolate (KX674369) with 99.61% nucleotide and amino acid identity (i.e., six nucleotide and two amino acid differences). Although their nucleotide sequences were slightly divergent, at the amino acid level, the South African M2746 isolate was also closely related to the Japanese isolate (AP018021) and the G1 American isolate (EF065641) with 99.81% sequence identity and one amino acid difference.

The seven G4-like South African isolates were most closely related to the G4 American isolate (AF033818) and the G4 Russian isolate (JN695878), sharing 98.51-98.71% nucleotide sequence identities (19-23 nucleotide differences) and 98.64-98.84% amino acid sequence identities (6-8 amino acid differences).

#### 2.3.2.2. Pair-wise comparison of the BLV partial Env sequences

To further assess the genetic variabilities of the BLV Env sequences between the G1- and G4-like South African isolates and the G1 and G4 isolates from other geographic regions, pair-wise comparison of the partial Env nucleotide and amino acid sequences was performed (Table 2.6 and Table S2.3 for the full data). This analysis was consistent with the phylogenetic analyses based on the partial *env* nucleotide sequences where one South African isolate belongs to G1 and the seven remaining South African isolates belong to G4 and they were most closely related to the G4 Zambian isolate (LC193462).

**Table 2.6: Nucleotide and amino acid differences in the BLV partial Env sequences between South Africa isolates and genotype-1 and genotype-4 isolates**

| | | | South Africa | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|
| | | | K1170 | K1194 | M1878 | M2746 | P591 | P2152 | P2677 | L3401 |
| | K1170 | South Africa | | | | | | | | |
| | K1194 | South Africa | 0 (0) | | | | | | | |
| | M1878 | South Africa | 1 (1) | 1 (1) | | | | | | |
| | M2746 | South Africa | 13 (4) | 13 (4) | 14 (5) | | | | | |
| | P591 | South Africa | 0 (0) | 0 (0) | 1 (1) | 13 (4) | | | | |
| | P2152 | South Africa | 0 (0) | 0 (0) | 1 (1) | 13 (4) | 0 (0) | | | |
| | P2677 | South Africa | 1 (0) | 1 (0) | 2 (1) | 14 (4) | 1 (0) | 1 (0) | | |
| | L3401 | South Africa | 1 (0) | 1 (0) | 2 (1) | 14 (4) | 1 (0) | 1 (0) | 2 (0) | |
| | AP018021 | Japan | 12 (3) | 12 (3) | 13 (4) | 3 (1) | 12 (3) | 12 (3) | 13 (3) | 13 (3) |
| G1 | FJ808571 | Argentina | 12 (4) | 12 (4) | 13 (5) | 3 (2) | 12 (4) | 12 (4) | 13 (4) | 13 (4) |
| G1 | D00647 | Australia | 12 (3) | 12 (3) | 13 (4) | 3 (1) | 12 (3) | 12 (3) | 13 (3) | 13 (3) |
| G1 | LC075568 | Bolivia | 13 (3) | 13 (3) | 14 (4) | 4 (1) | 13 (3) | 13 (3) | 14 (3) | 14 (3) |
| G1 | AF399703 | Brazil | 11 (4) | 11 (4) | 10 (3) | 4 (2) | 11 (4) | 11 (4) | 12 (4) | 12 (4) |
| G1 | EF065637 | Costa Rica | 11 (3) | 11 (3) | 12 (4) | 4 (1) | 11 (3) | 11 (3) | 12 (3) | 12 (3) |
| G1 | KX674372 | Dominica | 12 (3) | 12 (3) | 13 (4) | 3 (1) | 12 (3) | 12 (3) | 13 (3) | 13 (3) |
| G1 | EU266061 | Iran | 13 (4) | 13 (4) | 14 (5) | 4 (2) | 13 (4) | 13 (4) | 14 (4) | 14 (4) |
| G1 | EF065646 | Japan | 11 (3) | 11 (3) | 12 (4) | 2 (1) | 11 (3) | 11 (3) | 12 (3) | 12 (3) |
| G1 | LC075558 | Paraguay | 11 (3) | 11 (3) | 12 (4) | 2 (1) | 11 (3) | 11 (3) | 12 (3) | 12 (3) |
| G1 | LC075543 | Peru | 12 (3) | 12 (3) | 13 (4) | 3 (1) | 12 (3) | 12 (3) | 13 (3) | 13 (3) |
| G1 | KX674369 | St. Kitts and Nevis | 11 (3) | 11 (3) | 12 (4) | 2 (1) | 11 (3) | 11 (3) | 12 (3) | 12 (3) |
| G1 | KJ707242 | South Korea | 16 (5) | 16 (5) | 17 (6) | 8 (3) | 16 (5) | 16 (5) | 17 (5) | 17 (5) |
| G1 | KU233528 | Thailand | 12 (3) | 12 (3) | 13 (4) | 3 (1) | 12 (3) | 12 (3) | 13 (3) | 13 (3) |
| G1 | FM209468 | Uruguay | 13 (4) | 13 (4) | 12 (3) | 5 (2) | 13 (4) | 13 (4) | 14 (4) | 14 (4) |
| G1 | EF065641 | USA | 13 (3) | 13 (3) | 14 (4) | 4 (1) | 13 (3) | 13 (3) | 14 (3) | 14 (3) |
| G1 | MH170027 | Vietnam | 12 (3) | 12 (3) | 13 (4) | 3 (1) | 12 (3) | 12 (3) | 13 (3) | 13 (3) |
| G1 | MH170028 | Vietnam | 13 (4) | 13 (4) | 14 (5) | 4 (2) | 13 (4) | 13 (4) | 14 (4) | 14 (4) |
| G4 | HQ902258 | Belarus | 6 (3) | 6 (3) | 7 (4) | 13 (3) | 6 (3) | 6 (3) | 7 (3) | 7 (3) |
| G4 | K02251 | Belgium | 4 (3) | 4 (3) | 5 (4) | 13 (3) | 4 (3) | 4 (3) | 5 (3) | 5 (3) |
| G4 | AY515279 | Chile | 9 (5) | 9 (5) | 10 (6) | 16 (5) | 9 (5) | 9 (5) | 10 (5) | 10 (5) |
| G4 | M35238 | France | 2 (1) | 2 (1) | 3 (2) | 13 (3) | 2 (1) | 2 (1) | 3 (1) | 3 (1) |
| G4 | LC060792 | Mongolia | 3 (2) | 3 (2) | 4 (3) | 10 (2) | 3 (2) | 3 (2) | 4 (2) | 4 (2) |
| G4 | AF067081 | Poland | 4 (2) | 4 (2) | 5 (3) | 13 (2) | 4 (2) | 4 (2) | 5 (2) | 5 (2) |
| G4 | JN695878 | Russia | 5 (3) | 5 (3) | 6 (4) | 14 (3) | 5 (3) | 5 (3) | 6 (3) | 6 (3) |
| G4 | JQ696091 | Russia | 6 (3) | 6 (3) | 7 (4) | 15 (3) | 6 (3) | 6 (3) | 7 (3) | 7 (3) |
| G4 | AF033818 | USA | 4 (3) | 4 (3) | 5 (4) | 13 (3) | 4 (3) | 4 (3) | 5 (3) | 5 (3) |
| G4 | LC193462 | Zambia | 1 (0) | 1 (0) | 2 (1) | 12 (4) | 1 (0) | 1 (0) | 2 (0) | 2 (0) |

Amino acid sequence identities and differences are shown in brackets. Each cell in the table is highlighted according to amino acid differences.

Differences 0 1 2 3 4 5 6 >7

The NJ and ML trees based on the partial Env nucleotide sequences could not identify G1 global isolate(s) that was most closely related to the G1-like South African isolate (M2746) as the G1-like South African isolate was closely related to fourteen G1 isolates with 39%

bootstrap value. However, pair-wise comparison of the partial *env* nucleotide and amino acid sequences revealed that the G1-like South African isolate was most closely related to the Japanese (EF065646), Paraguayan (LC075558) and St Kitts and Nevis (KX674369) isolates, sharing 99.55% nucleotide sequence identity (two nucleotide differences) and 99.32% amino acid sequence identity (one amino acid difference).

Three G4-like South African isolates (M1878, P2677 and L3401) and other four G4-like South African isolates (K1170, K1194, P591 and P2152) shared 99.55% (two nucleotide differences) and 99.77% nucleotide sequence identities (one nucleotide difference) with the Zambian isolate (LC193462), respectively but all seven G4-like South African isolates except the M1878 isolate shared 100% amino acid sequence identity with the Zambian isolate. The M1878 isolate shared 99.32% amino acid sequence identity with the Zambian isolate, which is only one amino acid difference between these isolates.

#### 2.3.2.3. Pair-wise comparison of the BLV Gag nucleotide and amino acid sequences between the South African and global isolates

Consistent with the phylogenetic analyses based on the NJ and ML trees, pair-wise comparison of the BLV full-length Gag nucleotide and amino acid sequences revealed that the G-1 like South African isolate (M2746) was most closely related to the G1 Vietnamese isolate (MH170028) and the seven remaining South African isolates were most closely related to the G4 isolates (Table 2.7, Figure 2.6 and Table S2.4 for the full data). The *gag* sequences amongst the seven G4-like South African isolates appeared slightly more variable than the *env* sequences. Furthermore, as evident in heat maps, the Gag amino acid sequences are generally more conserved than the *gag* nucleotide sequences (Figure 2.6 A and B) as well as the Env amino acid sequences (Figure 2.5 B).

Whilst the seven G4-like South African isolates showed up to four nucleotide and two amino acid differences in the *env* sequences with each other, their *gag* sequences had up to nine nucleotide and four amino acid differences with each other. Despite these some variabilities, the Gag sequences of the seven G4-like South African isolates still showed high sequence identities, sharing 99.24-99.92% nucleotide and 98.73-100.00% amino acid sequence identities with each other. The K1170 and P2677 isolates shared 100.00% amino acid sequence identity and the P2152 and L3401 isolates also shared 100.00% amino acid sequence identity.

**Table 2.7: Nucleotide and amino acid differences in the BLV full-length Gag sequences between South Africa isolates and global isolates representing nine genotypes**

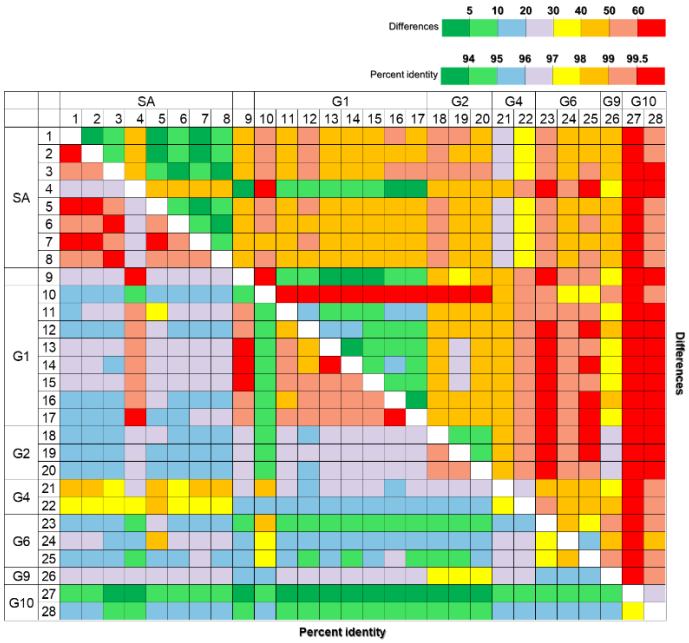
| | | | South Africa | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|
| | | | K1170 | K1194 | M1878 | M2746 | P591 | P2152 | P2677 | L3401 |
| | K1170 | South Africa | | | | | | | | |
| | K1194 | South Africa | 5 (1) | | | | | | | |
| | M1878 | South Africa | 8 (3) | 9 (3) | | | | | | |
| | M2746 | South Africa | 46 (9) | 45 (10) | 47 (10) | | | | | |
| | P591 | South Africa | 4 (2) | 5 (3) | 10 (5) | 44 (9) | | | | |
| | P2152 | South Africa | 7 (2) | 8 (2) | 3 (1) | 44 (9) | 8 (3) | | | |
| | P2677 | South Africa | 2 (0) | 3 (1) | 8 (3) | 44 (9) | 1 (1) | 7 (2) | | |
| | L3401 | South Africa | 6 (2) | 7 (2) | 2 (1) | 45 (9) | 7 (3) | 1 (0) | 6 (2) | |
| | AP018021 | Japan | 44 (8) | 43 (9) | 45 (9) | 5 (1) | 43 (9) | 43 (8) | 42 (8) | 43 (8) |
| G1 | D00647 | Australia | 51 (23) | 50 (24) | 54 (26) | 66 (25) | 50 (24) | 52 (25) | 49 (23) | 52 (25) |
| | AB934282 | Japan | 48 (9) | 47 (10) | 47 (8) | 9 (2) | 47 (10) | 47 (9) | 46 (9) | 47 (9) |
| | LC005615 | Japan | 50 (11) | 51 (12) | 51 (12) | 7 (2) | 51 (12) | 49 (11) | 50 (11) | 49 (11) |
| | LC080651 | Paraguay | 46 (9) | 45 (10) | 47 (10) | 7 (2) | 45 (10) | 45 (9) | 44 (9) | 45 (9) |
| | HE967301 | Uruguay | 47 (9) | 46 (10) | 48 (10) | 8 (2) | 46 (10) | 46 (9) | 45 (9) | 46 (9) |
| | EF600696 | USA | 46 (9) | 45 (10) | 47 (10) | 7 (3) | 45 (10) | 45 (9) | 44 (9) | 45 (9) |
| | MH170027 | Vietnam | 50 (11) | 49 (12) | 51 (12) | 5 (2) | 49 (12) | 49 (11) | 48 (11) | 49 (11) |
| | MH170028 | Vietnam | 49 (10) | 48 (11) | 50 (11) | 4 (1) | 48 (11) | 48 (10) | 47 (10) | 48 (10) |
| G2 | FJ914764 | Argentina | 52 (11) | 51 (12) | 53 (12) | 44 (8) | 51 (12) | 51 (11) | 50 (11) | 51 (11) |
| | LC080655 | Paraguay | 50 (8) | 49 (9) | 51 (9) | 40 (5) | 49 (9) | 49 (8) | 48 (8) | 49 (8) |
| | LC080654 | Peru | 49 (8) | 48 (9) | 50 (9) | 43 (5) | 48 (9) | 48 (8) | 47 (8) | 48 (8) |
| G4 | KT122858 | Belgium | 22 (6) | 23 (7) | 25 (9) | 44 (9) | 23 (7) | 24 (8) | 22 (6) | 23 (8) |
| | AF033818 | USA | 30 (6) | 31 (7) | 31 (7) | 52 (8) | 31 (7) | 32 (8) | 30 (6) | 31 (8) |
| G6 | MF580991 | China | 53 (11) | 50 (10) | 54 (13) | 63 (12) | 54 (12) | 54 (12) | 53 (11) | 54 (12) |
| | LC080656 | Paraguay | 45 (6) | 46 (7) | 48 (9) | 56 (7) | 46 (7) | 46 (8) | 45 (6) | 46 (8) |
| | MH170030 | Vietnam | 49 (8) | 48 (9) | 50 (9) | 60 (9) | 48 (9) | 50 (10) | 47 (8) | 50 (10) |
| G9 | LC080659 | Bolivia | 43 (8) | 44 (9) | 44 (9) | 37 (5) | 44 (9) | 42 (8) | 43 (8) | 42 (8) |
| G10 | MF580994 | China | 69 (21) | 70 (22) | 72 (23) | 75 (20) | 70 (22) | 70 (23) | 69 (21) | 70 (23) |
| | LC154848 | Myanmar | 57 (12) | 58 (13) | 60 (15) | 65 (11) | 58 (13) | 58 (14) | 57 (12) | 58 (14) |

Amino acid sequence identities and differences are shown in brackets. Each cell in the table is highlighted according to amino acid differences.

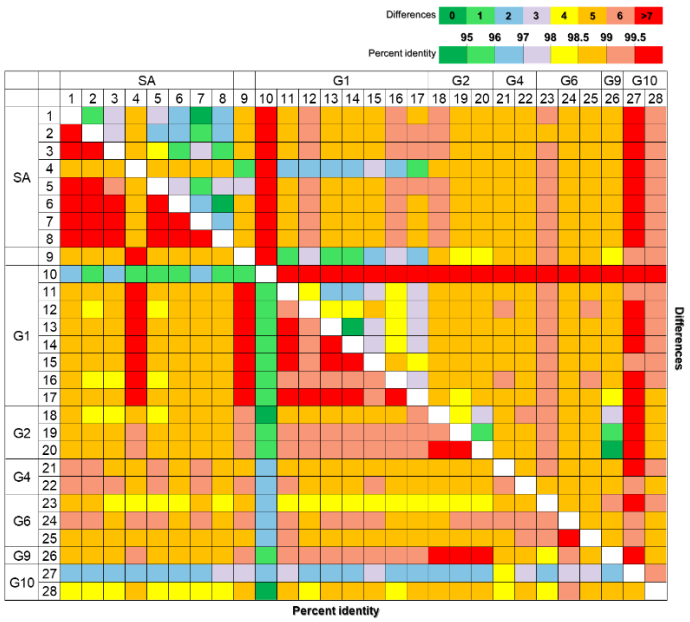
Differences 0 1 2 3 4 5 6 >7

Consistent with the results from the pair-wise comparison of the BLV Env sequences, the G1-like South African isolate (M2746) had fewer sequence variabilities with G1 isolates than the sequence variabilities between the G4-like South African isolates and the G4 isolates. The G1-like South African isolate (M2746) shared more than 99.00% nucleotide and amino acid sequence identities (<10 nucleotide differences and <4 amino acid differences) with all other G1 isolates except the Australian isolate (D000647) (c.f., the Australian isolate has 3-nucleotide deletion and is more divergent from other G1 sequences). The G1-like South African isolate shared the highest nucleotide sequence identity (99.66% identity or four nucleotide differences) and the highest amino acid identity (99.75% or one amino acid difference) with G1 Vietnamese isolate (MH170028). The G1-like South African isolate also shared with 99.75% amino acid identity and one amino acid difference with the Japanese isolate (AP018021).

**A. Nucleotide sequence**




**B. Amino acid sequence**




**C**

| | GenBank Accession number | Country of origin |
|---|---|---|
| 1. | K1170 | South Africa |
| 2. | K1194 | South Africa |
| 3. | M1878 | South Africa |
| 4. | M2746 | South Africa |
| 5. | P591 | South Africa |
| 6. | P2152 | South Africa |
| 7. | P2677 | South Africa |
| 8. | L3401 | South Africa |
| 9. | AP018021 | Japan |
| 10. | D00647 | Australia |
| 11. | AB934282 | Japan |
| 12. | LC005615 | Japan |
| 13. | LC080651 | Paraguay |
| 14. | HE967301 | Uruguay |
| 15. | EF600696 | USA |
| 16. | MH170027 | Vietnam |
| 17. | MH170028 | Vietnam |
| 18. | FJ914764 | Argentina |
| 19. | LC080655 | Paraguay |
| 20. | LC080654 | Peru |
| 21. | KT122858 | Belgium |
| 22. | AF033818 | USA |
| 23. | MF580991 | China |
| 24. | LC080656 | Paraguay |
| 25. | MH170030 | Vietnam |
| 26. | LC080659 | Bolivia |
| 27. | MF580994 | China |
| 28. | LC154848 | Myanmar |

**Figure 2.6*:* Heat map showing percent identities and differences of nucleotide and amino acid sequences in the BLV full-length Gag sequences from South Africa and other geographic regions worldwide.** Percent identities (lower matrix) and differences (upper matrix) of BLV full-length Gag (A) nucleotide and (B) amino acid sequences were compared between eight South African isolates (No. 18) and 20 sequences representing six genotypes (G1, G2, G4, G6, G9 and G10) from other geographic regions worldwide (No. 9-28). (C) A table summarises sequence number, GenBank accession number and country of origin used in Figures A and B.

In contrast, the G4-like South African isolates shared only 97.29-98.14% nucleotide identities and 97.72-98.48% amino acid identities with the G4 Belgian isolates (KT122858) and American isolate (AF0033818), which corresponds to 22-32 nucleotide differences and 6-9 amino acid differences.

### 2.3.3. Genetic variabilities of the BLV Env and Gag sequences

To assess genetic divergence of different BLV strains, mean intragenotype and intergenotype genetic distances were estimated amongst and between the South African sequences and 115 full-length Env and 56 full-length Gag sequences from BLV isolates from various geographic regions obtained from the NCBI [381].

#### 2.3.3.1. Genetic variabilities of the BLV Env sequences

Using 115 full-length Env sequences with ten genotypes available from the NCBI [381] and the eight South African Env sequences, mean intragenotype and intergenotype nucleotide and amino acid distances were estimated (Table 2.8). This analysis was consistent with the results from the pair-wise comparison of the full-length *env* sequences showing that the Env sequences of the G4-like South African isolates themselves were more conserved than other genotypes. The analysis also confirmed that the seven G4-like South African isolates and one G1-like South African isolate belong to G4 and G1, showing the lowest mean intergenotype distances. The full-length Env sequences of the G1-like and G4-like South Africa isolates had mean nucleotide distances of 0.008 and 0.015 with the G1 and G4 global isolates, respectively whereas their mean amino acid distances were 0.006 and 0.014, respectively.

Mean intragenotype distance of the G4-like South African Env sequences were 0.001 for both nucleotide and amino acid sequences, which was the lowest amongst all genotypes and indicates that the Env sequences of the G4-like South African sequences were less variable than other genotypes. Furthermore, the analysis revealed that whilst the mean intragenotype nucleotide distance of the G4 isolate (0.014) was twenty times higher than that of G1 (0.007), their amino acid distances of G1 and G4 isolates were comparable (0.007 and 0.009, respectively). This indicates that the full-length Env amino acid sequences of G1 and G4 isolates including the G4-like South African isolates were conserved.

In contrast, Env sequences of G6 and G10 isolates appeared to be more variable than other genotypes. The G6 and G10 Env sequences showed mean intragenotype nucleotide distances of 0.026 and 0.027, respectively, and mean intragenotype amino acid distances of 0.022 and 0.032, respectively. Lastly, average genetic distances in the Env nucleotide and

amino acid sequences amongst all genotypes including South African isolates were calculated and they were 0.038 and 0.032, respectively.

**Table 2.8: Mean nucleotide and amino acid distances in the BLV full-length Env sequences within (intragenotype) and between (intergenotype) BLV strains from South Africa and other geographic regions worldwide**

| | G1-like | G4-like | G1 | G2 | G3 | G4 | G5 | G6 | G7 | G9 | G10 | Avarage intergenotype distance |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| G1-like | N/A | 0.031 ± 0.008 | **0.006 ± 0.002** | 0.031 ± 0.007 | 0.023 ± 0.007 | 0.028 ± 0.007 | 0.035 ± 0.007 | 0.038 ± 0.008 | 0.027 ± 0.007 | 0.023 ± 0.006 | 0.043 ± 0.008 | |
| G4-like | 0.039 ± 0.006 | 0.001 ± 0.001<br>0.001 ± 0.001 | 0.032 ± 0.007 | 0.036 ± 0.007 | 0.026 ± 0.007 | **0.014 ± 0.004** | 0.033 ± 0.007 | 0.031 ± 0.006 | 0.028 ± 0.007 | 0.029 ± 0.007 | 0.037 ± 0.006 | |
| G1 | **0.008 ± 0.002** | 0.036 ± 0.005 | 0.007 ± 0.001<br>0.007 ± 0.001 | 0.032 ± 0.006 | 0.022 ± 0.006 | 0.028 ± 0.006 | 0.036 ± 0.007 | 0.039 ± 0.007 | 0.027 ± 0.006 | 0.023 ± 0.006 | 0.044 ± 0.007 | |
| G2 | 0.035 ± 0.005 | 0.038 ± 0.005 | 0.033 ± 0.004 | 0.011 ± 0.002<br>0.020 ± 0.005 | 0.018 ± 0.004 | 0.033 ± 0.006 | 0.045 ± 0.008 | 0.044 ± 0.007 | 0.035 ± 0.007 | 0.019 ± 0.004 | 0.049 ± 0.007 | |
| G3 | 0.030 ± 0.005 | 0.034 ± 0.005 | 0.029 ± 0.004 | 0.025 ± 0.004 | 0.005 ± 0.001<br>0.001 ± 0.001 | 0.024 ± 0.006 | 0.037 ± 0.008 | 0.036 ± 0.007 | 0.027 ± 0.007 | 0.012 ± 0.005 | 0.041 ± 0.007 | |
| G4 | 0.039 ± 0.005 | **0.015 ± 0.003** | 0.037 ± 0.005 | 0.039 ± 0.005 | 0.035 ± 0.004 | 0.014 ± 0.002<br>0.009 ± 0.003 | 0.033 ± 0.008 | 0.031 ± 0.007 | 0.023 ± 0.006 | 0.025 ± 0.006 | 0.037 ± 0.006 | 0.032 |
| G5 | 0.047 ±0.005 | 0.038 ± 0.005 | 0.040 ±0.005 | 0.046 ± 0.005 | 0.046 ± 0.005 | 0.039 ± 0.005 | 0.016 ± 0.002<br>0.002 ± 0.004 | 0.043 ± 0.007 | 0.033 ± 0.008 | 0.038 ± 0.008 | 0.048 ± 0.007 | |
| G6 | 0.047 ± 0.005 | 0.041 ± 0.005 | 0.045 ± 0.005 | 0.050 ± 0.005 | 0.050 ± 0.005 | 0.043 ± 0.004 | 0.049 ± 0.005 | 0.026 ± 0.003<br>0.022 ± 0.004 | 0.033 ± 0.006 | 0.037 ± 0.007 | 0.030 ± 0.005 | |
| G7 | 0.030 ± 0.005 | 0.032 ± 0.005 | 0.038 ± 0.005 | 0.042 ± 0.005 | 0.040 ± 0.005 | 0.035 ± 0.005 | 0.045 ± 0.005 | 0.045 ± 0.005 | 0.008 + 0.002<br>0.008 ± 0.003 | 0.028 ± 0.007 | 0.037 ± 0.006 | |
| G9 | 0.031 ± 0.005 | 0.035 ± 0.005 | 0.031 ± 0.005 | 0.024 ± 0.004 | 0.022 ± 0.004 | 0.036 ± 0.005 | 0.045 ± 0.006 | 0.045 ± 0.005 | 0.041 ± 0.006 | 0.002 ± 0.001<br>0.001 ± 0.001 | 0.040 ± 0.007 | |
| G10 | 0.049 ± 0.006 | 0.044 ± 0.005 | 0.048 ± 0.005 | 0.051 ± 0.005 | 0.045 ± 0.005 | 0.045 ± 0.005 | 0.029 ± 0.005 | 0.029 ± 0.003 | 0.049 ± 0.005 | 0.047 ± 0.005 | 0.027 + 0.003<br>0.032 ± 0.005 | |
| Avarage intergenotype distance | 0.038 | | | | | | | | | | | |

Genetic distances in each column are shown with standard errors. Lower matrix (highlighted in blue) shows intergenotype nucleotide distance and the upper matrix (highlighted in yellow) shows intergenotype amino acid distance. Diagonal columns (highlighted in green) show intragenotype nucleotide (top) and amino acid (bottom) distances. Average nucleotide and amino acid intergenotype distance from all genotypes are shown in the bottom row and right column, respectively. Values in bold indicate minimum genetic divergence with the South African isolates. N/A, not applicable.

### 2.3.3.2. Genetic variabilities of the BLV Gag sequences

Using 56 full-length Gag sequences with six genotypes available from the NCBI [381] and the eight South African Gag sequences, mean intragenotype and intergenotype nucleotide and amino acid distances were estimated (Table 2.9). The analysis indicated that the G4-like South African Gag sequences were more variable than the G4-like South African Env sequences. Mean intragenotype nucleotide and amino acid distances of the G4-like South African Gag sequences were 0.005 for both sequences whilst the mean intragenotype nucleotide and amino acid distance of the G4-like South African Env sequences were 0.001 (Table 2.8).

The average genetic distances in the Gag nucleotide and amino acid sequences amongst all genotypes were 0.043 and 0.029, respectively. This is consistent with the results from the pair-wise comparison of the Gag sequences showing that the Gag amino acid sequences are more conserved than the *gag* nucleotide sequences. It appeared that the nucleotide changes in the *gag* gene did not dramatically change Gag amino acid sequences, which is reflected by the lower average amino acid distance (0.029) than the average nucleotide distance (0.043).

Consistent with the genetic variabilities of the G4-like South African full-length Env sequences, mean nucleotide and amino acid distances between the G4-like South African isolates and the G4 isolates were lowest amongst all nine genotypes, showing 0.023 and 0.020,

respectively, which were slightly more divergent than the mean nucleotide and amino acid sequence distances in the full-length Env sequences between the G4-like South African and G4 isolates (0.015 and 0.014, respectively).

Mean nucleotide and amino acid distances between the G1-like South African isolates and the G1 isolates were 0.009 and 0.021, respectively. However, mean intergenotype amino acid distance between the G1-like South African isolate and G9 isolates showed 0.013, which was the lowest value amongst all nine genotypes, indicating that the G1-like South African isolate has a G9-like Gag amino acid sequence.

**Table 2.9: Mean nucleotide and amino acid distances in the BLV full-length Gag sequences within (intragenotype) and between (intergenotype) BLV strains**

| | G1-like | G4-like | G1 | G2 | G4 | G6 | G9 | G10 | Avarage intergenotype distance |
|---|---|---|---|---|---|---|---|---|---|
| G1-like | N/A | 0.025 ± 0.007 | 0.021 ± 0.003 | 0.020 ± 0.006 | 0.023 ± 0.007 | 0.028 ± 0.007 | **0.013 ± 0.006** | 0.035 ± 0.008 | 0.029 |
| G4-like | 0.039 ± 0.000 | 0.005 ± 0.001<br>0.005 ± 0.003 | 0.041 ± 0.008 | 0.030 ± 0.008 | **0.019 ± 0.006** | 0.029 ± 0.007 | 0.023 ± 0.007 | 0.039 ± 0.008 | |
| G1 | **0.009 ± 0.003** | 0.041 ± 0.000 | 0.013 ± 0.002<br>0.038 ± 0.005 | 0.037 ± 0.007 | 0.038 ± 0.007 | 0.044 ± 0.007 | 0.030 ± 0.006 | 0.051 ± 0.008 | |
| G2 | 0.038 ± 0.001 | 0.044 ± 0.000 | 0.039 ± 0.001 | 0.005 ± 0.001<br>0.011 ± 0.004 | 0.029 ± 0.007 | 0.031 ± 0.007 | 0.008 ± 0.003 | 0.037 ± 0.008 | |
| G4 | 0.042 ± 0.002 | **0.023 ± 0.001** | 0.023 ± 0.001 | 0.043 ± 0.001 | 0.015 ± 0.005<br>0.011 ± 0.004 | 0.024 ± 0.006 | 0.022 ± 0.006 | 0.034 ± 0.007 | |
| G6 | 0.055 ± 0.002 | 0.046 ± 0.001 | 0.053 ± 0.001 | 0.055 ± 0.001 | 0.043 ± 0.001 | 0.028 ± 0.003<br>0.020 ± 0.004 | 0.027 ± 0.007 | 0.030 ± 0.006 | |
| G9 | 0.034 ± 0.000 | 0.039 ± 0.001 | 0.035 ± 0.000 | 0.026 ± 0.000 | 0.038 ± 0.000 | 0.049 ± 0.000 | 0.002 ± 0.000<br>0.0003 ± 0.000 | 0.033 ± 0.007 | |
| G10 | 0.059 ± 0.002 | 0.053 ± 0.001 | 0.058 ± 0.001 | 0.059 ± 0.001 | 0.050 ± 0.001 | 0.043 ± 0.002 | 0.055 ± 0.000 | 0.025 ± 0.005<br>0.026 ± 0.005 | |
| Avarage intergenotype distance | 0.043 | | | | | | | | |

Genetic distances in each column are shown with standard errors. Lower matrix (highlighted in blue) shows intergenotype nucleotide distance and the upper matrix (highlighted in yellow) shows intergenotype amino acid distance. Diagonal columns (highlighted in green) show intragenotype nucleotide (top) and amino acid (bottom) distances. Average nucleotide and amino acid intergenotype distance from all genotypes are shown in the bottom row and the right column, respectively. Values in bold indicate minimum genetic divergence with the South African isolates. N/A, not applicable.

### 2.3.4. Alignment of the BLV Env and Gag amino acid sequences

To assess whether nucleotide substitutions altered amino acid sequences in the South African isolates and to identify genotype-specific amino acid substitutions previously described [249, 328-330] as well as amino acid substitutions unique to the South African isolates, the full-length Env and Gag deduced amino acid sequences of the South African isolates were aligned with a total of 282 Env amino sequences and 211 Gag amino acid sequences available from the NCBI [381].

### 2.3.4.1. Alignment of the BLV Env amino acid sequences

Alignment of the Env amino acid sequences of the South African isolates with the 282 Env amino acid sequences obtained from the NCBI [381] confirmed the presence of the G1- and

G4-specific amino acid substitutions in the M2746 isolate and the seven other South African isolates, respectively. Table 2.10 shows the alignment of Env amino acid sequences of the South African isolates with those of the 29 selected isolates representing ten genotypes. The analysis showed that the South African isolates contained G1-specific and G4-specific amino acid substitutions at 14 residues (at positions 29, 48, 56, 73, 74, 82, 121, 132, 134, 144, 254, 479, 480 and 504) in their Env sequences. Furthermore, three amino acid substitutions in the Env sequences (at positions 59, 153 and 476) that were present exclusively in the South African sequences were detected. An isoleucine-to-leucine substitution at position 59 (I59L) and a histidine-to-glutamine substitution at position 153 (H153Q) were only present in the K1194 and M2746 isolates, respectively and thus, this could be a random mutation or sequencing errors. In contrast, an E476D substitution was consistently found in the seven G4-like South African isolates and thus, this amino acid substitution appeared to be unique to the G4-like South African isolates. A D134N substitution was found only in the G4-like South African M1878 isolate and this appeared to be a G1-like substitution.

### 2.3.4.2. Alignment of the BLV Gag amino acid sequences

Alignment of the Gag amino acid sequences of the South African isolates with 212 Gag amino acid sequences available on the NCBI [381] also confirmed the presence of the G1- and G4-specific amino acid substitutions in the M2746 isolate and the seven other South African isolates, respectively. Table 2.11 shows the alignment of Gag amino acid sequences of the South African isolates with those of the 16 selected isolates representing six genotypes. The analysis showed that the South African isolates contained G1-specific and G4-specific amino acid substitutions at four residues (at positions 63, 69, 88 and 365) in their Gag sequences.

In this study, seven amino acid substitutions that had not been previously described were detected in the South African isolates and these were mainly found from the G4-like South African isolates. These were found at positions 29, 107, 108, 278, 318, 341 and 343 in the Gag sequences.

**Table 2.10: Amino acid substitutions in the BLV env gene of the South African sequences and 25 selected sequences**

| | | gp51 SU | | | | | | | | | | | | | | gp30 TM | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | Epitopes and motifs | Leader sequence | G epitope | H epitope | | G epitope | | | | | ND2 | | | D epitope | | | | | PXXP motif |
| | Amino acid position | 29 | 48 | 56 | 59 | 73 | 74 | 82 | 121 | 132 | 134 | 144 | 153 | 254 | 476 | 479 | | 480 | 504 |
| | Reference | d | a,d | a | | a,b | a,d | a,d | a,c | a | a,d | a,d | | a,d | | d | | d | d |
| | K1170 | Q | T | F | I | P | R | F | H | R | D | T | H | L | D | F | | P | T |
| | K1194 | Q | T | F | L | P | R | F | H | R | D | T | H | L | D | F | | P | T |
| | M1878 | Q | T | F | I | P | R | F | H | R | N | T | H | L | D | F | | P | T |
| | M2746 | R | A | S | I | A | K | S | R | Q | D | I | Q | S | E | L | | T | V |
| | P591 | Q | T | F | I | P | R | F | H | R | D | T | H | L | D | F | | P | T |
| | P2152 | Q | T | F | I | P | R | F | H | R | D | T | H | L | D | F | | P | T |
| | P2677 | Q | T | F | I | P | R | F | H | R | D | T | H | L | D | F | | P | T |
| | L3401 | Q | T | F | I | A | R | F | H | R | D | T | H | L | D | F | | P | T |
| | AP018021 JP | R | A | S | I | A | K | S | R | Q | D | I | H | S | E | L | | T | V |
| G1 | AF399703 BR | Q | A | S | I | A | K | F | R | Q | N | I | H | S | E | F | | T | V |
| G1 | KX674639 KN | R | A | S | I | A | K | S | R | Q | D | I | H | S | E | L | | T | V |
| G1 | EF065646 JP | Q | A | S | I | A | K | S | R | Q | D | I | H | S | E | L | | T | V |
| G1 | LC075558 PY | - | - | - | - | - | - | - | R | Q | D | I | H | - | - | - | | - | - |
| G1 | HE967301 UY | R | A | S | I | A | K | S | R | Q | N | I | H | S | E | L | | T | V |
| G1 | EF065641 US | R | A | S | I | A | K | S | R | Q | D | I | H | S | E | L | | T | V |
| G2 | AF257515 AR | Q | A | S | I | A | K | F | R | Q | D | I | H | L | E | L | | A | T |
| G2 | LC080655 PY | Q | A | S | I | A | K | F | R | Q | D | I | H | L | E | F | | A | T |
| G2 | LC080654 PE | Q | V | S | I | A | K | F | R | Q | D | I | H | L | E | F | | A | T |
| G3 | EF065650 JP | Q | A | S | I | A | K | F | R | Q | D | I | H | L | E | F | | A | T |
| G3 | EF065647 US | Q | A | S | I | A | K | F | R | Q | D | I | H | L | E | F | | A | T |
| G4 | M35238 FR | Q | T | F | I | P | R | F | H | Q | D | T | H | L | E | F | | P | T |
| G4 | AF067081 PL | - | - | - | - | - | - | - | H | Q | D | I | H | - | - | - | | - | - |
| G4 | JN695878 RU | Q | T | S | I | P | R | F | H | Q | D | I | H | L | E | F | | P | T |
| G4 | AF033818 US | Q | T | S | I | P | R | F | H | Q | D | I | H | L | E | F | | P | T |
| G4 | LC193462 ZM | - | - | - | - | - | - | - | H | R | D | T | H | - | - | - | | - | - |
| G5 | AF399702 BR | - | - | - | - | - | - | - | R | R | D | I | H | - | - | - | | - | - |
| G5 | EF065635 CR | R | T | S | I | A | R | F | R | R | D | I | H | L | E | F | | T | T |
| G6 | LC075571 BO | - | - | - | - | - | - | - | R | Q | D | T | H | - | - | - | | - | - |
| G6 | AY185360 BR | Q | T | S | I | A | R | F | R | Q | D | T | H | L | E | F | | T | T |
| G6 | MF580991 CN | Q | T | S | I | A | R | F | R | Q | D | T | Y | L | E | F | | T | T |
| G6 | KU233530 TH | - | - | - | - | - | - | - | R | Q | D | T | H | - | - | - | | - | - |
| G7 | KF801457 MD | Q | I | S | I | A | R | F | R | Q | D | I | H | L | E | F | | T | A |
| G7 | JN695879 RU | Q | T | S | I | A | R | F | R | Q | D | I | H | L | E | F | | T | A |
| G7 | S83530 IT | - | - | - | - | - | - | - | R | Q | D | I | H | - | - | - | | - | - |
| G8 | GU724606 HR | - | - | - | - | - | - | - | R | Q | D | I | H | - | - | - | | - | - |
| G8 | JF713455 RU | - | - | - | - | - | - | - | R | Q | D | I | H | - | - | - | | - | - |
| G9 | LC080659 BO | Q | A | S | I | A | K | L | R | Q | D | I | H | L | E | F | | A | T |
| G9 | LC080664 BO | Q | A | S | I | A | K | F | R | Q | D | I | H | L | E | F | | A | T |
| G10 | LC154848 MM | Q | T | S | I | A | R | F | R | Q | D | T | H | L | E | F | | T | T |
| G10 | KU233527 TH | - | - | - | - | - | - | - | H | Q | D | T | H | - | - | - | | - | - |

a, amino acid substitutions described by Rodriguez et al. (2009) [328]; b, genotype-specific amino acid substitution described by Zhao and Buehring (2007) [249]; c, genotype-specific amino acid substitution previously described by Lee *et a*l. (2016) [329]; d, genotype-specific amino acid substitutions described by Polat *et al.* (2016) [330]. SU, surface protein; TM, transmembrane protein; ND 2, neutralising domain 2; PXXP motif, proline-rich motif for the cell signalling; –, not available due to the partial *env* sequences. Amino acid substitutions that are previously not described are highlighted in blue. G1 and G4 sequences are highlighted in yellow for comparison. The country of origin is indicated by 2-letter codes. AR, Argentina; BE, Belgium; BO, Bolivia; BR, Brazil; HR, Croatia; IT, Italy; JP, Japan; KN, Saint Kitts and Nevis; MM, Myanmar; MD, Moldova; PE, Peru; PL, Poland; PY, Paraguay; RU, Russia; TH, Thailand; US, USA; UY, Uruguay; ZM, Zambia.

It appeared that four of these seven amino acid substitutions were unique to the South African sequences. The amino acid alignment showed that N29D, I278V and P343S substitutions were only found in the seven G4-like South African sequences, though the I278V substitution was also detected in an Iranian isolate (LC193727), which has not been genotyped. Although previously not described, our analysis indicates that the presence of methionine at position 318 in the M2746 sequence and isoleucine at the same position in the seven G4-like South African sequences could be genotype-specific. A K341Q substitution was only found in the South African P591 isolate and it is uncertain whether this is a spontaneous mutation or a sequencing error.

**Table 2.11: Amino acid substitutions in the BLV Gag gene of the South African sequences and 16 selected sequences**

| | | p15 MA | | | | | | p24 CA | | p12 NC | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | Amino acid position | 29 | 63* | 69* | 88* | 107 | 108 | 278 | 318 | 341 | 343 | 365* |
| | K1170 | D | A | K | E | A | V | V | I | K | S | T |
| | K1194 | D | A | K | E | V | V | V | I | K | S | T |
| | M1878 | D | A | R | G | S | I | V | I | K | S | T |
| | M2746 | N | T | K | G | A | V | I | M | K | P | A |
| | P591 | D | A | K | E | A | V | V | I | Q | S | T |
| | P2152 | D | A | K | G | S | V | V | I | K | S | T |
| | P2677 | D | A | K | E | A | V | V | I | K | S | T |
| | L3401 | D | A | K | G | S | V | V | I | K | S | T |
| | AP018021 | N | T | R | G | A | V | I | M | K | P | A |
| G1 | D00647 AU | N | A | K | E | A | V | I | T | K | P | T |
| | AB934282 JP | N | T | R | G | A | I | I | M | K | P | A |
| | LC005615 JP | N | T | R | G | A | V | I | M | K | P | A |
| | LC080651 PY | N | T | R | G | A | V | I | M | K | P | A |
| | MH170027 VN | N | T | R | G | A | V | I | M | K | P | A |
| | MH170028 VN | N | T | R | G | A | V | I | M | K | P | A |
| | EF600696 US | N | T | R | G | A | V | I | V | K | P | A |
| | HE967301 UY | N | T | R | G | A | V | I | M | K | P | A |
| G2 | FJ914764 AR | N | T | R | G | A | V | I | I | K | P | T |
| | LC080655 PY | N | T | R | G | A | V | I | I | K | P | T |
| | LC080654 PE | N | T | R | G | A | V | I | I | K | P | T |
| G4 | KT122858 BE | N | A | K | E | A | V | I | I | K | P | T |
| | AF033818 US | N | A | K | E | A | I | I | V | K | P | T |
| G6 | MF580991 CN | N | V | K | E | V | V | I | T | K | P | T |
| | LC080656 PY | N | V | K | E | A | V | I | T | K | P | T |
| | MH170030 VN | N | V | K | E | A | I | I | T | K | P | T |
| G9 | LC080659 BO | N | T | R | G | A | V | I | I | K | P | T |
| | LC080664 BO | N | T | R | G | A | V | I | I | K | P | T |
| G10 | MF580994 CN | N | V | K | E | A | D | I | V | K | P | A |
| | LC154848 MM | N | V | K | E | A | V | I | V | K | P | A |

* genotype-specific amino acid substitutions previously described by Polat *et al*. (2016) [330]. Amino acid substitutions that are previously not described are highlighted in blue. G1 and G4 sequences are highlighted in yellow for comparison. MA, matrix; CA, capsid; NC, nucleocapsid. The country of origin is indicated by 2-letter codes. AR, Argentina; AU, Australia; BE, Belgium; BO, Bolivia; CN, China; JP, Japan; MM, Myanmar; PE, Peru; PY, Paraguay; US, USA; UY, Uruguay; VN, Vietnam.

A genotype-specific glycine-to-glutamic acid substitution at position 88 (G88E) was previously described [330] and in their study, the presence of the glycine residue at this position was identified as a G1-specific and the presence of the glutamic acid residue as G4-specific. However, in our study, the G4-like M1878, P2152 and L3401 isolates had the glycine residue at this position. This could be a mutation conserved amongst these G4-like South African isolates, random mutation or a sequencing error.

A V108I substitution was exclusively found in the M1878 sequence and this mutation can be G4-specific but AF033818-like change or a sequencing error. On the other hand, an A107V substitution in the K1194 isolate and an A107S substitution in the M1878, P2152 and L3401 isolates could be either a sequencing error or a random mutation as these mutations did not appear to be genotype-specific.

## 2.4. DISCUSSION

In this study, full-length *env* and *gag* genes were sequenced for the first time from some South African BLV isolates and phylogenetic analyses revealed that at least two genotypes, genotype 1 and genotype 4, are present in South Africa. Pair-wise comparison of the full-length and partial *env* sequences between the South African isolates and those from other geographic regions showed that the *env* sequences of the G4-like South African isolates were significantly similar to the Zambian sequence whereas the *env* sequence of the G1-like South African isolate was similar to the Japanese, Paraguayan and St Kitts and Nevis isolates. Furthermore, the analyses indicated that the G1-like South African isolate is more similar to the other global G1 isolates whereas the G4-like South African isolates are more divergent from the other global G4 isolates. Lastly, amino acid sequence alignment identified genotype-specific as well as novel amino acid substitutions in the South African isolates.

In our study, neighbour-joining (NJ) and maximum-likelihood (ML) methods were used to analyse the evolutionary relationships between the South Africa isolates and global isolates. The results from the NJ trees were confirmed by the results obtained from the ML trees. The NJ method is one of the simplest methods to assess evolutionary relationships, but this method does not assume each nucleotide position as a discrete character. In contrast, the ML method takes a substitution rate at each nucleotide position into account and thus, it is considered to be a more robust method. During our analysis using partial *env* sequences, we found that one Iranian isolate (EU266061) as well as three Iranian isolates (EU266060, EU266062 and EU266063), which had been previously grouped into G1 and G8, respectively, by Matsumura *et al* [385], were all grouped into G1 (data not shown). Our analysis was consistent with the studies conducted by Rodríguez *et al.* [328] and Rola-Łuszczak [151] using Bayesian and ML or NJ methods, where they also classified these Iranian isolates (EU266060 to EU266063) as G1. The discrepancy in these results is that Matsumura *et al* [385] used the UPGMA method [386], which assumes a uniform substitution rate amongst different isolates and thus, is a less robust method. All our phylogenetic analyses based on the NJ and ML methods were thus, consistent with previous studies using the same or Bayesian models and supported by the similar bootstrap values. Similarly, all isolates used in our study were

correctly grouped as previous studies demonstrated [45, 151, 329, 330, 341-347]. In our study, phylogenetic analysis based on the partial *env* nucleotide sequences was supported by low to moderate bootstrap values, indicating that this analysis could be less reliable. However, phylogenetic analyses supported by low bootstrap values were also noted in previous studies [344, 347, 352] but these studies showed congruent tree topology amongst different studies and with our study. Furthermore, evolutionary relationships of BLV isolates using partial *env* nucleotide sequences were further assessed by pair-wise sequence comparison, whose results were in agreement with the phylogenetic analysis. Our analyses for the mean intergenotype and intragenotype genetic distances were also consistent with the previous studies [343, 346]. Lastly, our results reflect recent data as we included in our analyses not only the South Africa isolates but also isolates recently sequenced from China and Vietnam. This has allowed more comprehensive studies on the BLV genetic variants from diverse geographic regions. It should be noted that the collection dates are unknown for the majority of the isolates used in this study. Therefore, we could not perform the analysis and interpretation of the BLV evolutionary history for ten genotypes as well as G1- and G4-like South African isolates.

Our phylogenetic analyses based on the *env* and *gag* nucleotide sequences as well as sequence comparison revealed that the G1-like South African isolate (M2746) was more similar to the G1 isolates from the other geographic regions whereas the G4-like South African isolates appeared more divergent from other G4 global isolates except for the Zambian isolate. Although G4 isolates themselves appeared less conserved compared to the G1 isolates, the Env and Gag sequences of the seven G4-like South African isolates were conserved amongst themselves. This is consistent with the previous studies showing that G1 is the most conserved amongst other genotypes [329, 343] and that sequences from the same geographic area tend to be conserved [249, 328].

Since the only isolate sequenced from Africa (Zambia) belongs to G4, we predicted that the South African isolates might also belong to G4. We also speculated that the South African cattle might be infected with BLV with different genotype(s) as multiple genotypes are currently circulating in single countries or geographic areas worldwide (see Section 2.1) [151, 249, 330, 339, 353]. As speculated, our analysis identified two genotypes, G1-like and G4-like strains, from the South African isolates. Furthermore, not surprisingly, the phylogenetic analysis based on the partial *env* nucleotide sequences and sequence comparison revealed that the seven G4-like South African isolates and the Zambian isolate are significantly close to each other, differing by only 0.002 nucleotide substitutions per site. Furthermore, six of these seven G4-like South African isolates and the Zambian isolate showed the identical amino acid sequence and the one other G4-like South African isolate showed only one amino acid difference with

the Zambian isolate. Although the phylogenetic analyses and sequence comparison demonstrated with high bootstrap values and percent similarities that the G1-like South African isolate (M2746) is closely related to the G1Japanese, Paraguayan and St Kitts and Nevis isolates, this does not necessarily indicate that the cow infected with the M2746 isolate originates from these countries. However, South Africa and these countries may import cattle or semen/embryos from the same source. Furthermore, the coexistence of the G1 strain with the G4 strain in South Africa may indicate that cattle or semen/embryos have been imported from multiple countries [340, 357]. It is uncertain whether the G4 strain is already circulating extensively in South Africa and whether the presence of the G1 strain in South Africa is sporadic. However, our results highlight an urgent need for stringent disease management to prevent and control the BLV spread across the country.

Our analyses further revealed that the Japanese isolate (AP018021) used to design the recombinant LSDV vaccines (see Chapter 4) was closely related to the G1-like South African isolate (M2746) with only one amino acid difference in its Env and Gag sequences. During our study, the genotype of the Japanese isolate (AP018021) was unknown. However, the paper that described this isolate later became available [347] and consistent with our analyses, their study identified this Japanese isolate as G1. Since there has not been any study on the cross-protection between different BLV genotypes so far, it is unknown whether our recombinant LSDV vaccines could confer the cross-protection to animals infected with G4 strains. Yet, our results have provided valuable data for the future development of an efficacious BLV vaccine that is tailored to protect against local strains.

The analyses of the BLV Gag sequences provided an additional finding. The G4-like *gag* nucleotide sequences appeared prone to mutations and more variable than their *env* nucleotide sequences, but their amino acid sequences were comparable. Whilst the *gag* nucleotide sequences of the G4-like South African isolates showed 22 to 32 nucleotide differences with the global G4 isolates, their *env* nucleotide sequences differ by 19 to 28 nucleotides. However, both Gag and Env amino acid sequences differ by six to eight amino acids between the G4-like South African isolates and the global G4 isolates. This is in agreement with the analyses on the mean nucleotide and amino acid distances in the Env and Gag sequences. The mean intergenotype distances in the Gag sequences between the G4-like South African isolates and the global G4 isolates were 0.023 and 0.019 for the nucleotide and amino acid sequences, respectively whereas those in the Env sequences were 0.015 and 0.014 for the nucleotide and amino acid sequences, respectively. The same trend was also observed in Gag and Env sequences in general. The average nucleotide and amino acid distances in the Env sequences amongst all genotypes were 0.038 and 0.032, respectively whereas those in the Gag sequences were 0.043 and 0.029, respectively. Although *gag*

nucleotide sequences are generally more variable than *env* nucleotide sequences, these nucleotide changes in the *gag* gene did not cause significant changes in their amino acid sequences. These findings are consistent with a previous study [185] showing that the Gag sequences are subject to purifying selection. Mutagenesis studies on Gag variants have not been conducted and it remains unclear how BLV Gag mutations impact viral infectivity, fitness and persistence. However, a recent study identified two BLV haplotypes based on the *gag* sequences from 780 leukemic and aleukemic cows and demonstrated that the *gag* haplotypes are associated with the maintenance of high proviral load [387]. Furthermore, studies on the HIV Gag mutants demonstrated a correlation with drug resistance (e.g., protease inhibitors) [388-392], suggesting a role of retroviral Gag mutations in the viral persistence inside their hosts. Therefore, changes in Gag amino acid sequences could have a significant impact on viral infectivity and persistence and as a result, Gag amino acid sequences are less prone to mutations.

Seventeen and eleven amino acid substitutions in the South African Env and Gag sequences, respectively were detected by the amino acid sequence alignment between the South African isolates and global isolates. These substitutions include genotype-specific substitutions as well as those unique to the South African isolates. The amino acid substitution at position 476 in the Env sequences as well as the amino acid substitutions at positions 29, 278 and 343 in the Gag sequences were consistently detected from the seven G4-like South African isolates. These substitutions are absent in the global G4 isolates as well as any other global isolates. Importantly, these amino acid substitutions unique to the G4-like South African isolates appear to be conservative substitutions and thus, they are unlikely to cause functional changes to the proteins. No insertions and deletions were detected in the South African sequences.

The genotype-specific amino acid substitutions in the Env sequence detected in this study include those at position 29 in the signal sequence, positions 48, 73, 74 and 82 in the G epitope, position 56 in the H epitope, positions 134 and 144 in the second neutralising domain (ND2), position 254 in the D epitope and position 504 in the PXXP motif. The Env mutations in the South African isolates appeared to be concentrated in the conformational epitopes (F, G and H) and the ND2. These results are consistent with previous studies showing that the G epitopes, particularly resides 48, 74 and 82, are under positive selection and most polymorphic sites [185, 249, 333, 334]. Homology modelling of the BLV Env protein has suggested that the conformational epitopes and the neutralising domains are located on the surface of the virus [236, 249]. Therefore, it is not surprising that these epitopes and domains are targets of NAbs [238, 240, 241, 246] and under the strong selection pressure [249], leading to the antigenic variations in these regions [241].

There are eight conformational epitopes (F, G and H) [239, 331, 332] on the Env gp51 surface protein and they are important for cell fusion and syncytium during viral dissemination [238]. In our study, four point mutations in the G epitopes and one point mutation in the H epitope were detected from the South African isolates. Since functional studies on the Env protein and Env mutants are scarce, functional consequences of the Env mutations found in our studies are unknown. However, non-conservative mutations in the conformational epitopes and the ND2 would affect the recognition of NAbs to these epitopes. A previous study which identified the S56F substitution in the H epitope showed that this substitution altered the recognition of a mAb to this epitope owing to the drastic changes from the small serine residue to the large hydrophobic phenylalanine residue [348]. This effect would also apply to the S82F substitution in the G epitope. Mutations in the conformational epitopes appear to benefit viral persistence through antigenic changes and immune escape. Previous studies demonstrated that BLV natural variants lacking one or two of the conformational epitopes were viable and appear to have evolved these mutations to evade host immune responses by altered antibody recognition [241, 348]. Yet, BLV mutants lacking all conformational epitopes have not been detected and these deletion mutants retained at least one of the three conformational epitopes [241, 348]. These observations indicate that the simultaneous loss of these epitopes would be deleterious and possibly lose infectivity [238].

The BLV Env protein contains three neutralising domains (ND1 to ND3) [246] and these domains also play a role in viral infectivity (cell fusion and syncytium) as well as immunogenicity [237, 246, 353]. Previous studies demonstrated that the ND2 is particularly prone to multiple point mutations [328, 341] but it was also shown that the ND2 is under the purifying selection [249, 334], reflecting its functional significance. Rodríguez *et al*. [328] identified eleven point mutations within the ND2 from BLV strains from a variety of geographic regions. In contrast, interestingly, the ND1 and ND3 appear to be less prone to mutations [328, 339]. Using homology modelling, Moratorio *et al*. [353] demonstrated that the D134N substitution changed net charge in a loop of the Env protein, possibly disturbing the immunogenicity and/or fusogenic properties of the ND2. One G4-like South African isolate (M1878) had the G1-like D134N substitution in this ND2. It is unclear whether this is a spontaneous mutation or a sequencing error. Although the chromatograms showed high-quality trace data at this position (data not shown), to confirm whether this could be a spontaneous mutation or a sequencing error, the sequencing needs to be performed thrice.

Eleven amino acid substitutions were found in the South African Gag sequences and the majority of the substitutions (six out of eleven substitutions) were concentrated in the p15 matrix (MA) protein whereas only two amino acid substitutions were found in the p24 capsid (CA) protein. Since the CA protein is the second major target for the NAbs next to the gp51

surface protein [93], this protein can be under the selection pressure. Yet, it is possible that mutations in the CA can render fitness cost to the virus and cause deleterious effects to the viral replication. A genetic footprinting analysis on the Moloney murine leukemia virus demonstrated that the majority of the MA regions were tolerant of insertions whereas the N-terminal region of the CA was susceptible to insertions [393]. They further demonstrated that although Gag mutants harbouring mutations in the MA and N-terminal region of the CA all produced virions, CA mutants produced an abundance of immature virions [393]. Furthermore, whilst the MA mutants showed reverse transcriptase (RT) and nuclear transport activities and appeared infectious, the RT and nuclear transport activities were not detected in the CA mutants, indicating the defect of uncoating or viral entry [393].

Three amino acid substitutions were detected in the p12 nucleocapsid (NC) protein. The retroviral NC protein contains conserved zinc-finger motifs [259, 394-396] and basic residues [397-399] for RNA packaging and virion assembly [400, 401]. The K341Q substitution in the NC protein was only detected from the South African P591 isolate, not from any other South African nor global isolates. This mutation might be a spontaneous mutation or a sequencing error, which needs to be confirmed by re-sequencing. Yet, this amino acid change may cause functional changes in the virion assembly. Wang *et al*. [260] performed alanine-scanning mutagenesis study for the basic residues in the BLV NC and demonstrated that although the K341A substitution in the NC did not affect RNA packaging, the K341A mutant had two- to three-fold reduction in virion production. Although lysine can be substituted with other polar amino acids including glutamine, it is unknown whether the loss of charge may also affect virion production.

This study served as the first phylogenetic analysis of the BLV *env* and *gag* complete sequences from some South African isolates and has contributed to a more comprehensive study on the global BLV genetic diversity. Furthermore, the detection of two genotypes (G1 and G4) in South Africa highlights the urgent need for the disease management and development of an efficacious vaccine against local strains. Since the sample size in this study was small and only cattle from a single herd were assessed, further studies investigating BLV variants and seroprevalence of multiple herds from different provinces will be desirable.

# CHAPTER 3

# ASSESSMENT OF THE ACTIVITY OF FIVE POXVIRUS PROMOTERS IN CELLS INFECTED WITH LUMPY SKIN DISEASE VIRUS (LSDV)

## 3.1. INTRODUCTION

Recombinant poxvirus vectors have been widely used as a next-generation vaccine platform for human and animal pathogens [402-410] and a therapeutic tool for various cancers [411-414]. Recombinant poxvirus-vectored vaccines can express foreign antigens that induce immune responses. Several factors are essential in the design of recombinant poxvirus-vectored vaccines such as types of poxviruses [415], host range of vector viruses [416] and poxvirus promoters used to drive transgene expression [417].

Poxvirus promoters are particularly important in determining the strength and timing of antigen expression in recombinant poxvirus vaccines, thereby playing a role in the induction of desired immune responses. Linked to the stages of the viral life cycle, poxvirus promoters are classified into five temporal classes: immediate-early, early, early-late, intermediate and late promoters. Temporal gene expressions, switching from early to late gene expressions, are not unique to poxvirus but ubiquitous to all types of viruses, whether DNA or RNA viruses. However, poxvirus transcription is a cascading pathway in which gene expression is temporally regulated at levels of transcriptional initiation and by phase-specific transcription factors (TFs), which are products of the preceding gene expression (Figure 3.1) [418]. Early genes are expressed from early promoters by the control of early TFs that have been packaged in virion cores. Early genes encode proteins necessary for DNA replication and transcription of intermediate genes. Following the uncoating of viral cores, DNA replication initiates, which results in expression of intermediate genes from intermediate promoters and expression of proteins required for late gene expression and morphogenesis. Upon initiation of intermediate transcription, transcription of late genes commences from late promoters.

One classic early-late poxvirus promoter is that of the 7.5 kilo Dalton (kDa) polypeptide gene (p7.5) of vaccinia virus (VACV) [419], which is the first poxvirus promoter sequenced [420] and has been used widely in recombinant poxvirus vectors [94, 327, 421-424]. In poxvirus-infected cells, the p7.5 promoter is activated at the early stage of the viral life cycle and re-activated in the later stage [425].

Much focus has been on the selection of strong poxviruses promoters to enhance the level of antigen expression to improve immunogenicity of vaccines [426, 427]. However, accumulating evidence suggests that not only the strength of poxvirus promoter *per se* is critical for the immunogenicity but also the timing of antigen expression driven by different temporal classes of poxviruses promoters determines the types of immune responses elicited.

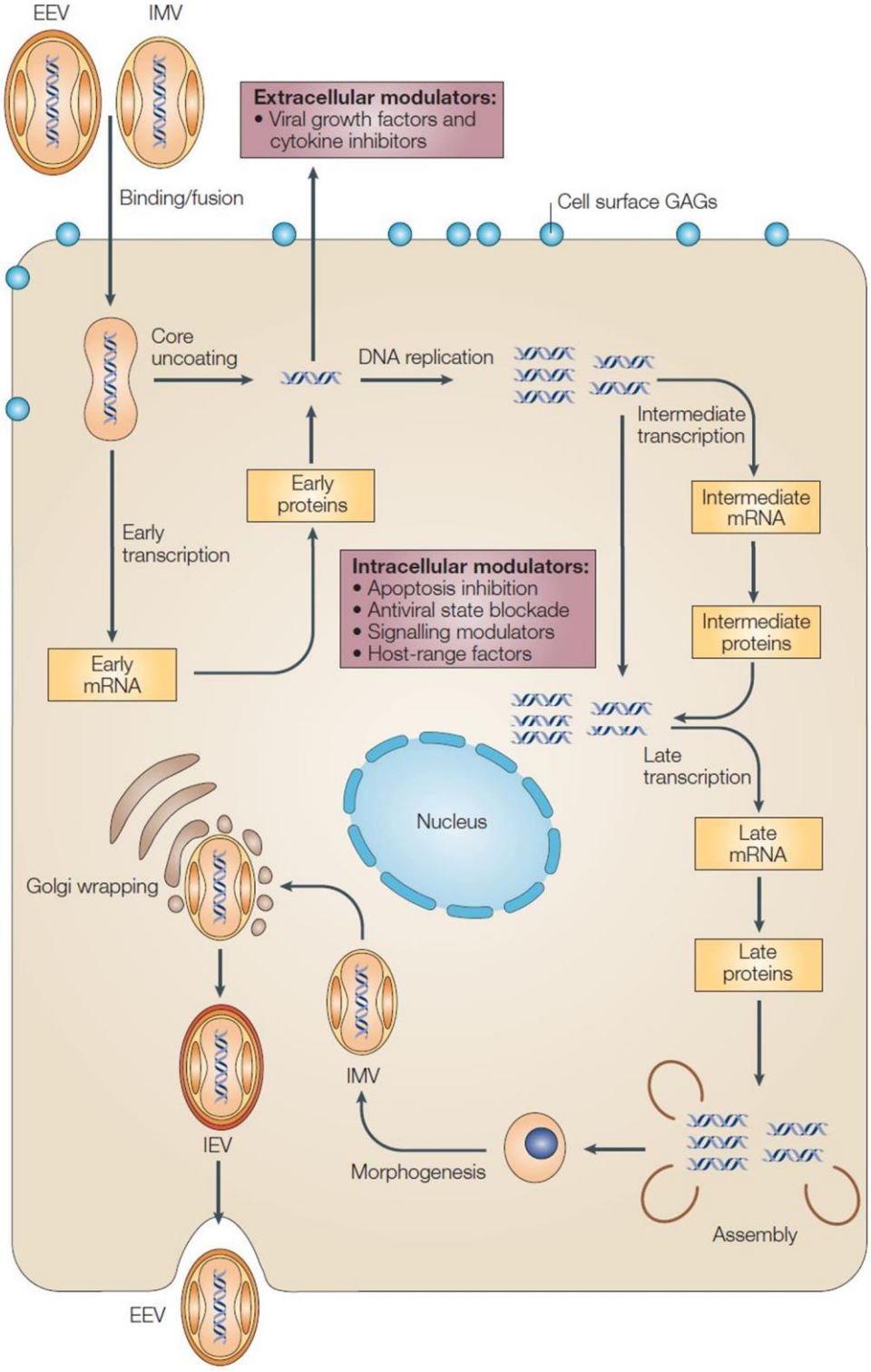



**Figure 3.1: Lifecycle of poxvirus.** Replication cycle and transcription pathways of poxviruses are conserved amongst the *Poxviridae* family. Infectious virions, extracellular enveloped virus (EEV) or intracellular mature virus (IMV) bind to cell surface proteins to fuse with the cell membrane, which results in the release of the viral core into the cytoplasm. Transcription of immediate-early and early genes leads to the uncoating of viral cores and DNA replication in viral factories (not shown in the figure). Transcription of intermediate and late genes follows DNA replication. In the viral factory, immature virions are assembled and form IMVs ((not shown in the figure). Some IMVs are transported to the Golgi apparatus, where they are wrapped with a double membrane to form intracellular enveloped viruses (IEVs). The IEVs are transported to the cell membrane, and thereby released as EEVs to initiate infection of uninfected cells. Taken from McFadden. G (2015) [416].

It has been shown that early-activity of poxvirus promoters is important to induce cytotoxic T cell responses *in vivo* [428-431] whereas genes expressed from intermediate and/or late promoters induced both antibody and $CD4^+$ T cell responses [432-434]. In particular, early expression of antigens relates to the induction of antigen-presenting dendritic cells [435] as well as the sustained production of memory $CD4^+$ and $CD8^+$ T cells, which play a crucial role in the secondary immune responses [428, 435-437] in vaccinated individuals.

The aim of this chapter is to examine the activity of five selected poxvirus promoters in cells infected with LSDV by the detection of transient expression of an enhanced green fluorescent protein (eGFP) reporter gene driven by each poxvirus promoter from a reporter plasmid. The poxvirus promoters tested included a modified early fowlpox virus promoter (mFP) [438, 439], the p7.5 promoter [419], a synthetic early-late promoter of VACV (pS) [426], a modified early-late promoter of the H5 gene of VACV (mH5) [440] and a synthetic early-late optimised promoter (LEO) of VACV [436], which all have either an early promoter element alone or an early-late promoter element.

## 3.2. MATERIALS AND METHODS

### 3.2.1. Source of plasmids

Plasmids pBLV-Env-Gag, pLSDV_K1L_eGFP (a generous gift from Ruzaiq Omar) and pSSPExSHIVgp150I-Pgag (a generous gift from Dr. Ros Chapman) (Figure 3.2) were used to construct the reporter plasmid vectors that contained one of the five poxvirus promoters upstream of the eGFP gene.

### 3.2.2. Source of poxvirus promoters

Sequences of the p7.5, pS, mH5 and LEO promoters were taken from original studies that characterised these promoters [419, 426, 436, 440]. The early mFP promoter was designed by Ruzaiq Omar [438] from a native early-late fowlpox virus promoter [439] by replacing the late promoter element with an antisense poxvirus terminator sequence (AGAAAAA).

### 3.2.3. Source of LSDV

A recombinant Neethling lumpy skin disease virus, nLSDVSODis-UCT [441] was used for the infection of baby hamster kidney (BHK-21) cells.

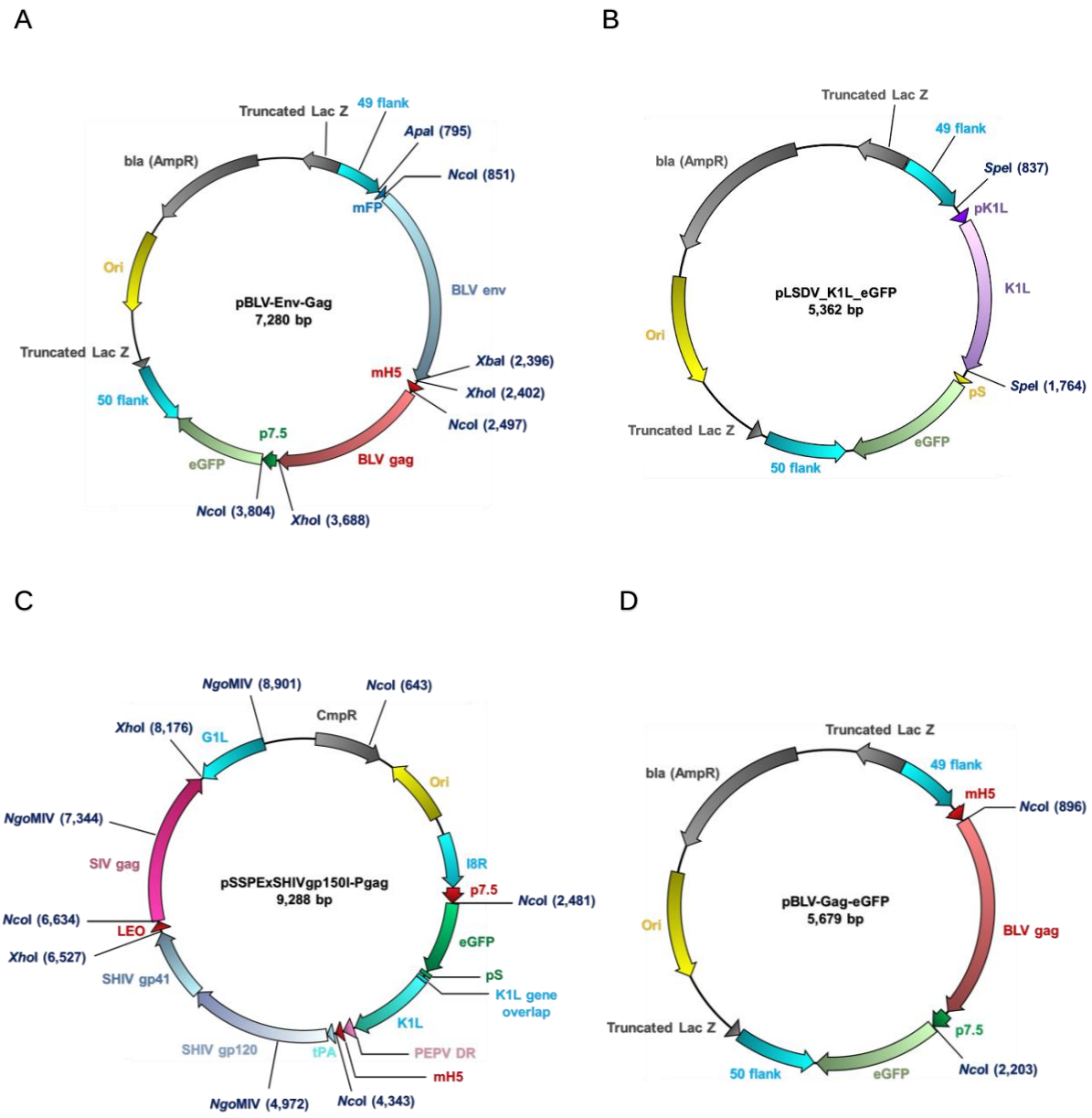



**Figure 3.2: Plasmid maps of the source and intermediate plasmids used to construct the five poxvirus promoter plasmid vectors containing different promoters. (A)** pBLV-Env-Gag plasmid. **(B)** pLSDV_K1L_eGFP plasmid. **(C)** pSSPExSHIVgp150I-Pgag-1. **(D)** pBLV-Gag-eGFP is an intermediate plasmid vector constructed by excision of the mFP promoter and BLV *env* gene from the pBLV-Env-Gag plasmid. This intermediate plasmid was used to construct the pmH5-eGFP and pLEO-eGFP plasmids. Nucleotide positions of restriction sites are shown in brackets. Arrows, arrowheads and boxes indicate genes. **mFP**, modified early fowlpox virus promoter; **p7.5**, promoter for the 7.5 kDa polypeptide gene of vaccinia virus (VACV); **pS**, synthetic early-late promoter of VACV; **mH5**, a modified promoter of the H5 gene of VACV; **LEO**, synthetic early-late optimised promoter of VACV, **49 flank**, 356bp of the 3' end of *049* ORF from Neethling lumpy skin disease virus; **50 flank**, 440bp of the 3' end of *050* ORF from Neethling lumpy skin disease virus; **BLV env**, *envelope* gene of bovine leukaemia virus (BLV); **BLV gag**, *gag* gene of BLV; eGFP, gene of enhanced green fluorescent protein; **K1L**, *K1L* gene of VACV; **I8R**, 3' portion of *I8R* gene of VACV; **G1L**, 3' portion of *G1L* gene of VACV; **PEPV DR**, penguin poxvirus direct repeat; **tPA**, leader sequence of a human tissue-type plasminogen activator gene; **SHIV gp120**, *env gp120* gene of human immunodeficiency virus (HIV); **SHIV gp41**, *env gp41* gene of HIV; **SIV gag**, *gag* gene of simian immunodeficiency virus; **bla (AmpR)**, *beta-lactamse* (*bla*) gene; **CmpR**, chloramphenicol resistance gene ; **Ori**, ColE1 origin of replication.

The Neethling vaccine strain was modified such that ORF *131*, a truncated superoxide dismutase (SOD) was deleted and replaced with a modified SOD homologue gene which resembled that of Herbivac LS, a vaccine strain of LSDV derived from Neethling (manufactured by Deltamune, Pretoria, South Africa) [442]. The resultant nLSDVSODis-UCT had a stable *131* gene which encoded a protein with the identical amino acid sequence to that of the Herbivac LS SOD homologue. The nLSDVSODis-UCT ("is" referring **i**mproved **s**tability) recombinant was designed such that it would be unlikely to mutate in a region of dinucleotide repeats and therefore be more stable. There are no selection and/or marker gene(s) present in nLSDVSODis-UCT.

### 3.2.4. Cell culture

#### 3.2.4.1. Sources of cells

The baby hamster kidney (BHK-21) adherent cell line (CCL-10) was obtained from the American Type Culture Collection (ATCC).

#### 3.2.4.2. Thawing and recovery of cell lines from deep-frozen stocks

A cryovial containing $4.0x10^6$ BHK-21 cells was removed from the -80°C freezer and immediately placed in the 37°C water bath. The cells were thawed by agitating the vial gently in the water bath for up to 1 minute. Then, the cells were re-suspended in 5ml of pre-warmed complete Dulbecco's Modified Eagle Medium (cDMEM) (Appendix A) contained in a 15ml conical centrifuge tube to dilute the dimethyl sulfoxide (DMSO) (Lonza, USA). After the cell suspension was centrifuged briefly for 3 minutes at low speed (< 500g), the supernatant was removed, and the cell pellet was resuspended using the residual cell medium. Lastly, 5ml of cDMEM (Appendix A) was added and the cell suspension was transferred into a $25cm^2$ cell culture flask (T-25). Cell viability was examined using the trypan blue staining method as described in Section 3.2.4.4. If excessive dead cells were observed, they were removed carefully once the majority of the viable cells settled. Then, the T-25 flask was incubated for 2 to 3 days in a $CO_2$ incubator set at 37°C, 70% relative humidity and 5% $CO_2$ (standard growth conditions) to stabilise the cells before transferring the cells into a $75cm^2$ tissue culture (T-75)

#### 3.2.4.3. Growth and maintenance of adherent cell line

The method was adapted from Phelan (2007) [443] and Cotter et al. (2015) [444]. The BHK-21 cells were sub-cultured regularly or when the cells formed a confluent monolayer. The

whole volume of culture medium was aspirated, and cells were washed with 10ml of 1x phosphate-buffered saline (PBS) (Thermo Fisher Scientific, USA). The PBS was discarded, and the cells were overlaid with 2ml of 1x trypsin-EDTA (0.25%) (Thermo Fisher Scientific, USA). After a 3-minute incubation in the $CO_2$ incubator to enhance the dissociation of cells, the dissociation of the adherent cells was further facilitated by tapping the flask gently several times. Then, 8ml of cDMEM (Appendix A) was added to the trypsinised cells and the cell suspension was mixed thoroughly by pipetting up and down several times to dissociate cell clumps. Lastly, the cell suspension was diluted with cDMEM at a ratio of 1:5 and incubated in the $CO_2$ incubator under standard growth conditions until the next sub-culturing. Cell growth and viability was monitored daily by examining the cells with an optical microscope under brightfield illumination (Olympus, Japan) and the colour change of the culture media.

#### 3.2.4.4. Cell counting

Cell count of viable cells was performed by a trypan blue staining method [443, 445]. When the cells formed a confluent monolayer, cells were washed with 1x PBS and trypsinised (See Section 3.2.4.3) Then, the cell suspension and Trypan Blue Stain (0.4%) (Thermo Fisher Scientific, USA) were mixed thoroughly in a ratio of 1:10 in a 1.5ml Eppendorf tube and 10µl of the mixture was loaded on a Neubauer Chamber with coverslip in place (Marienfeld, Germany). Transparent, viable cells on $1mm^2$ grids at the four corners were counted under the optical microscope at 100x magnification (Olympus, Japan). The following equation was used to obtain total cells per ml:

*Total cells/ml = The average number of cells obtained from the Neubauer Chamber x $10^4$ x Dilution factor (i.e., 10)*

### 3.2.5. Construction of pmFP-eGFP, p7.5-eGFP, pmH5-eGFP, pS-eGFP and pLEO-eGFP reporter plasmids

#### 3.2.5.1. Construction of plasmid pmFP-eGFP

pBLV-Env-Gag plasmid DNA was digested with restriction endonuclease *Nco*I, and a 4,327bp fragment containing the vector backbone, the 49 flank, mFP promoter, eGFP gene and 50 flank was gel purified as described in Appendix B1.1. One hundred nanograms of the gel-purified plasmid DNA was then re-ligated and transformed into *E. coli* competent cells (see Appendix B1.3 and B1.4). Twenty single colonies were picked from the plates for small scale plasmid DNA isolation (see Appendix B1.5). The plasmid DNA was then digested with restriction endonucleases FastDigest *Hin*dIII and FastDigest *Kpn*I (Thermo Fisher Scientific,

USA), as described in Appendix B1.1 and was subject to ethidium-bromide (0.5µg/ml) stained 0.8% agarose gel electrophoresis in 1xTBE at 100V for 1 hour. The desired plasmid was amplified as described in Appendix B1.6. Preliminary confirmation of the correct plasmid construct was made by restriction endonuclease mapping of the plasmid DNA and final confirmation by Sanger sequencing using M13F and M13R universal primers (Table 3.1).

**Table 3.1: A set of primers used to sequence the five reporter plasmid vectors**

| Primer | Orientation | Primer sequence | Binding site |
|---|---|---|---|
| M13F | Forward | 5'- GTTTTCCCAGTCACGAC - 3' | Lac Z outside the 49 flank |
| M13R | Reverse | 5'- CAGGAAACAGCTATGAC - 3' | Lac Z outside the 50 flank |

### 3.2.5.2. Construction of plasmid p7.5-eGFP

pBLV-Env-Gag plasmid DNA was digested with restriction endonucleases *Apa*I and *Xho*I (Thermo Fisher Scientific USA), and a 4,387bp fragment containing the vector backbone, the 49 flank, p7.5 promoter, eGFP gene and 50 flank was gel purified as described in Appendix B1.1. Approximately 1,140ng of the gel-purified plasmid DNA was blunted and re-ligated (see Appendix B1.2 and B1.3). For the ligation reaction, two reaction mixtures were prepared. One reaction mixture contained five times more plasmid DNA than the manufacturer's recommendation whereas the other contained two times more T4 DNA ligase than the manufacturer's recommendation. Then, re-ligated plasmids were transformed into *E. coli* competent cells (see Appendix B1.4). Sixteen single colonies were picked from the plates for small scale plasmid DNA isolation (see Appendix B1.5). The plasmid DNA was then digested with restriction endonuclease FastDigest *Nco*I (Thermo Fisher Scientific, USA), as described in Appendix B1.1 and was subject to ethidium-bromide (0.5µg/ml) stained 0.8% agarose gel electrophoresis in 1xTBE at 100V for 1 hour. The desired plasmid was amplified as described in Appendix B1.6. Preliminary confirmation of the correct plasmid construct was made by restriction endonuclease mapping of the plasmid DNA and final confirmation by Sanger sequencing using M13F and M13R universal primers (Table 3.1).

### 3.2.5.3. Construction of plasmid pS-eGFP

pLSDV_K1L_eGFP plasmid DNA was digested with endonuclease *Spe*I (New England Biolabs, USA) and a 5,326bp fragment containing the vector backbone, the 49 flank, pS promoter, eGFP gene and 50 flank was gel purified as described in Appendix B1.1. Approximately 50ng of the gel-purified plasmid DNA was then re-ligated and transformed into

*E. coli* competent cells (see Appendix B1.3 and B1.4). For the ligation reaction, two reaction mixtures were prepared. One reaction mixture was prepared as per the manufacturer's recommendation whereas the other contained five times more plasmid DNA than the manufacturer's recommendation. Then, re-ligated plasmids were transformed into *E. coli* competent cells (see Appendix B1.4). Sixteen single colonies were picked from the plates for small scale plasmid DNA isolation (see Appendix B1.5). The plasmid DNA was then digested with restriction endonucleases FastDigest *Xho*I and FastDigest *Bam*HI (Thermo Fisher Scientific, USA), as described in Appendix B1.1 and was subject to ethidium-bromide (0.5µg/ml) stained 0.8% agarose gel electrophoresis in 1xTBE at 100V for 1 hour. The desired plasmid was amplified as described in Appendix B1.6. Preliminary confirmation of the correct plasmid construct was made by restriction endonuclease mapping of the plasmid DNA and final confirmation by Sanger sequencing using M13F and M13R universal primers (Table 3.1).

### 3.2.5.4. Construction of the pBLV-Gag-eGFP intermediate plasmid

pBLV-Env-Gag plasmid DNA was digested with restriction endonucleases *Apa*I and *Xba*I (Thermo Fisher Scientific USA), and a 5,679bp fragment containing the vector backbone, the 49 flank, mH5 promoter, BLV *gag* gene, p7.5 promoter, eGFP gene and 50 flank was gel purified as described in Appendix B1.1. Approximately 30ng of the gel-purified plasmid DNA was blunted and re-ligated. (see Appendix B1.2 and B1.3) Two ligation mixtures were prepared as described in Section 3.2.5.2. Then, re-ligated plasmids were transformed into *E. coli* competent cells (see Appendix B1.4). Sixteen single colonies were picked from the plates for small scale plasmid DNA isolation (see Appendix B1.5). The plasmid DNA was then digested with restriction endonuclease FastDigest *Nco*I (Thermo Fisher Scientific, USA), as described in Appendix B1.1 and was subject to ethidium-bromide (0.5µg/ml) stained 0.8% agarose gel electrophoresis in 1xTBE at 100V for 1 hour. The desired plasmid was amplified as described in Appendix B1.6. Preliminary confirmation of the correct plasmid construct was made by restriction endonuclease mapping of the plasmid DNA.

### 3.2.5.5. Construction of plasmid pmH5-eGFP

pBLV-Gag-eGFP intermediate plasmid DNA was digested with restriction endonuclease *Nco*I (Thermo Fisher Scientific USA), and a 4,368bp fragment containing the vector backbone, the 49 flank, mH5 promoter, eGFP gene and 50 flank was gel purified as described in Appendix B1.1. Approximately 41.5ng of the gel-purified plasmid DNA was re-ligated and transformed into *E. coli* competent cells (see Appendix B1.3 and B1.4). Two ligation mixtures were prepared as described in Section 3.2.5.2. Sixteen single colonies were picked from the plates

for small scale plasmid DNA isolation (see Appendix B1.5). The plasmid DNA was then digested with restriction endonucleases *Sma*I and *Sal*I (New England Biolabs, USA), as described in Appendix B1.1 and was subject to ethidium-bromide (0.5µg/ml) stained 0.8% agarose gel electrophoresis in 1xTBE at 100V for 1 hour. The desired plasmid was amplified as described in Appendix B1.6. Preliminary confirmation of the correct plasmid construct was made by restriction endonuclease mapping of the plasmid DNA and final confirmation by Sanger sequencing using M13F and M13R universal primers (Table 3.1).

#### 3.2.5.6. Construction of plasmid pLEO-eGFP

Since the LEO promoter sequence is only 82bp, it was decided it would be difficult to purify the 82bp fragment from an agarose gel. Therefore, ligation of the LEO promoter DNA fragment into the reporter plasmid backbone was conducted by "shot-gun cloning".

Five micrograms of plasmid pSSPExSHIVgp150I-Pgag were digested with 1µl of *Ngo*MIV, *Nco*I and *Xho*I restriction endonucleases (New England BioLabs, USA) (see Appendix B1.1). Following the incubation at 37°C for 1 hour, the reaction was heat-inactivated at 80°C for 20 minutes as per the manufacturer's recommendations. To generate the vector backbone for the cloning, 1.4µg of the pBLV-Gag-eGFP intermediate plasmid was digested with 1µl of *Nco*I and *Xho*I (Thermo Fisher Scientific, USA). For both reactions, reaction mixtures without the restriction endonucleases were also prepared as a negative control. Following a 1-hour incubation at 37°C, the pBLV-Gag-eGFP intermediate plasmid DNA digested with the *Nco*I and *Xho*I restriction endonucleases was subject to ethidium-bromide (0.5µg/ml) stained 0.8% agarose gel electrophoresis in 1x TBE buffer (Appendix A) at 100V for 1 hour. A 4,277bp fragment containing the vector backbone, the 49 flank, eGFP gene and 50 flank was gel purified as described in Appendix B1.1.

For the "shot-gun cloning," two experimental reactions and two controls were prepared. The two experimental reaction mixtures consisted of 20µl and 40µl. The insert (pSSPExSHIVgp150I-Pgag plasmid DNA digested with *Ngo*MIV, *Nco*I and *Xho*I) was ligated with the gel-purified plasmid backbone using T4 DNA Ligase as described in Appendix B1.3. For the controls, one reaction mixture without the insert plasmid DNA and one without both the insert plasmid DNA and T4 DNA ligase were prepared. The first control was included to determine the levels of singly-cut or undigested plasmid DNA and the second control was included to detect the quantity of undigested vector. See Appendix B1.3 for the detailed method of ligation. Following the incubation of the reaction mixtures at room temperature overnight, the transformation of the chemically competent *E. coli* cells was conducted (see Appendix B1.4). Eighteen bacterial colonies were selected from the agar plates and inoculated

into an LB-broth (Appendix A) containing carbenicillin (50µg/ml) (Thermo Fisher Scientific, USA) for colony PCR. These 18 bacterial cultures were incubated overnight on at 37°C shaking incubator at 250 rpm.

#### 3.2.5.6.1. Colony PCR

Colony PCR was used to identify the recombinants containing the LEO promoter. Colony PCR was performed using 1µl of each of the overnight bacterial culture, 1.25µl of the LEO fwd 4 and eGFP rev 5 primers (Table 3.2) and 12.5µl of KAPA 2G Robust HotStart ReadyMix PCR Kit (KAPA BIOSYSTEMS, USA) in 25µl reaction mixtures as per manufacturer's protocol. A reaction mixture without bacterial culture was included as a PCR control. The reaction conditions were as follows: initial denaturation at 95°C for 5 minutes followed by 30 cycles of denaturation at 95°C for 15 seconds, annealing at 52°C for 15 seconds and extension at 72°C for 15 seconds.

**Table 3.2: Primers used for the colony PCR**

| Primer | Orientation | Primer sequence | Binding site |
|---|---|---|---|
| LEO fwd 4 | Forward | 5'- AGACGCTAGACTAGTACC -3' | LEO promoter |
| eGFP rev 5 | Reverse | 5'- GCGGATCTTGAAGTTCAC -3' | 5' end of eGFP gene |

A large-scale plasmid DNA isolation was carried out using one of the recombinants found to contain the LEO promoter (see Appendix B1.6). Preliminary confirmation of the correct plasmid construct was made by restriction endonuclease mapping of the plasmid DNA and final confirmation by Sanger sequencing using M13F and M13R universal primers (Table 3.1).

### 3.2.6. Fluorescence microscopy analysis of transient eGFP expression in baby hamster kidney (BHK-21) cells infected with LSDV and transfected with a reporter plasmid

A 12-well tissue culture plate was seeded with 1ml of BHK-21 cells at a concentration of $1.0x10^5$ cells/ml and incubated overnight under standard growth conditions. On the following day, a 70% confluent monolayer of the cells was infected with 5µl of the recombinant Neethling lumpy skin disease virus nLSDVSODis-UCT ($1.0x10^{7.25}$ $TCID_{50}$/ml) at a multiplicity of infection (MOI) of 0.5 and incubated for 2 hours under standard growth conditions. Following a 2-hour incubation, the culture media was aspirated from wells and the cells were transfected with a transfection mixture containing 500µl of DMEM (Thermo Fisher Scientific, USA), 1µl of X-tremeGene (Roche, Switzerland) and 2µg of poxvirus reporter plasmid DNA.

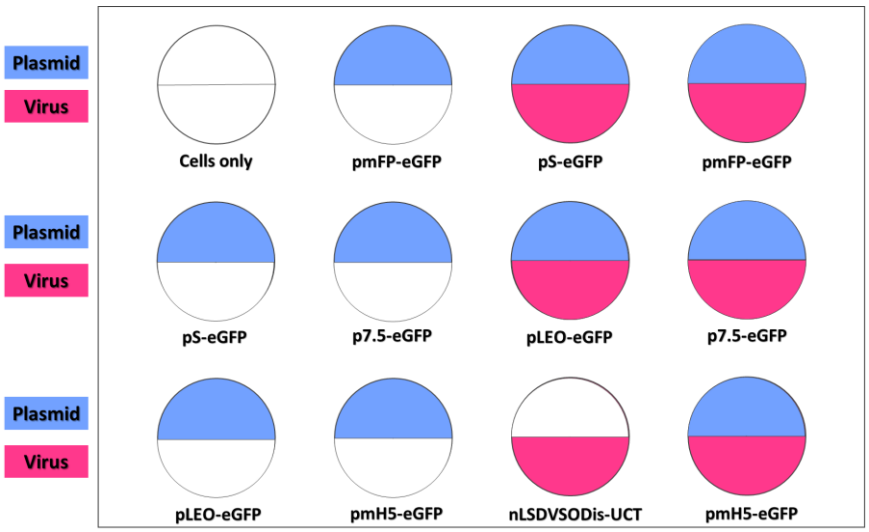



**Figure 3.3: Layout of a 12-well plate for the infection and transfection of BHK-21 cells with LSDV and poxvirus promoter plasmid DNA.** A schematic diagram shows seven control wells and five experimental wells. One well was left uninfected and untransfected (Cells only). In another control well, BHK-21 cells were only infected with LSDV. The remaining five control wells were only transfected with one of the five poxvirus reporter plasmids. In the experimental wells, BHK-21 cells were infected and transfected with LSDV and each of the poxvirus promoter plasmid DNA. Wells where cells were infected with the LSDV are shown in pink semicircles, those transfected with the poxvirus promoter plasmid DNA are shown in blue semicircles and uninfected and/or untransfected wells are shown in white semicircles.

One experimental control well of uninfected and untransfected cells, one infection control well of the LSDV-infected cells only and five plasmid control wells of the transfected cells only were also prepared (Figure 3.3). eGFP expression was monitored daily for 72 hours post-infection/transfection using an epifluorescent inverted microscope (Zeiss, Germany).

## 3.3. RESULTS

In this study, five reporter plasmid vectors that contained each of the selected poxvirus promoters were constructed. To assess whether these promoters were expressed in the LSDV-infected cells, BHK-21 cells were infected with LSDV and transfected with each of the reporter plasmid vectors. eGFP was transiently expressed from all poxvirus promoter plasmid vectors over the 72-hour study period. Figure 3.4 outlines the experimental procedure and a mechanism for transient eGFP expression driven by the poxvirus promoter and viral transcription complex in the LSDV infected and transfected BHK-21 cells

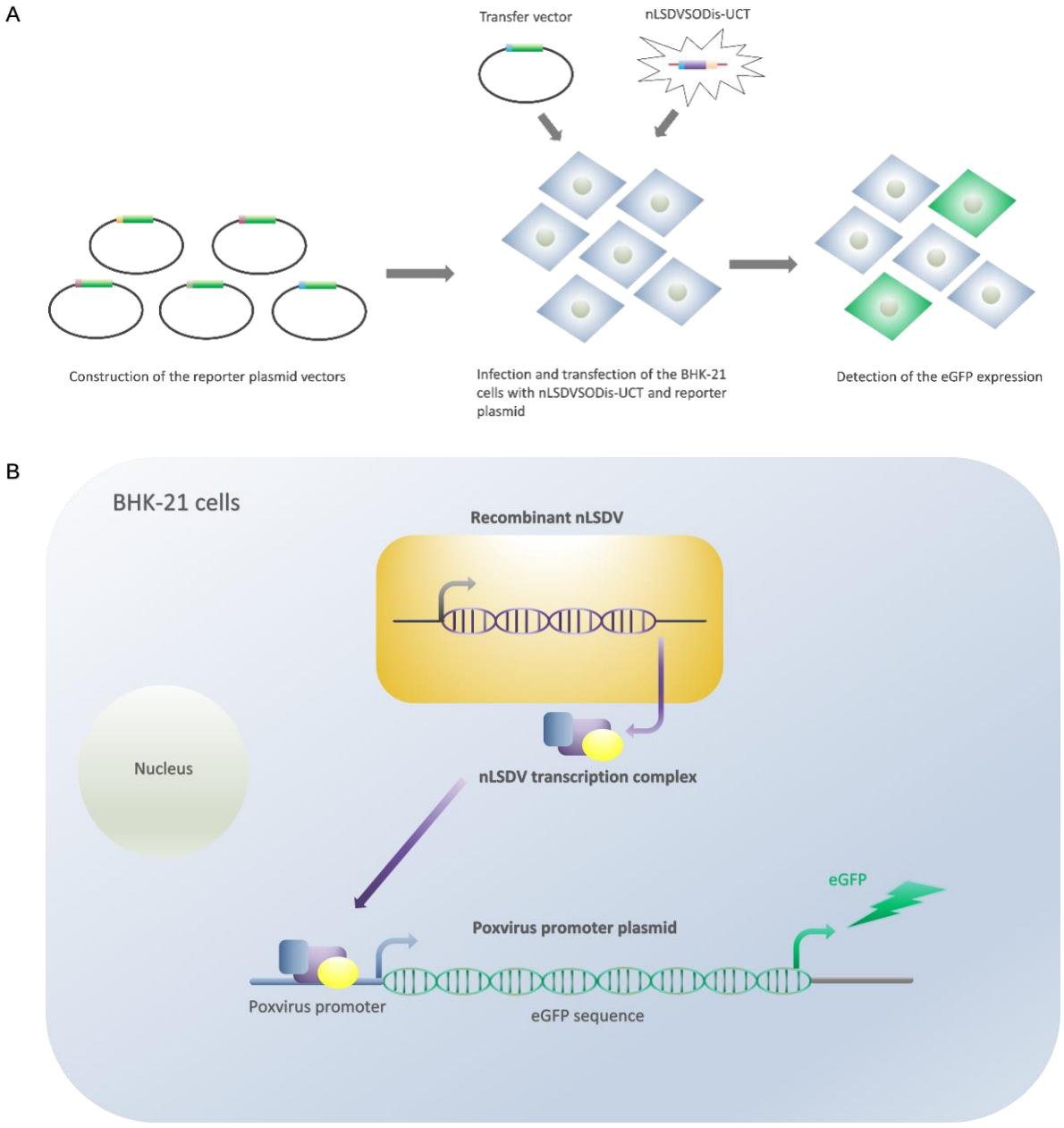



**Figure 3.4: Flow diagram of the experimental procedure and schematic diagram of a mechanism for transient eGFP expression in LSDV infected and transfected BHK-21 cells. (A)** Five reporter plasmid vectors were constructed, and BHK-cells were infected with LSDVT and transfected with one of the reporter plasmids. eGFP expression was examined using an epifluorescent inverted microscope. **(B)** In BHK-21 cells that were infected with LSDV and transfected with a reporter plasmid DNA, the LSDV expresses a viral transcription complex that binds to the poxvirus promoter of the reporter plasmid DNA, driving transient expression of eGFP.

### 3.3.1. Construction of the poxvirus promoter-eGFP plasmid vectors

#### 3.3.1.1. Enzymatic manipulation of the plasmids

Five reporter plasmid vectors were constructed as described in the Methods and shown in Table 3.3 and the schematic diagrams in Figure 3.5. The resultant plasmids each contained one of the poxvirus promoters immediately upstream of the eGFP gene. Table 3.4 outlines the promoter sequences used in this study.

**Table 3.3: Table showing the details of the plasmids and restriction endonucleases used to construct the five reporter plasmids**

| Promoter | Source plasmid | Restriction enzyme | Resultant plasmid |
|---|---|---|---|
| mFP | pBLV-Env-Gag | *Nco*I | pmFP-eGFP |
| p7.5 | pBLV-Env-Gag | *Apa*I and *Xho*I | p7.5-eGFP |
| mH5 | pBLV-Env-Gag | *Apa*I and *Xba*I | pBLV-Gag-eGFP |
| | pBLV-Gag-eGFP | *Nco*I | pmH5-eGFP |
| pS | pLSDV_K1L_eGFP | *Spe*I | pS-eGFP |
| LEO | pSSPExSHIVgp150I-Pgag | *Xho*I, *Nco*I and *Ngo*MIV | pLEO-eGFP |
| | pBLV-Gag-eGFP | *Xho*I and *Nco*I | |

**Table 3.4: Sequences of the five poxvirus promoters used in this study**

| Promoter | Sequence | Size | References |
|---|---|---|---|
| LEO | TTTTATTTTTTTTTTTTTGGAATATAAATATCCGGTAAAATTGAAAAAA TATACACTAATTAGCGTCTCGTTTCAGACGCTAG | 82bp | [436] |
| mH5 | AAAAATTGAAAATAAATACAAAGGTTCTTGAGGGTTGTGTTAAATTG AAAGCGAGAAATAATCATAAATAA | 71bp | [440] |
| mFP | AGAAAAATATCCTAAAATTGAATTGTAATTATCGATAATAA | 41bp | [438, 439] |
| p7.5 | TCCAAACCCACCCGCTTTTTATAGTAAGTTTTTCACCCATAAATAAT AAATACAATAATTAATTTCTCGTAAAAGTAGAAAATATATTCTAATTT ATTGCACGG | 104bp | [419] |
| pS | AAAATTGAAATTTTATTTTTTTTTTTTGGAATATAAATA | 39bp | [426] |

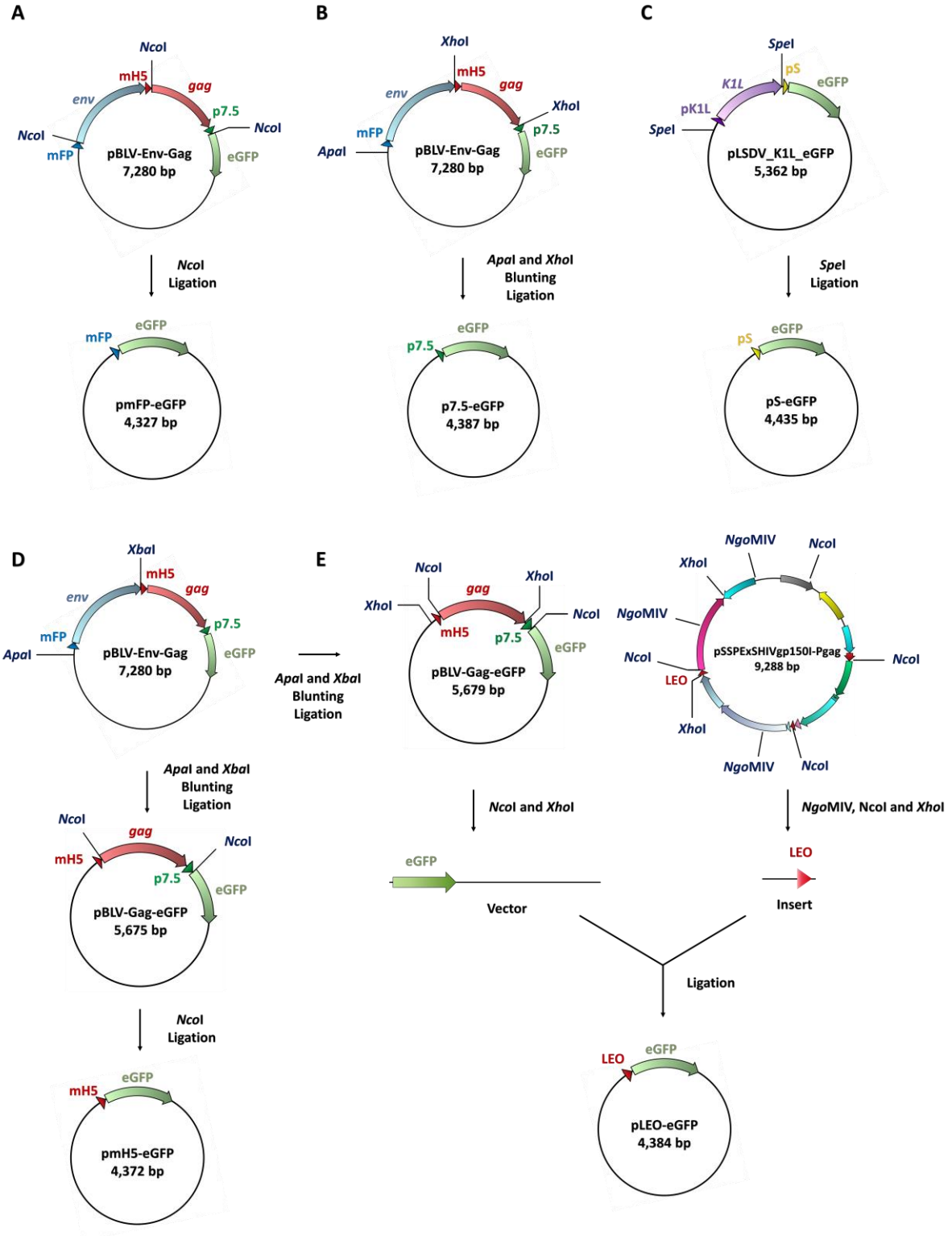



**Figure 3.5: Construction of the five poxvirus reporter plasmids. (A)** pmFP-eGFP **(B)** p7.5-eGFP **(C)** pS-eGFP **(D)** pmH5-eGFP **(E)** pLEO-eGFP plasmids. Poxvirus promoter sequences are represented by triangles and arrows indicate ORFs. The *bla* and *lacz* genes and Ori derived from the pUC57 simple plasmid cloning vector and 49 and 50 flanks are omitted from the plasmid maps.

### 3.3.1.2. Confirmation of the integrity of the poxvirus reporter plasmids

Restriction endonuclease mapping was used to confirm the integrity of plasmids pmFP-eGFP, p7.5-eGFP, pS-eGFP, pBLV-Gag-eGFP and pmH5-eGFP. Table 3.5 summarises the restriction endonucleases used for the restriction endonuclease mapping and the expected sizes of the fragments generated.

**Table 3.5: Restriction endonucleases used to confirm the integrity of the poxvirus reporter plasmids**

| Plasmid construct | Restriction enzyme | Size of restriction fragments |
|---|---|---|
| pmFP-eGFP | *Apa*I and *Kpn*I | 3,079bp and 1,248bp |
| p7.5-eGFP | *Nco*I and *Kpn*I | 3,195bp and 1,192bp |
| pS-eGFP | *Xho*I and *Bam*HI | *2,*742bp and 1,693bp |
| pBLV-Gag-eGFP | *Nco*I | 4,368bp and 1,307bp |
| pmH5-eGFP | *Nco*I and *Sal*I | 3,599bp and 773bp |

Restriction endonuclease digestion of plasmids pmFP-eGFP, p7.5-eGFP, pS-eGFP, pBLV-Gag-eGFP and pmH5-eGFP yielded DNA fragments of the correct sizes (Figure 3.6 A-E).

Amplification of a 525bp fragment using LEO fwd 4 and eGFP rev 5 primers from the pLEO-eGFP plasmid and not from the pBLV-Gag-eGFP intermediate plasmid or the pmH5-eGFP plasmid (which were included as negative controls) provided preliminary confirmation that the correct pLEO-eGFP plasmid was constructed (Figure 3.6 F).

The integrity of all plasmids was also confirmed by Sanger sequencing using M13F and M13R universal primers (Table 3.1).

## 3.3.2. Analysis of transient eGFP expression in BHK-21 cells infected with LSDV and transfected with the different reporter plasmids

To confirm that all five poxvirus promoters were active in LSDV, BHK-21 cells were infected with nLDSVSODis-UCT (LSDV) and transfected with each of the reporter plasmids, and expression of the eGFP was monitored using an epifluorescent inverted microscope. All five poxvirus promoters appeared to be functional in LSDV-infected cells as eGFP expression was consistently detectable throughout the 72-hour study period (Figure 3.8 to Figure 3.11).

No eGFP expression was detected from any of the experimental controls over the 72-hour study period (Figure 3.7). The BHK cells that were not infected and transfected remained viable, forming a healthy monolayer of cells.

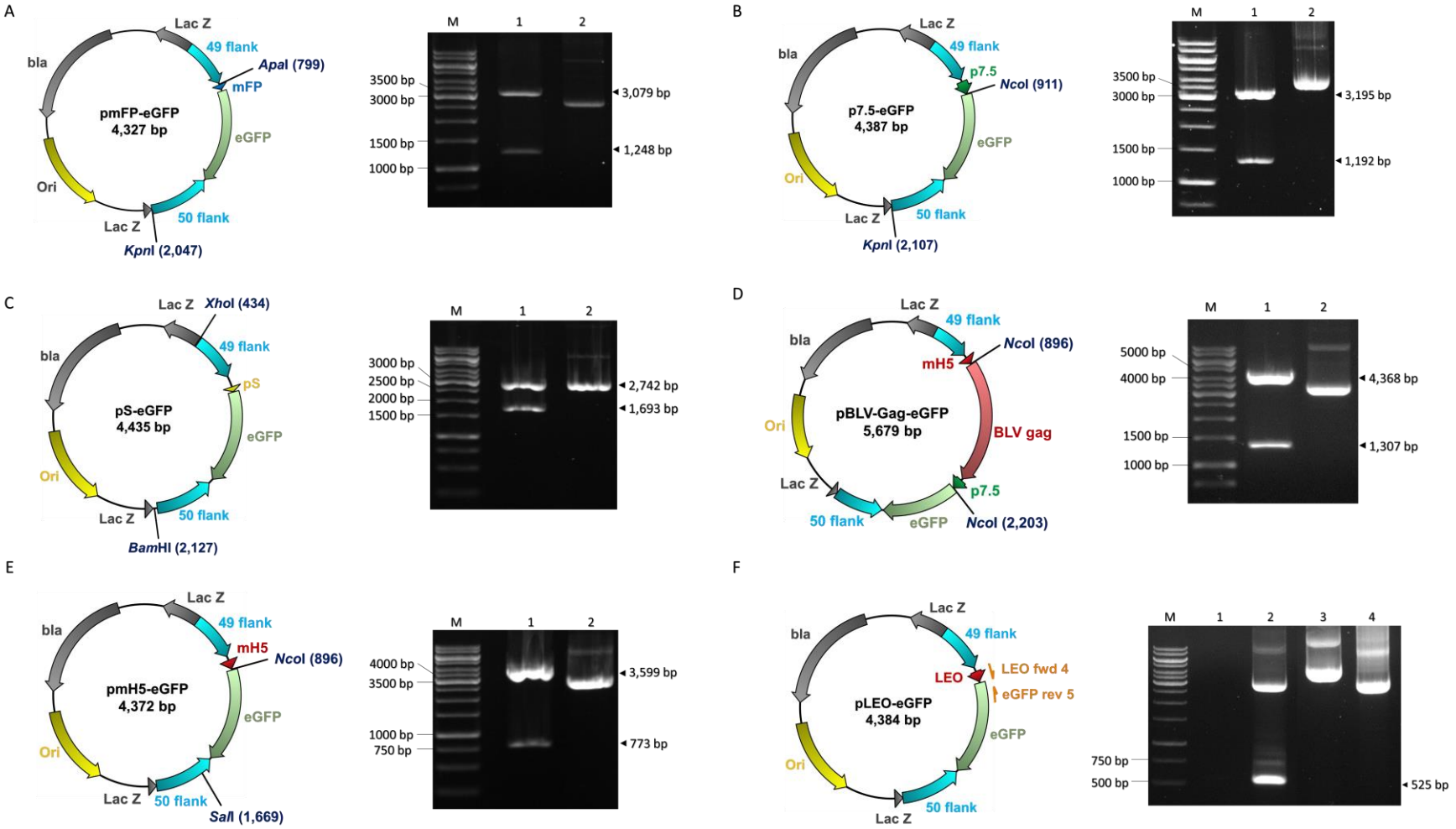



**Figure 3.6: Preliminary confirmation of the integrity of the five poxvirus reporter plasmids.** Plasmids **(A)** pmFP-eGFP, **(B)** p7.5-eGFP, **(C)** pS-eGFP, **(D)** pBLV-Gag-eGFP and **(E)** pmH5-eGFP were digested with restriction endonucleases (lane 1) or without restriction endonucleases (lane 2) and analysed by 0.8% agarose gel electrophoresis with ethidium-bromide staining (0.5µg/ml). **M**, 1kb DNA ladder (Thermo Scientific, USA). A 525bp fragment was amplified from **(F)** pLEO-eGFP plasmid using LEO fwd 4 and eGFP rev 5 primers. **1**, PCR control without template DNA; **2**, pLEO-eGFP plasmid DNA as a template; **3**, pBLV-Gag-eGFP plasmid DNA as a template; **4**, pmH5-eGFP plasmid DNA as a template. Restriction sites are shown on the plasmid maps with their nucleotide positions shown in brackets. Binding sites of LEO fwd 4 and eGFP rev 5 primer (yellow arrows) are indicated on the pLEO-eGFP plasmid map. Black arrowheads in the images of agarose gels indicate the sizes of DNA fragments digested with restriction endonucleases.

The BHK cells that were infected with LSDV at an MOI of 0.5 were heavily infected and numerous cells were rounded up at 72 hours post-infection/transfection. At 72 hours post-transfection, the BHK cells in all transfection control wells were sparser and less viable than the infection control and uninfected cells due to the toxicity of a transfection reagent to the cells.

The eGFP expression from the LSDV-infected cells that were transfected with each of the five poxvirus promoter plasmids was first monitored at 3 hours post-infection/transfection and no eGFP was detectable from any of the reporter plasmids. However, at 6 hours post-infection/transfection, weak eGFP expression was detected from some cells transfected with the pmH5-eGFP reporter plasmid (Figure 3.8).

At 9 hours post-infection/transfection, slightly more cells transfected with the pmH5-eGFP plasmid showed eGFP expression but no eGFP expression was detected from the other four reporter plasmids (data not shown).

At 24 hours post-infection/transfection, eGFP expression was also detected from the other four poxvirus promoter plasmids (Figure 3.9) but their eGFP expression was still weaker than the eGFP expression driven by the pmH5-eGFP reporter plasmid. Despite the weaker eGFP expression from the other four reporter plasmids, most cells were transfected and expressing eGFP. Interestingly, although eGFP expression from the p7.5-eGFP reporter plasmid was also only first detectable at 24 hours post-infection/transfection, its eGFP expression appeared stronger than the pS-eGFP, pLEO-eGFP and pmFP-eGFP reporter plasmids. Furthermore, eGFP expression from the mFP-eGFP reporter plasmid appeared much weaker than the other four reporter plasmids.

At 48 hours post-infection/transfection, strong eGFP expression was observed from the pS-eGFP, pLEO-eGFP and p7.5-eGFP reporter plasmids (Figure 3.10) and almost all cells appeared to be expressing eGFP in all experimental wells. In particular, a large amount of the eGFP product appeared to be accumulating in the LSDV-infected cells that were transfected with the p7.5-eGFP and pmH5-eGFP reporter plasmids, resulting in brighter eGFP expression than other reporter plasmids. In contrast, eGFP expression from the pmFP-eGFP reporter plasmid remained low even at 48 hours post-infection/transfection.

At 72 hours post-infection/transfection, more eGFP appeared to be accumulating in all experimental wells but eGFP expression from the pmFP-eGFP reporter plasmid was still much lower than the other four reporter plasmids (Figure 3.11). Nonetheless, stable eGFP expression was detected at the 72-hours post-infection/transfection.

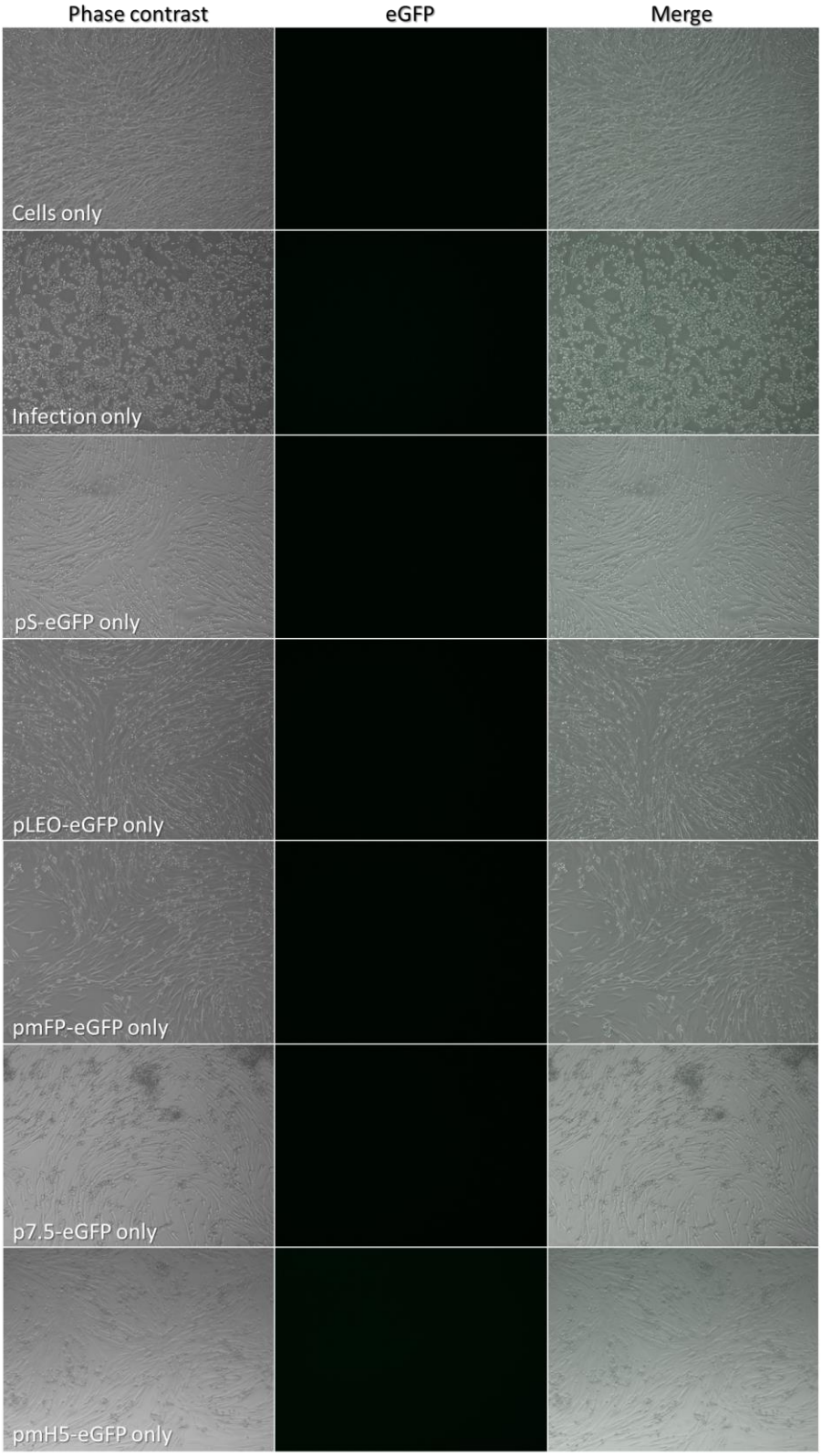



**Figure 3.7: Fluorescent images of the experimental, infection and transfection controls. Cells only:** Baby hamster kidney (BHK-21) cells were not infected or transfected; **Infection only:** BHK-21 cells were infected with LSDV at an MOI of 0.5; **Transfection only:** BHK-21 cells were transfected with 2µg of pS-eGFP, pLEO-eGFP, pmFP-eGFP, p7.5-eGFP or pmH5-eGFP plasmid DNA. All images were taken at 6 hours post-infection/transfection using a Zeiss Axiovert 200M fluorescence inverted microscope (Zeiss, Germany) at 100x magnification.

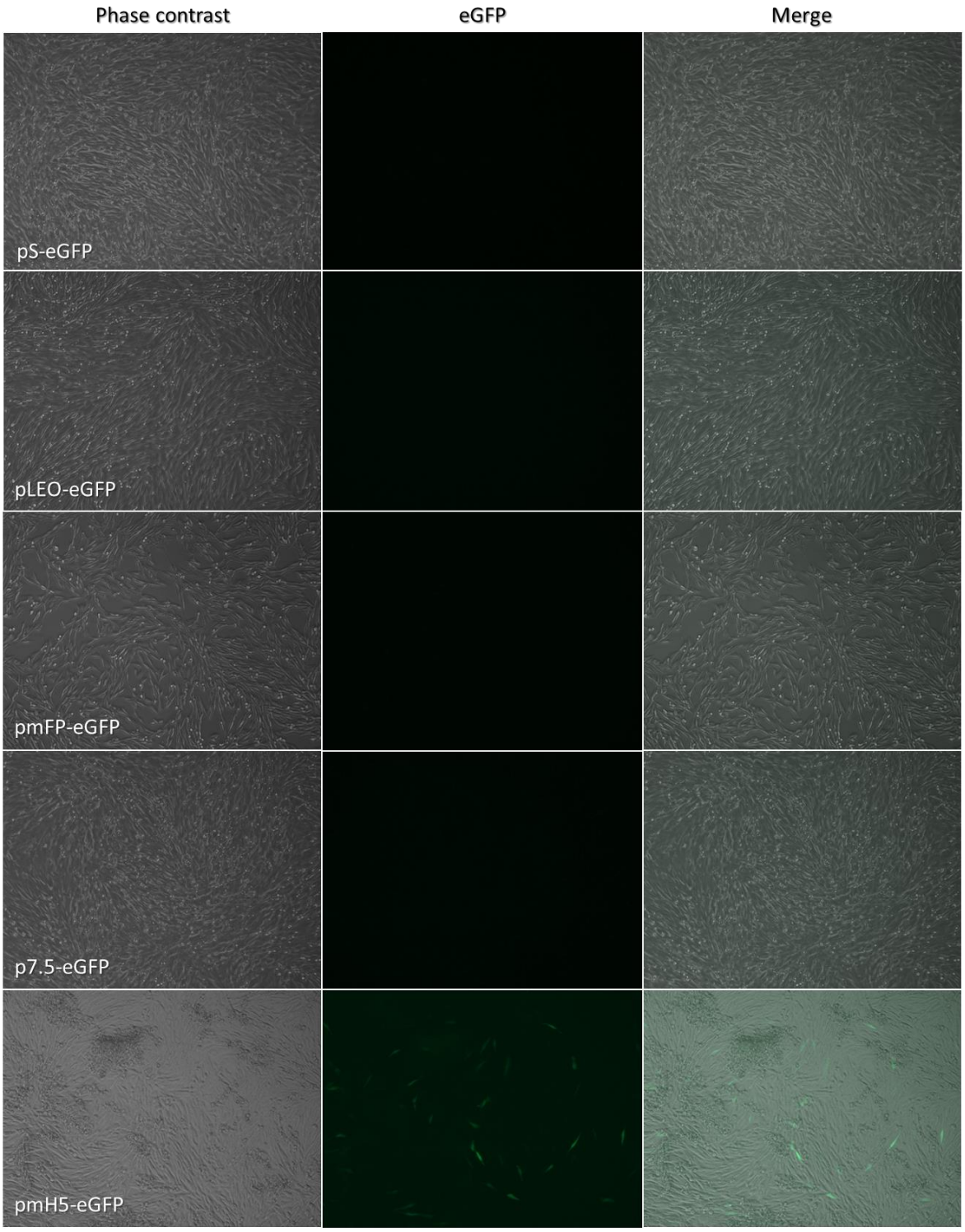



**Figure 3.8: Transient eGFP expression in BHK-21 cells infected with LSDV and transfected with a reporter plasmid at 6 hours post-infection/transfection.** Baby hamster kidney (BHK-21) cells ($2.0x10^5$ cells per well) were infected with LSDV at an MOI of 0.5 and transfected with 2µg of pS-eGFP, pLEO-eGFP, pmFP-eGFP, p7.5-eGFP or pmH5-eGFP plasmid DNA. All images were taken at 6 hours post-infection/transfection using a Zeiss Axiovert 200M fluorescence inverted microscope at 100x magnification.

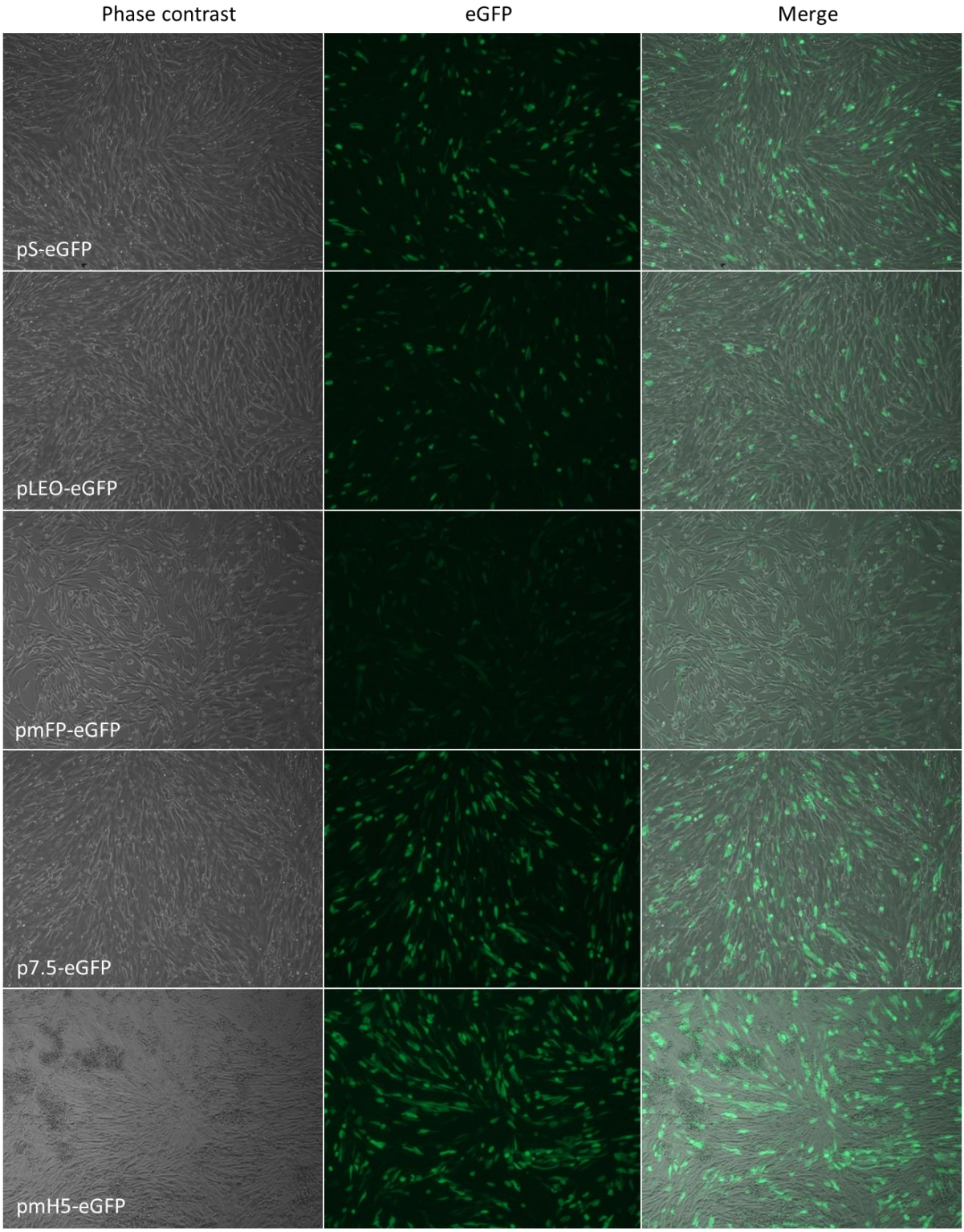



**Figure 3.9: Transient eGFP expression in BHK-21 cells infected with LSDV and transfected with a reporter plasmid at 24 hours post-infection/transfection.** Baby hamster kidney (BHK-21) cells ($2.0x10^5$ cells per well) were infected with LSDV at an MOI of 0.5 and transfected with 2µg of pS-eGFP, pLEO-eGFP, pmFP-eGFP, p7.5-eGFP or pmH5-eGFP plasmid DNA. All images were taken at 24 hours post-infection/transfection using a Zeiss Axiovert 200M fluorescence inverted microscope at 100x magnification.

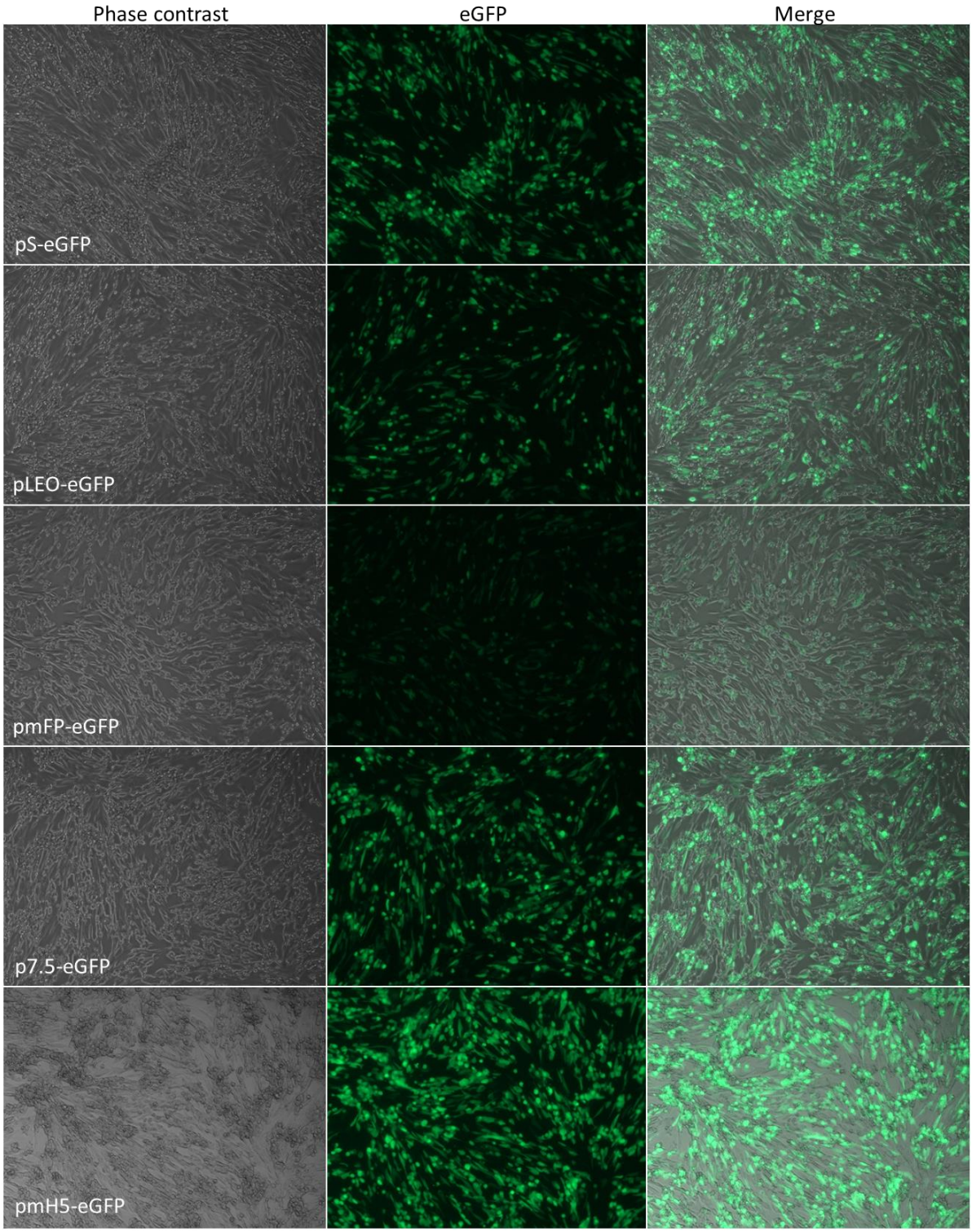



**Figure 3.10: Transient eGFP expression in BHK-21 cells infected with LSDV and transfected with a reporter plasmid at 48 hours post-infection/transfection.** Baby hamster kidney (BHK-21) cells ($1.0x10^5$ cells per well) were infected with LSDV at an MOI of 0.5 and transfected with 2µg of pS-eGFP, pLEO-eGFP, pmFP-eGFP, p7.5-eGFP or pmH5-eGFP plasmid DNA. All images were taken at 48 hours post-infection/transfection using a Zeiss Axiovert 200M fluorescence inverted microscope at 100x magnification.

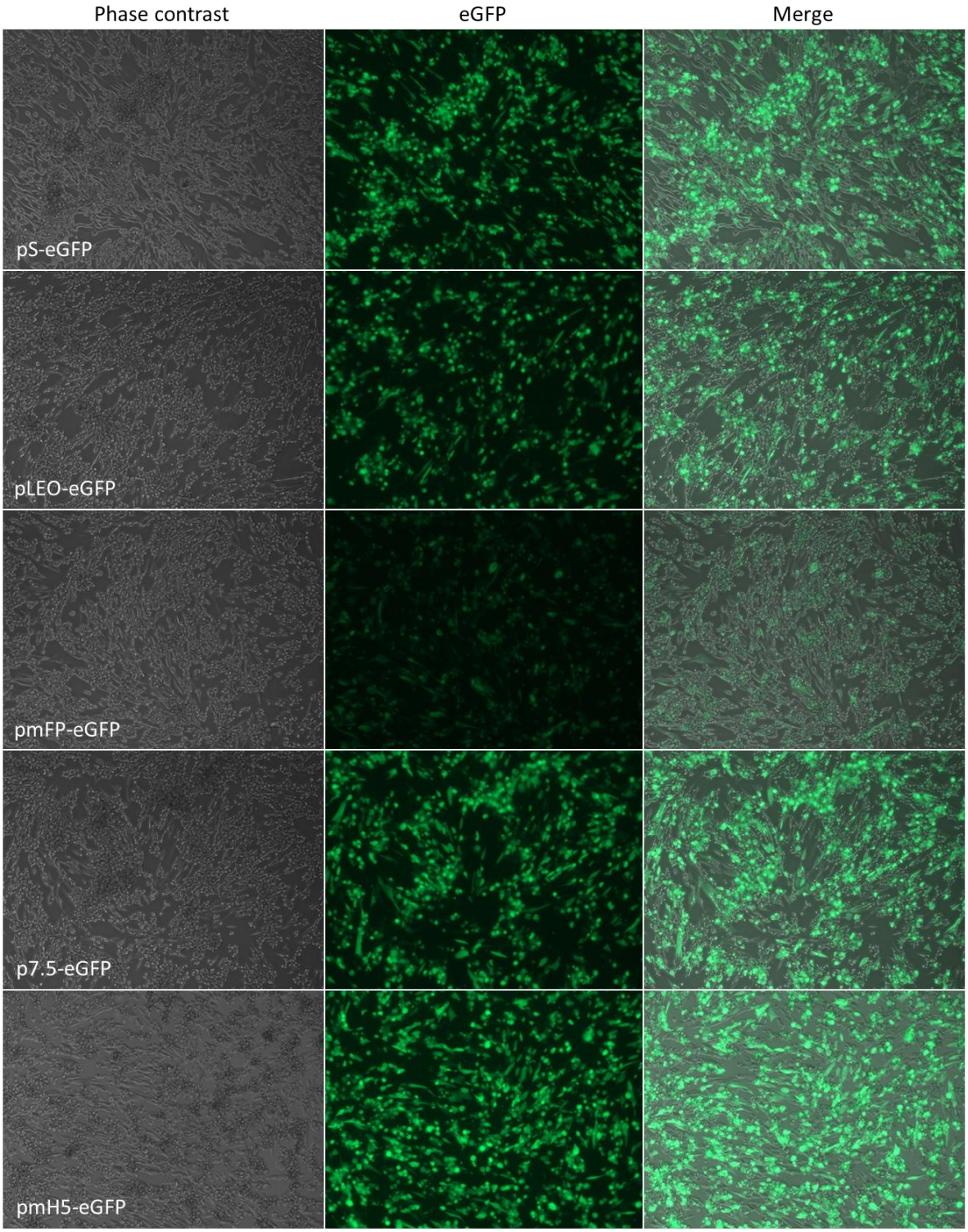



**Figure 3.11: Transient eGFP expression in BHK-21 cells infected with LSDV and transfected with a reporter plasmid at 72 hours post-infection/transfection.** Baby hamster kidney (BHK-21) cells ($1.0x10^5$ cells per well) were infected with LSDV at an MOI of 0.5 and transfected with 2µg of pS-eGFP, pLEO-eGFP, pmFP-eGFP, p7.5-eGFP or pmH5-eGFP plasmid DNA. All images were taken at 72 hours post-infection/transfection using a Zeiss Axiovert 200M fluorescence inverted microscope at 100x magnification.

## 3.4. DISCUSSION

In this study, the activity of five poxvirus promoters (mFP, pS, LEO, p7.5 and mH5 promoters) was examined by the detection of the transient eGFP expression driven by each of the five poxvirus promoters to assess whether the five selected poxvirus promoters were functional in the LSDV-infected cells. We demonstrated that all poxvirus promoters tested in this study were functional in the LSDV-infected cells.

The five poxvirus promoters selected in this study all contained an early promoter element and four of the promoters also contained a late promoter element (pS, LEO, p7.5 and mH5). As early promoter expression is considered to occur immediately following infection and prior to DNA replication, eGFP expression was monitored first at 3 hours and at 6 hours post-infection/transfection as poxviral DNA replication communes within 2 hours post-infection and terminates at approximately 8 hours post-infection [428, 437]. At 3 hours post-infection/transfection, no eGFP was detected from any of the five reporter plasmids. At 6 hours post-infection/transfection, low levels of eGFP expression were detected from the pmH5-eGFP reporter plasmid and at 9 hours post-infection/transfection, eGFP expression was still undetectable from the four remaining reporter plasmids. It should be noted that the monitoring of the activity of the poxvirus promoters relied solely on the visible detection of eGFP and the detection of eGFP is only possible when the amount of the eGFP produced reaches a detectable level. Therefore, the absence of the eGFP expression from all reporter plasmids at 3 hours post-infection/transfection and from the pmFP-eGFP, pS-eGFP, pLEO-eGFP and p7.5-eGFP reporter plasmids at 6 hours does not necessarily indicate that all promoters commenced transcription later than 3 hours post-infection/transfection. It could be simply because the amount of the eGFP was below the detectable levels using fluorescent microscopy.

By 24 hours post-infection/transfection, eGFP was detected from the other four promoters and most cells were fluorescing in all experimental wells. At 48 hours post-infection/transfection, almost all cells were fluorescing in all experimental wells and eGFP expression from all reporter plasmids except for the pmFP-eGFP reporter plasmid appeared brighter than the eGFP observed at 24 hours post-infection/transfection. At 72 hours post-infection/transfection, a further increase in the eGFP intensity was observed in the infected/transfected cells, except for cells transfected with the pmFP-eGFP reporter plasmid, whose eGFP expression remained weak at 72 hours post-infection/transfection. Since almost all cells were transfected and fluorescing, this weak eGFP expression is likely due to the weak activity of the mFP promoter. These results together demonstrated that five poxvirus promoters were able to drive the stable expression of eGFP during the study period.

A previous study conducted by Baur *et al*. [429] with MVA demonstrated that similar to the earlier expression of the mH5 promoter observed in our study, their strong hybrid early-late promoter (pHyb) showed detectable eGFP expression as early as 30 minutes post-infection/transfection whereas mean fluorescence intensity of eGFP driven by p7.5 and pS promoters only reached an equivalent expression level at 6 hours post-infection. Furthermore, they observed slightly earlier eGFP expression driven by the p7.5 promoter than the pS promoter [429]. However, these expression levels were not significantly different.

The modified early fowlpox (mFP) promoter was designed from the native early-late fowlpox promoter [439] at our laboratory to retain the early promoter element by replacing the late promoter element with the poxvirus terminator sequence [438]. The mFP promoter was previously utilised in our recombinant LSDV vaccine to drive the expression of the Rift Valley fever virus (RVFV) nucleocapsid (NC) gene [438]. The expression of the NC protein from this recombinant LSDV was confirmed by Western blotting and immunofluorescence assay [438]. In our study, eGFP expression driven by the mFP promoter appeared to be lower than the other four promoters even at 72 hours post-infection/transfection. It should be noted that there was no internal control to determine if the lower expression level was due to low transfection efficiency. However, it is possible that the removal of the late promoter element had an impact on the overall promoter activity, thereby leading to lower eGFP expression. A previous study demonstrated that a substitution of a G residue to an A, C or T residue in the late promoter initiation sequence (TAAATG) resulted in a significant decrease in promoter activity with approximately 80-90% reduction of the reporter gene expression [446]. Since the late promoter initiation sequence is absent in the mFP promoter, this could explain the low eGFP expression observed in our study. It is also possible that since the mFP promoter has early promoter activity only, the eGFP production terminates after DNA replication, i.e., ~8 hours post-infection, which may lead to a smaller amount of eGFP production compared to the other four promoters. The other four poxvirus promoters contain a late promoter element, and this may drive sustained transcription after DNA replication. It should be noted that strong promoters are not necessarily advantageous. Wyatt *et al.* [440] observed that irrespective of types of recombinant viruses, cell lines, the number of transgenes and insertion sites, the use of the pS promoter in recombinant vaccinia viruses to express parainfluenza virus type 3 antigen(s) resulted in the inhibition of replication of the recombinant vaccinia viruses with titres ranging from $6x10^0$ to $6x10^4$ pfu/ml after four or five passages. Whereas recombinants constructed using the same VACV vector expressing the same transgenes inserted at the same locus but under the control of the p7.5 promoter replicated to higher titres and were immunogenic in vaccinated animals [447]. They reasoned that the overexpression of the transgene(s) by the pS promoter was too toxic for the recombinant vaccinia viruses to

propagate efficiently [440]. Therefore, the possibly weaker mFP promoter might be useful to drive expression of genes that could be toxic.

It may be possible to enhance early promoter activity of the mFP promoter to an appropriate level by introducing tandem repeats of an early promoter sequence [429, 448, 449]. However, caution must be taken as tandem repeats are prone to induce mutations and inter- and intramolecular homologous recombination [450-452]. In addition, a previous study [453] showed that the overexpression of a transgene driven by a strong promoter harbouring tandem repeats of the early element of the p7.5 promoter in the replication-competent vaccinia virus LC16m8Δ resulted in the increased neurovirulence of the vaccinia virus. Apart from the modification and optimisation of the promoter sequence itself, it has been reported that a spacer length between a promoter and a gene of interest could enhance antigen expression and immunogenicity [437, 448].

The synthetic early-late optimised promoter (LEO) of VACV is a new synthetic vaccinia virus promoter designed by Pilato *et at.* [436, 437] to improve early gene expression and antigen-specific T cell responses by using a late promoter element of the pS promoter and an early promoter consensus sequence identified from the alignment of 45 genes belonging to the immediate-early and E1.1 classes. To characterise the LEO promoter activity, they constructed recombinant MVA containing the eGFP or a *Leishmania* LACK protein under the control of LEO, pS or mH5 promoters [436, 437]. They demonstrated that *in vitro* and *in vivo* expression of the eGFP and the LACK protein driven by the LEO promoter was higher at 4 hours post-infection than the expression driven by the pS and mH5 promoters, which correlated to the enhanced expression of antigen-specific multifunctional memory $CD4^+$ and $CD8^+$ T cells [436, 437]. Our results demonstrated that the LEO promoter was functional in the LSDV-infected cells and indicate that the LEO promoter will be functional in recombinant LSDV.

In the present study, to remove possible confounding factors, monitoring of the promoter activity was conducted under the same molecular, biological and technical contexts where possible. Firstly, the five reporter plasmids were constructed to have a similar size (4,327bp - 4,435bp) and the same plasmid backbone. All reporter plasmids also contained the same *egfp* gene optimised for bovine cells. Secondly, to equalise the amount of plasmid DNA used for the transfection, the concentrations of the five plasmid DNA were more accurately estimated based on the absolute density of the plasmid DNA resolved on an agarose gel, which enables the separation of the plasmid DNA and RNA contained in each sample. Thirdly, the same recombinant LSDV at the same MOI was used to infect the same batch of BHK-21 cells at a concentration of $1.0x10^5$ cells/ml. Fourthly, to detect expression of the eGFP driven by each of the reporter plasmids, the same area of the cells was monitored during the 72-hour study

and all fluorescence images were taken by normalising the exposure time based on the exposure time used to take images of the cells transfected with the p7.5-eGFP reporter plasmid. This allowed us to monitor the activity of the five poxvirus promoters under as similar contexts as possible. However, the use of plasmid DNA and a virus in this reporter system essentially introduces experimental variables both from the plasmid and the virus.

It should be noted that the results of this experiment are qualitative and not quantitative. The aim was to determine whether the promoters would be recognised by LSDV or not. Promoter activity was not quantified. In order to quantitate expression, one would require an internal transfection control. The use of an internal control would allow for measuring of timing and strength of the five poxvirus promoters relative to this internal control. In addition, transfection efficiency, which is another experimental variable, was not evaluated in this study. In our study, no comparison of the strength of promoter activities was made to avoid misinterpretation.

Another possible limitation in this study is the use of eGFP as a reporter gene. The use of fluorescent proteins (FPs) in reporter gene systems is a well-established method and myriads of mutant FPs with improved photochemical properties have been engineered, allowing them for multiple labelling and wide use in various applications. As opposed to enzyme-based assays, the use of FPs allows direct monitoring of live cells noninvasively (i.e., no fixation, permeabilization and lysis of cells) without the need for cofactors and substrates. However, it is important to take into account their limitations. It is also important to understand biochemical properties of FP to make correct interpretations of experimental results.

eGFP is one of the mutant variants and was engineered from the wild-type *Aequorea* GFP by introducing two point mutations (S65T and F64L) [454]. The resultant eGFP is well recognised for its increased brightness [454], which is one of the reasons for its popularity amongst researchers. In addition, eGFP is superior to the wild-type *Aequorea* eGFP due to its thermostability [455] and pH resistance [456]. eGFP also exhibits faster maturation time, improved sensitivity of detection and slower photobleaching rates [456-458]. Since eGFP is more tolerant of photobleaching, this feature is useful for time-lapse imaging [459]. Yet, consideration should also be given to the fact that intensive illumination can result in cell damage and cell death [460].

Since one eGFP molecule emits one photon, the signal cannot be amplified. This means that detection of eGFP expression largely relies on the amount of eGFP produced above threshold levels. Approximately $10^4$ to $10^6$ eGFP molecules per cell are generally required for detection [456]. To achieve these copy numbers, one of the simplest ways is the use of strong or constitutive promoters or transcription regulators. In addition to the threshold, eGFP intensity should exceed that of cellular autofluorescence [461].

The use of the FPs is useful for an “all-or-none” type of reporter assays, which was utilised in our study. There are several methods to estimate the strength and timing of the promoter activity. For example, the number of eGFP positive cells and fluorescence intensity of the eGFP positive cells could be measured using flow cytometry [429, 436, 437] at various time points up to 8 hours post-infection to monitor the activity of the early promoters. Alternatively, a luciferase assay [462] or quantification of the eGFP mRNA by real-time PCR [437] would be more sensitive. There are some advantages to the use of luciferase as a reporter over FPs. FPs are stable proteins and thus, tend to accumulate in cells [455] whereas the low stability of luciferases [463] means less accumulation in cells and so small changes in expression levels are more easily detected. In addition, only viable cells expressing luciferase can be bioluminescent and dead cells do not contribute any signal. This is due to the inherent properties of the enzymatic reaction where luciferases require cellular ATP from viable cells [464, 465]. Luciferase assays are free from autofluorescence and provide a better signal to noise ratio [466]. Lastly, posttranslational modifications (i.e., protein maturation) and photobleaching are absent in luciferase assays [464]. These features also enable instantaneous measurement of gene expression as well as detection of a wide range of expression levels.

In conclusion, stable and sustained activity of the five selected poxvirus promoters that contain early and late promoter elements were confirmed in the LSDV-infected cells under the transient expression system. Our results indicate that the five poxvirus promoters will be functional to drive the expression of transgenes in recombinant LSDV. Further study will be necessary to estimate the timing and strength of the poxvirus promoters in the recombinant LSDV.

# CHAPTER 4

# CONSTRUCTION AND CHARACTERISATION OF RECOMBINANT LSDV-VECTORED DUAL VACCINES AGAINST BOVINE LEUKAEMIA VIRUS AND LUMPY SKIN DISEASE VIRUS

## 4.1. INTRODUCTION

Poxviruses are one of the most well-studied and extensively-utilised viruses in the history of vaccination: from the classical use of cowpox virus by Benjamin Jesty and Edward Jenner to protect from smallpox in the 18th century [467, 468] to the use of vaccinia virus to eradicate smallpox in the 20th century and to the current use of recombinant poxviruses in various vaccines against animal and human diseases [402-406, 408-410, 469]. Poxviruses harbour a large genome, up to 370kb [470], and replicate in the cytoplasm of the host cell [471], enabling them to accommodate large transgene(s) [472, 473] without an issue of integration of transgenes into the host genome. The poxvirus genome contains numerous non-essential genes for its replication [474-476] as well as regions which have acquired deletions which resulted from successive and intensive passages [477-479]. These regions of the genome have been traditionally utilised as sites for transgenes in expression systems. Intergenic regions have also been recently utilised as insertion sites [480, 481]. Furthermore, it is possible to insert multiple transgenes at a single insertion site [29, 30, 482] or multiple insertion sites [479, 483, 484]. Most poxviruses have a similar genomic arrangement [485] and share the same transcriptional pathways [418]. Previous studies have shown that transcription factors and promoter functions are conserved across the *Poxviridae* family [486, 487]. These features make recombinant poxviruses useful as an expression vector as heterologous poxvirus promoters can be used in a recombinant poxvirus, and several different poxvirus promoters can be incorporated into a single recombinant poxvirus vector. It is also possible to fine-tune the timing and strength of transgene expression by placing these genes under the control of different poxvirus promoters [419, 426, 440, 488]. Host tropism of poxviruses is another useful feature that can be utilised in generating safe poxvirus-vectored vaccines. Poxvirus host tropism [416] is unique in that unlike other families of viruses, poxvirus host range is not determined by their binding to host cell receptors but regulated by host range genes [489, 490] and immunomodulatory genes [491]. For example, a poxvirus with a specific host range can infect and express its genes in restrictive cells but the replication is abortive at a later stage of the replication cycle (i.e., after protein synthesis) [492]. Therefore, the spill-over of host-restricted vaccine viruses to non-host animals is unlikely. Lastly, genetic engineering of recombinant poxviruses has enabled the generation of further versatile poxvirus vectors such as attenuated recombinant viruses [493-501], inducible recombinant vaccinia virus expression system [502, 503] and modified recombinant poxvirus vectors that contain genetic adjuvant(s) to enhance immunogenicity [504-506].

As homologous recombination (HR) naturally occurs between replicating poxviruses during viral replication, one of the conventional methods to generate recombinant poxviruses utilises this process to insert transgene(s) into the poxvirus genome [507-511] at a specific site (Figure

4.1). In this method, a transfer vector is designed to contain transgene(s) under the control of poxvirus promoter(s), homologous sequences to the poxvirus genome at the site of insertion (left and right flanks) and marker and/or selectable genes. Nowadays, genes expressing fluorescent proteins are frequently utilised as marker genes to identify recombinant viruses. Transgenes are placed between the left and right flanks so that these flanks guide the exchange of genomic sequences between the poxvirus genome and the flanking regions of the transfer vector through HR. This results in the site-directed insertion of the transgene(s) into the poxvirus genome and the generation of a recombinant poxvirus.

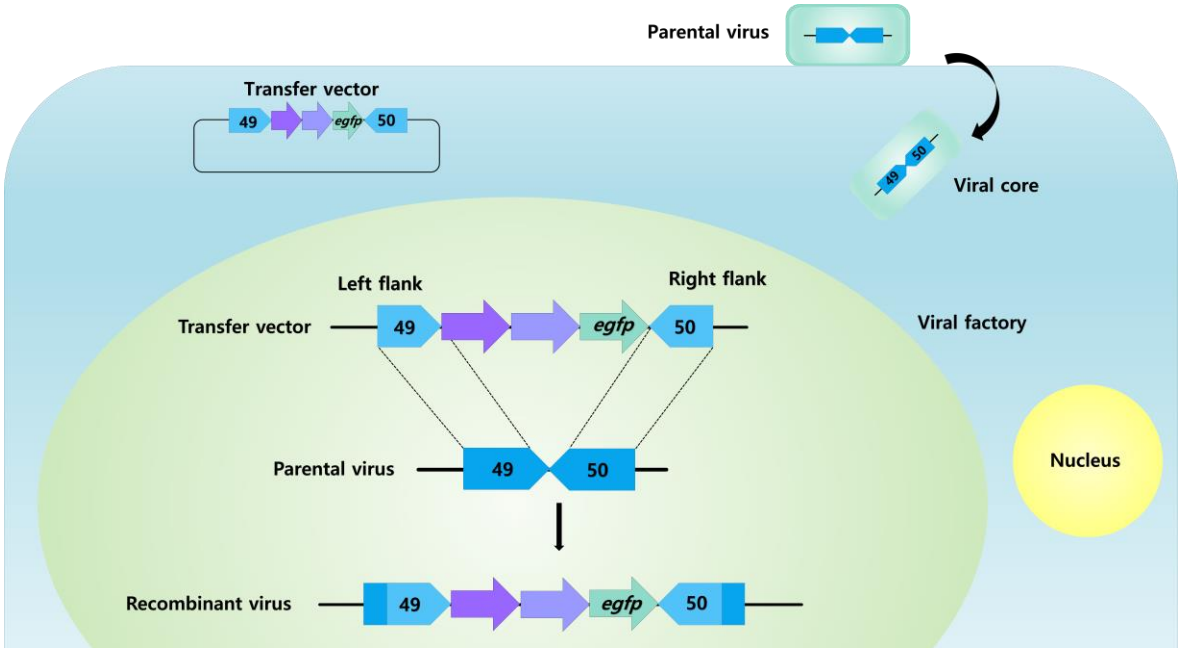



**Figure 4.1: Schematic diagram of the generation of a recombinant poxvirus by homologous recombination.** The diagram describes a method to generate a recombinant virus from parental virus and a transfer vector containing two antigen genes (purple arrows), an *egfp* gene as a marker gene (green arrow) as well as left and right flanks (49 and 50 flanks, respectively; blue arrows). **49**, left flank of the transfer vector, ±300bp of the 3' end of *049* ORF of the LSDV genome; **50**, right flank of the transfer vector, ±300bp of the 3' end of *050* ORF of the LSDV genome; ***egfp***, green-fluorescent protein gene.

The overall aim of this chapter was to construct and characterise a recombinant LSDV expressing BLV Env and Gag and another recombinant LSDV expressing BLV Env alone (collectively, recombinant LSDVs). BLV *env* gene was selected as an antigen based on previous studies that showed induction of humoral and cellular responses from recombinant vaccinia virus containing BLV *env* gene [90-95, 97, 326, 327]. BLV *gag* gene was selected as a second antigen as BLV Gag forms virus-like particles (VLPs) [264, 512-514], which are immunogenic [274, 277-279] and could serve as a platform to present the BLV Env proteins in dense and repetitive arrays [276].

The objectives included:

- to select BLV Env and Gag sequences for the BLV expression cassette based on the sequence alignment of BLV natural isolates.
- to determine whether the human tissue plasminogen activator (tPA) signal sequence is functional in bovine cells.
- to design and construct BLV transfer vectors to generate the recombinant LSDVs.
- to confirm the presence of BLV *env* and *gag* genes in the recombinant LSDVs by PCR and to confirm the integrity of these genes by sequencing.
- to confirm expression of BLV Env protein by immunofluorescence and BLV Gag protein by Western blotting.

## 4.2 MATERIALS AND METHODS

### 4.2.1. Design of the BLV transfer vectors

#### 4.2.1.1. Selection of a BLV natural isolate to design the BLV *env* and *gag* sequences of the BLV expression cassette

To design antigen sequences for the BLV expression cassette in the pBLV-Env-Gag and pBLV-Env transfer vectors (collectively BLV transfer vectors), full-length *env* and *gag* nucleotide sequences of a natural BLV isolate were selected as follows. Firstly, BLV complete genomic sequences from BLV natural isolates representing six genotypes were obtained from the GenBank nucleotide sequence database (Table S4.1). BLV complete genomic sequences with unknown genotype were also included to add sequence variations. The nucleotide sequences were then, translated into amino acid sequences using CLC Main Workbench (QIAGEN Bioinformatics, Denmark). These nucleotide and amino acid sequences were aligned to obtain consensus Env and Gag (Cons-Env and Cons-Gag, respectively) nucleotide and amino acid sequences, respectively, (Table S4.2 and Table S4.3). Then, nucleotide and amino acid sequences of the Cons-Env and Cons-Gag were aligned to those of BLV natural isolates available in the Basic Local Alignment Search Tool (BLAST) database [374]. Full-length Env and Gag nucleotide and amino acid sequences of candidate natural isolates were selected using 90% nucleotide and amino acid sequence identity as a cut-off and excluding isolates whose partial Env and Gag nucleotide and amino acid sequences had high identity with those of the Cons-Env and Cons-Gag. Env and Gag amino acid sequences of candidate

natural isolates were aligned back to the Cons-Env and Cons-Gag amino acid sequences using pair-wise alignment of EMBOSS Needle protein [515] for final confirmation.

#### 4.2.1.2. Assessment of functionality of the signal sequence of the human tissue plasminogen activator gene (tPA) in a bovine cell line

To assess whether the signal sequence of the BLV *env* gene in the BLV transfer vectors could be replaced with the signal sequence of the human tPA, amino acid sequences of the signal sequences of the human and bovine tPA were compared.

##### 4.2.1.2.1. Amino acid sequence comparison of the signal sequences of the human and bovine tPA

Amino acid sequences of the signal sequences of the human and bovine tPA were obtained from the UniProtKB database and the comparison of their amino acid sequences was performed using pair-wise alignment of EMBOSS Needle protein [515]. EBLOSUM62 with 10,0 value as gap penalty and 0.5 value as extend penalty were used.

##### 4.2.1.2.2. Assessment of the *in vitro* activity of the human tPA by Western blotting

###### 4.2.1.2.2.1. Source of an adherent cell line

A Madin-Darby bovine kidney (MDBK) adherent cell line (CCL-22) was obtained from the American Type Culture Collection (ATCC).

For the detailed methods of recovery of cell lines from deep-frozen stocks, maintenance of cell lines and cell counting, see Section 3.2.4.

###### 4.2.1.2.2.2. Source of a virus

Recombinant modified vaccinia virus Ankara (MVA) containing HIV-1 type C *env* and *gag* genes (MVA-GC5SS) was obtained from Shireen Galant (a member of our research group). The signal sequence of the HIV *env* gene in MVA-GC5SS has been replaced with that of the human tPA.

#### 4.2.1.2.2.3. Crude extraction of total proteins from MDBK cells infected with MVA-GC5SS

One millilitre of MDBK cells at a concentration of $2.0x10^5$ cells/ml were infected with MVA-GC5SS at a multiplicity of infection (MOI) of 1, 2 or 5 in 12-well tissue culture plates and the plates were incubated under standard growth conditions (37°C, 70% relative humidity and 5% $CO_2$) for 3 days with daily monitoring of the infected cells using an epifluorescent inverted microscope (Zeiss, Germany). At 72 hours post-infection, crude extraction of total proteins from the MVA-GC5SS infected cells was conducted as outlined in Appendix B2.1. The total proteins contained in the culture media and cell lysate were resolved by sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) and the resolved proteins were transferred onto polyvinylidene difluoride (PVDF) membranes as described in Appendix B2.2 and B2.3, respectively. Subsequently, HIV-1 Env glycoprotein was detected with a goat anti-HIV-1 Env polyclonal antibody (BioRad, USA) at a dilution of 1:1,000 followed by an anti-goat/sheep IgG monoclonal antibody conjugated with alkaline phosphatase (Sigma-Aldrich, USA) at a dilution of 1:10,000 as outlined in Appendix B2.3.

### 4.2.2. Construction of the BLV transfer vectors

#### 4.2.2.1. Insertion site, maker gene and promoters

An insertion site of the BLV expression cassette in the LSDV was determined based on the following criteria. Firstly, an insertion site should be genetically stable such that BLV *env* and *gag* genes and *egfp* gene would not be deleted and/or mutated. Secondly, an insertion site was selected so as not to disturb promoter regions and expression of adjacent genes.

Homologous sequences to the LSDV genome at the site of insertion (left and right flanks or flanking sequences) were included at the 5' and 3' ends of the BLV expression cassette to facilitate HR and correct insertion of the BLV expression cassette at a given viral genome.

An *egfp* gene was selected as a marker gene and obtained from Ruzaiq Omar's pBEFV_K1L_eGFP transfer vector.

Three heterologous poxvirus promoters were selected to control the expression of the BLV *env*, *gag* and *egfp* genes.

#### 4.2.2.2. Modification and optimisation of the sequences of the BLV expression cassette

Two modifications were made in the BLV *env* gene of a BLV natural isolate obtained in Section 4.2.1.1. The signal sequence of the BLV *env* gene (see Section 4.2.1.2) was replaced with the

signal sequence of the human tPA and the subtilisin/kesin-like convertase cleavage sequence (RVRR) located between BLV Env gp51 and gp30 sequences was replaced with a flexible linker $(GGGGS)_2$.

Another modification was the introduction of *Mfe*I and *Eco*RI restriction sites to allow the removal of the BLV *gag* gene from the BLV expression cassette (see Section 4.2.2.5).

Lastly, the *env* and *gag* nucleotide sequences of the BLV natural isolate selected in Section 4.2.1.1 were codon-optimised for the use in cattle prior to their inclusion into the pBLV-Env-Gag transfer vector.

#### 4.2.2.3. Preparation of the pBLV-Env-Gag transfer vector plasmid DNA

The pBLV-Env-Gag transfer vector was manufactured by GenScript and received as lyophilised plasmid DNA. The lyophilised pBLV-Env-Gag plasmid DNA was suspended in 20µl of high-performance liquid chromatography (HPLC) grade water (Merck, USA) and the solution was heated in a heating block at 50°C for 15 minutes to completely dissolve the plasmid DNA. The plasmid DNA concentration was adjusted to 2ng/µl using HPLC grade water prior to the transformation of chemically competent *E. coli* cells. One microliter of the plasmid DNA (2ng) was used for the transformation of *E. cloni* 10G chemically competent cells (Lucigen, USA). See Appendix B1.4 for the detailed method of transformation. Expression mixes from the transformation were plated on LB agar (Appendix A) containing 50µg/ml carbenicillin (Thermo Fisher Scientific, USA) and incubated at 37°C overnight. Two single bacterial colonies were picked from the LB-agar plates for medium-scale plasmid DNA isolation, which was conducted using ZymoPURE II Plasmid Midiprep Kit (Zymo Research, USA) as per the manufacturer's instructions. Concentration and purity of the extracted plasmids were measured using Nanodrop®1000 (Thermo Fisher Scientific, USA). The extracted plasmids were stored at -20°C until needed.

#### 4.2.2.4. Confirmation of the integrity of the pBLV-Env-Gag transfer vector

Restriction endonuclease mapping was used to make a preliminary confirmation of the integrity of the pBLV-Env-Gag transfer vector plasmid. Approximately, 280ng to 355ng of the pBLV-Env-Gag plasmids were digested with restriction endonuclease FastDigest *Nco*I (Thermo Fisher Scientific, USA) as described in Appendix B1.1 and subject to ethidium-bromide (0.5µg/ml) stained 0.8% agarose gel electrophoresis in 1xTBE at 100V for 1 hour. Final confirmation was made by Sanger sequencing using sequencing primers outlined in Table 4.1 (also see Table S4.4 and Figure S4.1).

**Table 4.1: Primers used to sequence the pBLV-Env-Gag plasmid vector**

| Primer | Orientation | Primer sequence | Binding site |
|---|---|---|---|
| M13F | Forward | 5'- GTTTTCCCAGTCACGAC - 3' | Lac Z |
| 49 fwd | Forward | 5'- TGGAAACGATGCATGTGCG - 3' | 49 flank |
| Env fwd 6 | Forward | 5'- TCCTTTAATACCACACAG - 3' | Env gp51 |
| Env fwd 7 | Forward | 5'- CCTGGAATTGGGATCTGG - 3' | Env gp30 |
| Gag fwd 1 | Forward | 5'- GACTTCACCGATCTGAAG - 3' | Gag |
| eGFP rev 5 | Reverse | 5'- GCGGATCTTGAAGTTCAC - 3' | eGFP |
| M13R | Reverse | 5'- CAGGAAACAGCTATGAC - 3' | Lac Z |

### 4.2.2.5. Construction of the pBVL-Env transfer vector

To construct the pBLV-Env transfer vector, the pBLV-Env-Gag transfer vector plasmid was enzymatically manipulated to remove the mH5 promoter and BLV *gag* gene. One microgram of the pBLV-Env-Gag plasmid was digested with restriction endonucleases *Mfe*I and *Eco*RI (New England Biolab, USA) as described in Appendix B1.1 and a 5,995bp fragment containing the vector backbone, the 49 flank, mFP promoter, BLV *env*, p7.5 promoter, eGFP gene and 50 flank was gel purified using the Zymoclean Gel DNA Recovery Kit (Zymo Research, USA) as per the manufacturer's protocol. Self-circularisation of the linearised DNA was performed using two ligation reaction mixtures (both in 20µl). The first reaction mixture contained 1µl (approx. 871.5ng) of the linearised plasmid DNA, 1µl of Quick T4 DNA Ligase (New England BioLabs, USA), 2µl of 10x T4 Ligase Reaction Buffer (New England BioLabs, USA) and HPLC water as per the manufacturer's recommendation. The second reaction mixture contained 5µl (approx. 4.35µg) of the linearised plasmid DNA (five times more plasmid DNA than the manufacturer's recommendation), 1µl of T4 DNA Ligase (New England BioLabs, USA), 2µl of 10x T4 Ligase Reaction Buffer (New England BioLabs, USA) and HPLC water. A reaction mixture without T4 DNA Ligase was included as a negative control. The reaction mixtures were then incubated at room temperature (20°C to 25°C) as described in Appendix B1.3.

Aliquots of 1.5µl of the ligation mixtures were used for the transformation of *E.cloni* 10G chemically competent cells (Lucigen, USA) (Appendix B1.4). Expression mixtures from the transformation were plated on LB agar (Appendix A) containing 100µg/ml ampicillin (VWR Life Science, USA) and incubated at 37°C overnight. Sixteen single colonies were picked from the plates for small scale plasmid DNA isolation (Appendix B1.5). The plasmid DNA was then digested with restriction endonucleases FastDigest *Hind*III and FastDigest *Sal*I (Thermo Fisher Scientific, USA), as described in Appendix B1.1 and subject to ethidium-bromide (0.5µg/ml) stained 0.8% agarose gel electrophoresis in 1xTBE at 100V for 1 hour. One millilitre of bacteria culture containing the correct *E. coli* recombinant (identified from the restriction endonuclease mapping) was used to prepare a 1.5ml starter culture for large scale plasmid

DNA isolation (see Appendix B1.6). Preliminary confirmation of the correct plasmid construct was made by restriction endonuclease mapping of the plasmid DNA and final confirmation by Sanger sequencing using M13F and M13R universal primers, 49 fwd, Env fwd 6 and Env fwd 7 primers (Table 4.1).

### 4.2.3. Construction of recombinant LDSV-BLV-Env-Gag and LSDV-BLV-Env

#### 4.2.3.1. Source of an adherent cell line

Primary foetal bovine testes (FBT) adherent cells were used for the initial transfection of the transfer vectors and infection of the LSDV to generate the recombinant LSDV and Madin-Darby bovine kidney (MDBK) adherent cells were used for further purification of the recombinant LSDV.

Primary FBT adherent cells were obtained from Ruzaiq Omar (a member of our research group) at the University of Cape Town, who prepared the cells from foetal bovine testes that had been obtained from the Paarl Abattoir in Cape Town, South Africa.

An MDBK adherent cell line (NBL-1) (CCL-22™) was obtained from the American Type Culture Collection (ATCC, USA).

For the detailed methods of recovery of cell lines from deep-frozen stocks, maintenance of cell lines and cell counting, see Section 3.2.4. As a minor modification, the primary FBT cells were specifically treated with cDMEM containing a 0.1% antifungal agent (10mg/ml) (InvivoGen, USA) (Appendix A).

#### 4.2.3.2. Source of parental LSDV viruses

Two recombinant Neethling lumpy skin disease virus (nLSDV) vectors generated by Henry Munyanduki were used in this study [441]; nLSDVSODis-M was used for the construction of LSDV-BLV-Env-Gag; and nLSDVSODis-UCT (see Section 3.2.3) was used for the construction of LSDV-BLV-Env. The nLSDVSODis-M has the same sequence as the nLSDVSODis-UCT except that it contains an *Escherichia coli* xanthine phosphoribosyltransferase (*Ecogpt*) selection gene and *mCherry* red fluorescent protein marker gene upstream and downstream of the *SODis* ORF, respectively at the *SODis* locus.

### 4.2.3.3. Generation of the recombinant LSDV-BLV-Env-Gag and LSDV-BLV-Env in primary foetal bovine testes (FBT) cells

To generate recombinant LSDV expressing the BLV Env and Gag antigens, a 12-well tissue culture plate was seeded with 1ml of primary FBT cells at a concentration of $2.0x10^5$ cells/ml and incubated overnight under the standard growth conditions. A 70% confluent monolayer of the cells was infected with the nLSDVSODis-M ($1.0x10^6$ $TCID_{50}$/ml) at an MOI of 0.01 or 0.1 and incubated for 2 hours under the standard growth conditions. Following a 2-hour incubation, the culture media was aspirated from wells and the cells were washed with DMEM (Thermo Fisher Scientific, USA). Then, the cells were transfected with a transfection mixture containing 500µl of DMEM (Thermo Fisher Scientific, USA), 2µl of X-tremeGene (Roche, Switzerland) and 6µg of the pBLV-Env-Gag transfer vector plasmid DNA. One experimental control well (cells only) of uninfected and untransfected cells, two infection control wells (nLSDVSODis-M only) where cells were infected with nLSDVSODis-M at an MOI of 0.1 or 0.01, and one plasmid control well (pBLV-Env-Gag only) of the transfected cells only were also prepared (Figure 4.2). eGFP and mCherry expressions were monitored daily using an epifluorescent inverted microscope (Zeiss, Germany). Figure 4.3 outlines a procedure used to generate the recombinant LSDV-BLV-Env-Gag in primary FBT cells.

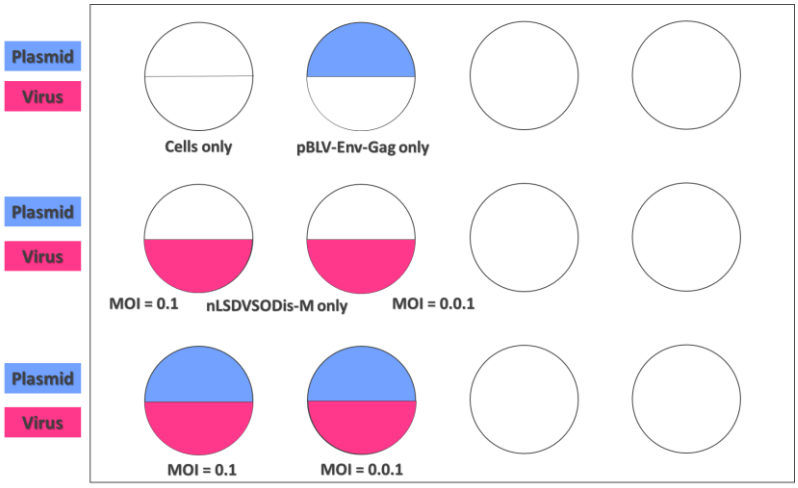



**Figure 4.2: Layout of a 12-well plate for the infection and transfection of primary FBT cells with nLSDVSODis-M and pBLV-Env-Gag transfer vector plasmid DNA to generate recombinant LSDV-BLV-Env-Gag.** The schematic diagram shows two experimental wells and four control wells. In experimental wells, primary FBT cells were transfected with the pBLV-Env-Gag transfer vector plasmid DNA and infected with the nLSDVSODis-M at an MOI of 0.1 or 0.01. In a control well (Cells only), cells were not infected or transfected. In a transfection control well (pBLV-Env-Gag only), cells were only transfected with the pBLV-Env-Gag transfer vector plasmid. For infection controls, cells were only infected with the nLSDVSODis-M at an MOI of 0.1 or 0.01. Wells where cells were transfected with the pBLV-Env-Gag transfer vector plasmid DNA are shown in blue semicircles, those infected with the nLSDVSODis-M are shown in pink semicircles and uninfected and/or non-transfected wells are shown in white semicircles.

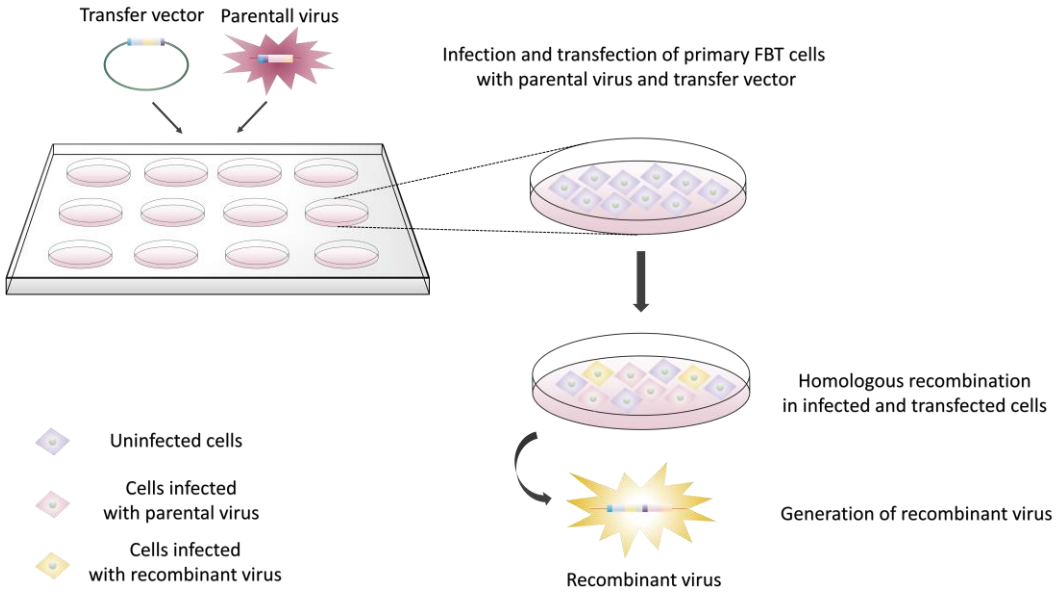



**Figure 4.3: Schematic outline of the generation of recombinant viruses in primary FBT cells.** The primary FBT cells were infected with the parental virus and transfected with the transfer vector. Following the incubation of the cells at several days, recombinant viruses resulted from the HR between the transfer vector and parental virus are detected as green and red fluorescing cells under the microscope whereas parental viruses are detected as red fluorescing cells.

A similar method was used to construct recombinant LSDV expressing the BLV Env antigen only with the following minor modifications. A 12-well tissue culture plate was seeded with 1ml of primary FBT cells at a concentration of $1.0x10^5$ cells/ml and incubated overnight under standard growth conditions. Then, 70% confluent monolayer cells were infected with nLSDVSODis-UCT ($1.0x10^{7.25}$ $TCID_{50}$/ml) at a MOI of 0.01, 0.05, 0.1 or 0.5 (Figure 4.4). Following a 2-hour incubation, transfection of the nLSDV-infected cells was performed using a transfection mixture containing 500µl of DMEM (Thermo Fisher Scientific, USA), 2µl of X-tremeGene (Roche, Switzerland) and 6µg of the pBLV-Env transfer vector plasmid DNA as described above. Seven control wells were also prepared. In an experimental control well (cells only), cells were not infected or transfected. In four infection control wells, cells were only infected with nLSDVSODis-UCT at an MOI of 0.01, 0.05, 0.1 or 0.5. In two plasmid control wells, cells were transfected with 6µg of the pBLV-Env transfer and 2µl of X-tremeGene (Roche, Switzerland), or 3µg of the pBLV-Env transfer and 1µl of X-tremeGene. eGFP expression was monitored daily using an epifluorescent inverted microscope (Zeiss, Germany).

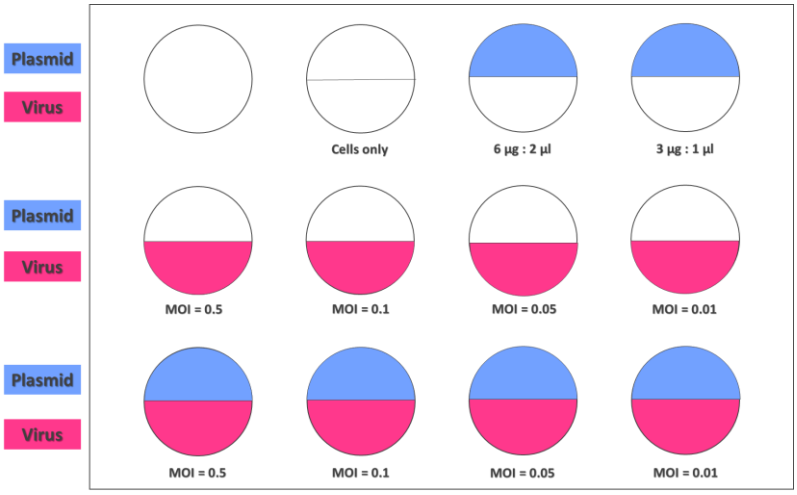



**Figure 4.4: Layout of a 12-well plate for the infection and transfection of primary FBT cells with nLSDVSODis-UCT and pBLV-Env transfer vector plasmid DNA to generate recombinant LSDV-BLV-Env.** The schematic diagram shows four experimental wells and seven control wells. In experimental wells, primary FBT cells were transfected with the pBLV-Env transfer vector plasmid DNA and infected with the nLSDVSODis-UCT at an MOI of 0.5, 0.1, 0.05 or 0.01. In a control well (Cells only), cells were not infected or transfected. In transfection control wells, cells were only transfected with 6µg or 3µg of the pBLV-Env transfer vector plasmid. In four infection control wells, cells were only infected with the nLSDVSODis-UCT at an MOI of 0.5, 0.1, 0.05 or 0.01. Wells where cells were transfected with the pBLV-Env transfer vector plasmid DNA are shown in blue semicircles, those infected with the nLSDVSODis-UCT are shown in pink semicircles and uninfected and/or non-transfected wells are shown in white semicircles.

### 4.2.4. Purification of the recombinant LSDV-BLV-Env-Gag and LSDV-BLV-Env in MDBK cells

Purification of the recombinant LSDV-BLV-Env-Gag and LSDV-BLV-Env was conducted by sequential passages of progeny viruses in MDBK cells. To release the virus from the cells, the plate containing the infected and transfected primary FBT cells were subjected to three cycles of freezing at - 80°C and thawing at 37°C. The crude cell lysate was then aspirated from the plates and transferred into 1.5ml Eppendorf tubes and either stored at -20°C until needed or vortexed briefly prior to the infection of MDBK cells.

One to two hundred microliters of the cell lysates were used to infect MDBK cells at a concentration of $2.0x10^5$ cells/ml in 12-well or 24-well tissue culture plates. The plates were incubated under standard cell growth conditions for several days to allow the virus to propagate. The infected MDBK cells were monitored daily using an epifluorescent inverted microscope (Zeiss, Germany) until mature foci were detected.

For the next round of infection (passage 2), either cell lysates or picked green fluorescent foci were used. To isolate an individual focus, culture media was aspirated from wells and cells were washed 3 to 5 times with 500µl to 1ml of 1x PBS (Thermo Fisher Scientific, USA). Then, the position of the green fluorescent focus was marked underneath the plate and a P10 pipette

tip was used to transfer the focus into a 1.5ml Eppendorf tube containing 50µl or 100µl of DMEM (Thermo Fisher Scientific, USA), which was then, frozen and thawed thrice at - 80°C and 37°C, respectively as described above to release the virus from the cells. Prior to the infection of MDBK cells, viral suspensions of picked foci were vortexed and centrifuged briefly at 12,100g. A volume of 5µl to 50µl of the viral suspensions of picked foci or 5µl to 200µl of cell lysates were used to infect MDBK cells ($2.0x10^5$ cells/ml) in 12-well or 24-well tissue culture plates.

Several more rounds of infection of MDBK cells with recombinant progeny viruses and propagation and isolation of recombinant viruses were performed using 6-well, 12-well, 24-well or 96-well tissue culture plates with different amounts and dilutions ($10^{-1}$ to $10^{-9}$ dilutions that varied depending on each passage and tissue culture plate used) of cell lysate or viral suspension of picked foci. Figure 4.5 outlines a general procedure used to passage the recombinant LSDV-BLV-Env-Gag in the MDBK cells using cell lysate or picked foci.

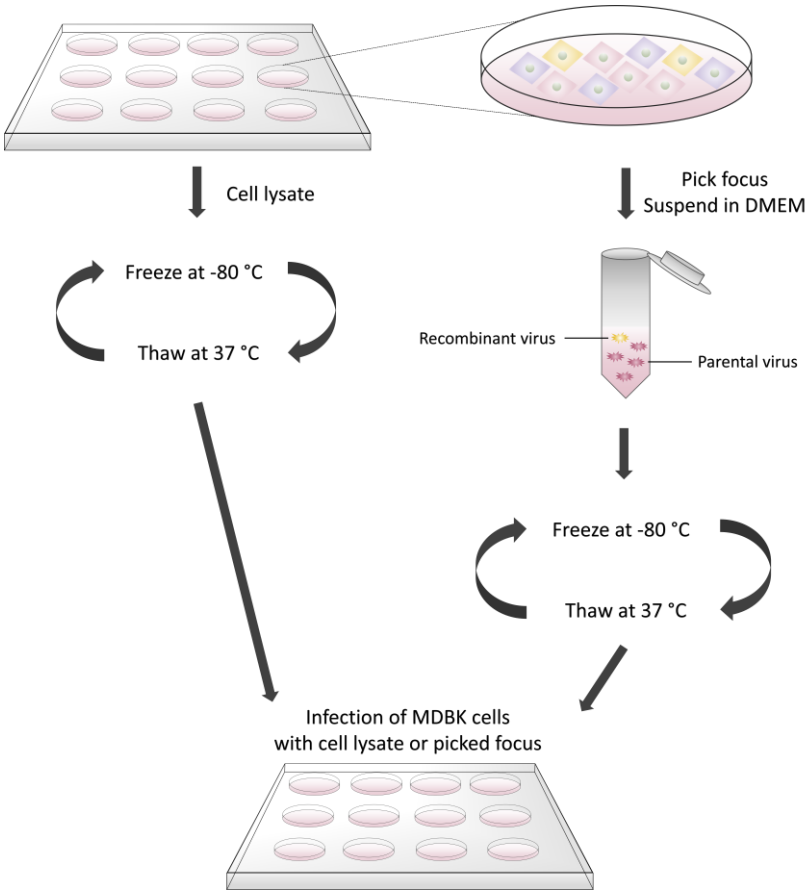



**Figure 4.5: Schematic outline of the purification of recombinant viruses by serial passages in MDBK cells.** To purify recombinant viruses, serial passages of the progeny viruses were performed by infecting the MDBK cells with cell lysate or picked foci. To release the viruses and infect the cells with the progeny viruses, the infected cells were subjected to three cycles of freezing at - 80°C and thawing at 37°C.

### 4.2.5. Characterisation of the recombinant LSDV-BLV-Env-Gag

#### 4.2.5.1. Confirmation of the presence of BLV *env* and *gag* genes by polymerase chain reaction (PCR)

##### 4.2.5.1.1. Confirmation of the presence of BLV env and gag genes in recombinant LSDV-BLV-Env-Gag by PCR

To determine whether the recombinant LSDV-BLV-Env-Gag contained the BLV *env* and *gag* genes at the correct insertion site and whether an intermediate recombinant containing the entire transfer vector or the final recombinant only containing the BLV *env* and *gag* genes and *egfp* gene had been obtained, PCR was performed at various passages using various sets of PCR primers (Table 4.2). See Appendix B3.1 for the detailed method of extraction of total DNA from infected MDBK cells and Appendix B3.3 for the preparation of PCR reaction mixtures.

The presence of a final recombinant or an intermediate recombinant was assessed using primer sets 1 and 2 (or 2B). The primer set 1 consisted of a primer that bound upstream of the 49 flank of the transfer vector in the *049* ORF of LSDV (57 fwd) and a BLV env-specific primer (gp51 rev). The primer set 2 or 2B consisted of a primer specific to *egfp* (eGFP fwd 2 or 3 fwd) and one which bound downstream of the 50 flank of the transfer vector in the *050* ORF of LSDV (94 rev). Primer set 3 (57 fwd and 94 rev) was used to determine the presence of the correct, final LSDV-BLV-Env-Gag recombinant, and to determine whether the final LSDV-BLV-Env-Gag stock contained the parental virus.

To determine whether the final stock of the LSDV-BLV-Env-Gag contained an intermediate recombinant virus containing the entire transfer vector, two sets of primers were used. A primer set 4 was used to determine whether the viral stock contained an intermediate LSDV-BLV-Env-Gag whereby recombination had only occurred in the *049* ORF. Primer set 5 was utilised to determine the presence or absence of an intermediate recombinant virus whereby recombination had only occurred in the *050* ORF.

#### 4.2.5.2. Confirmation of the BLV Env and Gag protein expression by Western blotting

To confirm the expression of the BLV Env and Gag proteins, Western blotting was performed. Total crude proteins were extracted from MDBK cells infected with 100µl of a viral stock of the recombinant LSDV-BLV-Env-Gag at 72 hours post-infection. At 24 hours, 48 hours and 72 hours post-infection, 500 µl of the culture media were transferred into 1.5ml Eppendorf tubes, and crude cell lysates were extracted as outlined in Appendix B2.1. Total proteins in the culture media and cell lysates were resolved by SDS-PAGE as described in Appendix B2.2 and

Western blotting was performed as outlined in Appendix B2.3, using mouse anti-BLV Env gp51 monoclonal antibody (Veterinary Medical Research and Development, USA) at a dilution of 1:5,000 and anti-BLV Gag p24 monoclonal antibody (Veterinary Medical Research and Development, USA) at a dilution of 1:5,000. Goat anti-mouse IgG H and L polyclonal antibody conjugated with alkaline phosphatase (Abcam, UK) at a dilution of 1:10,000 was used for the detection of BLV Env protein and the same secondary antibody at a dilution of 1:5,000 was used to detect BLV Gag.

**Table 4.2: Primer sets used to confirm the presence of the recombinant LSDV-BLV-Env-Gag**

| Set | Primer[a] | Primer sequence | Binding site | PCR thermocycling condition[b] | Passage number | Detection of target virus and product size |
|---|---|---|---|---|---|---|
| 1 | 57 fwd | 5'- GAGTGAAGCCTGGAACAT -3' | *049* ORF (upstream of 49 flank) | 30 x {94°C (30 sec), 55°C (30 sec), 68°C (1 min)}, 68°C (5 min)* | 7 | Final and intermediate recombinant virus<br>744bp |
| | | | | 35 x {95°C (15 sec), 54°C (15 sec), 72°C (15 sec)}, 72°C (1 min) † | 14 | |
| | gp51 rev 1 | 5'-TCGATGGAGATGCTGAAC -3' | BLV *env* gene | 35 x {95°C (15 sec), 54°C (15 sec), 72°C (1 min)}, 72°C (1 min) | 18 | |
| 2 | eGFP fwd 2 | 5'- AACCACTACCTGAGCACC -3' | *egfp* gene | 30 x {94°C (30 sec), 52°C (30 sec), 68°C (1 min)}, 68°C (5 min)* | 7 | Final and intermediate recombinant virus<br>937bp |
| | 94 rev | 5'- ATCTGGAAACTATGTGGC -3' | *050* ORF (upstream of 50 flank) | 30 x {95°C (15 sec), 54°C (15 sec), 72°C (15 sec)}, 72°C (1 min) † | 14 | |
| 2B | 3 fwd | 5'- GACGAGCTGTACAAGTAA -3' | *egfp* gene | 30 x {95°C (15 sec), 55°C (15 sec), 72°C (1 min)}, 72°C (1 min) † | 18 | Final and intermediate recombinant virus<br>834bp |
| | 94 rev | 5'- ATCTGGAAACTATGTGGC -3' | *050* ORF (upstream of 50 flank) | | | |
| 3 | 57 fwd | 5'- GAGTGAAGCCTGGAACAT -3' | *049* ORF (upstream of 49 flank) | 30 x {94°C (30 sec), 52°C (30 sec), 68°C (5 min)}, 68°C (5 min)* | 7 | Final recombinant virus and/or parental virus<br>5,084bp and/or 1,340bp |
| | | | | 35 x {95°C (15 sec), 50°C (15 sec), 72°C (3 min)}, 72°C (5 min) † | 14 | |
| | 94 rev | 5'- ATCTGGAAACTATGTGGC -3' | *050* ORF ORF (upstream of 50 flank) | 35 x {95°C (15 sec), 54°C (15 sec), 72°C (5 min)}, 72°C (7 min) † | 18 | |
| 4 | Lacz F | 5'- ATTGGAGATCGGTACTTCGC - 3' | *lacZ* within pUC57 region of transfer vector backbone | 30 x {95°C (15 sec), 60°C (15 sec), 72°C (1.5 min)}, 72°C (1.5 min) † | 17,18 | Intermediate recombinant virus (HR in *049* ORF only)<br>1,420bp |
| | 50R | 5'- GCATCTGGAAACTATGTGGC - 3' | *050* ORF (upstream of 50 flank) | | | |
| 5 | 57 fwd | 5'- GAGTGAAGCCTGGAACAT -3' | *049* ORF (upstream of 49 flank) | 30 x {95°C (15 sec), 54°C (15 sec), 72°C (1 min)}, 72°C (1 min)† | 17,18 | Intermediate recombinant virus (HR into *050* ORF only)<br>1,051bp |
| | M13 R2 | 5'- CAGGAAACAGCTATGACC -3' | M13 primer binding site within pUC57 region of the transfer vector backbone | | | |

a, fwd and F denote forward primers whereas rev and R denote reverse primers. b, the number of cycles, thermocycling conditions for denaturation, annealing, extension per cycle and final extension are shown. Note that thermocycling conditions with an asterisk (*) are specific to OneTaq DNA polymerase (New England BioLabs, USA) and those with dagger symbol (†) are specific to KAPA 2G HotStart DNA polymerase (KAPA BIOSYSTEMS, USA). PCR with OneTaq DNA polymerase initiated with denaturation at 94°C for 30 seconds and PCR with KAPA 2G HotStart DNA polymerase initiated with denaturation at 95°C for 3 minutes.

### 4.2.6. Characterisation of the recombinant LSDV-BLV-Env

#### 4.2.6.1. PCR screening of the presence of the possible final recombinant LSDV-BLV-Env

To perform PCR screening of the recombinant LSDV-BLV-Env at passage 15, the MDBK cells at a concentration of $2x10^5$ cells/ml were infected with 10µl of the cell lysate and incubated under the standard growth conditions until as many foci as possible were obtained. Once enough mature foci were obtained, all foci were picked and an individual focus was used to infect each well of a 24-well tissue culture plate which was seeded with the MDBK cells at a concentration of $2x10^5$ cells/ml. The plate was incubated under standard growth conditions for several days. When enough foci were obtained, total DNA was extracted as outlined in Appendix B3.2. PCR reaction mixtures were prepared as described in Appendix B3.3. PCR was performed using three sets of PCR primers (primer set 1, 2B and 3) (Table 4.3).

#### 4.2.6.2. Confirmation of the presence of the BLV *env* gene of the recombinant LSDV-BLV-Env by PCR

To determine whether the recombinant LSDV-BLV-Env contained the BLV *env* gene at the correct insertion site and whether an intermediate recombinant containing the entire transfer vector or the final recombinant only containing the BLV *env* gene and *egfp* gene had been obtained, PCR was performed at various passages using various sets of PCR primers (Table 4.3).

To determine whether the final stock of the LSDV-BLV-Env contained an intermediate recombinant virus containing the entire transfer vector, primer sets 4 and 5 (Table 4.2) and the same PCR conditions outlined in Section 4.2.5.1.1. Confirmation of the presence of BLV env and gag genes in recombinant LSDV-BLV-Env-Gag by PCR were used.

#### 4.2.6.3. Confirmation of the integrity of the recombinant LSDV-BLV-Env by Sanger sequencing

To confirm genetic integrity of the BLV *env* gene of the recombinant LSDV-BLV-Env, Sanger sequencing of a 2,244bp fragment containing a 260bp end of the 49 flank, an entire BLV *env* gene and a 365bp region of the beginning of the *egfp* gene was performed. Total crude DNA was extracted from MDBK cells infected with the recombinant LSDV-BLV-Env (see Appendix B3.1). To amplify a 2,244 bp fragment of the LSDV-BLV-Env, 49 fwd and eGFP rev 7 primers (Table 4.4) were utilised.

**Table 4.3: Primer sets used to confirm the presence of the recombinant LSDV-BLV-Env**

| Set | Primer[a] | Primer sequence | Binding site | PCR thermocycling condition[b] | Passage number | Detection of target virus and product size |
|---|---|---|---|---|---|---|
| 1 | 57 fwd<br>gp51 rev 1 | 5'- GAGTGAAGCCTGGAACAT -3'<br>5'-TCGATGGAGATGCTGAAC -3' | *049* ORF (upstream of 49 flank)<br>BLV *env* gene | 30 x {94°C (30 sec), 49°C (30 sec), 68°C (1 min)}, 68°C (5 min)* | 9, 13, 15, 17, 18 | Final and intermediate recombinant virus<br>744bp |
| 2A | eGFP F3<br>50 R | 5'- CAACCACTACCTGAGCACC -3'<br>5'- GCATCTGGAAACTATGTGGC - 3' | *egfp* gene<br>*050* ORF (upstream of 50 flank) | 30 x {95°C (15 sec), 60°C (15 sec), 72°C (1 min)}, 72°C (1 min) † | 9 | Final and intermediate recombinant virus<br>945bp |
| 2B | 3 fwd<br>94 rev | 5'- GACGAGCTGTACAAGTAA -3'<br>5'- ATCTGGAAACTATGTGGC -3' | *egfp* gene<br>*050* ORF (upstream of 50 flank) | 30 x {95°C (15 sec), 57°C (15 sec), 72°C (1 min)}, 72°C (1 min) † | 13, 15 | Final and intermediate recombinant virus<br>834bp |
| 2C | 3 fwd<br>39 rev | 5'- GACGAGCTGTACAAGTAA -3'<br>5'- GATGGTGTGCAAATAAATCATC -3' | *egfp* gene<br>*050* ORF (upstream of 50 flank) | 30 x {94°C (30 sec), 46°C (30 sec), 68°C (1 min)}, 68°C (5 min)* | 17,18 | Final and intermediate recombinant virus<br>720bp |
| 3 | 57 fwd<br>94 rev | 5'- GAGTGAAGCCTGGAACAT -3'<br>5'- ATCTGGAAACTATGTGGC -3' | *049* ORF (upstream of 49 flank)<br>*050* ORF ORF (upstream of 50 flank) | 35 x {94°C (30 sec), 47°C (30 sec), 68°C (4 min)}, 68°C (5 min)*<br>35 x {94°C (30 sec), 49°C (30 sec), 68°C (4 min)}, 68°C (6 min)* | 9<br>13, 15 | Final recombinant virus and/or parental virus<br>3,799bp and/or 1,340bp |
| 3B | 57 fwd<br>39 rev | 5'- GAGTGAAGCCTGGAACAT -3'<br>5'- GATGGTGTGCAAATAAATCATC -3' | *049* ORF (upstream of 49 flank)<br>*050* ORF ORF (upstream of 50 flank) | 35 x {94°C (30 sec), 47°C (30 sec), 68°C (4 min)}, 68°C (5 min)* | 17, 18 | Final recombinant virus and/or parental virus<br>3,537bp and/or 1,078bp |

a, fwd denotes forward primers whereas rev denotes reverse primers. b, the number of cycles, thermocycling conditions for denaturation, annealing, extension per cycle and final extension are shown. Note that thermocycling conditions with an asterisk (*) are specific to OneTaq DNA polymerase (New England BioLabs, USA) and those with dagger symbol (†) are specific to KAPA 2G HotStart DNA polymerase (KAPA BIOSYSTEMS, USA). PCR with OneTaq DNA polymerase initiated with denaturation at 94°C for 30 seconds and PCR with KAPA 2G HotStart DNA polymerase initiated with denaturation at 95°C for 3 minutes.

Sanger sequencing of the 2,244bp fragment of the LSDV-BLV-Env was conducted using sequencing primers outlined in Table 4.4 by Central Analytical Facilities at the University of Stellenbosch (South Africa).

**Table 4.4: Primers used to amplify and sequence a 2,244bp fragment of the LSDV-BLV-Env genome**

| Primers | Orientation | Binding site | Purposes |
|---|---|---|---|
| 49 fwd | Forward | *049* ORF | Amplification of a 2,244bp fragment |
| eGFP rev 7 | Reverse | *egfp* | |
| Env rev 1 | Reverse | Env gp51 | Sequencing of the 2,244bp PCR product |
| Env fwd 2 | Forward | Env gp51 | |
| Env fwd 6 | Forward | Env gp51 | |
| Env fwd 7 | Forward | Env gp30 | |

### 4.2.6.4. Confirmation of *in vitro* expression of the BLV Env protein by the recombinant LSDV-BLV-Env using immunofluorescence assay (IFA)

To confirm the *in vitro* expression of the BLV Env protein expressed by the recombinant LSDV-BLV-Env, an immunofluorescence assay (IFA) was conducted by live-cell staining of the MDBK cells infected with the recombinant LSDV-BLV-Env using the mouse anti-Env monoclonal antibody (anti-Env mAb) as outlined in Appendix B4. As a technical control for a donkey-anti-mouse IgG conjugated with Cy3 (anti-mouse IgG-Cy3), uninfected cells, cells infected with the parental nLSDVSODis-UCT virus and those infected with the recombinant LSDV-BLV-Env were incubated with anti-mouse IgG-Cy3 only. As an experimental control, uninfected cells and cells infected with the parental nLSDVSODis-UCT virus were incubated with anti-Env mAb at a dilution of 1:50 or 1:100 and anti-mouse IgG-Cy3 at a dilution of 1:5,000. Figure 4.6 outlines the layout of a 24-well plate used to perform the IFA.

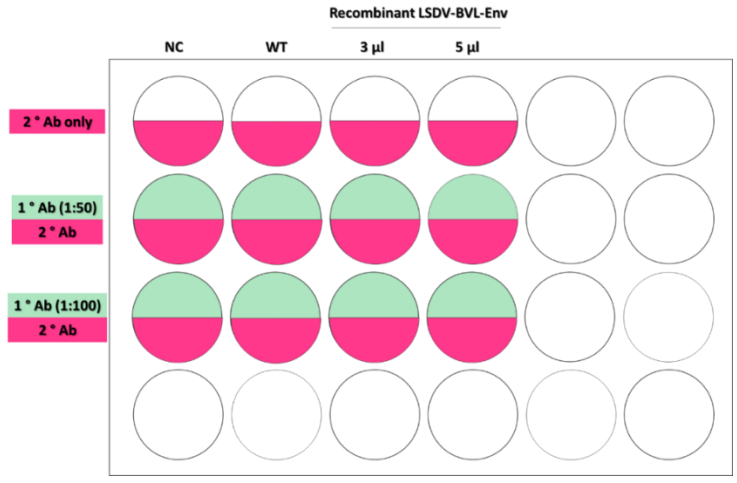



**Figure 4.6: Layout of a 24-well plate used for immunofluorescence assay of BLV Env expression from recombinant LSDV-BLV-Env.** MDBK cells were either not infected (NC; in the first column of the plate), infected with the parental LSDV virus (WT; the second column) or infected with 3µl or 5µl of the passage 16 viral stock of the recombinant LSDV-BLV-Env (fourth and fifth columns, respectively) Wells where cells were immunostained with the anti-Env mAb (1° Ab) are indicated by green semicircles and those immunostained with anti-mouse IgG-Cy3 (2° Ab) are indicated by pink semicircles.

## 4.3. RESULTS

### 4.3.1. Design of BLV transfer vectors

#### 4.3.1.1. Selection of BLV *env* and *gag* sequences

To design the BLV *env* and *gag* sequences of the BLV expression cassette, BLV *env* and *gag* sequences of a BLV natural isolate were selected as follows. Firstly, the nucleotide and amino acid sequences of consensus *env* and gag sequences (Cons-Env and Cons-Gag, respectively) were obtained by the alignment of BLV *env* and *gag* sequences of 62 BLV natural isolates representing six genotypes available in GenBank nucleotide database. Seven candidate BLV natural isolates whose BLV *env* and *gag* nucleotide and amino acid sequences were closest to those of the Cons-Env and Cons-Gag were selected from the BLAST database (Table 4.5), and their amino acid sequences were aligned back to those of the Cons-Env and Cons-Gag for the final confirmation. Consequently, a BLV natural isolate pvAK006 (GenBank accession number AP018021) was selected as its *env* and *gag* nucleotide sequences shared 99.5% and 99.8% identity, respectively, with those of the Cons-Env and Cons-Gag and they shared 100% identity of the Env and Gag amino acid sequences. This process is described in Figure 4.7 below.

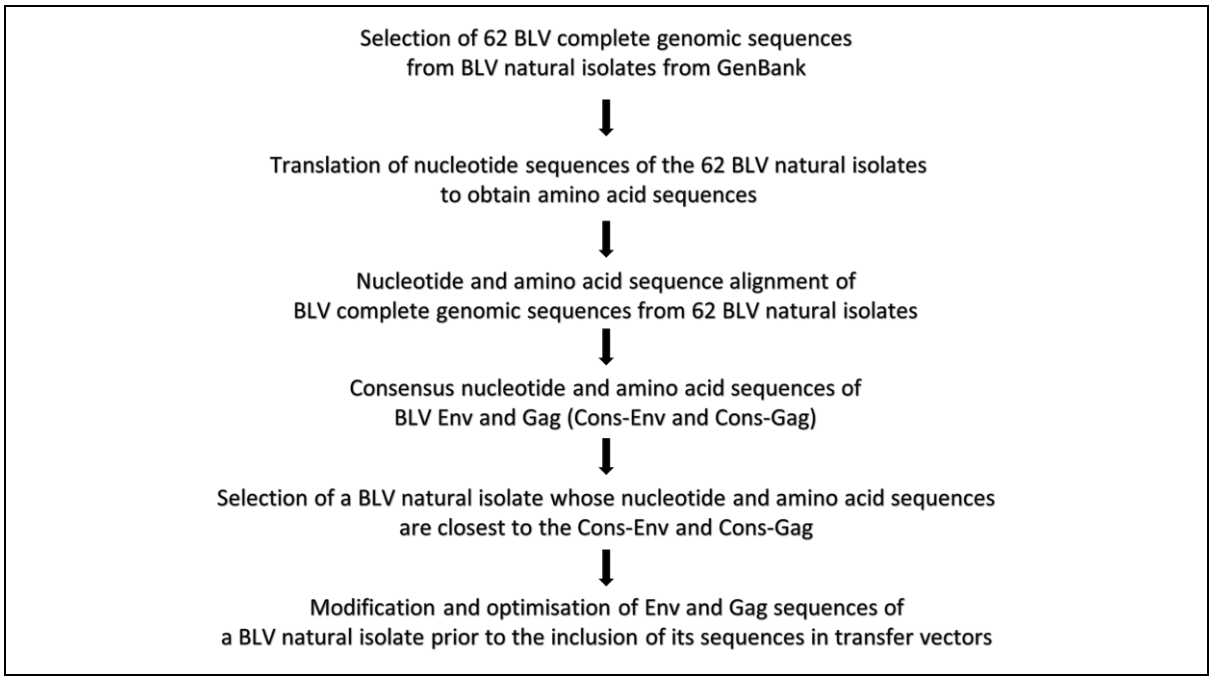



**Figure 4.7: Flow diagram outlining the selection of BLV Env and Gag amino acid sequences used to make LSDV recombinants.**

**Table 4.5: Summary of BLAST and EMBOSS results of the nucleotide and amino acid sequence alignment using the Cons-Env and Cons-Gag as a query sequence**

| | | Cons-Env | | Cons-Gag | | |
|---|---|---|---|---|---|---|
| GenBank accession number | Protein ID | Nucleotide sequence | Amino acid sequence | Nucleotide sequence | Amino acid sequence | Strain |
| LC164083.1 | BAV17954.1 | 99.8% (1545/1548bp) | Identity: 99.8% (514/515 aa)<br>Similarity: 100% (515/515 aa) | 99.8% (1180/1182bp) | Identity: 100% (389/392 aa) | Sublcone pBLV-FLK |
| AP018021.1 | BAX04183.1 | 99.5% (1541/1548bp) | Identity: 100% (515/515 aa)<br>Similarity: 100% (515/515 aa) | 99.8% (1180/1182bp) | Identity: 100% (393/393 aa) | pvAK006 |
| LC164085.1 | BAV17970.1 | 99.4% (1540/1548bp) | Identity: 99.4% (512/515 aa)<br>Similarity: 99.6% (513/515 aa) | 99.6% (1178/1182bp) | Identity: 99.7% (392/393 aa)<br>Similarity: 100% (393/393 aa) | pvAF967 |
| AP018032.1 | BAX04282.1 | 99. 8% (1546/1548bp) | Identity: 99.8% (514/515 aa)<br>Similarity: 100% (515/515 aa) | 99.6% (1178/1182bp) | Identity: 99.2% (390/393 aa)<br>Similarity: 99.5% (391/393 aa) | pvAN015 |
| AP018023.1 | BAX04201.1 | 99.4% (1540/1548bp) | Identity: 99.8% (514/515 aa)<br>Similarity 100% (515/515 aa) | 99.5% (1177/1182bp) | Identity: 99.7% (392/393 aa)<br>Similarity: 100% (393/393 aa) | pvAK011 |
| AP018029.1 | BAX04255.1 | 99. 6% (1542/1548bp) | Identity: 99.6% (513/515 aa)<br>Similarity: 99.8% (514/515 aa) | 99.4% (1176/1182bp) | Identity: 99.7% (392/393 aa)<br>Similarity: 100% (393/393 aa) | pvAN011 |
| AP018009.1 | BAX04075.1 | 99.4% (1540/1548bp) | Identity: 99.2% (511/515 aa)<br>Similarity: 99.8% (514/515 aa) | 99.4% (1176/1182bp) | Identity: 99.7% (392/393 aa)<br>Similarity: 100% (393/393 aa) | pvAF245 |

For the BLSAT results of the nucleotide sequences, sequence identities are shown. The number of nucleotides (bp) and amino acid (aa) matches between two sequences are shown in brackets. Each strain identifier (e.g., pvAN015, pvAK006) corresponds to a cow number (cow AN015 and AK009) from which the BLV was isolated.

#### 4.3.1.2. Assessment of functionality of the signal sequence of the human tissue plasminogen activator in bovine cells

Enhancement of antigen presentation on the cell membrane is important in the induction and improvement of immune responses in vaccines. One strategy that has been widely utilised in various expression systems [516-518] and vaccine modalities [519-523] is the modification of the signal sequence (also called signal peptide or leader sequence) of secretory and membrane proteins. Various studies have shown that the optimisation of the native signal sequence, particularly when the native signal sequence does not export proteins efficiently [524, 525], or the replacement of the native signal sequence with a heterologous signal sequence such as human tissue plasminogen activator (tPA) lead to efficient protein export and expression as well as enhancement of immunogenicity [517, 518, 522, 523, 526-529]. In our lab, the signal sequence of the human tPA has been utilised in our protein [530] and recombinant MVA-vectored [531] HIV-1 vaccines. However, the use of this signal sequence in bovine cells had not been tested. Therefore, the functionality of the human tPA signal sequence in bovine cells was assessed prior to its inclusion in the recombinant LSDV vaccines.

##### 4.3.1.2.1. Amino acid sequence comparison of the human and bovine tPA signal sequences

To determine whether the signal sequence of the BLV *env* gene could be replaced with the signal sequence of the human tPA, amino acid sequences of the human and bovine tPA signal sequences were compared.

Both the human and bovine tPA signal sequences are 21 amino acids in length and share a 71.4% identity (Figure 4.8).

```
Human tPA      M D A M K R G L C C V L L L C C A V F V S
               |   | | |               | | | | | | | | | | |
Bovine tPA   M M S A M K T E F L C V L L L C C A V V T
```

**Figure 4.8: Amino acid sequence alignment of human tPA signal sequence (protein ID; P00750) and bovine tPA signal sequence (protein ID; Q29819).**

##### 4.3.1.2.2. *In vitro* activity of the human tPA signal sequence in bovine cells

It was difficult to determine from the amino acid sequence comparison of the human and bovine tPA signal sequences whether the human tPA signal sequence would be functional in bovine cells and could be used in the BLV transfer vector. Therefore, to address these questions, extracellular expression of an HIV-1 Env glycoprotein from MVA-GC5SS, which

contains the HIV *env* gene with the human tPA signal sequence, was assessed by Western blotting. Extracellular expression of the HIV1 Env glycoprotein (Figure 4.9 A) was detected from Madin-Darby bovine kidney (MDBK) cells infected with MVA-GC5SS at a MOI of 5 and its intracellular expression from the cells infected with MVA-GC5SS at MOIs of 2 and 5 (Figure 4.9 B), indicating that the glycoprotein was successfully exported from the infected cells and that the human tPA signal sequence was functional in bovine cells. It should be noted that since the soluble Env protein (E) does not contain the transmembrane region and cytoplasmic tail, it is therefore slightly smaller (140 kDa) than the Env protein (150 kDa) expressed from MVA-GC5SS.

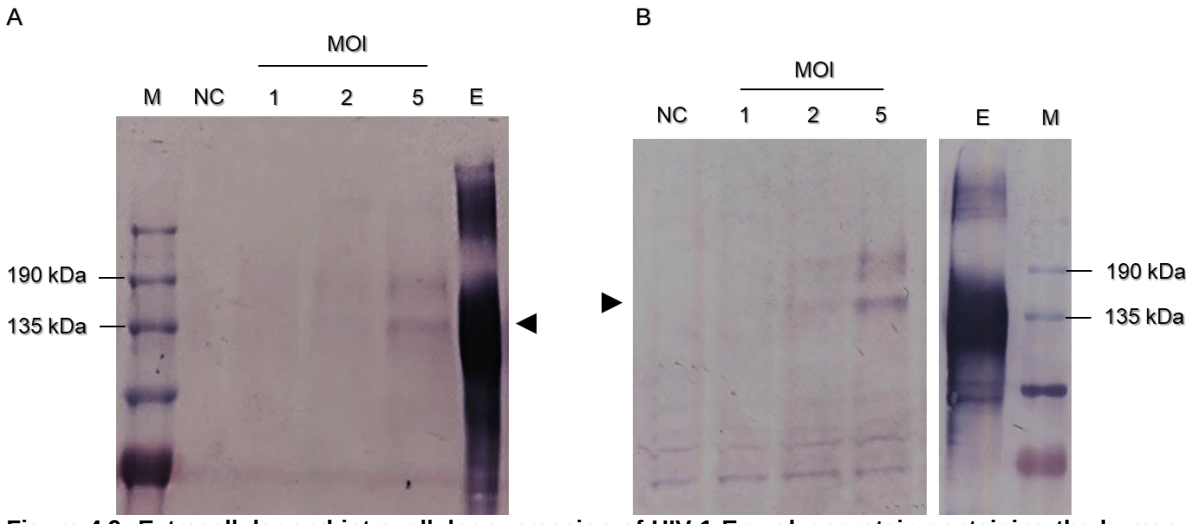



**Figure 4.9: Extracellular and intracellular expression of HIV-1 Env glycoprotein containing the human tPA signal sequence in bovine cells.** A confluent monolayer of MDBK cells at a concentration of $2.0x10^5$ cells/ml was infected with MVA-rGC5SS at an MOI of 1,2 or 5 and total protein was extracted at 72 hours post-infection from the culture media and cell lysate. The HIV-1 Env glycoprotein was detected with a goat anti-HIV-1 Env polyclonal antibody (BioRad, USA) followed by anti-goat/sheep IgG monoclonal antibody conjugated with alkaline phosphatase (Sigma-Aldrich, USA). **(A)** Extracellular expression of HIV-1 Env glycoprotein (arrowhead) extracted from the culture media from the MDBK cells infected with MVA-GC5SS at different MOIs. **(B)** Intracellular expression of HIV-1 Env glycoprotein (arrowhead) extracted from the cell from the MDBK cells infected with MVA-GC5SS at different MOIs. **M**, protein molecular weight ladder (11-245 kDa) (Thermo Fisher Scientific, USA); **E**, soluble HIV-1 Env protein; **NC**, protein extracted from uninfected cells.

### 4.3.1.3. Insertion site, marker gene and promoters

An intergenic region between open reading frames (ORFs) *049* and *050* in the central coding region of the LSDV genome (Figure 4.10) was selected as the site of insertion by Dr. Nicola Douglass based on the following reasons. Firstly, the central coding region of the LSDV genome is evolutionally conserved [532], and there is no intergenic region between ORFs *049* and *050*. Therefore, inserted genes in this region are less likely to be mutated and/or deleted. Secondly, insertion of genes in this region is unlikely to disrupt the expression of ORFs *049* and *050* as the two ORFs are convergent (i.e., one is transcribed in a sense direction and the

other in an antisense direction) and thus, promoter regions are unlikely to be disrupted by the insertion.

The same insertion site was used for both recombinant LSDV-BLV-Env-Gag and LSDV-BLV-Env, and 356bp and 440bp of the 3' ends of the *049* and *050* ORFs, respectively were included as flanking sequences (49 and 50 flanks, respectively) in the BLV transfer vectors. The 49 and 50 flanks correspond to positions 44542-44891 and 44887-45327 of the Neethling vaccine LW 1959 strain (GenBank accession number AF409138).

To differentiate the recombinant LSDV containing the BLV expression cassette from the parental viruses, an *egfp* gene was included as a marker gene.

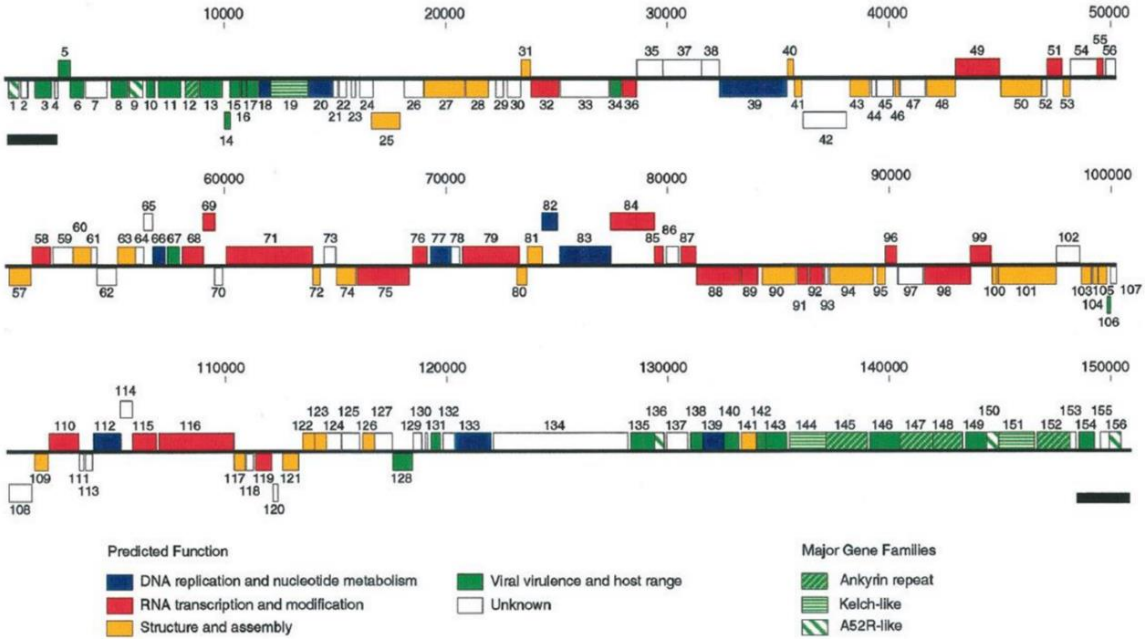



**Figure 4.10: A linear genomic map of an nLSDV NI-2490 isolate from Kenya (GenBank accession number AF325528).** LSDV genome consists of the central coding region, flanked by two identical inverted terminal repeat (ITR) regions. ITRs are shown as black bars with one below ORFs 01 and 04 and the other below ORFs 154 to 156. ORFs of LSDV genome are numbered from left to right along the genome. The ORFs on the sense strand, transcribed from left to right are shown above the horizontal line whereas those on the antisense strand, transcribed from right to left are shown below the horizontal line. Genes with similar functions are grouped and coloured in five groups and those within the same gene families are shown with one of three patterns. Taken from Tulman *et al*. (2001) [532].

The modified early fowlpox virus promoter (mFP) [438, 439], modified early-late promoter of the H5 gene of VACV (mH5) [440] and early-late promoter of the 7.5 kilodalton (kDa) polypeptide gene (p7.5) of vaccinia virus (VACV) [419] were selected to drive expression of the BLV *env*, *gag* and *egfp* genes, respectively. Since the assessment of the activity of the five poxvirus promoters including these three poxvirus promoters (see Chapter 3) was performed in parallel with this current study of the construction and characterisation of the

recombinant LSDVs, these three promoters were selected based on previous studies conducted in our lab as well as published data.

#### 4.3.1.4. Modification and optimisation of the BLV expression cassette sequences

Prior to the inclusion of the *env* and *gag* sequences of the BLV natural isolate pvAK006 (GenBank accession number AP018021.1) into the BLV expression cassette, these sequences were modified, and codon optimised for the optimal expression of the BLV *env* and *gag* genes under poxvirus gene expression systems and in cattle.

A few modifications were also made to the BLV *env* gene. The signal sequence of the BLV *env* gene was replaced with that of the human tissue plasminogen activator (tPA) for the efficient transport of the BLV Env protein to the cell membrane of infected cells and the sequence encoding the subtilisin/kesin-like convertase cleavage site (RVRR) located between the gp51 and gp30 subunits was replaced with a flexible linker sequence, $(GGGGS)_2$. This was done to avoid poor subtilisin/kesin-like convertase cleavage which could impair the structure of the protein. The linker could also possibly stabilise the gp51 and gp30 glycoprotein complex.

A Kozak sequence was introduced at translation initiation sites of the BLV *env* and *gag* sequences as well as the *egfp* sequence. Poxvirus early terminator sequences (TTTTTNT with N being any nucleotide) were included after the stop codons of all the genes, including the 49 and 50 flanks. Poxvirus termination signals were removed from all ORFs to prevent immature transcriptional termination.  shows a plasmid map of the pBLV-Env-Gag transfer vector.

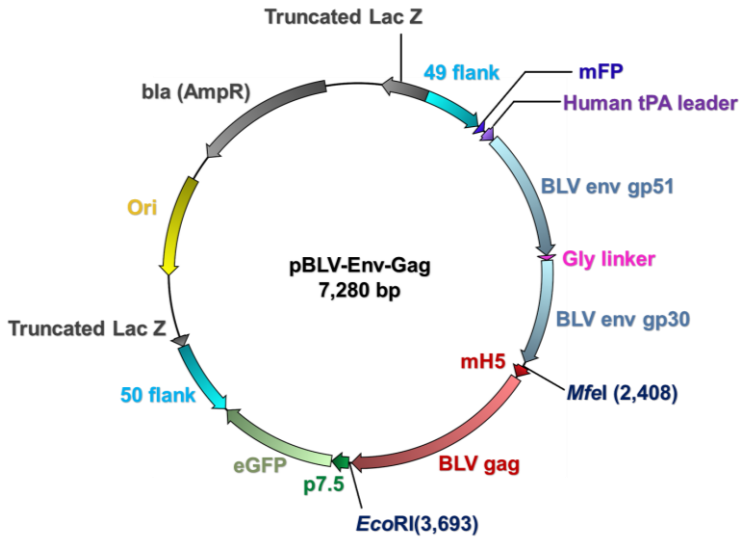



**Figure 4.11: Plasmid map of the pBLV-Env-Gag transfer vector. 49 flank**, 356bp 3' end of *049* ORF from Neethling lumpy skin disease virus; **50 flank**, 440bp 3' end of *050* ORF from Neethling lumpy skin disease virus; **mFP**, modified early fowlpox virus promoter; **mH5**, a modified promoter of the H5 gene of VACV; **p7.5**, promoter for the 7.5 kDa polypeptide gene of vaccinia virus (VACV); **BLV env**, *envelope* gene of bovine leukaemia virus (BLV); **BLV gag**, BLV *gag* gene **eGFP**, enhanced green-florescent protein gene; **tPA**, human tissue plasminogen activator signal sequence; **LacZ**, LacZ alpha fragment of beta-galactosidase gene; **bla (AmpR)**, beta-lactamase (*bla*) gene; **Ori**, ColE1 origin of replication.

### 4.2.1.5. Confirmation of the integrity of the pBLV-Env-Gag transfer vector

Prior to the enzymatic manipulation of the pBLV-Env-Gag plasmid to generate the pBLV-Env and its use to generate the recombinant LSDV-BLV-Env-Gag, the integrity of the pBLV-Env-Gag plasmid was confirmed by restriction endonuclease mapping and Sanger sequencing.

*E. coli* competent cells were transformed with the pBLV-Env-Gag plasmid from GanScript, and medium-scale plasmid DNA isolation was carried out with two of the transformants, C1 and C2. Restriction endonuclease digestion of these plasmids with *Nco*I yielded DNA fragments of the correct sizes (Figure 4.12). The C1 plasmid contained more supercoiled plasmid DNA than the C2 plasmid DNA, and therefore, the C1 plasmid was selected for the downstream experiments. The integrity of the C1 plasmid DNA was also confirmed by Sanger sequencing using sequencing primers outlined in Table 4.1.

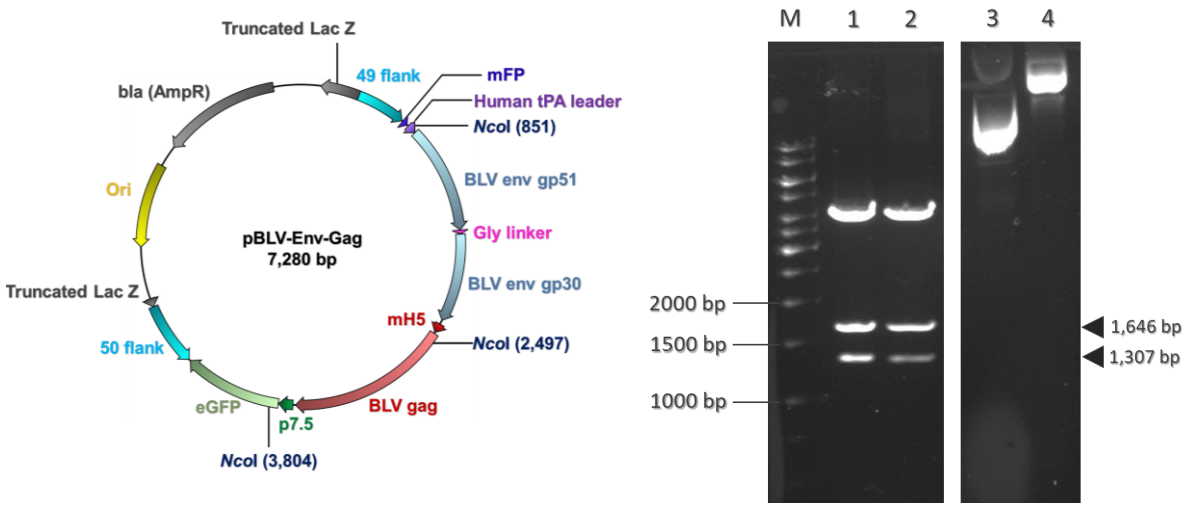



**Figure 4.12: Preliminary confirmation of the integrity of the pBLV-Env-Gag plasmids.** The pBLV-Env-Gag plasmid DNA was digested with restriction endonuclease *Nco*I and separated on 0.8% agarose TBE gel stained with ethidium bromide (0.5 µg/ml). **M**, 1 kb DNA ladder (Thermo Scientific, USA); **1**, the C1 plasmid digested with *Nco*I; **2,** the C2 plasmid digested with *Nco*I; **3**, uncut C1 plasmid; **4**, uncut C2 plasmid.

### 4.3.1.6. Construction of pBLV-Env transfer vector by enzymatic modification of the pBLV-Env-Gag plasmid

To construct the pBLV-Env transfer vector, the pBLV-Env-Gag plasmid was digested with *Mfe*I and *Eco*RI to remove the mH5 promoter and BLV *gag* gene (Figure 4.13). The integrity of the resultant plasmid was confirmed by restriction endonuclease mapping of the pBLV-Env with *Bam*HI and *Sal*I, yielding DNA fragments of the correct sizes (Figure 4.14). Final confirmation of the integrity of the pBLV-Env plasmid DNA was made by Sanger sequencing using M13F and M13R universal primers, 49 fwd, Env fwd 6 and Env fwd 7 primers (Table 4.1).

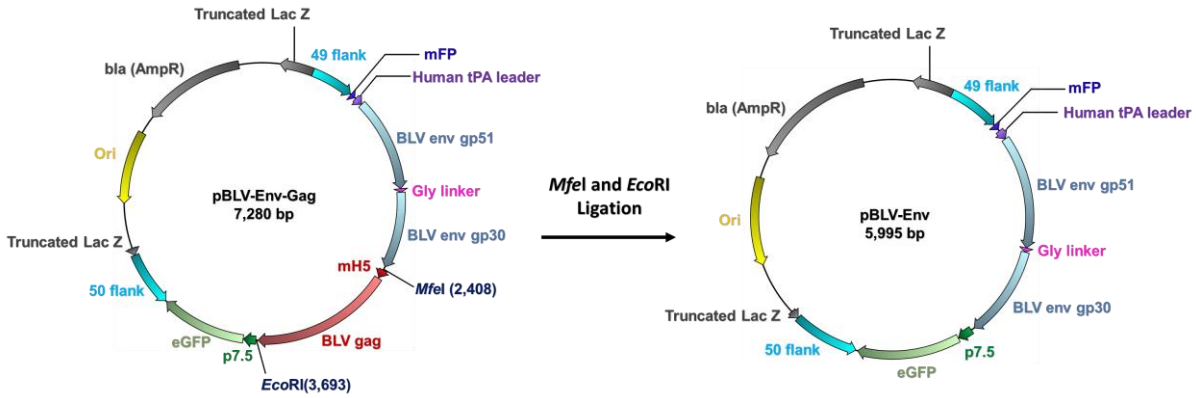



**Figure 4.13: Construction of the pBLV-Env transfer vector.** Restriction endonuclease digestion of the pBLV-Env-Gag with *Mfe*I and *Eco*RI and ligation of the vector backbone generated the pBLV-Env transfer vector.

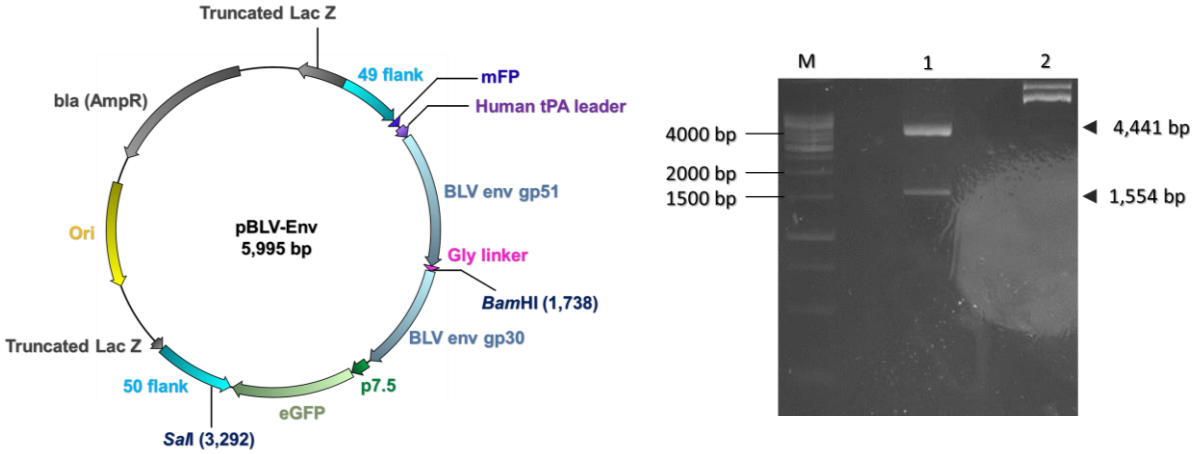



**Figure 4.14: Preliminary confirmation of the integrity of the pBLV-Env plasmid.** The pBLV-Env plasmid DNA was digested with restriction endonucleases *Bam*HI and *Sal*I and separated on 0.8% agarose TBE gel stained with ethidium bromide (0.5 µg/ml). **M**, 1 kb DNA ladder (Thermo Scientific, USA); **1**, pBLV-Env plasmid digested with *Bam*HI and *Sal*I; **2**, uncut pBLV-Env.

### 4.3.2. Construction and purification of the recombinant LSDV-BLV-Env-Gag

The recombinant LSDV harbouring the BLV *env* and *gag* genes as well as the *egfp* gene (LSDV-BLV-Env-Gag) was constructed by infecting and transfecting primary foetal bovine testes (FBT) cells with the nLSDVSODis-M and the pBLV-Env-Gag transfer vector, respectively. Although Madin-Derby bovine kidney (MDBK) cells are permissive for LSDV, transfection is inefficient in this cell line. Therefore, primary FBT cells were utilised to construct the recombinant LSDV-BLV-Env-Gag. The progeny viruses were then, isolated from the primary FBT cells and subjected to serial passages in MDBK cells to allow viral propagation and isolation. The parental nLSDVSODis-M contained a mCherry reporter gene and was

identified as red fluorescing cells by fluorescent microscopy whereas the recombinant LSDV-BLV-Env-Gag contained both red and green reporter genes and thus appeared yellow.

#### 4.3.2.1. Construction of the recombinant LSDV-BLV-Env-Gag in primary foetal bovine testes (FBT) cells

In the control wells, the primary FBT cells that were not infected or transfected (Figure 4.15 A) were slightly sparse at 48 hours post-infection but appeared to be viable enough. The primary FBT cells that were transfected only (Figure 4.15 B) appeared to be more sparse than the primary FBT cells that were not infected or transfected, due to the toxicity of the transfection reagent. Red fluorescing cells were observed from the primary FBT cells that were infected with the parental nLSDVSODis-M virus at a MOI of 0.1 (Figure 4.15 D) whereas such cells were not detected from those infected with the parental virus at an MOI of 0.01 (Figure 4.15 C) but several cells were rounded up and showed cytopathic effects (CPEs), indicating that the cells were infected by the parental virus.

At 48 hours post-infection, small green and red fluorescing cells were detected in the primary FBT cells that were transfected and infected with the transfer vector plasmid DNA and the parental nLSDVSODis-M virus at a MO of 0.1 and 0.01 (Figure 4.16). The cells were incubated further to obtain more and larger foci.

At 120 hours post-infection, in the experimental cells infected with the nLSDVSODis-M at MOIs of 0.01 and 0.1, more and larger foci were detected compared to those observed at 48 hours post-infection. Whilst some of the foci were fluorescing green and red from the entire foci (Figure 4.17 A), other foci were only partially fluorescing green (Figure 4.17 B) As a result of the extensive infection, the cells in the experimental wells had started to round up and die, indicating that further viral propagation was not possible. Therefore, at 120 hours post-infection, the cells were subjected to three cycles of freezing at -80°C and thawing at 37°C to release the viruses and cell lysates containing the released viruses were passaged in MDBK cells to purify the recombinant progeny viruses.

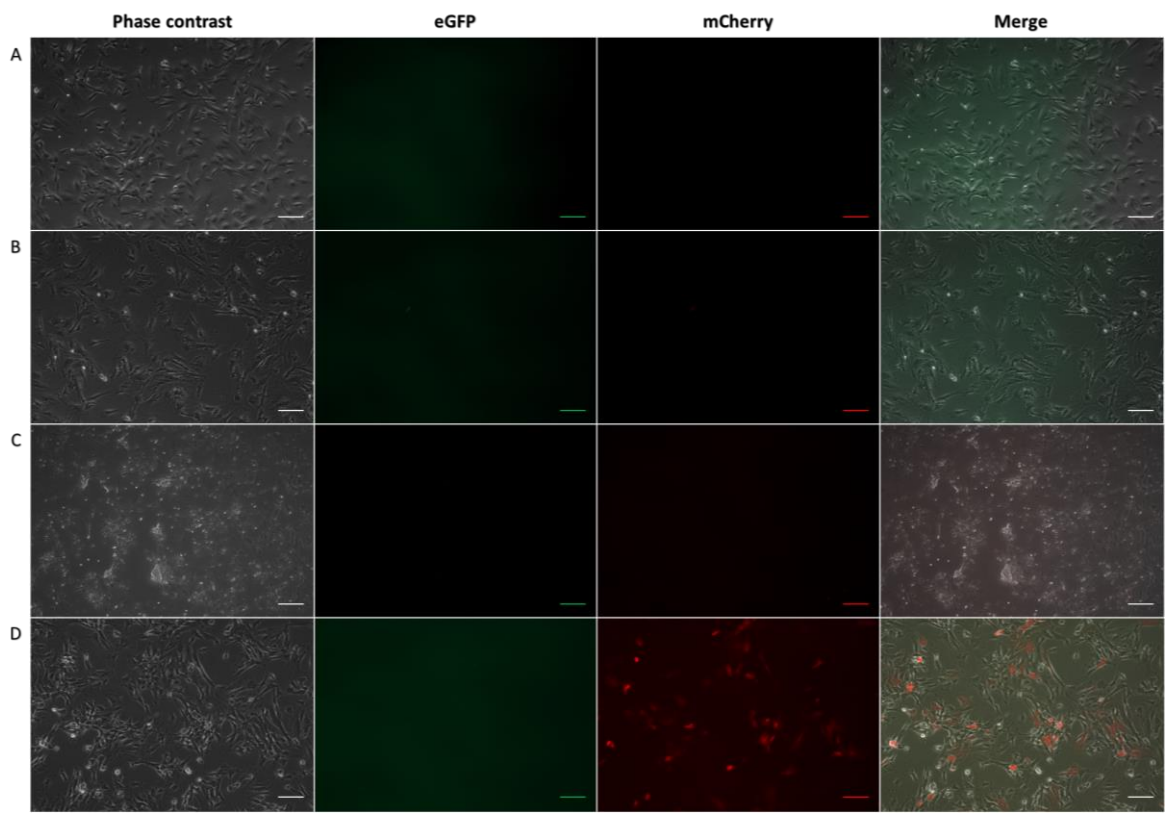



**Figure 4.15: Fluorescent images of the experimental, infection and transfection controls for the generation of recombinant LSDV-BLV-Env-Gag in primary foetal bovine testes (FBT) cells. (A) Cells only:** the primary FBT cells were not infected or transfected. (**B) Transfection control:** primary FBT cells were transfected with 6µg of the pBLV-Env-Gag plasmid DNA; **(C) Infection control 1,** primary FBT cells were infected with nLSDVSODis-M at an MOI of 0.01. **(D) Infection control 2:** primary FBT cells were infected with nLSDVSODis-M at an MOI of 0.1. Primary FBT cells infected with the parental nLSDVSODis-M were visualized by their mCherry expression (red). All images were taken at 48 hours post-infection using a Zeiss Axiovert 200M fluorescence inverted microscope (Zeiss, Germany) at 50x magnification. Scale bars represent 200µm.

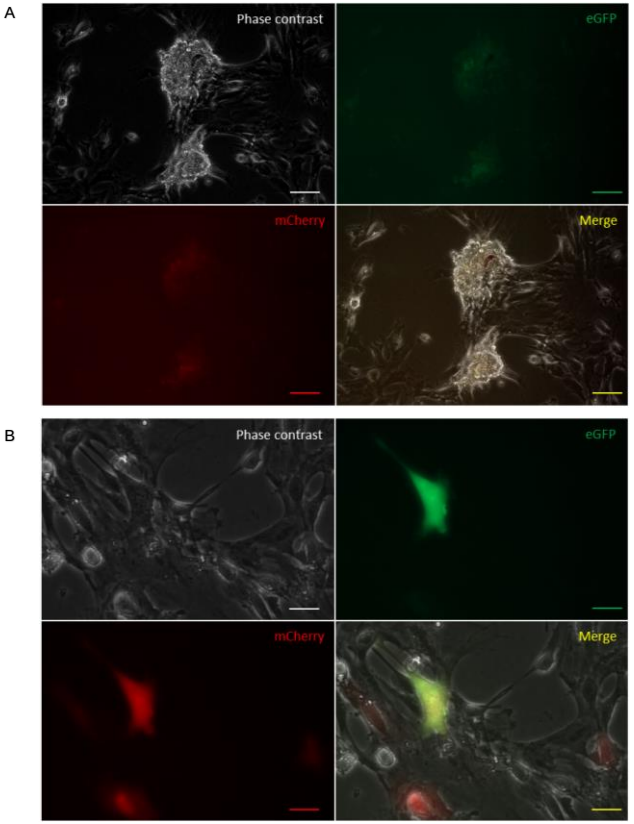



**Figure 4.16: Fluorescent images of primary FBT cells transfected with the pBLV-Env-Gag plasmid DNA and infected with the nLSDVSODis-M at 48 hours post-infection.** Cell morphology of the primary FBT cells at 48 hours post-infection that were transfected with the pBLV-Env-Gag plasmid DNA and infected with the nLSDVSODis-M at an MOI of **(A)** 0.01 and **(B)** 0.1. Primary FBT cells infected with the recombinant LSDV-BLV-Env-Gag were visualized by their eGFP (green) and mCherry expression (red). MDBK cells infected with the parental nLSDVSODis-M were visualized by their mCherry expression (red). All images were taken using a Zeiss Axiovert 200M fluorescence inverted microscope (Zeiss, Germany) at 200x magnification. Scale bars represent 50µm.

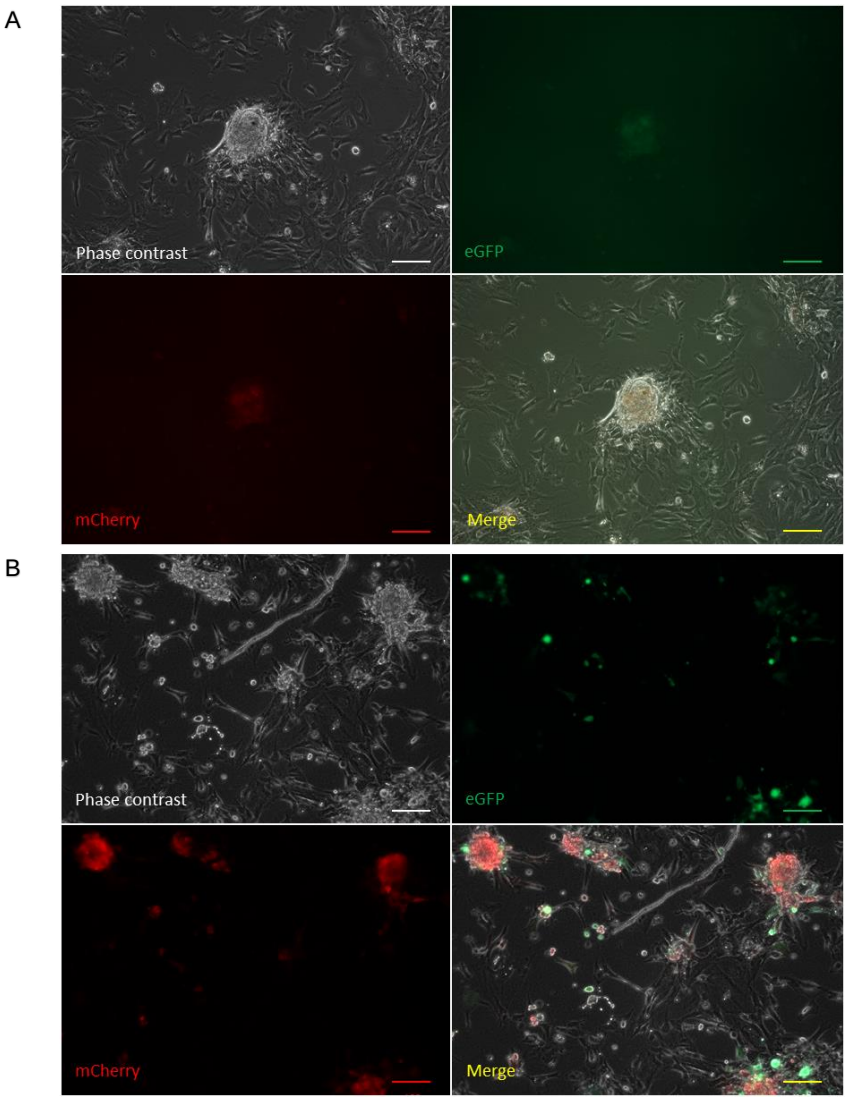



**Figure 4.17: Fluorescent images of primary FBT cells transfected with the pBLV-Env-Gag plasmid DNA and infected with the nLSDVSODis-M at 120 hours post-infection. (A)** Cell morphology of the primary FBT cells at 120 hours post-infection that were transfected with the pBLV-Env-Gag plasmid DNA and infected with the nLSDVSODis-M at an MOI of 0.01. (**B)** Cell morphology of the primary FBT cells at 120 hours post-infection that were transfected with the pBLV-Env-Gag plasmid DNA and infected with the nLSDVSODis-M at an MOI of 0.1. Primary FBT cells infected with the recombinant LSDV-BLV-Env-Gag were visualized by their eGFP (green) and mCherry expression (red). Primary FBT cells infected with the parental nLSDVSODis-M were visualized by their mCherry expression (red). All images were taken at 120 hours post-infection using a Zeiss Axiovert 200M fluorescence inverted microscope (Zeiss, Germany)) at 100x magnification. Scale bars represent 100µm.

### 4.3.2.2. Purification of the recombinant LDSV-BLV-Env-Gag by serial passages in Madin-Darby bovine kidney (MDBK) cells

To purify recombinant viruses away from parental viruses and allow them to propagate further, recombinant progeny viruses isolated in the primary FBT cells were sequentially passaged in MDBK cells. This process is to ensure that the recombinant progeny virus was a correct recombinant virus containing the BLV expression cassette between the *049* and *050* ORFs as the transfer vector may be transiently expressed in early passages. Figure 4.18 outlines tissue culture plates used at each passage and cell lysate or picked foci used for each passage.

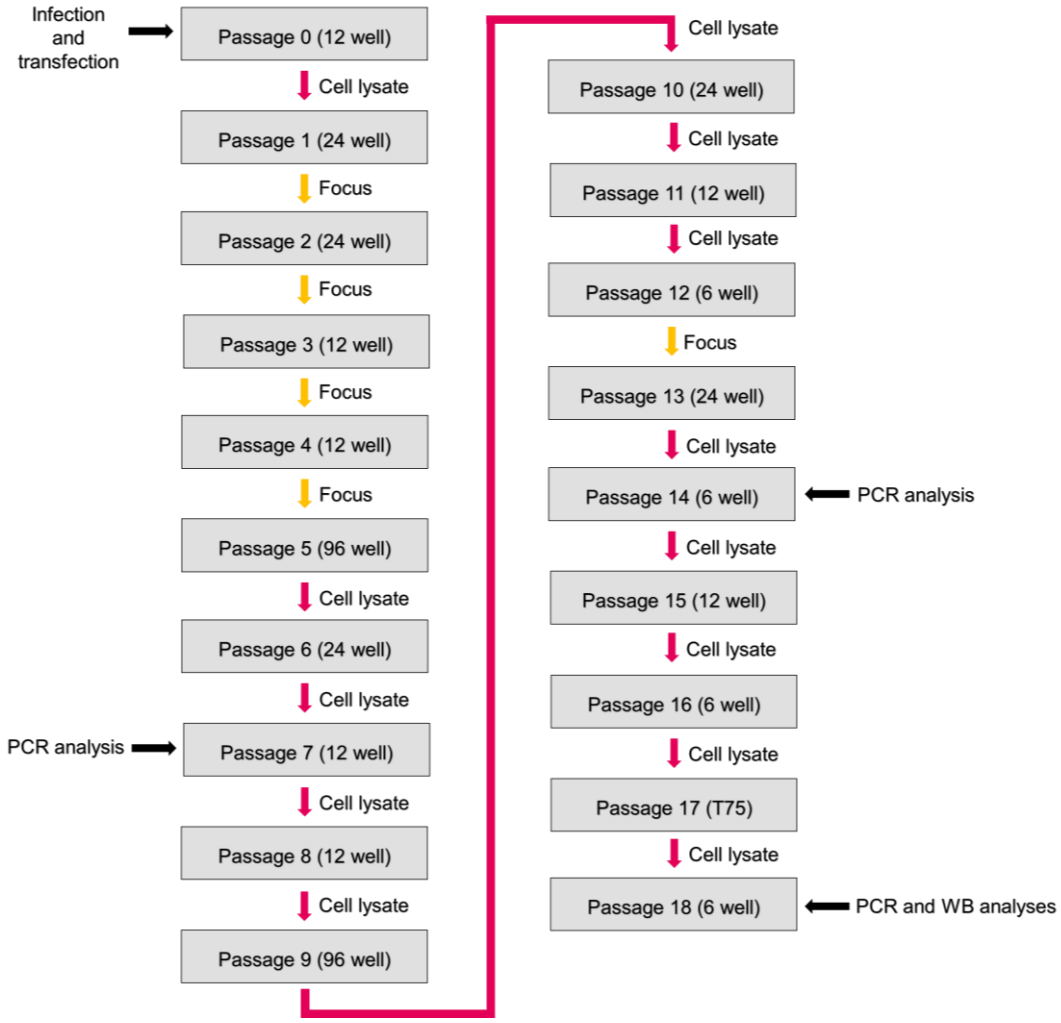



**Figure 4.18: Flow diagram outlining the purification of the recombinant LSDV-BLV-Env-Gag in MDBK cells.** The flow diagram outlines the tissue culture plates used at each passage and focus (yellow arrow) or cell lysate (pink arrow) isolated for the next passage. The viral stocks used to perform PCR and Western blotting (WB) are also indicated in this figure.

Cell lysates were isolated from the primary FBT cells and varying amounts of the cell lysates (5 to 200µl) were used to infect MDBK cells for the optimal growth and purification at passage 1. Cell lysate isolated from the transfected-primary FBT cells that were infected with the nLSDVSODis-M at an MOI of 0.01 in the initial plate did not produce any green and red fluorescing foci. In contrast, cell lysate isolated from the transfected-primary FBT cells that were infected with the nLSDVSODis-M at an MOI of 0.1 in the initial plate produced several green and red fluorescing foci at 96 hours post-infection. There were still a large number of red fluorescing foci (parental nLSDVSODis-M viruses) present. To isolate recombinant viruses away from the parental viruses, six large foci fluorescing both green and red were picked for the next passage. Of these six foci, five foci (Figure 4.19 A-E) did not grow in MDBK cells and only one focus (Figure 4.19 F) successfully grew and propagated extensively to produce 17 foci at passage 2.

From passages 2 to 5, picked foci were used to infect MDBK cells to further isolate recombinant progeny viruses out of populations of parental viruses. At passage 5, 96-well tissue culture plates were used to remove the parental viruses further and to purify recombinant progeny viruses specifically as a single focus without the presence of parental viruses in the same well. Following a 72-hour incubation, four single foci that were fluorescing both green and red were obtained from four different wells without parental viruses present in the same wells (Figure 4.20 A-D).

#### 4.3.2.2.1. PCR analysis of the presence of the recombinant LSDV-BLV-Env-Gag at passage 7

PCR was performed to determine whether the purification of the recombinant LSDV-BLV-Env-Gag was successful at passage 7 (Figure 4.21 A). Using primer sets 1 and 2, 744bp and 937bp fragments (Figure 4.21 C and D) were amplified from two samples (lanes 2 and 3) from the recombinant LSDV-BLV-Env-Gag. Since a sample in lane 4 was obtained from infected cells where only red fluorescing foci were detected, 744bp and 937bp fragments were not amplified. This result confirmed that the primer sets 1 and 2 were specific to the recombinant LSDV-BLV-Env-Gag genome.

Since 744bp and 937bp fragments were amplified from the 5'-end and 3'-end of the BLV expression cassette, their amplification indicates that the recombinant virus contained the BLV *env* and *egfp* genes at the correct insertion site, between the *049* and *050* genes of the LSDV-BLV-Env-Gag genome, but this could have resulted from a single crossover into *049* ORF only or *050* ORF only. Therefore, to determine the presence of a final recombinant virus that contained the entire BLV expression cassette in the insertion site by HR into both *049* and *050* ORFs, another PCR was performed using primer set 3. Amplification of a 5,084bp fragment covering the entire BLV expression cassette between the *049* and *050* genes of the LSDV-BLV-Env-Gag genome was not successful (Figure 4.21 E). This may have been due to the inefficient amplification of this large fragment. Using the same primer set 3, a 1,340bp fragment covering the *049* and *050* genes of the parental viral genome was amplified from recombinant LSDV-BLV-Env-Gag (Figure 4.21 E; lanes 2, 3 and 4), indicating the presence of the parental virus in the passage 7 viral stock.

It should be noted that since this was the first PCR conducted for the recombinant LSDV, primers and PCR results appeared to be suboptimal, showing faint 744bp and 937bp bands on the agarose gels. Therefore, modified versions of primers as well as different DNA polymerase and thermocycling conditions were utilised for subsequent PCR reactions.

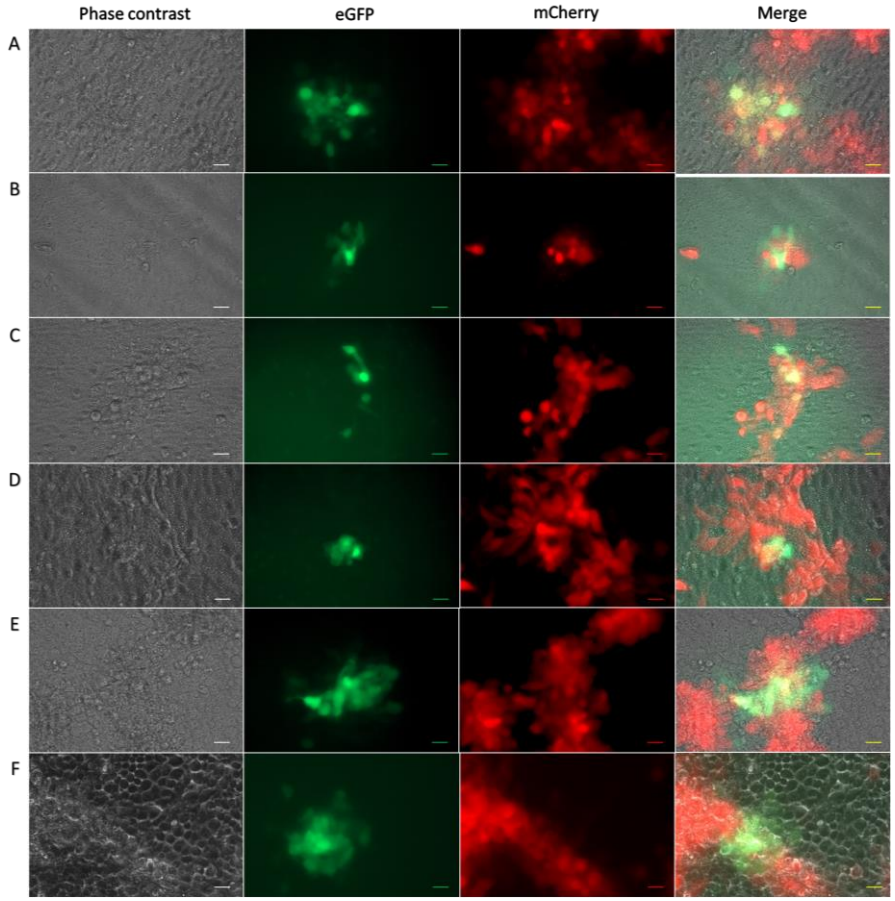



**Figure 4.19: Six green and red fluorescing foci of MDBK cells infected with putative recombinant LSDV-BLV-Env-Gag at passage 1. (A-F)** Green and red fluorescing foci isolated from a 24-well plate at passage 1. MDBK cells infected with the recombinant LSDV-BLV-Env-Gag were visualized by their eGFP (green) and mCherry expression (red). MDBK cells infected with the parental nLSDVSODis-M were visualized by their mCherry expression (red). All images were taken at 96 hours post-infection using a Zeiss Axiovert 200M fluorescence inverted microscope (Zeiss, Germany) at 400x magnification. Scale bars represent 20µm.

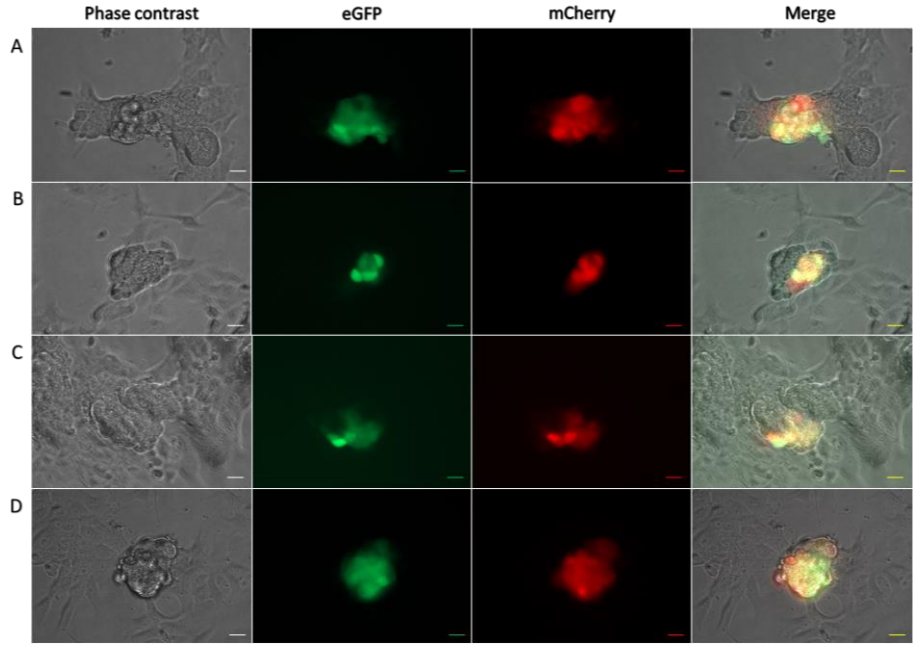



**Figure 4.20: Single green and red fluorescing foci of MDBK cells infected with recombinant LSDV-BLV-Env-Gag at passage 5.** (**A-D)** Single foci isolated from four wells of a 96-well plate at passage 5. MDBK cells infected with the recombinant LSDV-BLV-Env-Gag were visualized by their eGFP (green) and mCherry expression (red). MDBK cells infected with the parental nLSDVSODis-M were visualized by their mCherry expression (red). All images were taken at 72 hours post-infection using Zeiss Axiovert 200M fluorescent inverted microscope (Zeiss, Germany) at 400x magnification. Scale bars represent 200µm.

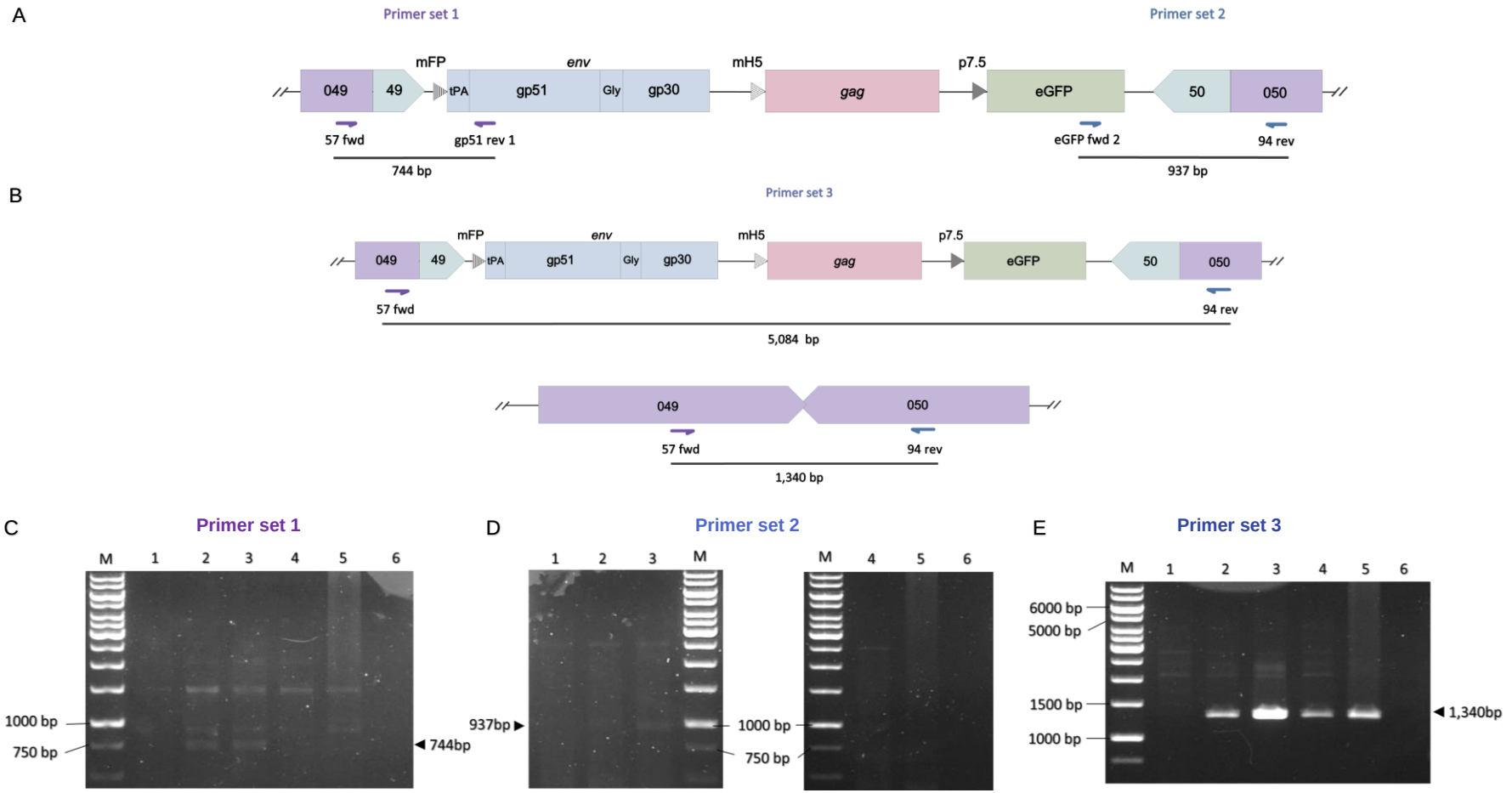



**Figure 4.21: Preliminary confirmation of the presence of the LSDV-BLV-Env-Gag at passage 7.** Schematic diagrams showing **(A)** binding sites of primer set 1 (purple arrows) and primer set 2 (blue arrows) on the LSDV-BLV-Env-Gag gnome and **(B)** binding sites of primer set 3 (purple and blue arrows) on the LSDV-BLV-Env-Gag gnome and parental nLSDVSODis-M genome. PCR products are shown as a line with expected product size. **49**, left flank; **50**, right flank; **049** and **050**, *049* and *050* ORFs of the LSDV genome; **mFP**, modified early fowlpox virus promoter; **mH5**, a modified promoter of the H5 gene of VACV; **p7.5**, promoter for the 7.5 kDa polypeptide gene of vaccinia virus (VACV); **BLV env**, *envelope* gene of bovine leukaemia virus (BLV); **BLV gag**, BLV *gag* gene; **eGFP**, green-florescent protein gene; **tPA**, human tissue plasminogen activator signal sequence. **(C)** Agarose gel showing a 744bp product amplified with primer set 1 to detect the presence of a final and intermediate recombinant LSDV-BLV-Env-Gag into *049* ORF. Agarose gel. **(D)** Agarose gel showing a 937bp product amplified with primer set 2 to detect the presence of a final and intermediate recombinant LSDV-BLV-Env-Gag into *050* ORF. **(E)** Agarose gel showing a 1,340bp product amplified with primer set 3 which detects the presence of a final recombinant LSDV-BLV-Env-Gag and/or parental nLSDVSODis-M. **M**, 1 kb DNA ladder (Thermo Scientific, USA); **1**, total DNA from uninfected cells; **2, 3 and 4**, total DNA from the passage 7 viral stock of recombinant LSDV-BLV-Env-Gag; **5**, parental viral DNA; **6**, PCR control without template DNA.

As expected from the PCR results, at passage 8, parental viruses were detected in all except one well of a tissue culture plate. Most of the parental viruses grew faster than recombinant viruses. In some wells, parental viruses outnumbered recombinant viruses. However, for unknown reasons, some parental viruses were only detected at 120 hours post-infection.

Careful monitoring of the viral growth revealed that some recombinant viruses were expressing eGFP only partially (Figure 4.22 A) and some of them appeared to have lost their eGFP expression almost entirely (Figure 4.22 B). These recombinant viruses could be intermediate recombinant viruses containing the entire transfer vector as a result of a single recombination event into *049* ORF or *050* ORF only. A second recombination event could then have resulted in the loss of the entire transfer vector and expression cassette, resulting in the regeneration of the parental virus.

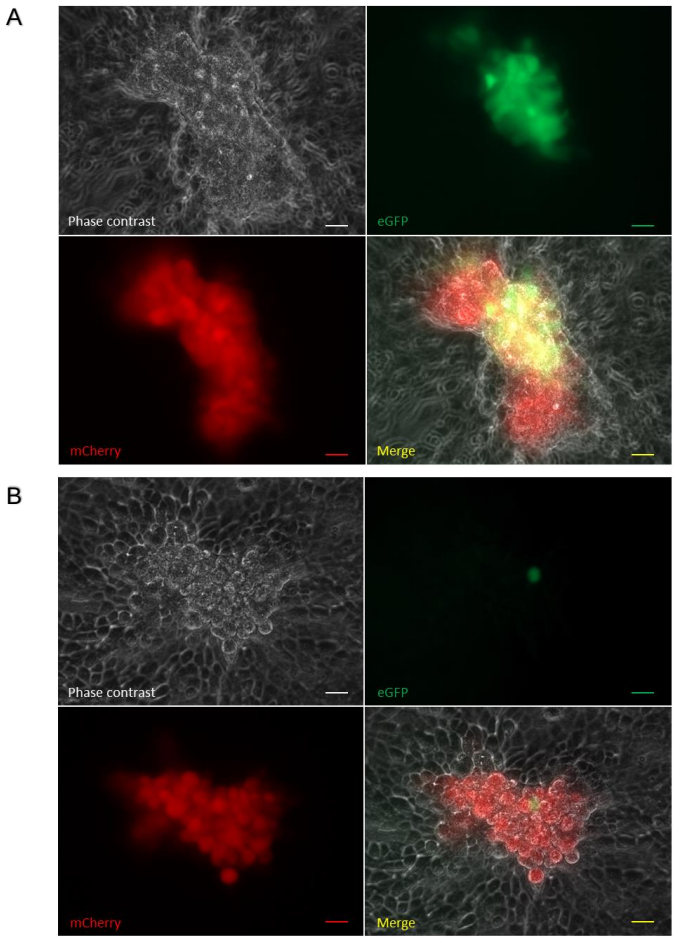



**Figure 4.22: Green and red fluorescing foci of MDBK cells infected with recombinant LSDV-BLV-Env-Gag that partially or almost entirely lost eGFP expression at passage 8. (A)** A representative image showing a recombinant virus showing the partial expression of eGFP. **(B)** A representative image showing recombinant virus that appeared to have lost its eGFP expression almost entirely. MDBK cells infected with the recombinant LSDV-BLV-Env-Gag were visualized by their eGFP (green) and mCherry expression (red). MDBK cells infected with the parental nLSDVSODis-M were visualized by their mCherry expression (red). All images were taken at 120 hours post-infection using Zeiss Axiovert 200M fluorescent inverted microscope (Zeiss, Germany) at 400x magnification. Scale bars represent 20µm.

#### 4.3.2.2.2. PCR analysis of the presence of the recombinant LSDV-BLV-Env-Gag at passage 14

Parental and recombinant viruses expressing partial eGFP expression persisted from passage 9 through to passage 11. Therefore, at passage 12, foci were once again picked from a 12-well tissue culture plate to specifically isolate recombinant viruses and to remove as many non-recombinant viruses as possible. Consequently, the number of parental viruses remained low up to passage 15. Since the presence of a possible final recombinant LSDV-BLV-Env-Gag had not been confirmed at passage 7, PCR was once again performed at passage 14 using the same primer sets 1, 2 and 3 but using different DNA polymerase and PCR conditions (Figure 4.23 A and B). Amplification of a 744bp fragment and no amplification of a 937bp fragment indicate the presence of a possible intermediate recombinant LSDV-BLV-Env-Gag into the *049* ORF only (Figure 4.23 C and D), though the latter result might have been due to the inefficient PCR using primer set 2. Using primer set 3, once again the presence of parental virus was confirmed by amplification of a 1,340bp fragment whereas a 5,084bp fragment indicating the presence of the final recombinant LSDV-BLV-Env-Gag was not amplified (Figure 4.23 E).

#### 4.3.2.2.3. Characterisation of the BLV Env and Gag in the recombinant LSDV-BLV-Env-Gag in the final viral stock

At passage 16, partial eGFP expression was still detected in foci. This recombinant virus was passaged twice to scale up the viral stock for downstream analyses of PCR (Section 4.3.2.2.3.1) and Western blotting (Section 4.3.2.2.3.2).

##### 4.3.2.2.3.1. Confirmation of the presence of the BLV *env* and *gag* genes in recombinant LSDV-BLV-Env-Gag at passage 18

Using primer sets 1 and 2B, 744bp and 843bp fragments (Figure 4.24 C and D) were amplified, indicating the presence of a possible final or intermediate recombinant LSDV-BLV-Env-Gag whereby HR occurred into *049* ORF only (primer set 1) and *050* ORF only (primer set 2B) in the passage 18 viral stock. Once again, using primer set 3, only a 1,340bp fragment was amplified from the recombinant LSDV-BLV-Env-Gag, indicating the presence of the parental virus (Figure 4.24 E).

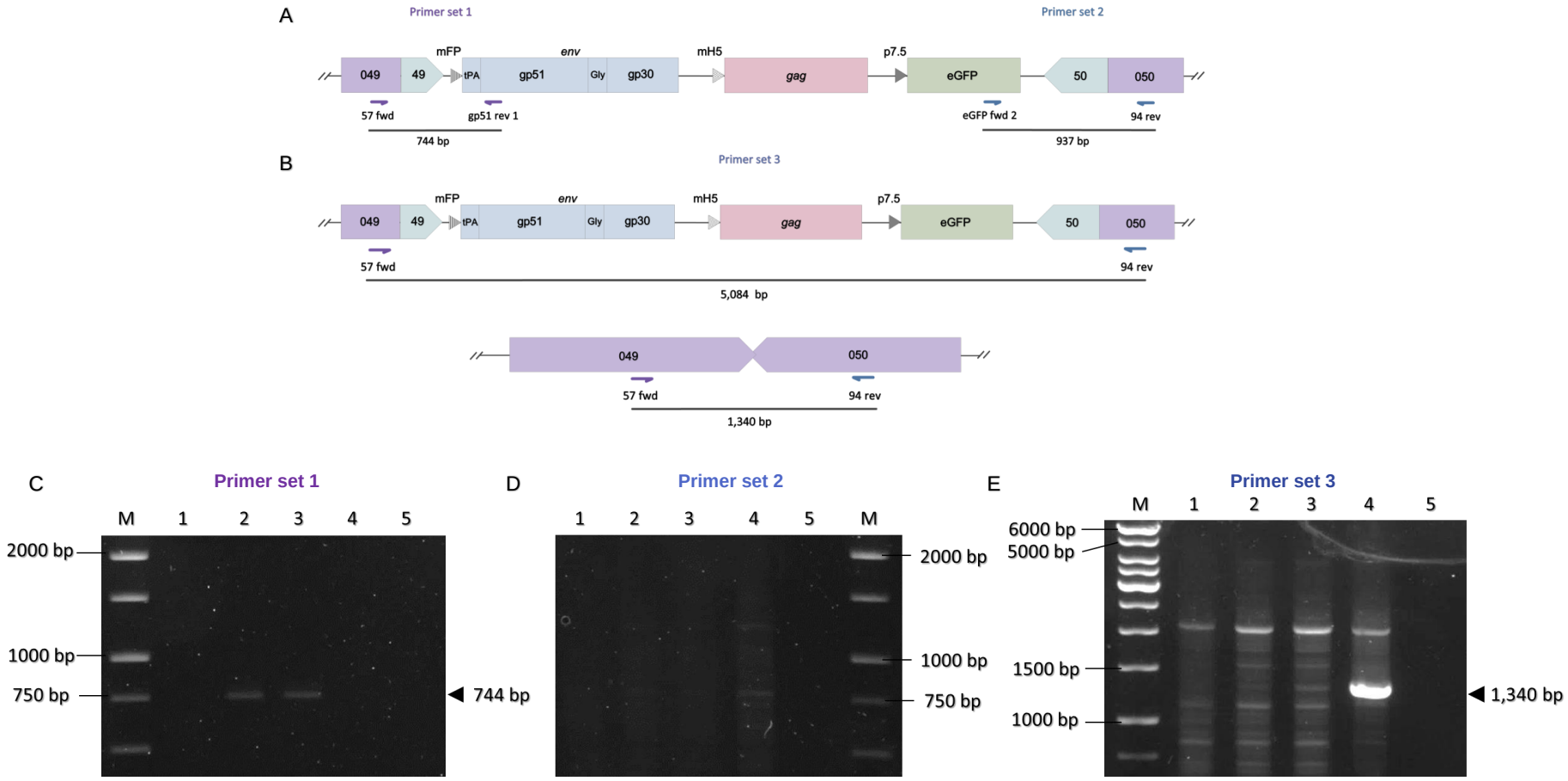



**Figure 4.23: PCR analysis of the putative LSDV-BLV-Env-Gag at passage 14.** Schematic diagrams showing **(A)** binding sites of primer set 1 (purple arrows) and primer set 2 (blue arrows) on the LSDV-BLV-Env-Gag gnome and **(B)** binding sites of primer set 3 (purple and blue arrows) on the LSDV-BLV-Env-Gag gnome and parental nLSDVSODis-M genome. PCR products are shown as a line with expected product size. See Figure 4.21 for the sequence annotation. **(C)** Agarose gel showing a 744bp product amplified with primer set 1 to detect the presence of a final and intermediate recombinant LSDV-BLV-Env-Gag into *049* ORF. **(D)** Agarose gel showing no amplification of a 937bp product with primer set 2 which detects the presence of a final and intermediate recombinant LSDV-BLV-Env-Gag into *050* ORF. **(E)** Agarose gel showing a 1,340bp product amplified with primer set 3 which detects the presence of a final recombinant LSDV-BLV-Env-Gag and/or parental nLSDVSODis-M. **M**, 1 kb DNA ladder (Thermo Scientific, USA); **1**, total DNA from uninfected cells; **2 and 3**, total DNA from the passage 14 viral stock of recombinant LSDV-BLV-Env-Gag; **4**, parental viral DNA; **5**, PCR control without template DNA.

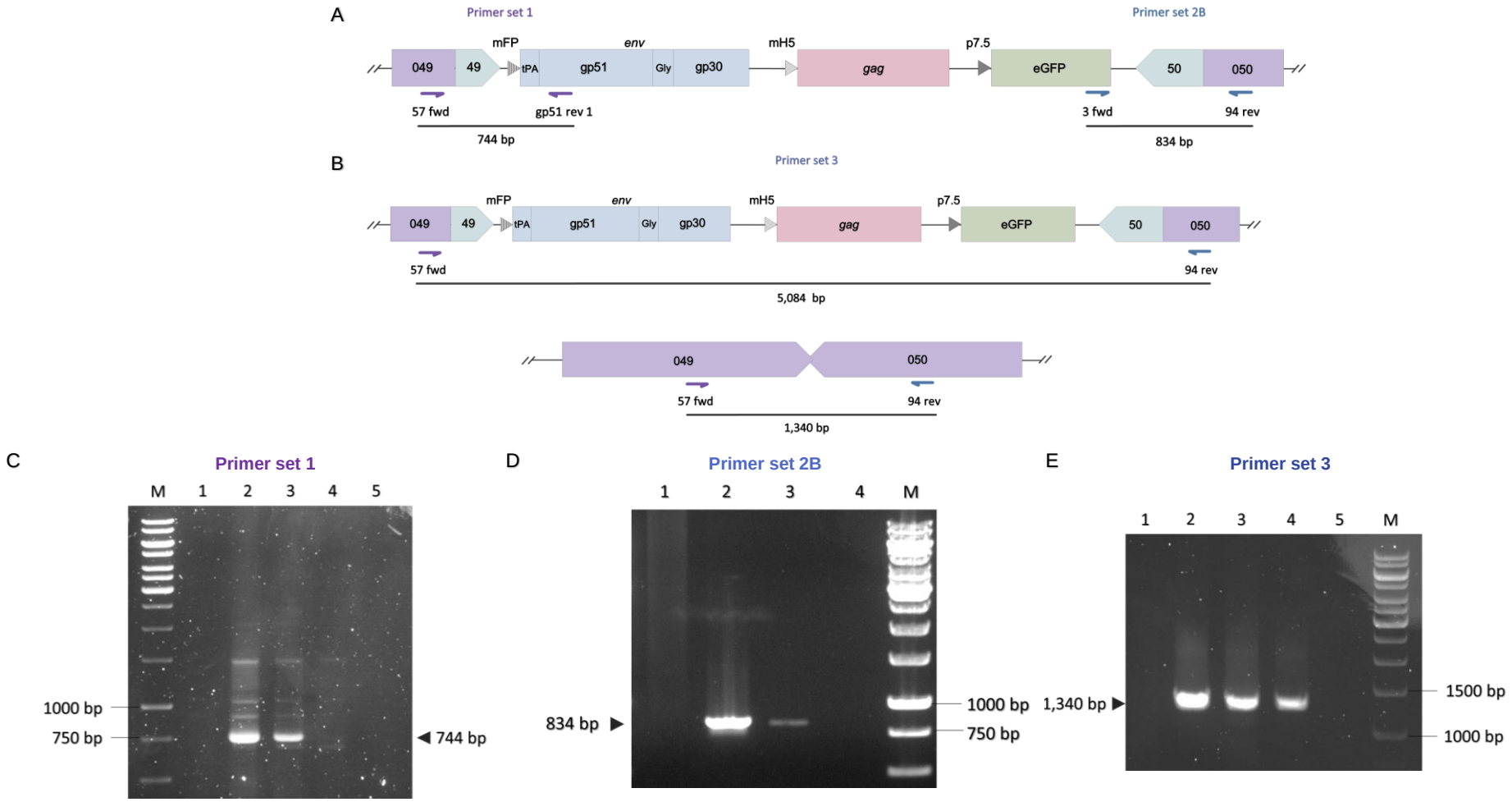



**Figure 4.24: PCR analysis of the putative LSDV-BLV-Env-Gag at passage 18**. Schematic diagrams showing **(A)** binding sites of primer set 1 (purple arrows) and primer set 2 (blue arrows) on the LSDV-BLV-Env-Gag gnome and **(B)** binding sites of primer set 3 (purple and blue arrows) on the LSDV-BLV-Env-Gag gnome and parental nLSDVSODis-M genome. PCR products are shown as a line with expected product size. See Figure 4.21 for the sequence annotation. **(C)** Agarose gel showing a 744bp product amplified with primer set 1 to detect the presence of a final and intermediate recombinant LSDV-BLV-Env-Gag into *049* ORF. **(D)** Agarose gel showing an 834bp product amplified with primer set 2B to detect the presence of a final and intermediate recombinant LSDV-BLV-Env-Gag into *059* ORF. **(E)** Agarose gel showing a 1,340bp product amplified with primer set 3 which detects the presence of a final recombinant LSDV-BLV-Env-Gag and/or parental nLSDVSODis-M. M, 1 kb DNA ladder (Thermo Scientific, USA); **1**, total DNA from uninfected cells; **2 and 3** total DNA from the passage 18 viral stock of recombinant LSDV-BLV-Env-Gag; **4**, parental viral DNA; **5**, PCR control without template DNA.

As foci that were partially fluorescing green were persistently detected during some passages, PCR confirmation of the presence or absence of an intermediate recombinant LSDV-BLV-Env-Gag virus in the passage 18 viral stock was performed using two sets of primers. Recombination into the *050* ORF only was confirmed by amplification of a 1,051bp fragment using primer set 5 (Figure 4.25 A and C). A 1,420bp fragment was not amplified using primer set 4 (Figure 4.25 A and B). This shows that a single crossover event took place such that the entire transfer vector was inserted into the *050* ORF. Because of the repeated flanking *050* sequence, the insert could either be excised in its entirety and the recombinant viruses reverts to the original parental virus, or a second crossover at the *049* ORF could result in the desired recombinant virus. In order to isolate the desired recombinant virus, extensive picking and screening of fluorescing foci are required. Due to time constraints, this could not be done.

#### 4.3.2.2.3.2. Confirmation of BLV Gag protein expression by Western blotting

To confirm that the recombinant LSDV-BLV-Env-Gag expressed BLV Gag protein, Western blotting was performed using total crude protein extracted at 24, 48 and 72 hours post-infection from MDBK cells infected with the recombinant LSDV-BLV-Env-Gag from the passage 18 viral stock. Crude total protein was also extracted from infected cells from three wells of a 6-well tissue culture plate at 72 hours post-infection and the protein from these wells was combined as a *B*ulk sample (72B in Figure 4.26 B). Figure 4.26 A shows MDBK cells from which the crude protein was extracted.

BLV Gag expression was detected at all time points both from the cell lysate and the culture media, indicating that the BLV Gag proteins were expressed intracellularly and extracellularly. Extracellular expression of the BLV Gag protein was detected even at 24 hours post-infection. Unexpectedly, the intensity of the BLV Gag band from the *B*ulk sample was not significantly different from those from proteins extracted at 48 and 72 hours post-infection.

Expression of the BLV Env protein from the recombinant LSDV-BLV-Env-Gag was not detected using Western blotting as the anti-BLV Env antibody available from Veterinary Medical Research and Development (USA) recognises a conformational epitope present on BLV Env and does not bind to the denatured protein separated by SDS-PAGE. It is, however, possible to detect BLV Env expression using immunofluorescence assay (IFA) by live-cell staining of the infected-cells. This assay was not done due to time constraints.

Table 4.6 summaries the findings in terms of fluorescing foci detected, PCR analysis and Western blot analysis of the potential recombinant virus.

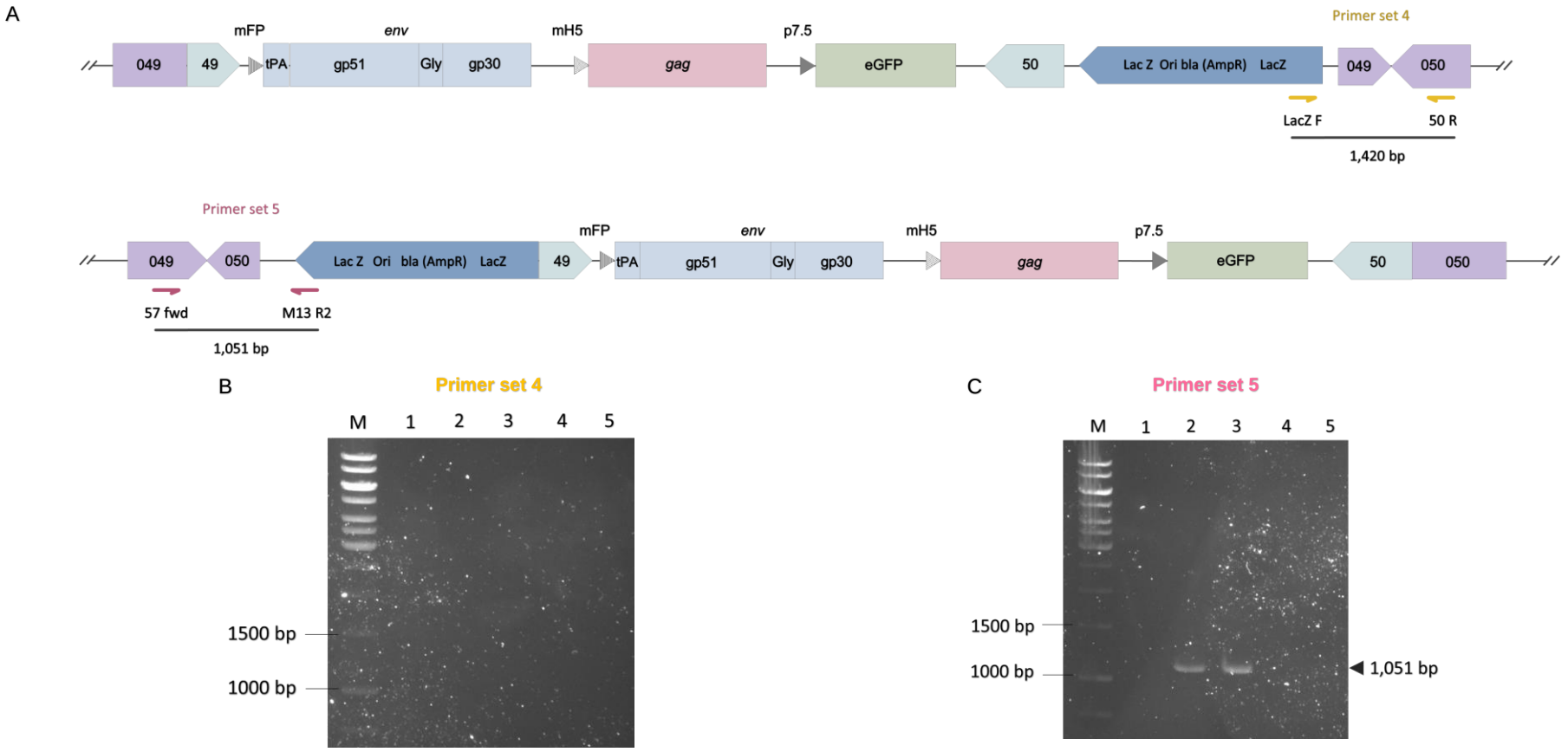



**Figure 4.25: PCR analysis of the putative intermediate LSDV-BLV-Env-Gag in the passage 18 viral stock. (A)** Schematic diagrams showing binding sites of primer set 4 (yellow arrows) and primer set 5 (red arrows) on the genome of a possible intermediate recombinant virus that recombined into the *049* ORF only (top) or into *050* ORF only (bottom). PCR products are shown as a line with expected product size. A fragment containing the pUC57 vector backbone is shown as a dark blue arrow box in this figure. **LacZ**, LacZ alpha fragment of the *beta-galactosidase* gene; **bla (AmpR)**, *beta-lactamase* (*bla*) gene; **Ori**, ColE1 origin of replication. **(B)** Agarose gel showing no amplification of a 1,420bp product with primer set 4 which detects the presence of intermediate recombinant LSDV-BLV-Env-Gag into *049* ORF only. **(C)** Agarose gel showing a 1,051bp product amplified with primer set 5 which detects the presence of intermediate recombinant LSDV-BLV-Env-Gag into *050* ORF only. **M**, 1 kb DNA ladder (Thermo Scientific, USA); **1**, total DNA from uninfected cells; **2 and 3,** total DNA from passage 18 viral stock of recombinant LSDV-BLV-Env-Gag; **4**, parental viral DNA; **5**, PCR control without template DNA.

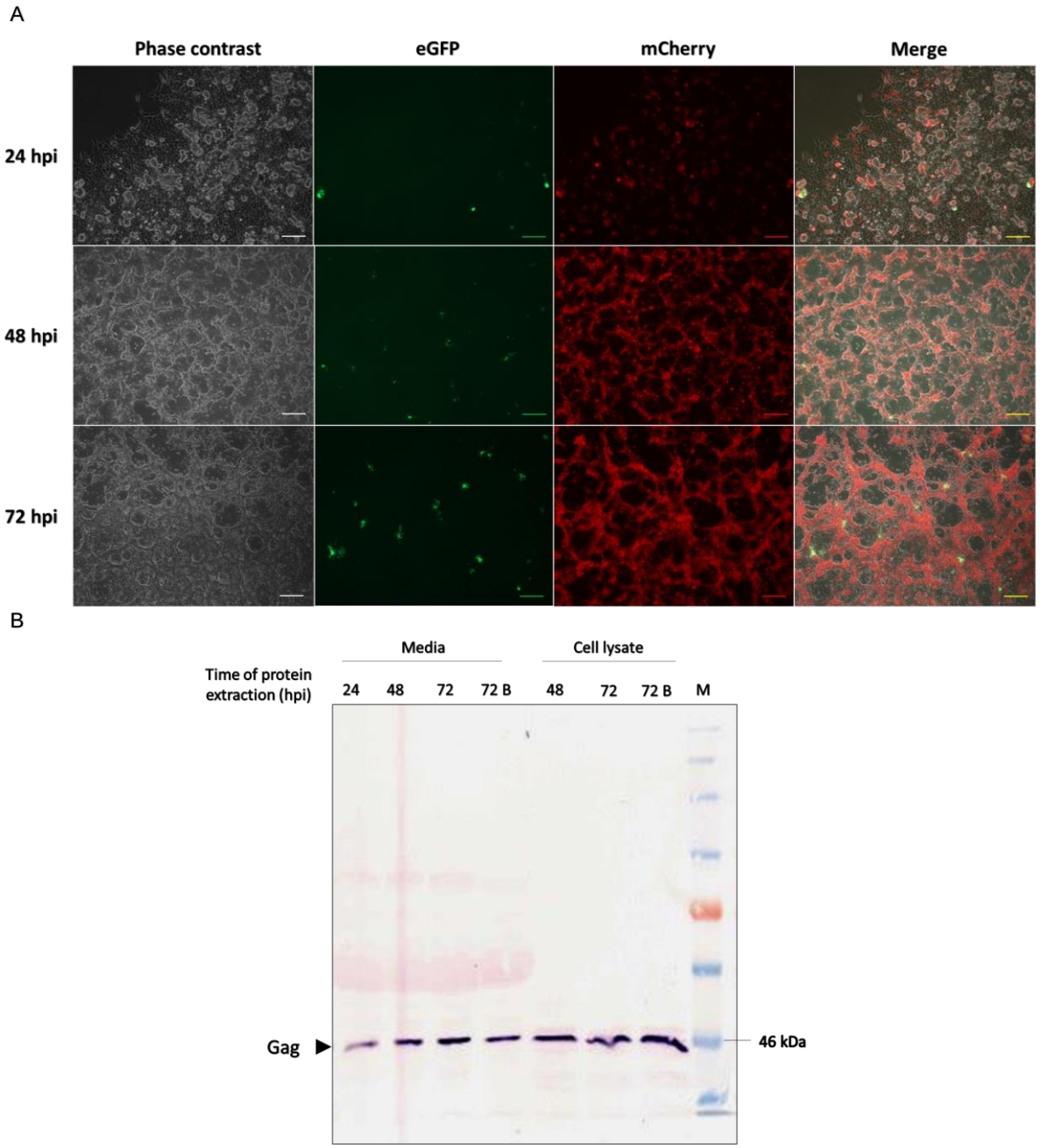



**Figure 4.26: Detection of *in vitro* expression of the BLV Gag proteins from the passage 18 viral stock of the recombinant LSDV-BLV-Env-Gag. (A)** Fluorescent images showing cell morphologies of the MDBK cells infected with the recombinant LSDV-BLV-Env-Gag at 24, 48 and 72 hours post-infection (hpi). MDBK cells infected with the recombinant LSDV-BLV-Env-Gag were visualized by their eGFP (green) and mCherry expression (red). MDBK cells infected with the parental nLSDVSODis-M were visualized by their mCherry expression (red). All images were taken using a Zeiss Axiovert 200M fluorescence inverted microscope (Zeiss, Germany) at 50x magnification at 24, 48 and 72 hours post-infection. Scale bars represent 200µm. **(B)** Western blotting showing extracellular and intracellular expression of the BLV Gag protein. **NC**, total protein extracted from uninfected cells; **24**, **48** and **72**, total protein extracted at 24, 48 and 72 hours post-infection, respectively; **72B**, total proteins were extracted from three wells of a 6-well tissue culture plate at 72 hours post-infection which were combined as a "*B*ulk" sample. Cell lysate at 24 hours post-infection is not available. BLV Gag protein with the expected size of 45 kDa is indicated by a black arrowhead.

**Table 4.6: Table summarising results from passaging potential recombinant LSDV-BLV-Env-Gag and assays to characterise BLV Env and Gag antigens**

| | Fluorescent foci | | PCR analysis[c] | | | | | | Antigen expression |
|---|---|---|---|---|---|---|---|---|---|
| Passage No. | Possible recombinant LSDV[a] | Parental virus[b] | Intermediate or final recombinant LSDV | | Final recombinant LSDV | Parental virus | Intermediate recombinant LSDV | | Gag[d] (WB) |
| | | | HR into 049 ORF | HR into 050 ORF | | | HR into 049 ORF | HR into 050 ORF | |
| 1-4 | ✓ (Figure 4.19) | +++ | | | | | | | |
| 5 | ✓ (Figure 4.20) | - | | | | | | | |
| 6 | ✓ | ++ | | | | | | | |
| 7 | ✓ | - | ✓ (Figure 4.21) | ✓ (Figure 4.21) | ✖ (Figure 4.21) | ✓ (Figure 4.21) | | | |
| 8 | ✓ (Figure 4.22) | +++ | | | | | | | |
| 9-13 | ✓ | + | | | | | | | |
| 14 | ✓ | + | ✓ (Figure 4.23) | ✓ (Figure 4.23) | ✖ (Figure 4.23) | ✓ (Figure 4.23) | | | |
| 15-16 | ✓ | ++ | | | | | | | |
| 17 | ✓ | +++ | | | | | | | |
| 18 | ✓ | +++ | ✓ (Figure 4.24) | ✓ (Figure 4.24) | ✖ (Figure 4.24) | ✓ (Figure 4.24) | ✖ (Figure 4.25) | ✓ (Figure 4.25) | ✓ (Figure 4.26) |

a, Checkmark (✓) indicates the presence of possible recombinant LSDV-BLV-Env-Gag. b, the relative number of the parental virus was shown by a plus sign (+, ++ and +++) and the minus sign (-) indicates no detection of the parental virus in any wells. c, Checkmark represents amplification of expected PCR products which confirm the presence of a possible final or intermediate recombinant virus or parental virus and cross (✖) indicates no amplification of expected PCR products. d, Checkmark indicates the confirmation of the BLV Gag protein expression by Western blotting (WB). HR, homologous recombination; ORF, open reading frame.

### 4.3.3. Construction and purification of the recombinant LSDV-BLV-Env

Recombinant LSDV harbouring the BLV *env* and *gag* genes as well as the *egfp* gene (LSDV-BLV-Env-Gag) was constructed as shown in Section 4.3.2. However, the parental virus persisted and eventually outgrew the recombinant virus after 18 passages. In addition, an intermediate recombinant virus was detected in the final viral stock at passage 18. Therefore, to assess whether the expression of BLV Gag might have inhibited the growth of recombinant LSDV-BLV-Env-Gag, recombinant LSDV harbouring the BLV *env* gene and the *egfp* gene (LSDV-BLV-Env) was constructed. For the generation of this recombinant LSDV, nLSDVSODis-UCT was used as a parental virus. This parental virus does not contain an *Escherichia coli* xanthine phosphoribosyltransferase (*Ecogpt)* selection gene or *mCherry* fluorescent protein marker gene. Since the presence of *Ecogpt* gene is not desirable in vaccines, this nLSDVSODisUCT was selected to construct the recombinant LSDV-BLV-Env.

#### 4.3.3.1. Construction of the recombinant LDSV-BLV-Env in primary foetal bovine testes (FBT) cells

To generate the recombinant LSDV-BLV-Env, primary foetal bovine testes (FBT) cells were transfected with the pBLV-Env transfer vector plasmid and infected with the parental nLSDVSODis-UCT virus at an MOI of 0.01, 0.05, 0.1 or 0.5.

Green fluorescing cells were detected from all experimental wells at 48 hours post-infection (Figure 4.27) and a great number of green fluorescing cells were detected from the transfected-cells that were infected with the nLSDVSODis-UCT at MOIs of 0.05, 0.1 and 0.5. Some of the green fluorescing cells showed characteristic cytopathic effects (CPEs), forming large distinctive foci at 48 hours post-infection.

#### 4.3.3.2. Purification of the recombinant LDSV-BLV-Env by serial passages in Madin-Darby bovine kidney (MDBK) cells

For the purification of the recombinant LSDV-BLV-Env-Gag, one of the problems was the presence of a great number of parental viruses at most passages. To address this issue, individual green fluorescent foci of the recombinant LSDV-BLV-Env were picked and used to infect MDBK cells in 6-well, 12-well or 24-well tissue culture plates and when the number of the parental virus started decreasing significantly from the viral stock, cell lysates were used to infect cells in a 96-well plate to dilute out parental viruses. Figure 4.28 outlines tissue culture plates used at each passage and cell lysate or picked foci used for each passage.

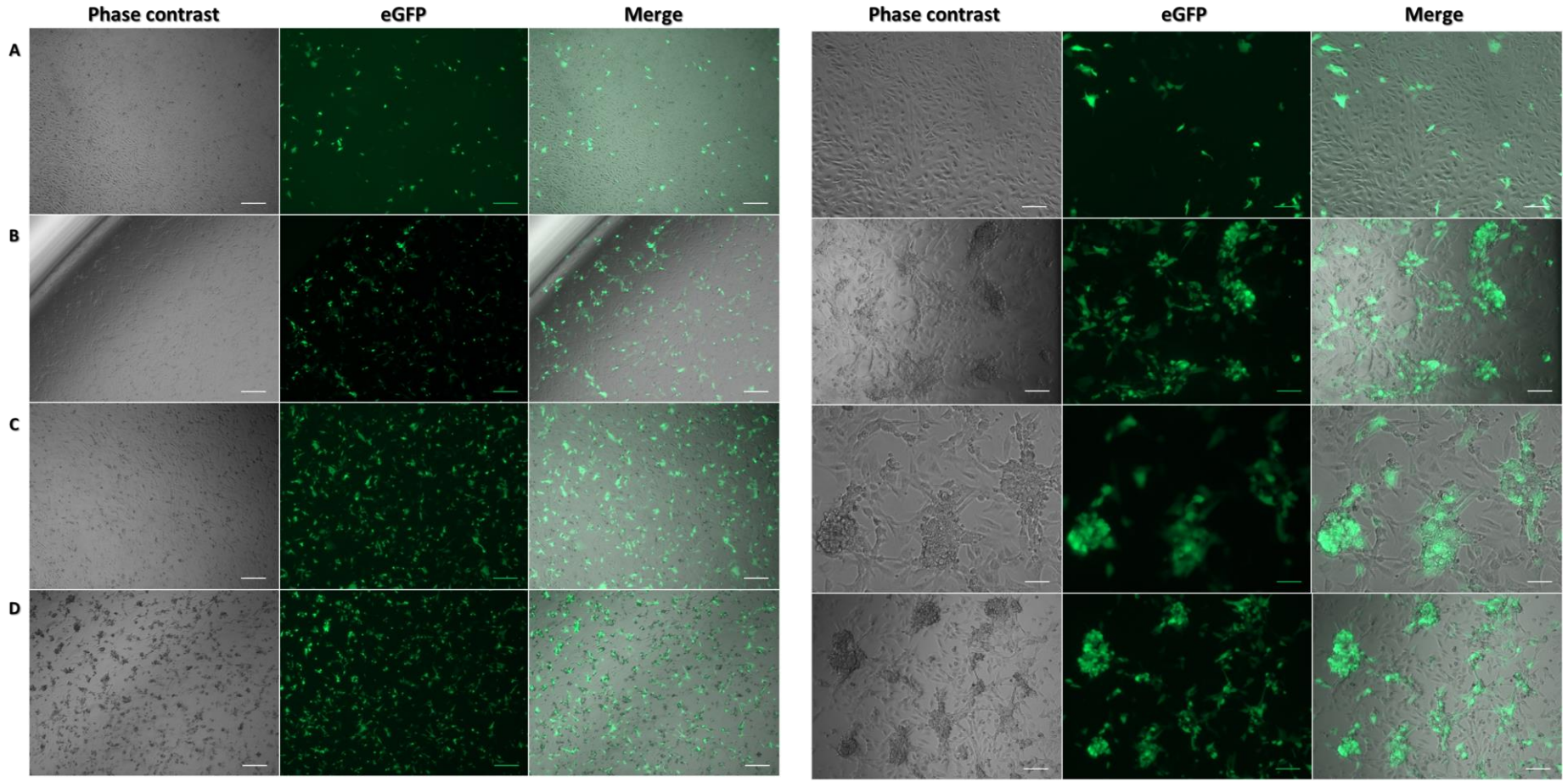



**Figure 4.27: Fluorescent images of primary FBT cells transfected with the pBLV-Env plasmid DNA and infected with nLSDVSODis-UCT.** Primary FBT cells were infected with the nLSDVSODis-UCT at MOIs of **(A)** 0.01, **(B)** 0.05, **(C)** 0.1 or **(D)** 0.5 and transfected with 6µg of pBLV-Env-Gag plasmid. Primary FBT cells infected with the recombinant LSDV-BLV-Env were visualized by their eGFP expression (green). All images were taken at 48 hours post-infection using a Zeiss Axiovert 200M fluorescence inverted microscope (Zeiss, Germany) at 50x for the left panel and at 100x for the right panel. Scale bars represent 200µm for the images on the left panel and 100µm for the images on the right panel.

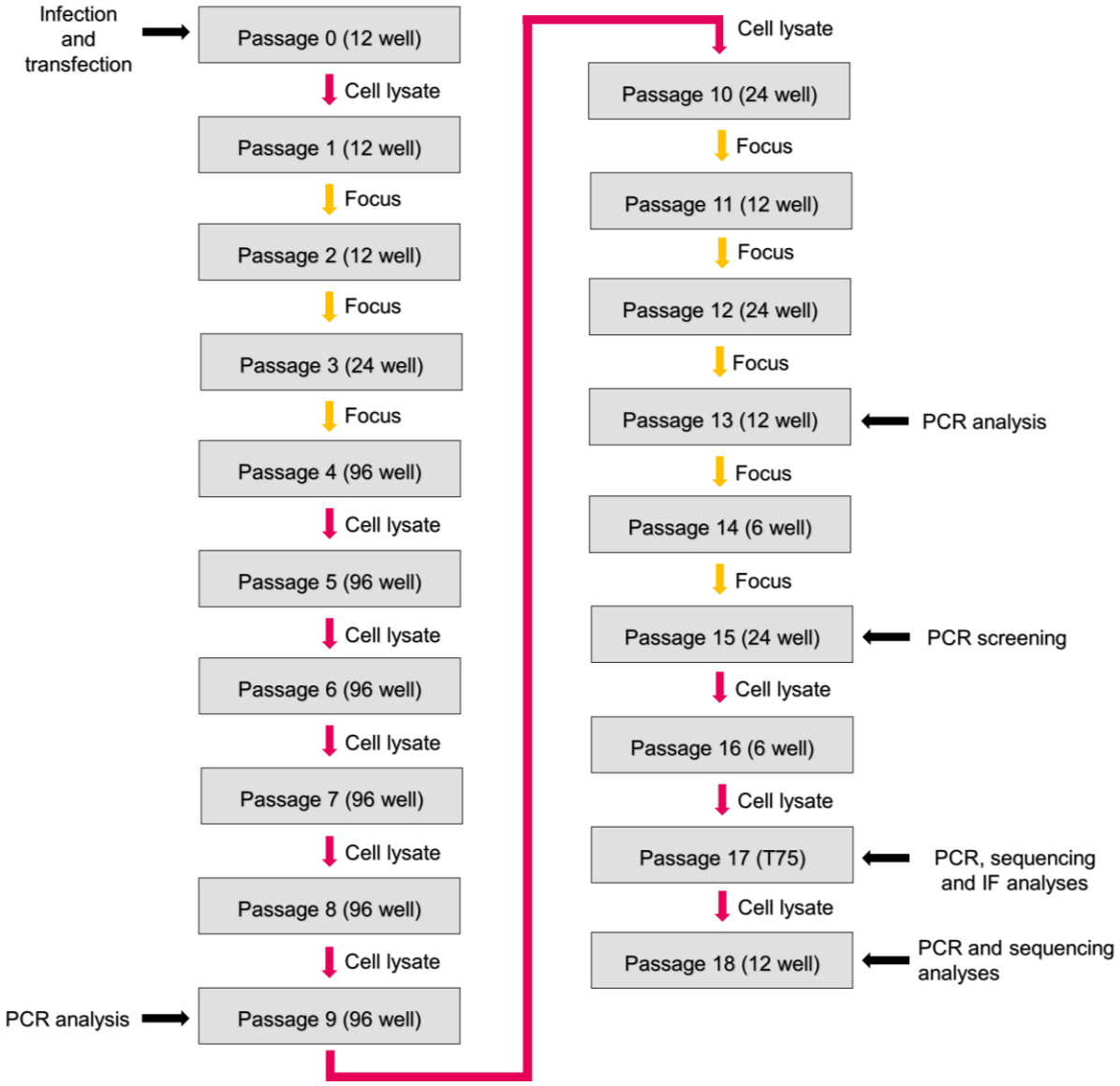



**Figure 4.28: Flow diagram outlining the purification of the recombinant LSDV-BLV-Env in MDBK cells.** Flow diagram outlines the tissue culture plates used at each passage and focus (yellow arrow) or cell lysate (pink arrow) isolated for the next passage. Detection of parental viruses as a whole is indicated as plus signs and their relative numbers are represented by + to +++. ND indicates no detection of the parental virus in any wells. Passages at which PCR, sequencing and immunofluorescence (IF) analyses were performed are indicated in this figure.

#### 4.3.3.2.1. Plaque purification of the LSDV-BLV-Env in MDBK cells from passage 1 to passage 3

Cell lysates were isolated from the primary FBT cells from the initial plate to infect MDBK cells at passage 1. At 48 hours post-infection, some of the green fluorescing foci observed at 24 hours post-infection disappeared, indicating that they were expressing eGFP transiently or they were still unstable. No green fluorescing foci were detected from some other wells after a 120-hour incubation, indicating that they were also expressing eGFP transiently or they were too unstable to grow.

Green fluorescing foci that did not have non-fluorescing foci (parental viruses) in their proximity were carefully picked used to infect MDBK cells consecutively at passages 1, 2 and 3 (Figure 4.29 A-C). Whilst some foci also resulted in no growth at passages 2 and 3, other foci were small, and others were large or appeared to be spreading and infecting adjacent cells. At passage 3, less parental viruses were detected and two wells appeared to contain a single green fluorescing focus without the presence of parental viruses (Figure 4.29 C), indicating that the use of the picked foci for the infection appeared to be effectively reducing parental viruses.

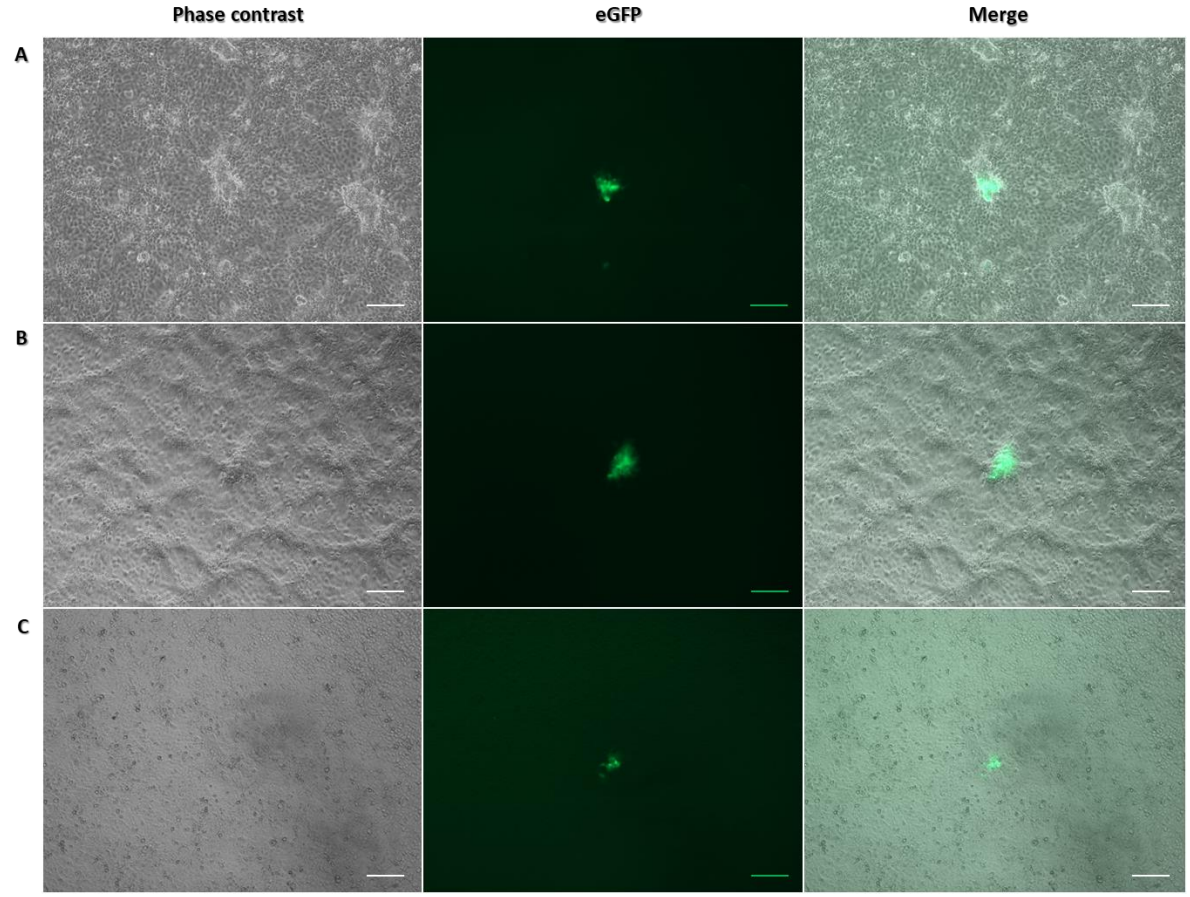



**Figure 4.29: Green fluorescing foci of MDBK cells infected with recombinant LSDV-BLV-Env at passages 1, 2 and 3.** A single focus from **(A)** passage 1 (images taken at 72 hours post-infection), **(B)** passage 2 (images taken at 96 hours post-infection) and **(C)** passage 3 (images taken at 96 hours post-infection). MDBK cells infected with the recombinant LSDV-BLV-Env were visualized by their eGFP expression (green). All images were taken using a Zeiss Axiovert 200M fluorescence inverted microscope (Zeiss, Germany) at 100x magnification. Scale bars represent 100µm.

### 4.3.3.2.2. Purification of the LSDV-BLV-Env in MDBK cells using serial dilutions of cell lysates from passage 4 to passage 9

Although non-fluorescing foci formed by parental viruses were not present in two wells at passage 3, parental viruses could be present in the culture media as an extracellular

enveloped virus (EEV), which exists in the extracellular space after budding out of the infected cells [533]. EEV is not visually detectable and detection of the parental viruses are only possible when parental viruses infect cells and form non-fluorescing foci, Therefore, from passage 4 to passage 9, MDBK cells were infected in a 96-well plate using serial dilutions of cell lysates to dilute out undetectable parental viruses and to isolate a single focus per well.

Careful monitoring of the infected cells at passage 4 revealed that for unknown reasons, some non-fluorescing foci were only detected after an extended period of incubation (~6 days) and thus the presence of the parental nLSDVSODis-UCT virus could be missed out if the incubation of infected cells was terminated before the formation of non-fluorescing foci.

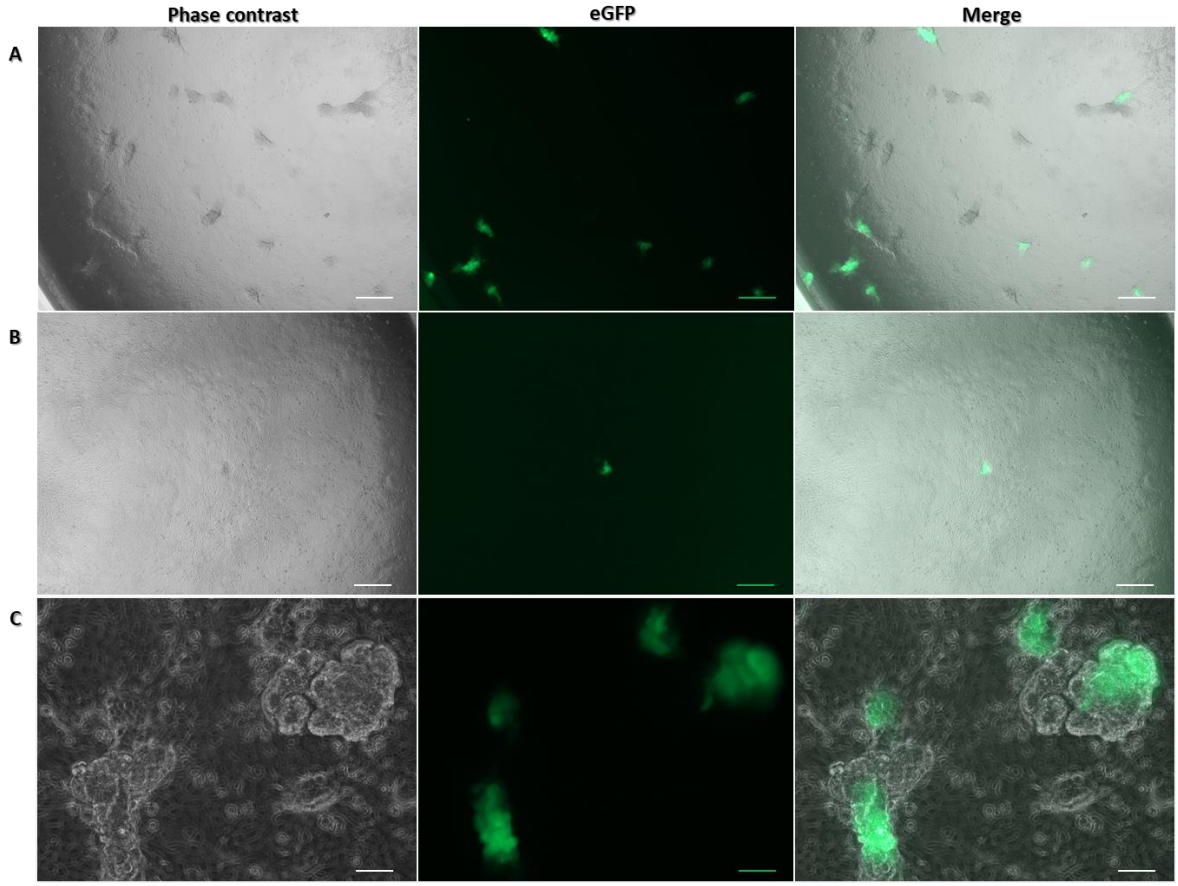



**Figure 4.30: Detection of parental nLSDVSODis-UCT, recombinant LSDV-BLV-Env as well as recombinant LSDV-BLV-Env that partially lost eGFP expression at passage 9. (A)** A representative image showing non-fluorescing parental viruses. **(B)** A representative image showing green fluorescing foci detected in a well where parental virus appeared to be absent. **(C)** A representative image showing green fluorescing foci that partially lost eGFP expression. MDBK cells infected with the recombinant LSDV-BLV-Env were visualized by their eGFP expression (green). All images were taken at 96 hours post-infection using a Zeiss Axiovert 200M fluorescence inverted microscope (Zeiss, Germany) at 50x magnification. Scale bars represent 200µm.

At passage 9, following the five consecutive passages in 96-well plates, whilst numerous parental viruses were still detected in many other wells (Figure 4.30 A), no parental viruses

were detected in several wells after the isolation of green fluorescing foci at 96 hours post-infection (Figure 4.30 B). However, in another tissue culture plate prepared at passage 9, green fluorescent foci that had partially lost eGFP expression was detected in some wells (Figure 4.30 C), indicating the presence of possible intermediate recombinant LSDV-BLV-Env whereby HR occurred into *049* ORF only or *050* ORF only.

#### 4.3.3.2.2.1. PCR analysis of the presence of the recombinant LSDV-BLV-Env at passage 9

Due to the presence of possible final and intermediate recombinant LSDV-BLV-Env as well as parental viruses at passage 9, PCR was performed to assess whether the final recombinant LSDV-BLV-Env was present at passage 9 (Figure 4.31). Amplification of a 744bp fragment using primer set 1 indicates that HR occurred into both *049* and *050* ORFs or into *049* ORF only whereas no amplification of a 945bp fragment using primer set 2A suggests either inefficient PCR or the absence of HR into *050* ORF (Figure 4.31 C and D). As these results were inconclusive to determine the presence of the final recombinant LSDV-BLV-Env, another PCR was performed to aim at amplification of a 3,799bp fragment across the BLV expression cassette using primer set 3 (Figure 4.31E). However, an expected 3,799bp fragment was not amplified. Instead, a 1,340bp fragment indicating the presence of the parental nLSDVSODis-UCT was amplified using the same primer set 3 (Figure 4.31E).

Since the amplification of a 744bp fragment using primer set 1 and no amplification of a 945bp fragment using primer set 2 from the 5'-end and 3'-end of the BLV expression cassette, respectively, only suggest, but cannot conclusively show, the possible presence of an intermediate recombinant virus whereby HR occurred into *049* ORF only, to confirm these results, primer sets 4 and 5 were used (Figure 4.32 A). The presence of a possible intermediate recombinant virus whereby HR occurred into *050* ORF only and not into *049* ORF was confirmed by amplification of a 1,051bp using primer set 5 (Figure 4.32 A and C). A 1,420bp fragment was not amplified using primer set 4 (Figure 4.32 A and B), indicating either the absence of a possible intermediate recombinant LSDV-BLV-Env whereby HR occurred into *049* ORF only or inefficient PCR (i.e, possible presence of this type of intermediate virus).

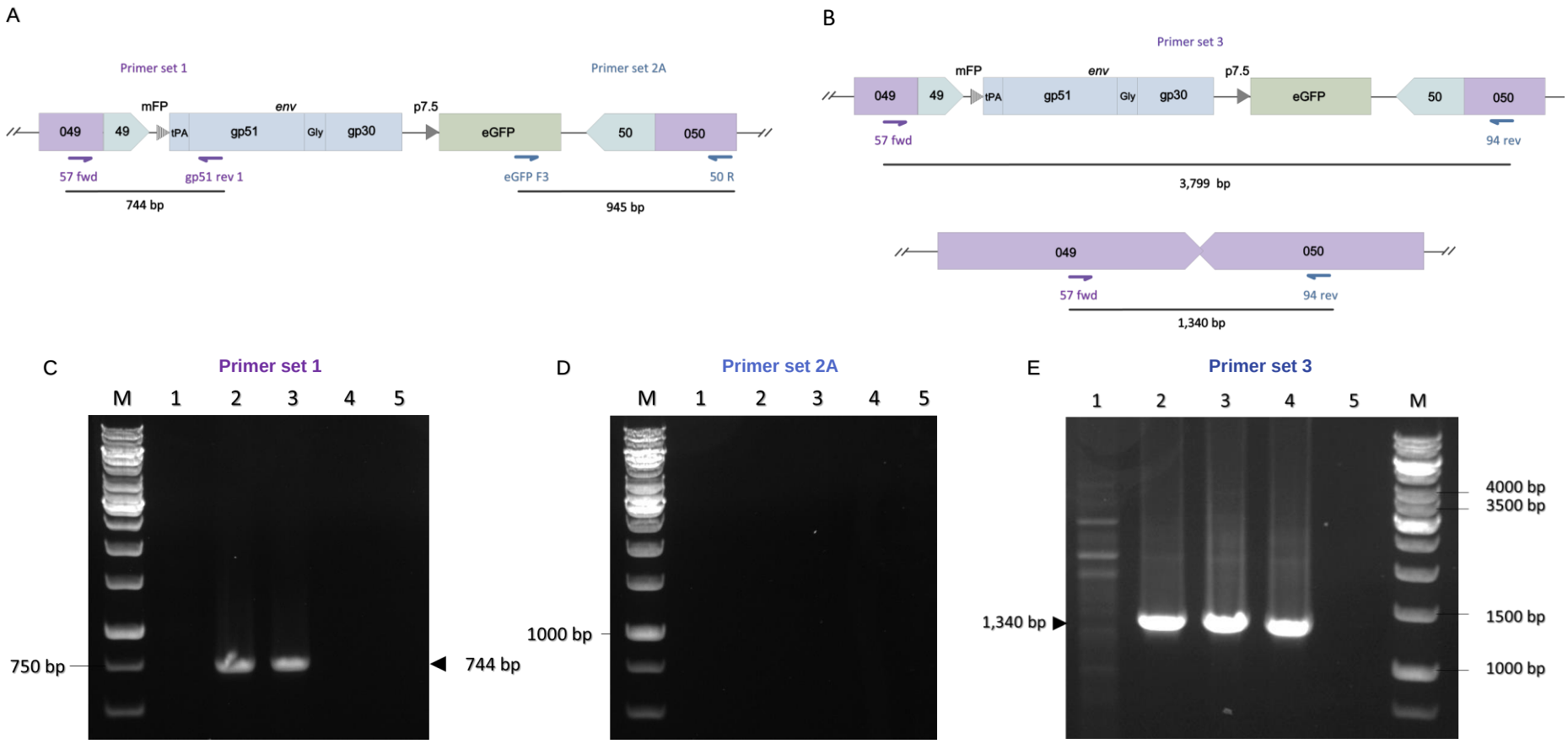



**Figure 4.31: Preliminary confirmation of the presence of the LSDV-BLV-Env at passage 9.** Schematic diagrams showing **(A)** binding sites of primer set 1 (purple arrows) and primer set 2 (blue arrows) on the LSDV-BLV-Env gnome and **(B)** binding sites of primer set 3 (purple and blue arrows) on the LSDV-BLV-Env gnome and parental nLSDVSODis-UCT genome. PCR products are shown as a line with expected product size. See Figure 4.21 for the sequence annotation. **(C)** Agarose gel showing a 744bp product amplified with primer set 1 to detect the presence of a final and intermediate recombinant LSDV-BLV-Env into *049* ORF. **(D)** Agarose gel showing no amplification of a 945bp product with primer set 2A which detects the presence of a final and intermediate recombinant LSDV-BLV-Env into *050* ORF. **(E)** Agarose gel showing a 1,340bp product amplified with primer set 3 which detects the presence of a final recombinant LSDV-BLV-Env- and/or parental nLSDVSODis-UCT. **M**, 1 kb DNA ladder (Thermo Scientific, USA); **1**, total DNA from uninfected cells; **2 and 3,** total DNA from the passage 9 viral stock of recombinant LSDV-BLV-Env; **4**, parental viral DNA; **5**, PCR control without template DNA.

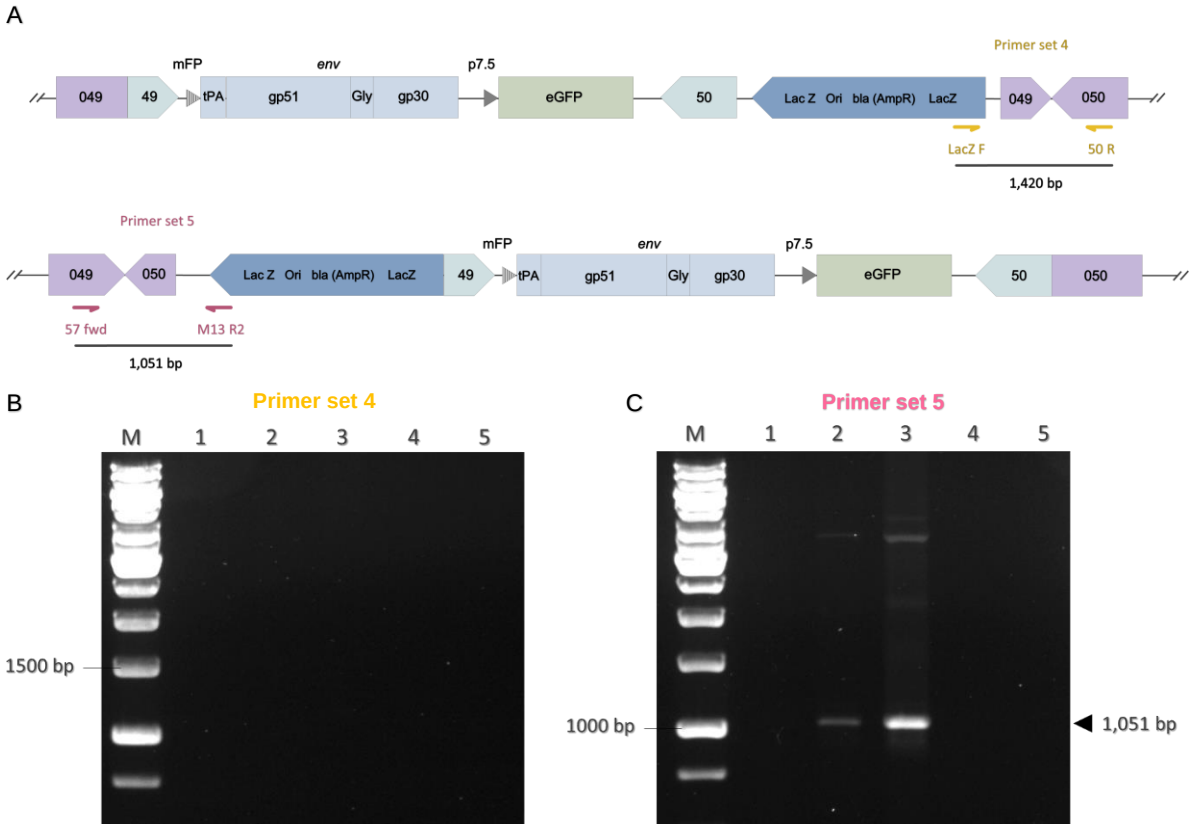



**Figure 4.32: Preliminary confirmation of the presence of an intermediate LSDV-BLV-Env at passage 9. (A)** Schematic diagrams showing binding sites of primer set 4 (yellow arrows) and primer set 5 (red arrows) on genome of possible intermediate recombinant virus that recombined into the *049* ORF only (top) or into *050* ORF only (bottom). PCR products are shown as a line with expected product size. See Figure 4.25 A for the sequence annotation. **(B)** Agarose gel showing no amplification of a 1,420bp product with primer set 4 which detects the presence of intermediate recombinant LSDV-BLV-Env into *049* ORF only. **(C)** Agarose gel showing a 1,051bp product amplified with primer set 5 which detects the presence of intermediate recombinant LSDV-BLV-Env into *050* ORF only. **M**, 1 kb DNA ladder (Thermo Scientific, USA); **1**, total DNA from uninfected cells; **2 and 3,** total DNA from passage 9 viral stock of recombinant LSDV-BLV-Env; **4**, parental viral DNA; **5**, PCR control without template DNA.

#### 4.3.3.2.3. Plaque purification of the LSDV-BLV-Env in MDBK cells from passage 10 to passage 15

Cell lysates were isolated from the passage 9 plate where no parental viruses were detected and used to infect MDBK cells to allow the recombinant LSDV-BLV-Env to propagate further without the presence of the parental virus. The passaging of the recombinant viruses continued by infecting picked foci at passages 10, 11 and 12. At passage 13, only a single parental virus was detected in one well of a tissue culture plate, suggesting that the passaging of picked foci after the consecutive passages of serial dilutions of cell lysates in a 96-well tissue culture plate appeared to be successfully reducing the number of the parental viruses.

#### 4.3.3.2.3.1. PCR analysis of the presence of the recombinant LSDV-BLV-Env at passage 13

Since the purification of the recombinant LSDV-BLV-Env had appeared to be successful, the presence of a possible final recombinant LSDV-BLV-Env at passage 13 was once again assessed by PCR. Amplification of 744bp, 834bp and 1,340bp fragments using primer sets 1, 2B and 3, respectively indicates the presence of the parental virus but this time again, the presence of a final recombinant LSDV-BLV-Env was not confirmed (Figure 4.33 C-E). Since the presence of a possible intermediate recombinant LSDV-BLV-Env whereby HR occurred into *050* ORF was detected at passage 9, the presence of this type of intermediate recombinant virus was assessed at passage 13 using primer set 5. The absence of this intermediate recombinant virus was noted for no amplification of a 1,051bp fragment (Figure 4.34 B).

At passage 14, some parental viruses were detected. Nevertheless, a total of twenty green fluorescing foci were picked and each focus was used to infect MDBK cells in each well of a tissue culture plate. This passage 15 served as a preparation for the PCR screening of the presence of the final recombinant viruses.

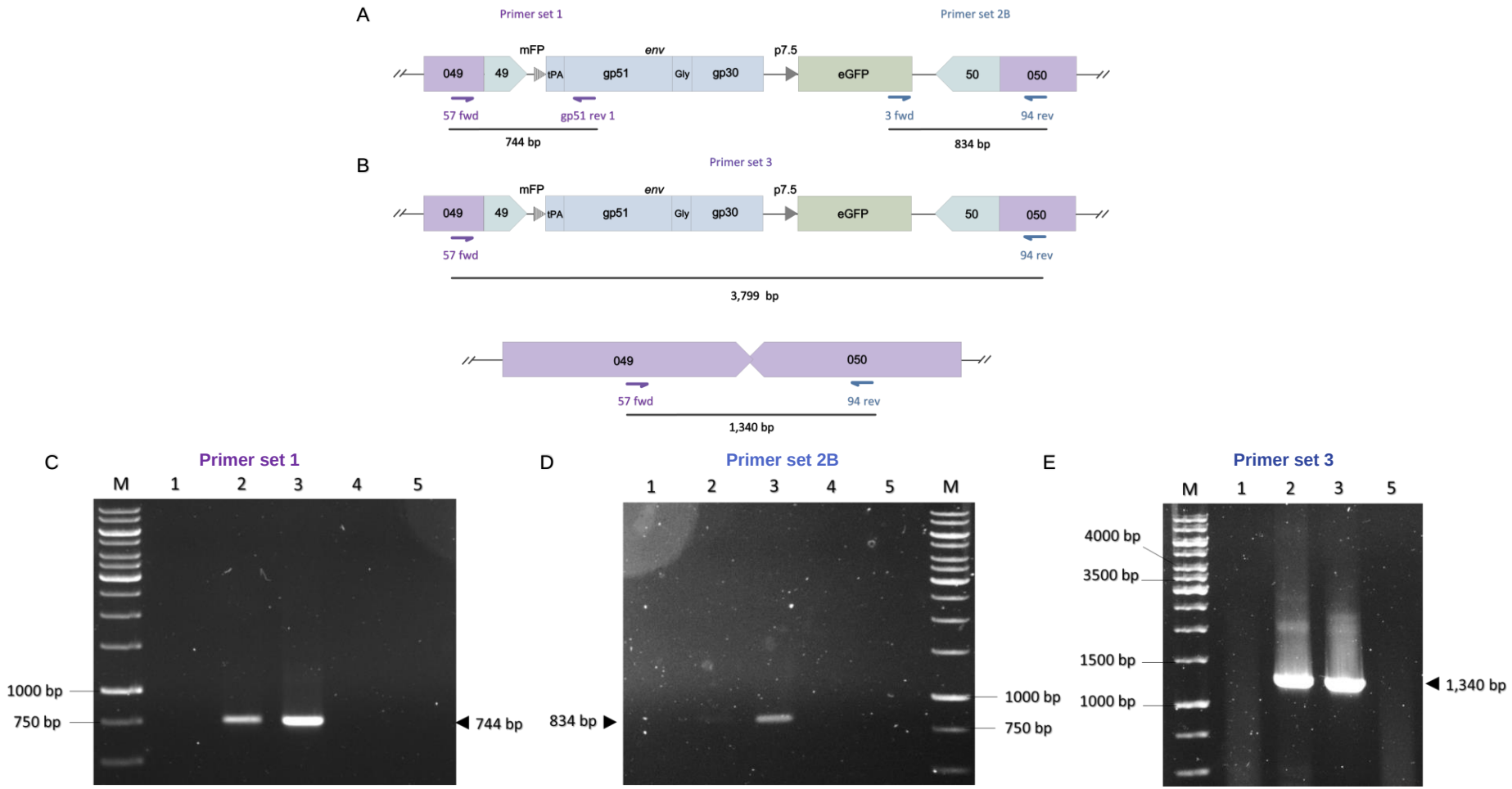



**Figure 4.33: PCR analysis of the putative LSDV-BLV-Env at passage 13.** Schematic diagrams showing **(A)** binding sites of primer set 1 (purple arrows) and primer set 2B (blue arrows) on the LSDV-BLV-Env gnome and **(B)** binding sites of primer set 3 (purple and blue arrows) on the LSDV-BLV-Env gnome and parental nLSDVSODis-UCT genome. PCR products are shown as a line with expected product size. See Figure 4.21 for the sequence annotation. **(C)** Agarose gel showing a 744bp product amplified with primer set 1 to detect the presence of a final and intermediate recombinant LSDV-BLV-Env into *049* ORF. **(D)** Agarose gel showing an 834bp product amplified with primer set 2B to detect the presence of a final and intermediate recombinant LSDV-BLV-Env into *050* ORF. **(E)** Agarose gel showing a 1,340bp product amplified with primer set 3 which detects the presence of a final recombinant LSDV-BLV-Env- and/or parental nLSDVSODis-UCT. **M**, 1 kb DNA ladder (Thermo Scientific, USA); **1**, total DNA from uninfected cells; **2 and 3,** total DNA from the passage 13 viral stock of recombinant LSDV-BLV-Env; **4**, parental viral DNA; **5**, PCR control without template DNA.

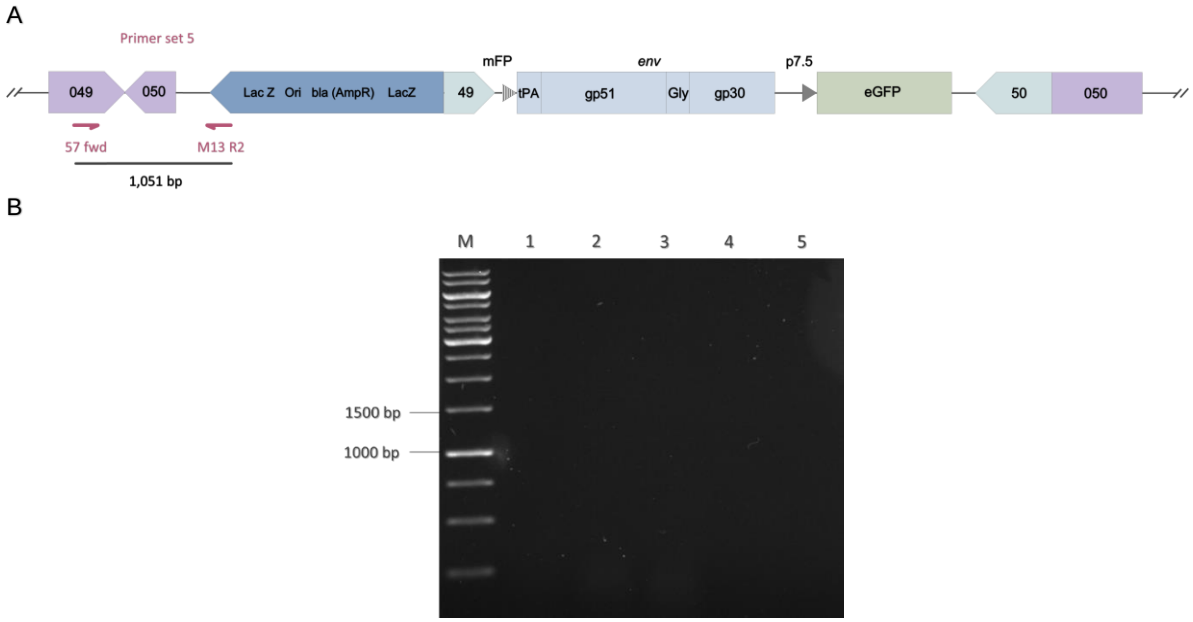



**Figure 4.34: Preliminary confirmation of the absence of an intermediate LSDV-BLV-Env that recombined into *050* ORF only at passage 13. (A)** A schematic diagram showing binding sites of primer set 4 (yellow arrows) and primer set 5 (red arrows) on genome of possible intermediate recombinant virus that recombined into the *049* ORF only (top) or into *050* ORF only (bottom). PCR products are shown as a line with expected product size. See Figure 4.25 A for the sequence annotation. **(B)** Agarose gel showing no amplification of a 1,051bp fragment with primer set 5 which detects the presence of an intermediate LSDV-BLV-Env that recombined into the *050* ORF only. **M**, 1 kb DNA ladder (Thermo Scientific, USA); **1**, total DNA from uninfected cells; **2 and 3,** total DNA from passage 13 viral stock of the LSDV-BLV-Env; **4**, parental viral DNA; **5**, PCR control without template DNA.

### 4.3.3.2.3.2. PCR screening of the presence of the final recombinant LSDV-BLV-Env at passage 15

PCR screening of the many possible recombinant viruses was conducted in order to isolate those with no parental viruses in the same well. To do this, twenty foci that did not have parental viruses in their proximity were isolated from a 6-well tissue culture at passage 14 and the individual foci were used to infect MDBK cells in each well of a 24-well tissue culture plate. Some foci (Figure 4.35) propagated extensively forming large foci at 96 hours post-infection but parental viruses were detected in all wells. Ten out of twenty wells that contained more recombinant viruses and less parental viruses were selected to perform PCR screening using the primer sets outlined in Table 4.3.

Using primer set 1, a 744bp fragment was amplified from all ten samples (Figure 4.36 B), indicating recombination into the *049* ORF had occurred. An 834bp fragment using primer set 2B was amplified only from samples 2, 6, 8 and 9 (Figure 4.36 C), indicating that recombination into the *050* ORF had occurred.

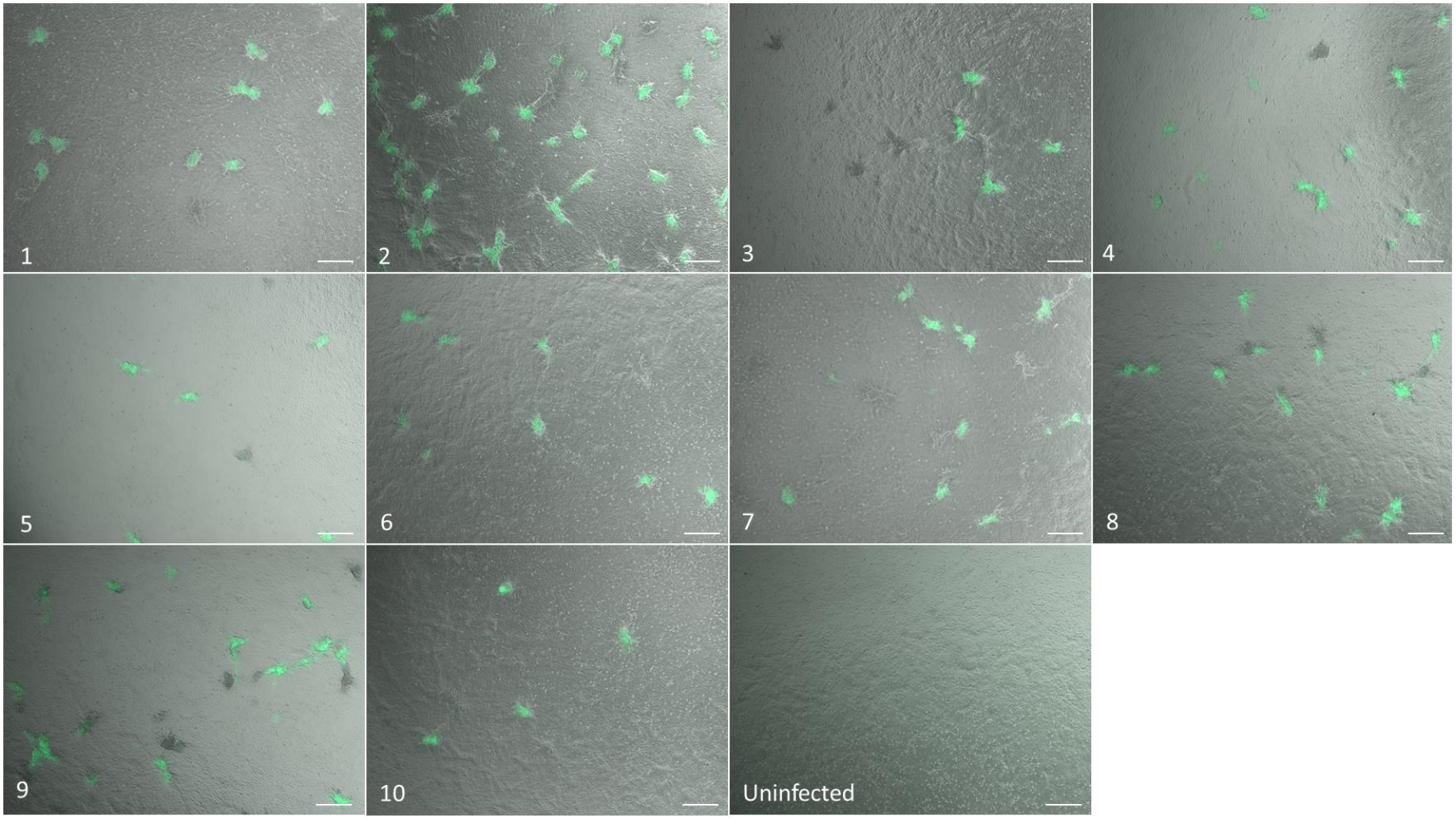



**Figure 4.35: Fluorescent images showing the presence of parental nLSDVSODis-UCT and recombinant LSDV-BLV-Env in ten wells selected for the PCR screening of recombinant LSDV-BLV-Env at passage 15. (1-10)** Fluorescent images showing ten wells of the passage 15 plate, from which total crude DNA was extracted at 96 hours post-infection. Note that foci detected in each well originated from single foci from the passage 14 plate. MDBK cells infected with the recombinant LSDV-BLV-Env were visualized by their eGFP expression (green). All images were taken at 96 hours post-infection using a Zeiss Axiovert 200M fluorescence inverted microscope (Zeiss, Germany) at 50x magnification. Scale bars represent 200µm. **Uninfected,** uninfected cells.

Although an 834bp fragment was not amplified from other samples, this does not mean that those samples did not contain the recombinant viruses. It possible that the primer set 2 did not amplify as efficiently and thus may not have detected low levels of recombinants in this region. Using primer set 3, a 1,340bp fragment was amplified from all samples, indicating the presence of the parental virus in all the well (Figure 4.36 D). No 3,799bp fragment indicating the presence of the recombinant LSDV-BLV-Env was amplified in any of the samples (Figure 4.36 D). This may have been due to the inefficient amplification of this large fragment.

Well number 2 was selected for the downstream analyses as amplification of the 834bp fragment using both primer sets 1 and 2 was successful (Figure 4.36 C lane 2).

Although the parental virus was still detected, cell lysate was further passaged into a 6-well tissue culture plate and then into a 75 $cm^2$ tissue culture (T-75) flask to perform a PCR analysis to assess the presence of the final recombinant virus and IFA to confirm the expression of the BLV Env protein from the recombinant LSDV-BLV-Env. Unlike the passage 17 and passage 18 viral stocks of the recombinant LSDV-BLV-Env-Gag, the parental virus did not outgrow the recombinant LSDV-BLV-Env and the recombinant LSDV-BLV-Env propagated extensively and formed large foci.

The eGFP expressing recombinant LSDVs from the parental virus could have been purified by fluorescence-activated cell sorting (FACS) but further steps to further purify the final recombinant LSDVs from intermediate recombinant viruses by passaging would be required. Due to time constrains, this process was not done.

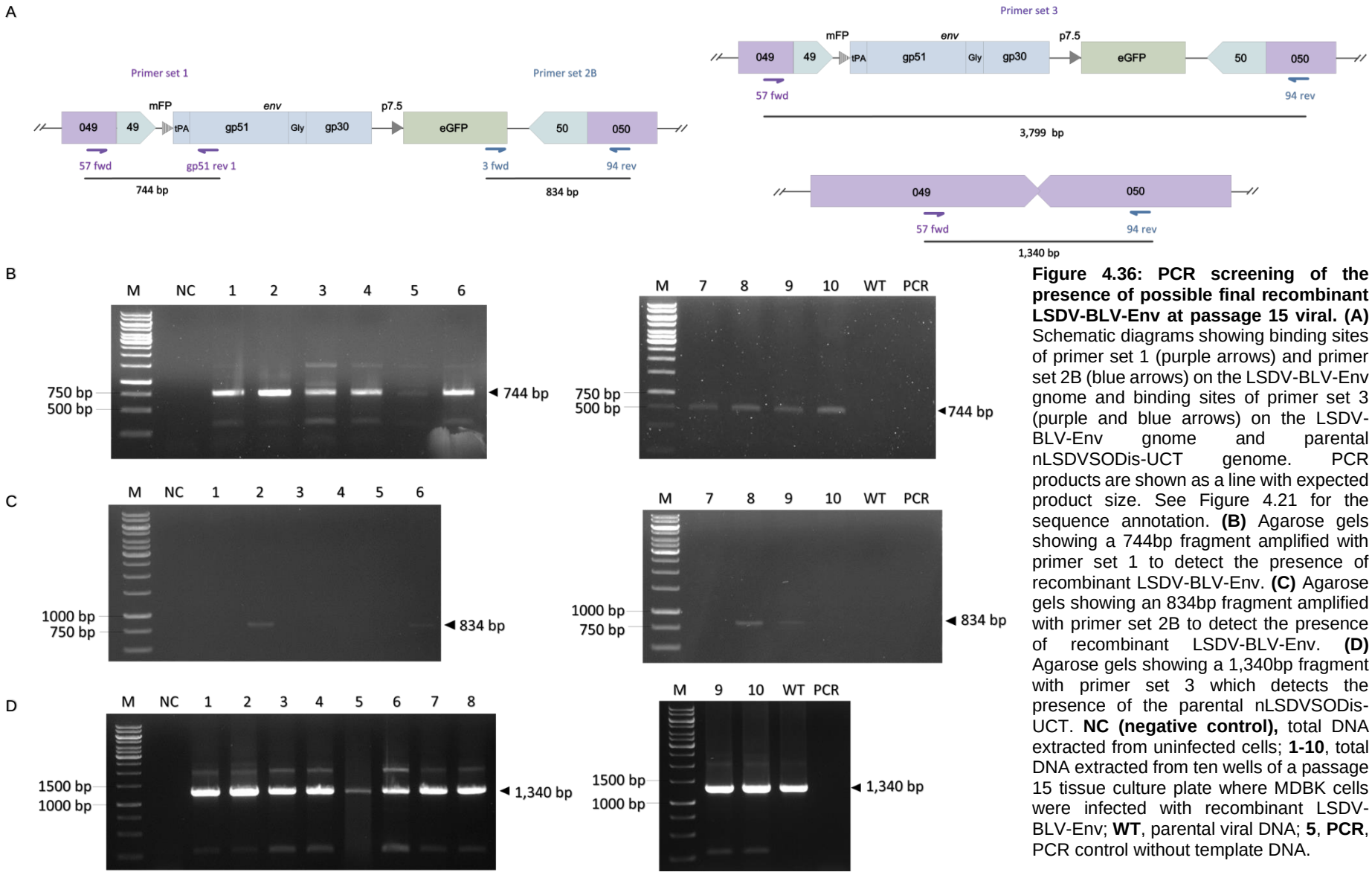



**Figure 4.36: PCR screening of the presence of possible final recombinant LSDV-BLV-Env at passage 15 viral. (A)** Schematic diagrams showing binding sites of primer set 1 (purple arrows) and primer set 2B (blue arrows) on the LSDV-BLV-Env gnome and binding sites of primer set 3 (purple and blue arrows) on the LSDV-BLV-Env gnome and parental nLSDVSODis-UCT genome. PCR products are shown as a line with expected product size. See Figure 4.21 for the sequence annotation. **(B)** Agarose gels showing a 744bp fragment amplified with primer set 1 to detect the presence of recombinant LSDV-BLV-Env. **(C)** Agarose gels showing an 834bp fragment amplified with primer set 2B to detect the presence of recombinant LSDV-BLV-Env. **(D)** Agarose gels showing a 1,340bp fragment with primer set 3 which detects the presence of the parental nLSDVSODis-UCT. **NC (negative control),** total DNA extracted from uninfected cells; **1-10**, total DNA extracted from ten wells of a passage 15 tissue culture plate where MDBK cells were infected with recombinant LSDV-BLV-Env; **WT**, parental viral DNA; **5**, **PCR**, PCR control without template DNA.

#### 4.3.3.2.4. Characterisation of the recombinant LSDV-BLV-Env

##### 4.3.3.2.4.1. Final confirmation of the presence of the BLV *env* gene in the recombinant LSDV-BLV-Env by PCR

To determine whether an intermediate recombinant containing the entire transfer vector or the final recombinant only containing the BLV *env* gene and the *egfp* gene had been obtained, PCR was performed with different sets of primers as outlined in Table 4.3 and using viral stocks from passage 17 and passage 18 of the recombinant LSDV-BLV-Env.

Using primer sets 1 and 2C, 744bp and 720bp fragments were amplified from the passage 17 and 18 viral stocks of the recombinant LSDV-BLV-Env (Figure 4.37 C and D). To confirm the presence of a possible final recombinant LSDV-BLV-Env, another PCR was performed using primer set 3B. Amplification of a 3,537bp fragment covering the entire BLV expression cassette between the 049 and 050 genes of the LSDV-BLV-Env genome was not successful. This may have been due to the inefficient amplification of this large fragment. Using the same primer set 3, a 1,078bp fragment was amplified, confirming the presence of the parental virus in the passage 17 and 18 viral stocks (Figure 4.37 E).

PCR confirmation of the presence or absence of an intermediate recombinant LSDV-BLV-Env virus in the passage 17 and 18 viral stocks was performed as no intermediate recombinant virus was detected in the passage 13 viral stock. The presence of an intermediate recombinant virus in the passage 17 and 18 viral stocks where recombination into the *050* ORF only had occurred was, however, confirmed by amplification of a 1,051bp fragment using primer set 5 (Figure 4.38 A and C). Once again, no intermediate recombinant virus in the passage 17 and 18 viral stocks where recombination into the *049* ORF only had occurred was detected as there was no amplification of a 1,420bp fragment using primer set 4 (Figure 4.38 A and B).

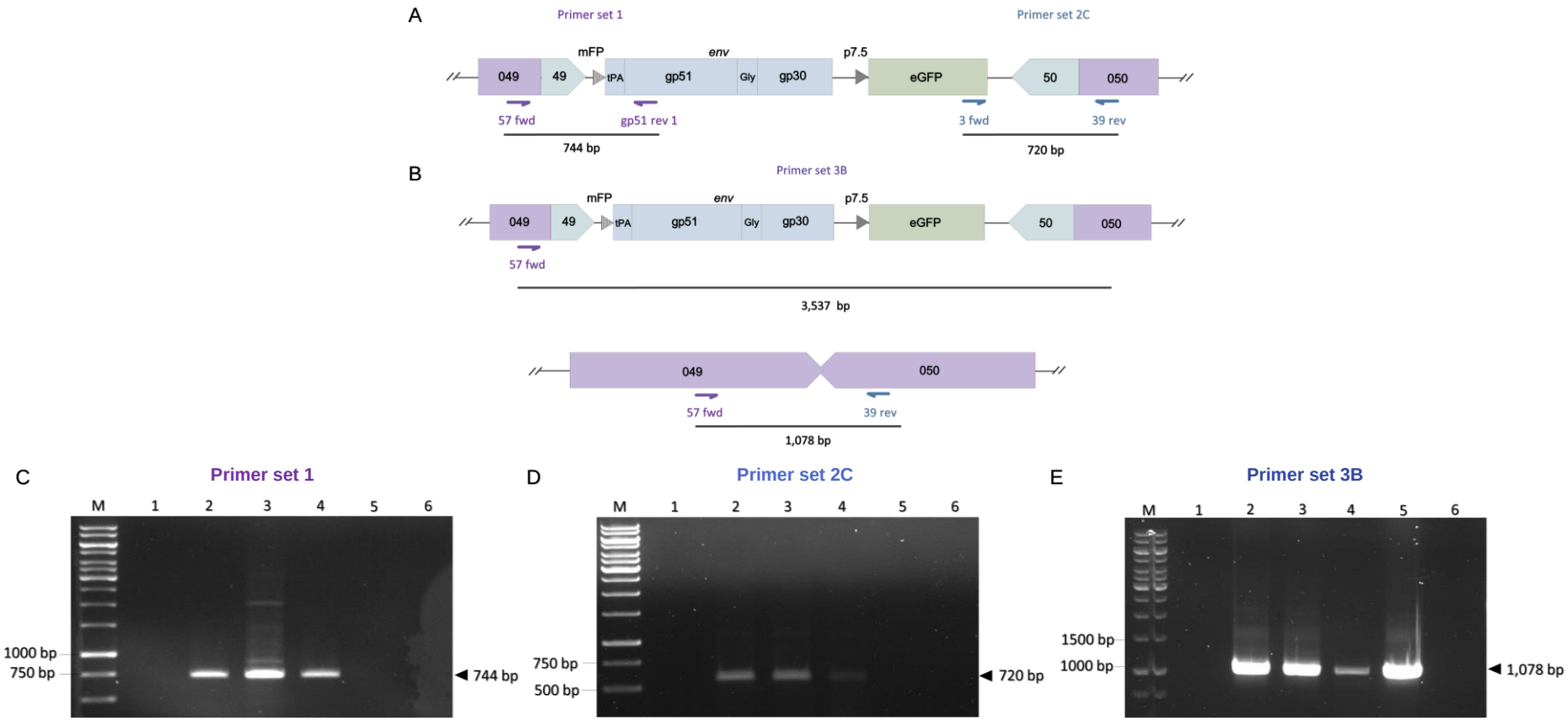



**Figure 4.37: PCR analysis of the putative LSDV-BLV-Env at passages 17 and 18.** Schematic diagrams showing **(A)** binding sites of primer set 1 (purple arrows) and primer set 2C (blue arrows) on the LSDV-BLV-Env gnome and **(B)** binding sites of primer set 3B (purple and blue arrows) on the LSDV-BLV-Env gnome and parental nLSDVSODis-UCT genome. PCR products are shown as a line with expected product size. See Figure 4.21 for the sequence annotation. **(C)** Agarose gel showing a 744bp product amplified with primer set 1 to detect the presence of a final and intermediate recombinant LSDV-BLV-Env into *049* ORF. **(D)** Agarose gel showing a 720bp product amplified with primer set 2C to detect the presence of a final and intermediate recombinant LSDV-BLV-Env into *050* ORF. **(E)** Agarose gel showing a 1,078bp product amplified with primer set 3B which detects the presence of a final recombinant LSDV-BLV-Env- and/or parental nLSDVSODis-UCT. **M**, 1 kb DNA ladder (Thermo Scientific, USA); **1**, total DNA from uninfected cells; **2,** total DNA from the passage 17 viral stock of recombinant LSDV-BLV-Env; **3,** total DNA from the passage 18 viral stock of recombinant LSDV-BLV-Env; **4**, total DNA from the passage 13 viral stock of recombinant LSDV-BLV-Env included as a positive control; **5,** parental viral DNA; 6, PCR control without template DNA.

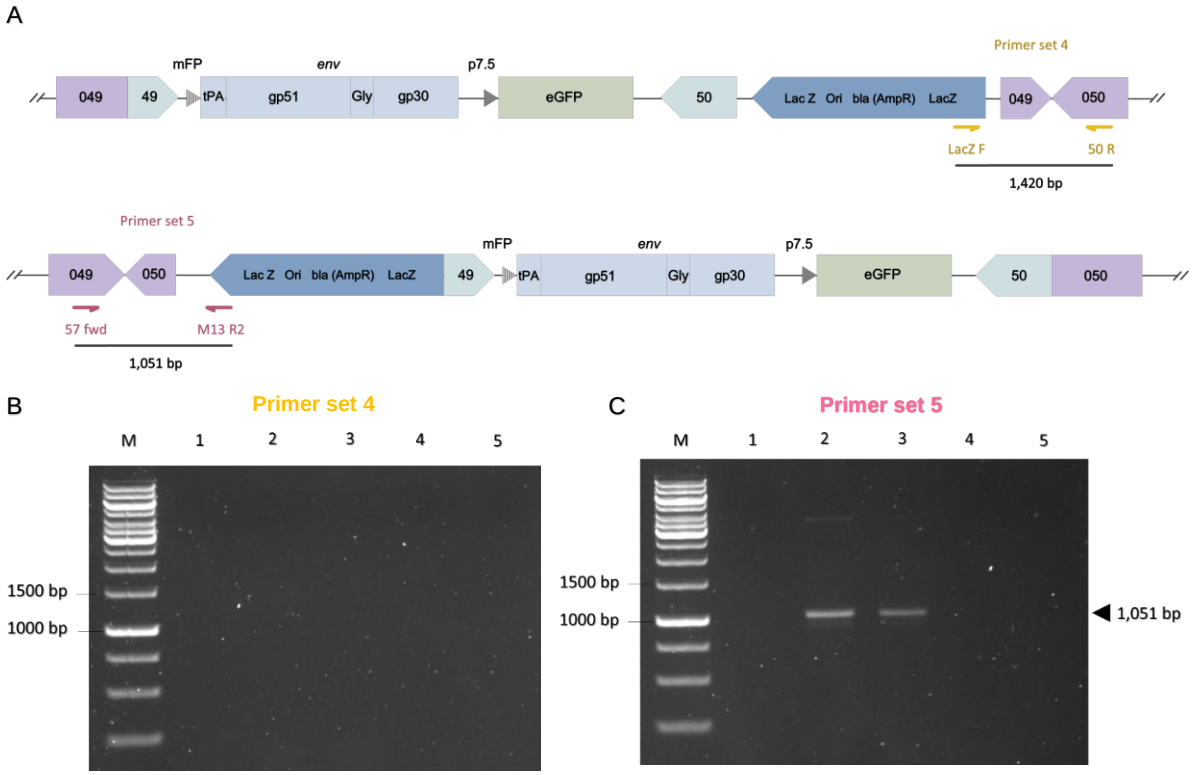



**Figure 4.38: PCR analysis of the putative intermediate LSDV-BLV-Env in passage 17 and 18 viral stocks.** **(A)** Schematic diagrams showing binding sites of primer set 4 (yellow arrows) and primer set 5 (red arrows) on the genome of a possible intermediate recombinant virus that recombined into the *049* ORF only (top) or into *050* ORF only (bottom). PCR products are shown as a line with expected product size. See Figure 4.25 A for the sequence annotation. **(B)** Agarose gel showing no amplification of a 1,420bp product using primer set 4. **(C)** Agarose gel showing amplification of a 1,051bp product with primer set 5. **M**, 1 kb DNA ladder (Thermo Scientific, USA); **1**, total DNA from uninfected cells**2,** total DNA from the passage 17 viral stock of recombinant LSDV-BLV-Env; **3,** total DNA from the passage 18 viral stock of recombinant LSDV-BLV-Env; **4**, parental viral DNA; **5**, PCR control without template DNA.

### 4.3.3.2.4.2. Confirmation of the integrity of the recombinant LSDV-BLV-Env by Sanger sequencing

To confirm that the recombinant LSDV-BLV-Env from the passage 17 and passage 18 viral stocks contained the correct BLV *env* gene, amplification of a 2,244bp fragment using 49 forward (fwd) and eGFP reverse (rev) 7 primers was performed (Table 4.7). This 2,244bp fragment containing 260bp of the 3' end of the 49 flank, the mFP promoter, the tPA signal sequence the entire BLV *env* gene and 365bp region of the beginning of the *egfp* gene (Figure 4.39 A) was selected for sequencing.

The 2,244bp fragment was successfully amplified from the passage 17 and 18 viral stocks of the recombinant LSDV-BLV-Env (Figure 4.39 B) and these PCR products were sequenced using five primers outlined in Table 4.7. The integrity of the mFP promoter, the tPA signal sequence, the BLV *env* gene as well as 260bp of the 49 flank and 365bp region of the beginning of the *egfp gene* were confirmed.

**Table 4.7: Primers used to amplify and sequence a 2,244bp fragment of the LSDV-BLV-Env genome**

| Primers | Orientation | Binding site | Purposes |
|---|---|---|---|
| 49 fwd | Forward | *049* ORF | Amplification of a 2,244bp fragment |
| eGFP rev 7 | Reverse | *egfp* | |
| Env rev 1 | Reverse | Env gp51 | Sequencing of the 2,244 PCR products |
| Env fwd 2 | Forward | Env gp51 | |
| Env fwd 6 | Forward | Env gp51 | |
| Env fwd 7 | Forward | Env gp30 | |

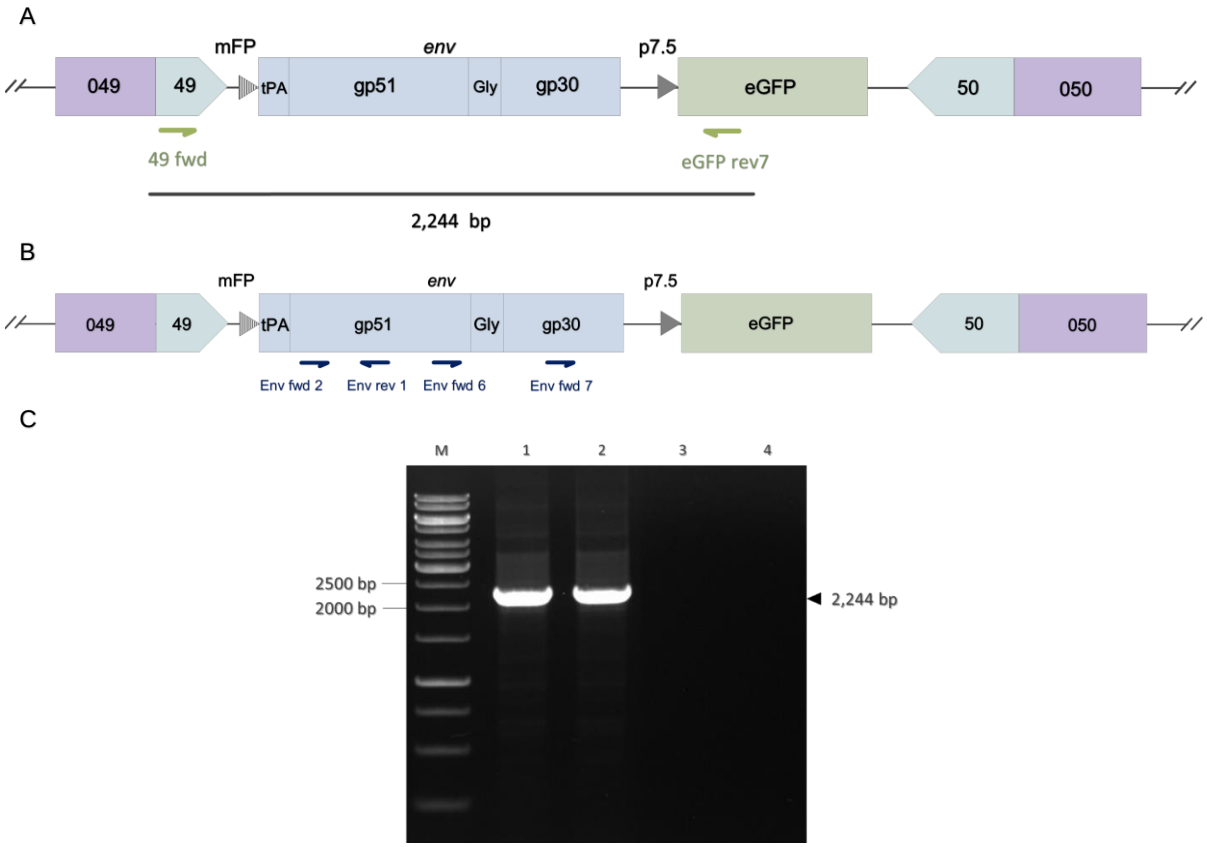



**Figure 4.39: Amplification of a 2,244bp fragment from the LSDV-BLV-Env genome for sequencing. (A)** A diagram showing binding sites of 49 forward (fwd) and eGFP reverse (rev) 7 primers (green arrows) on the recombinant LSDV-BLV-Env viral genome. PCR product is shown as a line with expected product size. **(B)** A diagram showing binding sites of five primers (blue arrows) on the recombinant LSDV-BLV-Env viral genome used to sequence the 2,244bp product. **(C)** Agarose gel showing amplification of a 2,244bp product with primer 49 fwd and eGFP rev7. **M**, 1 kb DNA ladder (Thermo Scientific, USA); **1**, total DNA from passage 17 viral stock **2,** total DNA from passage 18 viral stock of recombinant LSDV-BLV-Env; **3,** total DNA from uninfected cells; **4**, PCR control without template DNA.

### 4.3.3.2.4.3. Final confirmation of the BLV Env protein expression by immunofluorescence assay (IFA)

Since the anti-BLV Env gp51 monoclonal antibody (Veterinary Medical Research and Development, USA) utilised in this study recognises a conformational epitope on the Env gp51

glycoprotein, BLV Env expression from the recombinant LSDV-BLV-Env-Gag was not detected by Western blotting (See Section 4.3.2.2.3.2). Therefore, immunofluorescence assay (IFA) using the anti-BLV Env gp51 monoclonal antibody (anti-Env mAb) and anti-mouse IgG conjugated with Cy3 (anti-mouse IgG-Cy3) was performed to detect the BLV Env protein expression from the recombinant LSDV-BLV-Env. To allow the anti-BLV Env gp51 primary antibody to recognise the conformational epitope on the BLV Env protein, live-cell staining was performed by infecting MDBK cells with the passage 16 viral stock of the recombinant LSDV-BLV-Env.

In the technical control wells, no Cy3 expression was detected from MDBK cells that were not infected (Figure 4.40 A), infected with parental nLSDSODis-UCT virus (Figure 4.40 B) or infected with the recombinant LSDV-BLV-Env (Figure 4.40 C and D) and immunostained with the anti-mouse IgG-Cy3 only, confirming the absence of non-specific binding of the anti-mouse IgG-Cy3 to the MDBK cells, MDBK cells infected with parental nLSDVSODis-UCT virus and MSDBK cells infected with recombinant LSDV-BLV-Env. In experimental wells, no Cy3 expression was detected from MDBK cells that were not infected (Figure 4.40 E and G) and those infected with parental nLSDVSODis-UCT virus (Figure 4.40 F and H) when they were immunostained with the anti-Env mAb (1:50 or 1:100) and the anti-mouse IgG-Cy3, confirming the absence of non-specific binding of the anti-Env mAb against MDBK cells and the parental nLSDVSODis-UCT virus.

BLV Env expression was detected from all experimental wells, irrespective of the amount of the recombinant virus used for the infection and dilutions of the anti-Env mAb. Figure 4.41 shows two representative images of the BLV Env expression in MDBK cells infected with two different amounts (3µl or 5µl) of the recombinant virus and with two different dilutions of the anti-Env mAb (1:50 or 1:100). The anti-Env mAb appeared to be highly specific to the BLV Env protein only with an insignificant degree of background binding, which is consistent with IF results in the experimental controls. BLV Env expression was detected from almost all foci in each experimental well and the BLV Env expression appeared to be specifically concentrated in the foci. Furthermore, the BLV Env appeared to be actively transported from the infected cells or expressed on the surface of the infected cells.

Table 4.8 summaries the results from passaging potential recombinant LSDV-BLV-Env and assays to characterise the BLV env gene and its protein expression by PCR, sequencing and IFA.  potential recombinant LSDV-BLV-Env and assays to characterise the BLV env gene and its protein expression by PCR, sequencing and IFA.

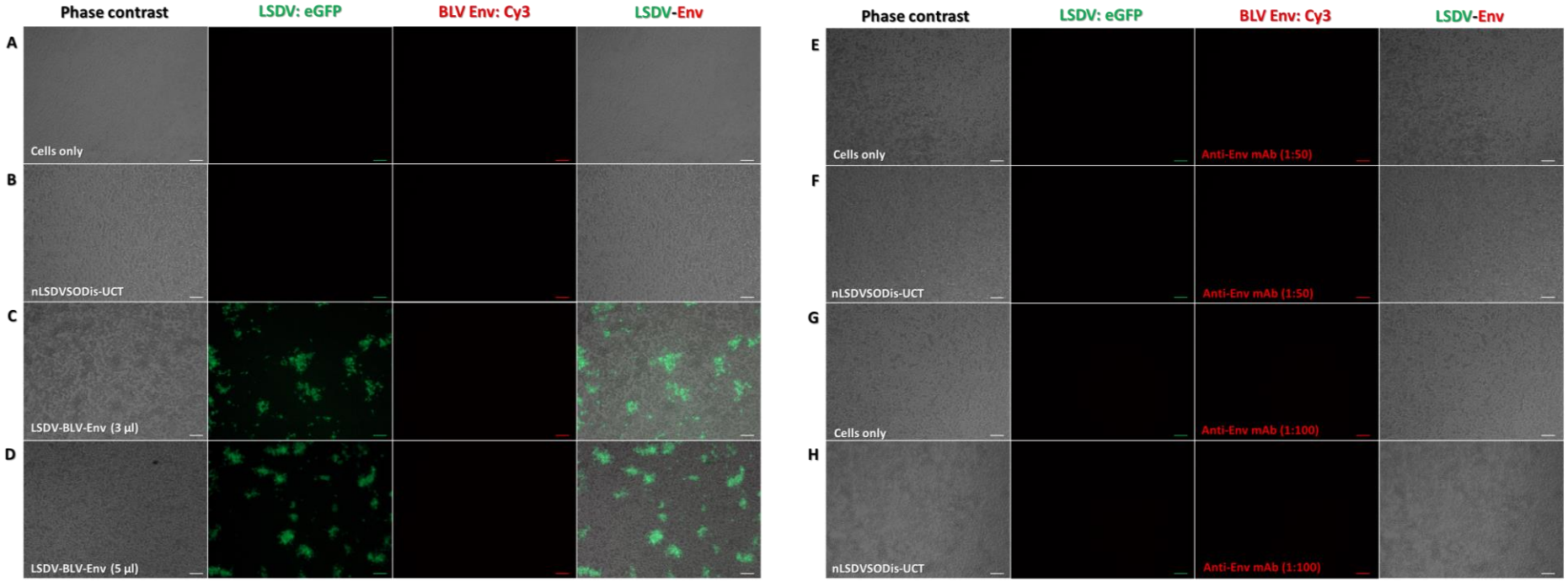



**Figure 4.40: Live-cell staining of technical controls.** MDBK cells were immunostained with **(A-D)** donkey anti-mouse Cy3-conjugated secondary antibody (anti-mouse IgG-Cy3) (1:500) only or **(E-H)** mouse anti-BLV Env gp51 primary antibody (anti-Env mAb) (1:50 or 1:100) followed by the anti-mouse IgG-Cy3. MDBK cells were either not infected (cells only), infected with nLSDVSODis-UCT at an MOI of 0.01 or infected with 3µl or 5µl of passage 16 viral stock of the recombinant LSDV-BLV-Env. MDBK cells infected with the recombinant LSDV-BLV-Env (LSDV) were visualized by their eGFP expression (green). All images were taken at 48 hours post-infection using a Zeiss Axiovert 200M fluorescence inverted microscope at 100x magnification. Scale bars represent 100µm.

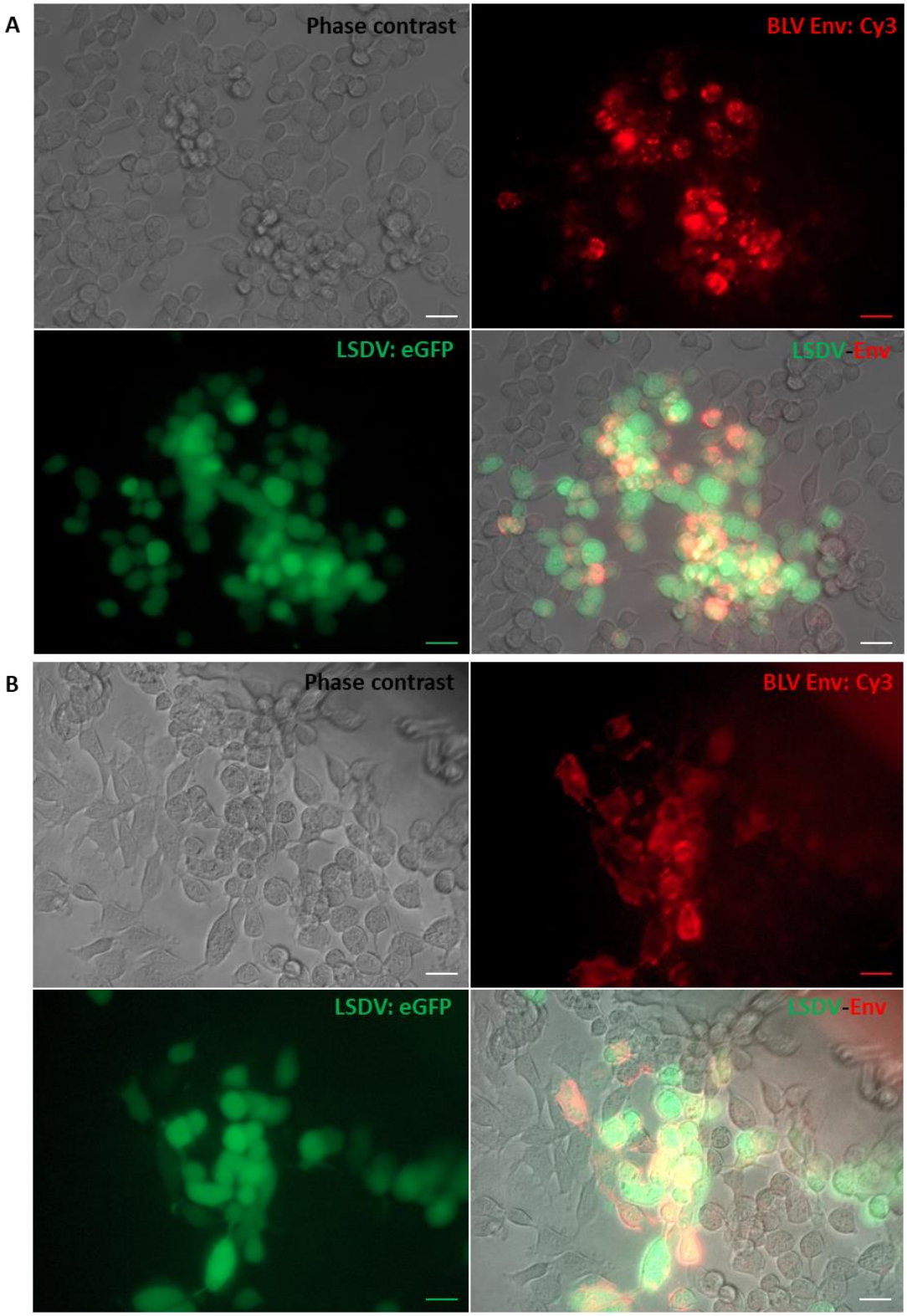



**Figure 4.41: Detection of the *in vitro* expression of the BLV Env protein in MDBK cells infected with passage 16 viral stock of the recombinant LSDV-BLV-Env**. **(A)** MDBK cells were infected with 3µl of the passage 16 viral stock of the recombinant LSDV-BLV-Env and immunostained with a mouse anti-BLV Env monoclonal antibody (anti-Env mAb) (1:50) followed by a donkey-anti-mouse IgG conjugated with Cy3 (anti-mouse IgG-Cy3) (1:500) **(B)** MDBK cells were infected with 5µl of the passage 16 viral stock of the recombinant LSDV-BLV-Env and immunostained with anti-Env mAb (1:100) followed by anti-mouse IgG-Cy3 (1:500). BLV Env protein expression was detected with the anti-mouse IgG-Cy3 (red). MDBK cells infected with the recombinant LSDV-BLV-Env (LSDV) were visualized by their eGFP expression (green). All images were taken at 48 hours post-infection using a Zeiss Axiovert 200M fluorescence inverted microscope at 400x magnification. Scale bars represent 200µm.

**Table 4.8: Table summarising results from passaging potential recombinant LSDV-BLV-Env and assays to characterise BLV Env antigen**

| | Fluorescent foci | | PCR analysis[c] | | | | | | Antigen integrity | Antigen expression |
|---|---|---|---|---|---|---|---|---|---|---|
| Passage No. | Possible recombinant LSDV[a] | Parental virus[b] | Intermediate or final recombinant LSDV | | Final recombinant LSDV | Parental virus | Intermediate recombinant LSDV | | Env (Sequencing)[d] | Env (IFA)[e] |
| | | | HR into 049 ORF | HR into 050 ORF | | | HR into 049 ORF | HR into 050 ORF | | |
| 1-3 | ✓ (Figure 4.29) | +++ | | | | | | | | |
| 4-6 | ✓ | + | | | | | | | | |
| 7 | ✓ | - | | | | | | | | |
| 8 | ✓ | ++ | | | | | | | | |
| 9 | ✓ (Figure 4.30) | ++ (Figure 4.30) | ✓ (Figure 4.31) | ✖ (Figure 4.31) | ✖ (Figure 4.31) | ✓ (Figure 4.31) | ✖ (Figure 4.32) | ✓ (Figure 4.32) | | |
| 10 | ✓ | ++ | | | | | | | | |
| 11-12 | ✓ | - | | | | | | | | |
| 13 | ✓ | ++ | ✓ (Figure 4.33) | ✓ (Figure 4.33) | ✖ (Figure 4.33) | ✓ (Figure 4.34) | | ✖ (Figure 4.34) | | |
| 14 | ✓ | + | | | | | | | | |
| 15 | ✓ (Figure 4.35) | ++ (Figure 4.35) | ✓ (Figure 4.36) | ✓ (Figure 4.36) | ✖ (Figure 4.36) | ✓ (Figure 4.36) | | | | |
| 16 | ✓ | ++ | | | | | | | | |
| 17 | ✓ | +++ | ✓ (Figure 4.37) | ✓ (Figure 4.37) | ✖ (Figure 4.37) | ✓ (Figure 4.37) | ✖ (Figure 4.38) | ✓ (Figure 4.38) | ✓ (Figure 4.39) | ✓ (Figure 4.41) |
| 18 | ✓ | +++ | ✓ (Figure 4.37) | ✓ (Figure 4.37) | ✖ (Figure 4.37) | ✓ (Figure 4.37) | ✖ (Figure 4.38) | ✓ (Figure 4.38) | ✓ (Figure 4.39) | |

a, Checkmark (✓) indicates the presence of possible recombinant LSDV-BLV-Env-Gag. b, Relative number of parental virus was shown by, +, ++ and +++. c, Checkmark represents amplification of expected PCR products which confirm the presence of a possible final or intermediate recombinant virus or parental virus and cross (✖) indicates no amplification of expected PCR products. d, Checkmark indicates the integrity of the BLV *env* gene by Sanger sequencing. e, Checkmark indicates the confirmation of the BLV Env protein expression by immunofluorescence assay (IFA) HR, homologous recombination; ORF, open reading frame.

## 4.4. DISCUSSION

Two recombinant LSDVs expressing BLV Env with (LSDV-BLV-Env-Gag) or without Gag (LSDV-BLV-Env) were constructed. The presence of BLV *gag* gene in the recombinant LSDV-BLV-Env-Gag was confirmed by Western blotting, which demonstrated extracellular and intracellular expression of the BLV Gag protein from the recombinant LSDV-BLV-Env-Gag. Due to time constraints, the BLV *env* and *gag* sequences in the recombinant LSDV-BLV-Env-Gag were not confirmed. The presence of the BLV *env* gene and the *in vitro* expression of the BLV Env protein from LSDV-BLV-Env was confirmed by PCR and sequencing and immunofluorescence assay (IFA), respectively. Parental viruses appeared to outgrow both the recombinants and thus, due to time constraints, purification of the final LSDV-BLV-Env-Gag and LSDV-BLV-Env recombinants was not achieved. Intermediate recombinant viruses were isolated whereby HR took place at the *050* ORF only and not at the *049* ORF. The implication of these results will be discussed, and some suggestions will be presented to address these issues.

### 4.4.1. Selection of the BLV *env* and *gag* antigen sequences of the recombinant LSDVs

The BLV *env* and *gag* nucleotide sequences of the BLV expression cassette were designed based on sequences from a Japanese isolate (GenBank accession number, AP018021). Precise design of antigen sequences is a first critical step in the development of efficacious vaccines but designing immunogenic antigens is one of the most difficult aspects in the vaccine development. Most pathogens have evolved to have various mechanisms to evade host immune responses, such as mutating their antigen sequences, reassorting their genome in co-infected cells and shielding surface antigens [534-536]. Consequently, some pathogens have extensive genetic diversity between and within genotypes or serotypes. It has been shown that despite being a member of the *Retroviridae* family, the BLV genome is less variable than HIV between different genotypes [341, 537]. Therefore, BLV Env and Gag antigen sequences were designed based on a sequence from a BLV natural isolate that was closest to the consensus sequence. The *env* and *gag* sequences of the selected Japanese isolate pvAK006 shared 99.5% and 99.8% nucleotide sequence identity, respectively and 100% amino acid sequence identity with the consensus Env and Gag sequences obtained by the alignment of 62 sequences of BLV natural isolates. Since the consensus Env and Gag sequences obtained from the sequence alignment are artificial sequences, they could be dissimilar to sequences that are currently circulating. Therefore, BLV *env* and *gag* sequences of a natural isolate that were closest to the consensus *env* and *gag* sequences were selected

for the design of the BLV transfer vectors to minimise sequence divergence between vaccine antigens and currently circulating strains (also see Chapter 2).

### 4.4.2. Selection of the insertion site in the nLSDVSODis genome

To construct the recombinant LSDVs, the BLV expression cassette was inserted into an intergenic region between *049* and *050* ORFs of the parental LSDV. The careful selection of an appropriate insertion site was made by analysing LSDV genomic sequence such that inserted transgenes would be expressed properly without altered functions or inactivation of protein products and the insertion of transgenes would not adversely affect the replication and gene expression of recombinant viruses (N. Douglass (University of Cape Town), personal communication). The LSDV genome has a central conserved region flanked by two identical inverted terminal repeats (ITRs) on both sides and contains 156 putative genes [532]. ORFs are located on both DNA strands [538]. A limited number of insertion sites in the poxvirus genome have been available as the central region of poxviruses has a complex structure of closely packed ORFs, which are continuous or partially overlapping or are spaced by short intergenic regions (IGRs) [532, 538, 539]. Transgenes can be inserted into loci of non-essential genes, major deletion regions or IGRs of poxvirus genomes.

Traditional insertion sites include non-essential genes such as *thymidine kinase* (*TK*) [510] and *haemagglutinin* (*HA*) genes [540, 541]. Since the parental LSDV viruses used in this study are based on the live-attenuated Neethling vaccine strain, we did not utilise traditional insertion sites to avoid possible insertional mutagenesis as well as a decrease in viral replication and immunogenicity. Furthermore, the use of the *TK* locus as an insertion site is restricted by the use of the murine LMTK$^-$ cell line, which is not permissive to LSDV.

Previously, Wyatt *et al*. [480] observed genetic instability of recombinant viruses and overgrowth of the recombinant variants as a result of deletions and mutations in transgenes inserted into the deletion (del) II and del III regions of the MVA genome, respectively. They postulated that since the deletion in the transgene extended into the non-essential sequences adjacent to the del II region, the deletion mutants were viable without selective pressure [480]. They also postulated that since no deletion but only mutations occurred in the del III region, which is flanked by essential genes encoding DNA ligase [542] and protein kinase [543], deletion affecting essential genes would be deleterious [480]. Based on this observation, they selected an intergenic region (IGRs) between two convergent ORFs of essential genes located in the conserved central region to improve the genetic stability of their recombinants [480]. They selected an intergenic region between the *I8R* gene encoding for the DNA/RNA helicase [544] and the *G1L* gene for the putative metalloproteinase [545, 546] and demonstrated that

the resultant recombinant virus was genetically stable [480]. We employed the same approach to aim at genetic stability of the recombinant LSDV and an intergenic region between convergent *049* and *050* genes, which are orthologues of the *vaccinia virus I8R* and *G1L* genes, located in the conserved central region of the LSDV genome was selected.

### 4.4.3. Selection of the heterologous poxvirus promoters and antigen expression

The BLV *env*, *gag* and *egfp* genes were placed under the control of the modified early fowlpox virus promoter (mFP) [438, 439], modified early-late promoter of the H5 gene of VACV (mH5) [440] and early-late promoter of a 7.5 kilodalton (kDa) polypeptide gene (p7.5) of vaccinia virus (VACV) [419], respectively.

The mFP promoter was designed from a native early-late FP promoter [439] to retain an early promoter activity by removing a late promoter element [438]. Since a previous study [446] demonstrated stronger activity of the native early-late FP promoter than the p7.5 promoter but weaker than the strong late p11 promoter (promoter for the 11kDa polyprotein of VACV), we speculated that the mFP promoter would also be a moderate or strong promoter, equivalent to the p7.5 promoter but weaker than the mH5 promoter and a compact synthetic promoter (pS). However, as described in Chapter 3, the activity of the mFP promoter appeared to be weaker than that of the p7.5 promoter. At that stage, it was uncertain if this may have had an impact on the expression of the BLV Env protein. However, the immunofluorescence assay demonstrated abundant expression of the BLV Env protein on the surface of cells infected with the recombinant LSDV-BLV-Env.

The BLV *gag* gene was placed under the control of the mH5 promoter. As the retrovirus Gag protein is known to elicit T cell responses [244, 547-551] and the virion morphogenesis takes place at the late stage of the viral life cycle, the strong early-late mH5 promoter was selected for the expression of the BLV Gag from the recombinant LSDV-BLV-Env-Gag. Western blots confirmed the extracellular and intracellular BLV Gag expression at 24 hours post-infection and the sustained expression during the 72-hour study period. To confirm that the budding BLV Gag particles form virus-like particles, electron microscopy analysis will be necessary.

### 4.4.4. Challenges in the screening of the recombinant LSDVs

Two recombinant LSDVs were constructed using two different parental LSDV viruses. These parental viruses differ in the presence or absence of selectable genes. The first recombinant LSDV-BLV-Env-Gag was constructed using nLSDVSODis-M containing the *Ecogp* selection

gene and mCherry marker gene whereas the second recombinant LSDV-BLV-Env was constructed using nLSDVSODis-UCT, which does not contain any selectable genes.

It was difficult to detect the parental nLSDVSODis-UCT during the passaging of the second recombinant LSDV-BLV-Env. This parental virus did not contain a marker gene and the detection of the parental virus relied solely on the detection of non-fluorescing foci. As a result, some slow-growing parental viruses were only detectable at 6 days post-infection and in some cases, parental viruses were not detectable at all even after further incubation. Longer incubation might have helped the detection of non-fluorescing foci but also appeared to have enhanced the propagation of parental viruses. It was also difficult to determine whether the CPEs were true foci formed by parental viruses or were simply cell aggregation. Furthermore, some viruses infect cells but do not always form characteristic foci. These viruses are only detectable if they produce fluorescent proteins. Lülf *et al*. [552] also observed that more than 70% of the MVA virions existed as non-plaque forming viruses or single infected cells whereas only 20-30% of them resulted in the formation of plaques. This could be another reason why it was difficult to detect a small fraction of the non-fluorescing and non-plaque forming parental viruses during the purification of the recombinant LSDV-BLV-Env.

These issues together highlight the need for more effective ways to screen and “rescue” recombinant viruses out of a large population of parental viruses. One approach is the use of dominant host-range selection [423, 553, 554]. The dominant host-range selection is a marker-free selection method and is a derivative of the rescue techniques that utilise replication-incompetent or functionally defective viruses [555, 556]. The dominant host-range selection method takes advantage of the poxvirus host-range genes that limit the replication of poxviruses in specific hots or cell lines. Discrimination between recombinant and parental viruses is based on the presence of a host-range gene in recombinant viruses and cultivation of recombinant viruses in a cell line that is permissive to recombinant viruses but non-permissive to parental viruses. In the absence of the host-range gene, parental viruses are not able to replicate or only able to replicate less efficiently in the non-permissive cell line. To utilise this system, either a host-range gene can be deleted from the recombinant viral genome [553, 555, 556] or in the case of attenuated poxviruses, a host-range gene can be re-introduced into the recombinant viral genome [423, 554]. One of the well-known examples is the restoration of the vaccinia virus K1L gene into the MVA genome, thereby enabling the recombinant MVA to grow in rabbit kidney cells where the wild-type MVA cannot replicate [423]. This method is not only a stringent way to screen recombinant viruses but also a more efficient way to purify recombinant viruses without issues regarding outgrowth of parental viruses or selection pressure that will potentially cause mutations/fitness cost to recombinant viruses. The wild-type LSDV is already host-restricted and only able to replicate in ovine and

bovine cells. It has not been determined whether the introduction of a heterologous host-range gene (e.g. K1L gene of vaccinia virus) into the LSDV genome confers a new host range that can be utilised for the dominant host-range selection. However, it is worth assessing and this will expand the utility of the LSDV as an expression vector.

### 4.4.5. Challenges in the purification of the recombinant LSDVs

Apart from the delayed or no visible detection of parental viruses during the purification process, another issue was the persistence of the parental viruses after numerous passages. During the purification of both recombinant LSDVs, parental viruses persisted up to the final passages. In addition, a significant outgrowth of parental viruses over the recombinant virus was observed during the passaging of the LSDV-BLV-Env-Gag. We speculated that since the purification of the recombinant LSDV-BLV-Env-Gag was performed mostly by using cell lysates and cell lysates would have contained numerous parental viruses, the purification of the recombinant LSDV-BLV-Env was carried out mainly by picked foci as picking individual foci several times during the first several rounds prior to the confirmation of the absence of parental viruses has been shown to be effective in the removal of parental viruses [557, 558]. Agarose overlay method was also attempted to reduce the chance of picking both recombinant and parental viruses [559].Despite these improved methods, the parental virus persisted in the recombinant LSDV-BLV-Env viral stocks. As the aim of the construction of the second recombinant LSDV-BLV-Env was to assess the effect of the presence of Gag in the first recombinant LSDV-BLV-Env-Gag on the slow growth, we can conclude that the presence or absence of Gag was unlikely to contribute to the slow growth.

One possible explanation for the preferential growth of the parental viruses over the recombinant LSDVs concerns the fitness cost of the recombinant viruses. Since the parental viruses did not contain the BLV expression cassette as an extra metabolic burden, they had higher fitness than the recombinant viruses and were able to grow faster than recombinant viruses. Furthermore, the persistence and outgrowth of the parental viruses were also because there was no selective pressure that favoured the growth of slow-growing recombinant viruses or selective pressure that opposed the propagation of the fast-growing parental viruses (c.f., dominant host-range selection as described above).

The persistence and overgrowth of the parental viruses could be also due to the presence of the intermediate recombinant viruses (“single cross-overs”). An intermediate recombinant virus whereby recombination into *050* ORF only had occurred and not *049* ORF was detected from both recombinant LSDVs using primer set 5. An intermediate recombinant virus that had recombined into *049* ORF was not detected using primer set 4. However, it is possible that

this was due to an inefficient PCR amplification as there was no positive control available to confirm that the PCR was working. Unsuccessful amplification of an approximately 800bp region spanning the end of the *egfp* gene and the *050* ORF (e.g., passage 9, 13 and 15 and more PCR experiments whose data are not shown) of the recombinant LSDV-BLV-Env, even using modified versions of primer set 2 (i.e., primer set 2B and 2C) as well as different DNA polymerase and thermocycling conditions during most passages of both recombinant LSDVs indicates the presence of an intermediate recombinant virus whereby recombination into *049* ORF only had occurred.If this intermediate recombinant virus outnumbered final recombinant viruses, the primer set 2 would preferentially bind to this intermediate recombinant viral genome and would amplify an approximately 4.5 kb fragment (shown as a dashed line in Figure 4.42). However, the amplification of this large fragment using PCR conditions set to amplify an approximately 800bp fragment was impossible. Consequently, the remaining primers that had not bound to the intermediate recombinant virus bound to a small number of final recombinant LSDV genomes, thereby resulting in a smaller amount of the PCR products. If some of these intermediate recombinant viruses had a second intramolecular HR at the same locus used as the first HR, the entire BLV expression cassette would be excised and the virus would revert to the parental virus (Figure 4.42).

HR is a rare event where generally only 1 in $10^3$ to $10^4$ progeny viruses are a recombinant virus [557, 560, 561]. However, the persistence of the intermediate recombinant virus was enigmatic. Since the intermediate recombinant viruses contained the entire transfer vectors, this would result in an extra 4,227bp for the recombinant LSDV-BLV-Env-Gag and an extra 3,507bp for the recombinant LSDV-BLV-Env compared to the final recombinant viruses. It is also surprising that the consecutive plaque purification used to isolate the recombinant LSDV-BLV-Env was not effective in removing the intermediate recombinant virus.

HR efficiency can be influenced by sizes and types of transgenes. For example, insertion of a large sequence or multiple sequences as well as genes potentially toxic to cells, such as a protease, appears to be less efficient [562]. Since the intermediate recombinant virus was detected in both recombinant LSDVs, this indicates that neither the size of the BLV expression cassettes (3,732bp for the recombinant LSDV-BLV-Env-Gag *vs* 2,447bp for the recombinant LSDV-BLV-Env) nor the presence of the BLV Gag contributed to the incomplete HR.

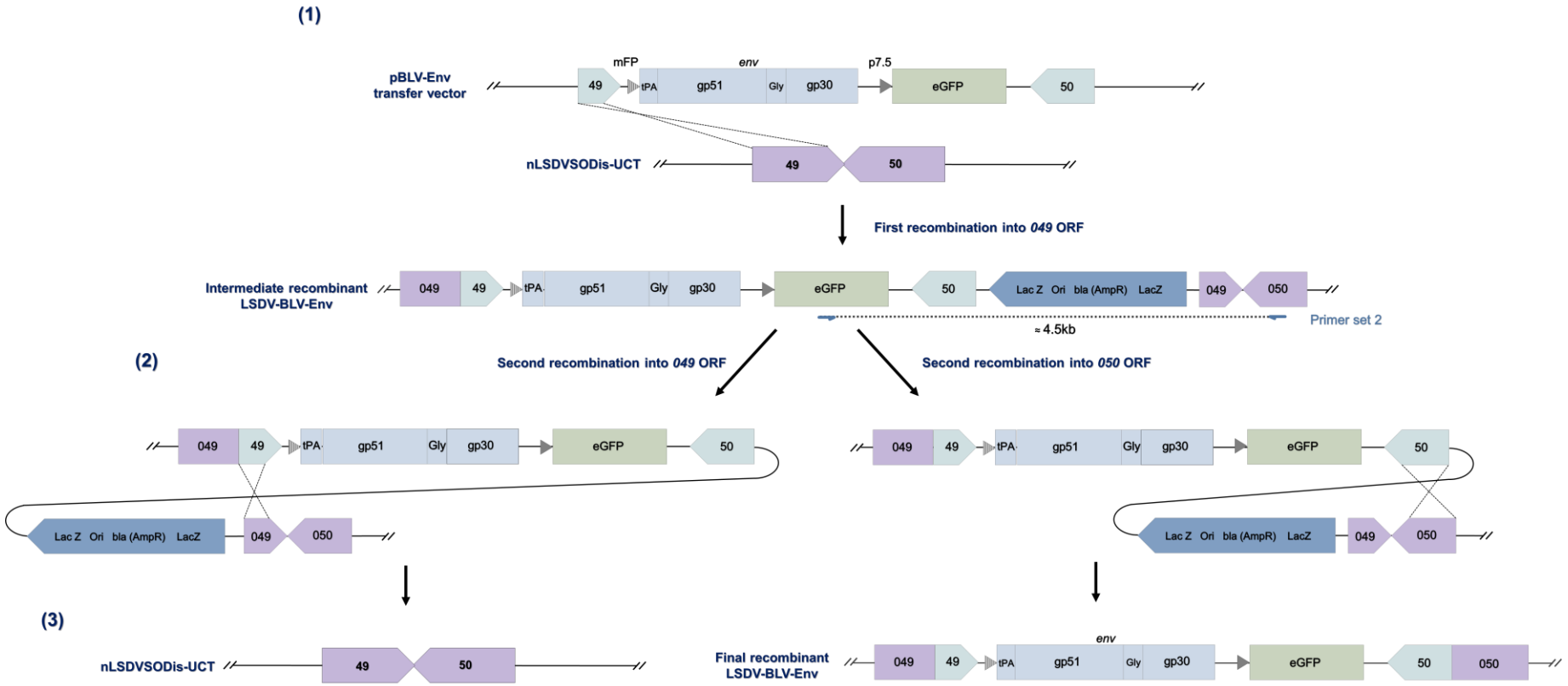



**Figure 4.42: A schematic diagram outlining a possible process of generation of intermediate recombinant viruses and the subsequent generation of the revertant parental virus and the final recombinant virus. (1)** When the first recombination (dashed lines) between the pBLV-Env transfer vector and the parental nLSDVSODis-UCT occurs into *049* ORF only, this results in the intermediate recombinant LSDV-BLV-Env that had incorporated the entire transfer vector, instead of BLV expression cassette only. Binding sites of primer set 2 (blue arrows) and an expected PCR product size (dashed line) are indicated in the diagram of a possible intermediate recombinant viral genome. **(2)** The second recombination can occur intermolecularly into *049* ORF or *050* ORF. **(3)** The second recombination once again into *049* ORF generates a revertant parental virus that has lost the entire transfer vector whereas the second recombination into *050* ORF generates final recombinant LSDV-BLV-Env that contain BLV expression cassette between *049* and *050* ORFs. Possible intermediate recombinant LSDV-BLV-Env that recombined into *049* only was used as an example.

To overcome the low efficiency of HR and to circumvent the generation of intermediate recombinant variants, the HR system can be optimised. The topology of plasmid DNA significantly affects HR efficiency and one possible manipulation is linearisation of transfer vector plasmid DNA. Yao *et al.* [563] compared HR efficiency using a set of plasmids in VACV-infected cells and demonstrated that HR efficiency between two liner plasmids was significantly high whereas the HR between two circular plasmids was 15 to 50 times less frequent than the linear-to-linear recombination. Since the poxviral genome is already linear, transfection of linearised plasmid DNA into poxvirus-infected cells is expected to increase the HR efficiency.

Apart from the manipulation of the plasmid DNA, the target viral DNA and the HR system can be manipulated. The first approach takes advantage of the intrinsic DNA-repair mechanism. Since HR efficiency significantly increases when the double-stranded break (DSB) is created in genomes during the viral life cycle [564], DSB can be artificially introduced into the target viral genome by an exogenous nuclease [565-567]. In this method, it is critical to reduce the chance of random excision and insertion into non-target insertion sites [568]. CRISPR/Cas9 system is able to meet both requirements of precise excision and insertion at target sites. Yet, another factor that may still potentially reduce HR efficiency is that DSB can be repaired either by HR or non-homologous end joining (NHEJ) [564]. These two DNA-repair pathways appear to compete with each other and the blockage of one pathway leads to the predominant use of the other pathway [569, 570]. NHEJ does not rely on homologous sequences to repair DSB and this DNA-repair mechanism is infamously known as an error-prone repair system where mutations can be introduced at the site of DSB [564]. This concerns the potency of vaccines. To circumvent this consequence, Hu *et al*. [571] and others [572-576] devised elegant methods utilising a cellular DNA ligase IV inhibitor SCR7 to block the NHEJ pathway. For example, they combined the use of the CRISPR/Cas9 system, cellular DNA ligase IV inhibitor SCR7 and single-stranded oligodeoxynucleotides (ssODN) as a guide template [577, 578] and demonstrated increased HR and decreased NHEJ compared to reaction without the SCR7 and/or ssODN [571]. This method is applicable in the development of recombinant poxviruses as despite cytoplasmic replication [579], poxviruses utilise the cellular DNA ligase IV in their NHEJ pathway [580]. By combining these methods, HR efficiency during the generation of recombinant viruses could be dramatically improved.

### 4.4.6. Concluding remarks and future work

This study provided an opportunity to prove how critical it is to understand the virology of poxviruses in order to design and develop a poxvirus-vectored vaccine as the resultant

vaccine needs to be stable, safe and potent. Two recombinant LSDVs were constructed and both recombinant LSDVs appeared to have a slower growth rate than the parental viruses. Furthermore, the purification of the recombinant LSDVs was challenging and the non-recombinant viruses (i.e., intermediate recombinant viruses and parental viruses) were detected in both recombinant LSDVs. Nevertheless, expression of the BLV Env and Gag antigens from the recombinant LSDVs were confirmed. Further purification of the recombinant LSDVs and confirmation of the formation of VLPs by confocal microscopy is necessary.

# APPENDICES

## APPENDIX A: REAGENT FORMULATIONS

**0.8% (w/v) agarose gel**

| | |
|---|---|
| Agarose | 3.2g |
| TBE | 400ml |
| Ethidium bromide | 20µl |

**1x Bjerrum Schafer-Nielsen transfer buffer (500ml)**

| | |
|---|---|
| Glycine | 1.465g |
| Tris | 2.91g |
| Methanol | 100ml |
| $ddH_2O$ | Adjust to 500ml |

**2.5% (w/v) BSA/PBS blocking buffer**

| | |
|---|---|
| BSA | 2.5g |
| 1x PBS | 100ml |

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

| | |
|---|---|
| Dulbecco's Modified Eagle Medium (DMEM) | 500ml |
| 1% Penicillin-Streptomycin (Pen-Strep) | 5ml |
| 10% Heat-inactivated foetal calf serum (FCS) | 50ml |
| 0.1% Fungin (optional) | 0.5ml |

| **Freezing medium** | Concentration (v/v) |
|---|---|
| Heat-inactivated fetal calf serum (FCS) | 80% |
| Dimethyl sulfoxide (DMSO) | 10% |
| DMEM | 10% |

**Luria-Bertani agar (100ml)**

| | |
|---|---|
| Tryptone | 1g |
| Yeast extract | 0.5g |
| NaCl (200mM) | 0.5g |
| Agar | 1.5g |
| $ddH_2O$ | Adjust to 100ml |

**Luria-Bertani broth (100ml)**

| | |
|---|---|
| Tryptone | 1g |
| Yeast extract | 0.5g |
| NaCl (200mM) | 0.5g |
| $ddH_2O$ | Adjust to 100ml |

**Lysis solution (P2 buffer)**

| | |
|---|---|
| NaOH (200mM) | 8g in 900ml of $ddH_2O$ |
| 1% (v/v) SDS | 100ml of 10% SDS in 1L of $ddH_2O$ |

**Naturalising solution (P3 buffer) (100ml)**

| | |
|---|---|
| Potassium acetate (3.0M, pH 4.8-4.9) | 4.41g |
| Glacial acetate | 7.5ml |
| $ddH_2O$ | Adjust to 15ml |

**1x PCR buffer**

| | |
|---|---|
| KCl | 0.187g |
| Tris-HCl (1M) | 500µl |
| $MgCl_2 \cdot 6H_2O$ | 0.0255g |
| $ddH_2O$ | Adjust to 50ml |
| Proteinase K (1 mg/ml) | 500µl |

**1x phosphate-buffered saline Tween (PBST) (1000ml)**

| | |
|---|---|
| Tween 20 | 10ml |
| 10x PBS | 100ml |
| $ddH_2O$ | Adjust to 1000ml |

**4x protein loading buffer**

| | |
|---|---|
| 100% glycerol | 4ml |
| 1.5M Tris/HCl (pH 6.8) | 1.6ml |
| SDS | 0.8g |
| Beta-mercaptoethanol | 0.5ml |
| Bromophenol blue | 4mg |
| $ddH_2O$ | Adjust to 3.9ml |

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

| | |
|---|---|
| 40% acryl-bisarylamide mix | 2ml |
| 1.5M Tris (pH 8.8) | 2.5ml |
| 10% SDS | 0.1ml |
| 10% APS | 0.1ml |
| TEMED | 6µl |
| $ddH_2O$ | 5.3ml |

**Resuspension solution (P1 buffer)**

| | |
|---|---|
| Tris-HCL (1 M, pH 8.0) | 2.5ml |
| 20% (w/v) glucose | 0.91 g of glucose in 4.55ml of $ddH_2O$ |
| EDTA (0.5 M, pH 8.0) | 2ml |
| $ddH_2O$ | 0.95ml |
| RNase (200µg/ml) | 400µl |

**10x SDS PAGE running buffer**

| | |
|---|---|
| SDS | 10g |
| Tris | 30.3g |
| Glycine | 29g |
| $ddH_2O$ | Adjust to 1000ml |

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

| | |
|---|---|
| 40% acryl-bisarylamide mix | 0.5ml |
| 1.5M Tris (pH 6.8) | 0.68ml |
| 10% SDS | 50µl |
| 10% APS | 50µl |
| TEMED | 5µl |
| $ddH_2O$ | 3.8ml |

**5x Tris-borate-EDTA (TBE) buffer (1L)**

| | |
|---|---|
| Tris base | 54g |
| Boric acid | 27.5g |
| 0.5 M EDTA (pH 8.0) | 20ml |
| $ddH_2O$ | Adjust to 1L |

**Tris-EDTA ($T_{10}E_1$) buffer**

| | |
|---|---|
| Tris-HCL (1 M) | 1ml |
| EDTA (0.5 M, pH 8.0) | 200µl |
| $ddH_2O$ | Adjust to 100ml |

# APPENDIX B: STANDARD PROTOCOLS

## B1. Molecular cloning

### B1.1. Restriction endonuclease digestion of plasmids

One microgram (5µg in the case of the cloning of the pLEO-eGFP) of plasmid DNA was digested with 1µl of restriction endonuclease(s) in a 25µl (60µl in the case of the cloning of the pLEO-eGFP) reaction mixture containing 2.5µl of 10x reaction buffer and double-distilled water (ddH2O). Following a one-hour incubation at 37°C, the digested plasmid DNA was resolved on an ethidium-bromide (0.5µg/ml) stained 0.8% agarose gel in 1x tris- borate-EDTA (TBE) buffer (Appendix A) at 100V for up to 2 hours (Nippon Genetics, Japan). DNA fragments were gel purified using the Zymoclean Gel DNA Recovery Kit (Zymo Research, USA) as per the manufacturer's protocol.

### B1.2. Blunting of restriction sites by T4 DNA polymerase

Where necessary T4 DNA polymerase was used to blunt the 5' and 3' DNA overhangs generated by restriction endonuclease digestion. Plasmid DNA was transferred into a 30µl reaction mixture containing 1µl of T4 DNA polymerase (New England BioLabs, USA), 3µl of 10x T4 DNA Ligase Reaction Buffer (New England BioLabs, USA), 1µl of Deoxynucleotide (dNTP) Solution Mix (10mM) (New England BioLabs USA) and $ddH_2O$. The reaction mixture was incubated at 12°C for 15 minutes. The blunting reaction was terminated by heating the reaction mixture to 75°C for 20 minutes.

### B1.3. DNA ligation

Linearised vector and insert plasmid DNA were ligated in a 50µl reaction mixture containing 1µl of T4 DNA Ligase (New England BioLabs, USA), 5µl of 10x T4 Ligase Reaction Buffer (New England BioLabs, USA) and $ddH_2O$ with 1-hour incubation at room temperature (20°C to 25°C). For the p7.5-eGFP, pBLV-Gag-eGFP, pmH5-eGFP and pS-eGFP reporter plasmids, two experimental ligation mixtures were prepared. A reaction mixture without T4 DNA Ligase was included as a negative control. Prior to transformation, the ligation reactions were heat-inactivated by incubating the reaction tubes in a heating block at 65°C for 10 minutes.

### B1.4. Transformation of plasmid DNA

Once the heat-inactivated plasmid DNA was cooled down to room temperature, transformation of chemically competent *E. coli* cells was conducted. An aliquot of 1.5µl of the ligation mixture

was transferred into an ice-cold 1.5ml Eppendorf tube containing 15µl of defrosted *E. cloni* 10G chemically competent cells (Lucigen, USA). The plasmid and *E. coli* cells were mixed gently, and the reaction mixture was left on ice for 20 minutes to allow the crystallisation of DNA molecules. The transformation was initiated with heat shock, in which the reaction mixture was first placed on the heating block at 42°C for 45 seconds and then immediately placed back on ice. The reaction mixture was left on ice for 2 minutes to allow the reaction to complete. An aliquot of 350µl of pre-warmed Recovery Medium (Lucigen, USA) was added to the transformed bacterial cells and the bacterial culture was incubated at 37°C for 1 hour. Then, the transformed bacterial cells were plated on Luria-Bertani agar plates (Appendix A) containing carbenicillin (50µg/ml) (Thermo Fisher Scientific, USA). The plates were incubated at 37°C overnight.

**B1.5. Small-scale plasmid DNA isolation**

Bacterial colonies were picked from the agar plates and inoculated into 1.6ml of the Luria-Bertani (LB) broth (Appendix A) containing carbenicillin (50µg/ml) (Thermo Fisher Scientific, USA) in 2ml Eppendorf tubes. The bacterial cultures were incubated overnight in a 37°C shaking incubator at 250 rpm.

Eight hundred microliter of each of the bacterial culture was transferred into sterile 2ml Eppendorf tubes and the bacterial cultures were centrifuged at 20,800g for 1 minute. After removing the supernatant, samples were placed on ice, and the bacteria pellets were re-suspended in 250µl of resuspension solution (P1 buffer) (Appendix A) and the cell pellet was re-suspended completely by pipetting the solution up and down. Then, 500µl of lysis solution (P2 buffer) (Appendix A) was added and the lysis of the cells was facilitated by inverting the tube gently several times. Samples were incubated on ice for 5 minutes. Finally, 375µl of neutralising solution (P3 buffer) (Appendix A) was added to the lysate and the sample was inverted several times to mix it thoroughly. Following a 10-minute incubation on ice, white aggregates of denatured protein were removed by centrifugation at 20,800g for 10 minutes, and 700µl of the supernatant containing plasmid DNA was carefully transferred into new 2ml Eppendorf tubes.

An aliquot of 700µl of ice-cold isopropanol was added to each of the reaction tubes to precipitate the plasmid DNA and the samples were mixed thoroughly by inverting the tubes gently several times. The reaction mixtures were kept on ice for 15 minutes to allow the reaction to complete. The plasmid DNA was then pelleted by centrifugation at 20,800g for 20 minutes. The supernatant was discarded, and the residual salt was removed from the pellet by rinsing it with 180µl of ice-cold 70% ethanol. The samples were centrifuged at 20,800g for

10 minutes and the 70% ethanol was discarded. After air-drying the pellet, the plasmid DNA was re-suspended in 50µl of TE buffer (Appendix A). The sample was stored at 4°C for future use.

### B1.6. Large-scale purification of plasmid DNA

Eight microliters of the bacterial culture were transferred into 5ml of LB broth (Appendix A) containing carbenicillin (50µg/ml) (Thermo Fisher Scientific, USA) and incubated for 6 to 8 hours in a 37°C shaking incubator at 250rpm. This starter culture was then, transferred into a 2-litre flask containing 150ml of LB broth (Appendix A) and carbenicillin (50µg/ml) (Thermo Fisher Scientific, USA). The flasks were incubated overnight at 37°C with shaking at 250rpm. Plasmid DNA was then extracted from overnight bacterial cultures using the ZymoPURE II Plasmid Maxiprep Kit (Zymo Research, USA) as per the manufacturer's instructions. Concentration and purity of the extracted plasmid DNA were measured using Nanodrop®1000 (Thermo Fisher Scientific, USA).

## B2. Western blotting

### B2.1. Crude extraction of total proteins from MDBK cell infected virus

An aliquot of 500µl of culture media was aspirated and transferred into 1.5ml Eppendorf tubes. The remaining culture media were discarded and each well was washed twice with 500µl of 1x phosphate-buffered saline (PBS) (Thermo Fisher Scientific, USA). Then, 100µl of 1x Glo lysis buffer (Promega, USA) was added to each well, and the plate was incubated at room temperature for 5 minutes. The entire volumes of the crude cell lysates were transferred into new 1.5ml Eppendorf tubes, and the cell lysates were centrifuged at 12,100g for 10 minutes. Following the centrifugation, the supernatant was carefully recovered and transferred into sterile 1.5ml Eppendorf tubes. Both culture media and cell lysates were stored at -20°C until needed.

### B2.2. Sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) and electroblotting

The total proteins contained in the culture media and cell lysate were resolved by sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) at 250 V, using discontinuous gels consisting of a 4% stacking gel (pH 6.8) and an 8% resolving gel (pH8.8) in a Tris-glycine buffer system (Appendix A). Following the completion of the SDS-PAGE, the

polyacrylamide gel was recovered and soaked in Bjerrum and Schafer-Nielsen transfer buffer (Appendix A) prior to the transfer of the resolved proteins onto polyvinylidene difluoride (PVDF) membranes. PVDF membranes were activated by soaking them briefly in methanol and immediately washing with the Bjerrum and Schafer-Nielsen transfer buffer. The resolved proteins were transferred onto the PVDF membranes using Trans-Blot SD Semi-Dry Transfer Cell (BioRad, USA) at 25V for 1 hour.

### B2.3. Western blotting

Upon completion of the electroblotting, the PVDF membranes were incubated in a 2.5% BSA/PBST blocking buffer (Appendix A) on a shaker with gentle agitation for 2 hours at room temperature. Then, the PVDF membranes were incubated overnight at room temperature with an antigen-specific primary antibody at an appropriate dilution. The PVDF membranes were subjected to five cycles of washing with phosphate-buffered saline containing 1% Tween 20 (PBST) (Appendix A) and a 15-minute incubation on a shaker with gentle agitation. Subsequently, the PVDF membranes were probed with an anti-goat/sheep IgG monoclonal antibody conjugated with alkaline phosphatase (Sigma-Aldrich, USA) at appropriate dilution and incubated at room temperature for 2 hours on a shaker with gentle agitation. The membranes were washed five times with PBST and incubated for 15 minutes each time as described above. Lastly, 5ml of BCIP/NTB phosphatase substrate (KPL/SeraCare, USA) was added to the PVDF membranes. The PVDF membranes were incubated at room temperature in the dark for 5 to 15 minutes prior to the termination of the reaction by washing the membrane with PBST. The membrane was left dry in the dark for 1 hour prior to scanning the membrane.

## B3. Standard protocol for PCR

### B3.1. Crude extraction of total DNA from MDBK cells infected with a virus by alkaline lysis

Culture media was removed and 500µl of resuspension solution (P1 buffer) (Appendix A) were added to each well. Then, 500µl of lysis solution (P2 buffer) (Appendix A) was added to each well and cells were lysed and collected by pipetting the solution several times over the cell layer. Crude cell lysates were transferred into 1.5ml Eppendorf tubes and 700µl of neutralising solution (P3 buffer) (Appendix A) was added to each tube. The contents were mixed by inverting the tubes several times. White aggregates of denatured protein were then, removed by centrifugation at 12,100g for 10 minutes and 800µl of the supernatant was carefully transferred into new 1.5ml Eppendorf tubes. An aliquot of 560µl of ice-cold isopropanol was

added to each of the reaction tubes to precipitate DNA and mixed thoroughly by inverting the tubes gently several times. Following the incubation of the samples at -20°C for 2 hours, the DNA was pelleted by centrifugation at 12,100g for 20 minutes. The supernatant was discarded, and the residual salt was removed from the pellet by rinsing it with 180µl of ice-cold 70% ethanol. The samples were centrifuged at 12,100g for 1 minute and the ethanol was carefully removed. After air-drying the pellet, the DNA was re-suspended in 50µl of HPLC grade water (Merck, USA). The sample was stored at -20°C for future use.

### B3.2. Crude extraction of total DNA from MDBK cells infected with a virus using PCR buffer

To isolate the total crude DNA, the culture media was aspirated, and the cells were washed with 500µl of 1x PBS. A volume of 600µl of 1x PCR buffer (Appendix A) containing 10µg/ml of proteinase K (Sigma-Aldrich, USA) was added to each well and the cells were frozen at - 80°C and thawed at 37°C. The cell lysate was transferred into 1.5ml Eppendorf tubes and incubated in a heating block at 56°C for 20 minutes. Then, the proteinase K was heat-inactivated at 85°C for 10 minutes. The reaction mixtures were centrifuged at 394g for 10 minutes and the supernatant was carefully transferred into new 1.5ml tubes.

### B3.3. Preparation of PCR reaction mixture

The 25µl (or 20µl) reaction mixture consisted of 12.5µl (or 10µl) of 2x KAPA 2G Robust HotStart ReadyMix (KAPA BIOSYSTEMS, USA) or OneTaq 2x Master Mix with Standard Buffer (New England BioLabs, USA), 3µl (or 2µl)of total crude DNA, 0.5µl of 50mM $MgCl_2$ (Thermo Fisher Scientific, USA) (where necessary), 1.25µl or 2µl of each primer (10µM each) depending on DNA polymerases and recommended concentration per manufacturer's protocol and HPLC water (Sigma-Aldrich, USA). A PCR control mixture was prepared using HPLC water instead of template DNA. The PCR products were analysed by gel electrophoresis on a 0.8% agarose gel stained with ethidium bromide (0.5µg/ml).

## B4. Live-cell staining of BLV Env glycoprotein using mouse anti-Env monoclonal antibody

A 24-well tissue culture plate was coated with 500µl of poly-L-lysine (Sigma-Aldrich, USA) at a dilution of 1:29. Following the incubation at 37°C for 1 hour, the solution was aspirated and the plate was washed twice with 500µl of 1x PBS. The plate was left to dry prior to use. The plate was then seeded with MDBK cells at a concentration of $2x10^5$ cells/ml and the cells were

infected with 3µl or 5µl of the cell lysate of the viral stock and incubated under the standard growth conditions for 48 hours. MDBK cells that were not infected and those infected with the parental nLSDVSODis-UCT virus ($1x10^{7.25}$ $TCID_{50}$) at an MOI of 0.01 were included as experimental controls.

Once enough mature foci were obtained, the culture media was aspirated and the cells were incubated with 250µl of the mouse anti-BLV Env gp51 monoclonal antibody (anti-Env mAb) (Veterinary Medical Research and Development, USA) diluted at 1:50 or 1:100 in cDMEM at room temperature for 1 hour. The primary antibody was aspirated, and the cells were washed thrice with 500µl of 1x PBS for 10 minutes at room temperature. The cells were then incubated with 250µl of the donkey-anti-mouse IgG conjugated with Cy3 (anti-mouse IgG-Cy3) diluted at 1:500 in cDMEM at room temperature for 30 minutes. The secondary antibody was aspirated, and the cells were washed twice as before. The cells were incubated with 500µl of the Hoechst nuclear stain diluted at 1:5,000 in 1x PBS at room temperature in the dark for 10 minutes. Following the removal of the Hoechst nuclear stain, the cells were washed with 1x PBS.

# APPENDIX C: SUPPLEMENTAL FIGURES FOR CHAPTER 2

**Table S2.1: GenBank accession number, the origin of the sequences and genotypes of the global isolates used for the phylogenetic analyses of the South African BLV env and gag sequences**

| GenBank accession number | Country of origin | Genotype | References |
|---|---|---|---|
| AB934282 | Japan | 1 | Direct submission by Mekata and Norimine (2014) |
| AF033818 | USA | 4 | Direct submission by Chappey (1997) |
| AF067081 | Poland | 4 | Direct submission by Mikiewicz *et al.* (1998) |
| AF257515 | Argentina | 2 | [247] |
| AF399702 | Brazil | 5 | [365] |
| AF399703 | Brazil | 1 | [365] |
| AF399704 | Brazil | 2 | [365] |
| AF503581 | Belgium | 4 | [253] |
| AP018021 | Japan | ND | [347] |
| AY151262 | Brazil | 1 | Direct submission by Stancek *et al*. (2002) |
| AY185360 | Brazil | 6 | Direct submission by Camargos *et al.* (2002) |
| AY515279 | Chile | 4 | [338] |
| AY515280 | Chile | 7 | [338] |
| D00647 | Australia | 1 | [189] |
| EF065635 | Costa Rica | 5 | [249] |
| EF065637 | Costa Rica | 1 | [249] |
| EF065641 | USA | 1 | [249] |
| EF065646 | Japan | 1 | [249] |
| EF065647 | USA | 3 | [249] |
| EF065650 | Japan | 3 | [249] |
| EF600696 | USA | 1 | [219] |
| EU262555 | Poland | 7 | Direct submission by Rola *et al.* (2016) |
| EU266061 | Iran | 1 | [53] |
| FJ808571 | Argentina | 1 | [328] |
| FJ808582 | Argentina | 6 | [328] |
| FJ914764 | Argentina | 2 | [364] |
| FM209468 | Uruguay | 1 | [353] |
| GU724606 | Croatia | 8 | Direct submission by Lojkic (2010) |
| HE967301 | Uruguay | 1 | Direct submission by Cristina (2012) |
| HM563764 | Ukraine | 8 | [151] |
| HQ902258 | Belarus | 4 | [151] |
| HQ902261 | Russia | 7 | [151] |
| JF713455 | Russia | 8 | Direct submission by Shaeva *et al*. (2011) |
| JN695878 | Russia | 4 | Direct submission by Lomakina and Gulyukin (2012) |
| JN695879 | Russia | 7 | Direct submission by Lomakina and Gulyukin (2012) |
| JQ686091 | Russia | 4 | Direct submission by Lomakina and Gulyukin (2012) |
| K02251 | Belgium | 4 | [581] |
| KF801457 | Moldova | 7 | [334] |
| KJ707242 | South Korea | 1 | [361] |
| KT122858 | Belgium | 4 | Direct submission by Rosewick *et al.* (2015) |
| KU233527 | Thailand | 10 | [329] |
| KU233528 | Thailand | 1 | [329] |
| KU233530 | Thailand | 6 | [329] |
| KX674369 | Saint Kitts and Nevis | 1 | [343] |
| KX674372 | Dominica | 1 | [343] |
| LC005615 | Japan | 1 | Direct submission by Okazaki *et al.* (2014) |
| LC060792 | Mongolia | 4 | [152] |

**Table S2.1: GenBank accession number, the origin of the sequences and genotypes of the global isolates used for the phylogenetic analyses of the South African BLV env and gag sequences (Continued)**

| GenBank accession number | Country of origin | Genotype | References |
|---|---|---|---|
| LC060801 | Mongolia | 7 | [152] |
| LC075543 | Peru | 1 | [330] |
| LC075544 | Peru | 2 | [330] |
| LC075549 | Peru | 6 | [330] |
| LC075553 | Paraguay | 6 | [330] |
| LC075558 | Paraguay | 1 | [330] |
| LC075563 | Bolivia | 9 | [330] |
| LC075568 | Bolivia | 1 | [330] |
| LC075571 | Bolivia | 6 | [330] |
| LC075577 | Bolivia | 2 | [330] |
| LC080651 | Paraguay | 1 | [330] |
| LC080654 | Peru | 2 | [330] |
| LC080655 | Paraguay | 2 | [330] |
| LC080656 | Paraguay | 6 | [330] |
| LC080659 | Bolivia | 9 | [330] |
| LC080664 | Bolivia | 9 | [330] |
| LC154064 | Myanmar | 10 | [341] |
| LC154848 | Myanmar | 10 | [341] |
| LC193462 | Zambia | 4 | [345] |
| M35238 | France | 4 | [348] |
| MF580991 | China | 6 | [344] |
| MF580994 | China | 10 | [344] |
| MH170027 | Vietnam | 1 | [342] |
| MH170028 | Vietnam | 1 | [342] |
| MH170030 | Vietnam | 6 | [342] |
| MH341516 | India | 6 | [359] |
| S83530 | Italy | 7 | [351] |

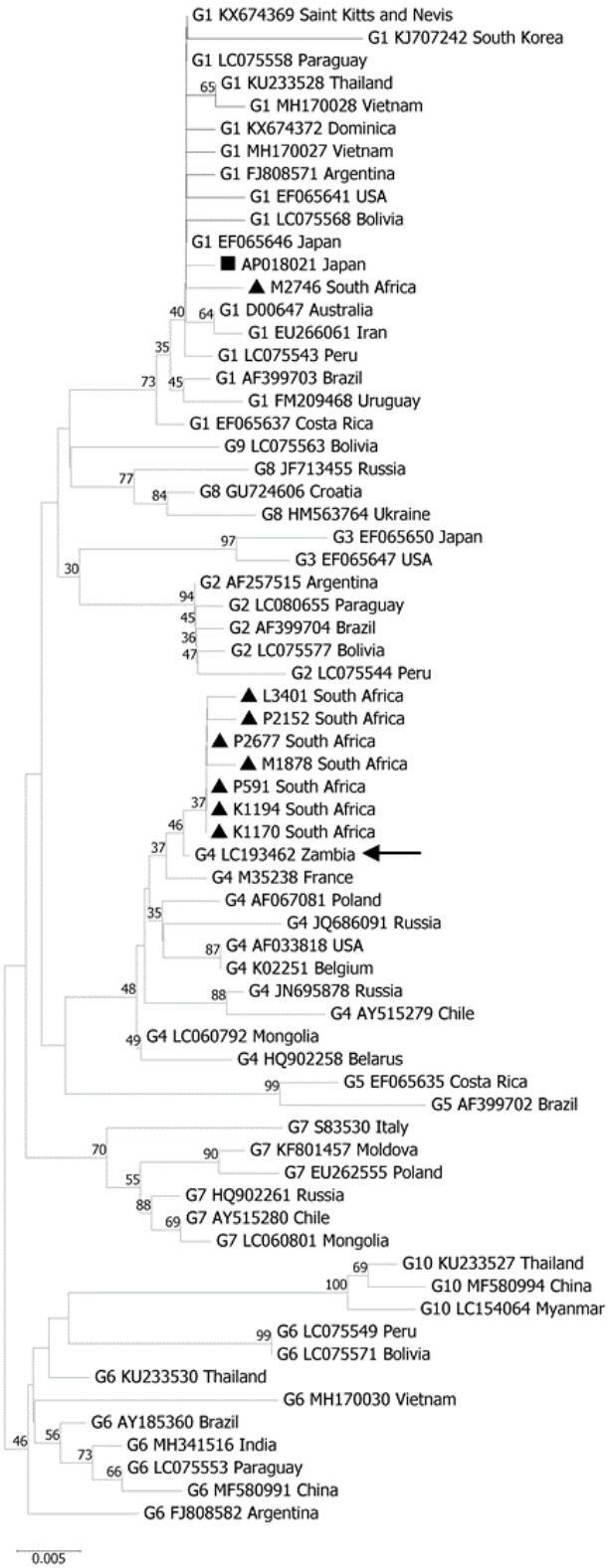



**Figure S2.1: Neighbour-joining phylogenetic tree based on the BLV partial *env* nucleotide sequences from South Africa and other geographic regions worldwide**. BLV strains identified in this study are indicated by filled triangles (▲) followed by their sample ID. The BLV strain used to design recombinant LSDV-BLV-Env-Gag and LSDV-BLV-Env is indicated by a filled square (■). A black arrow indicates the Zamibain isolate which is most closely related to the seven South African isolates. Other strains are indicated by their genotype, GenBank accession number and country of origin. Numbers at the branches denote bootstrap support (1000 iterations). The bar at the bottom of the figure denotes genetic distance.

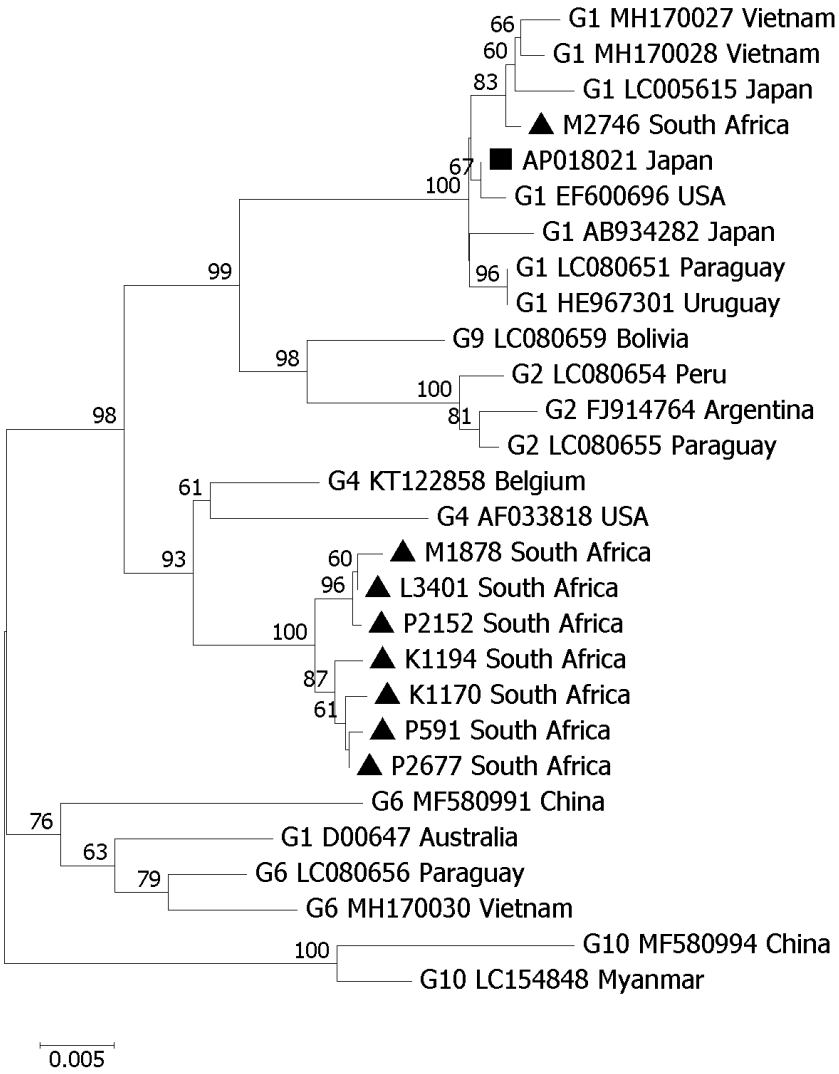
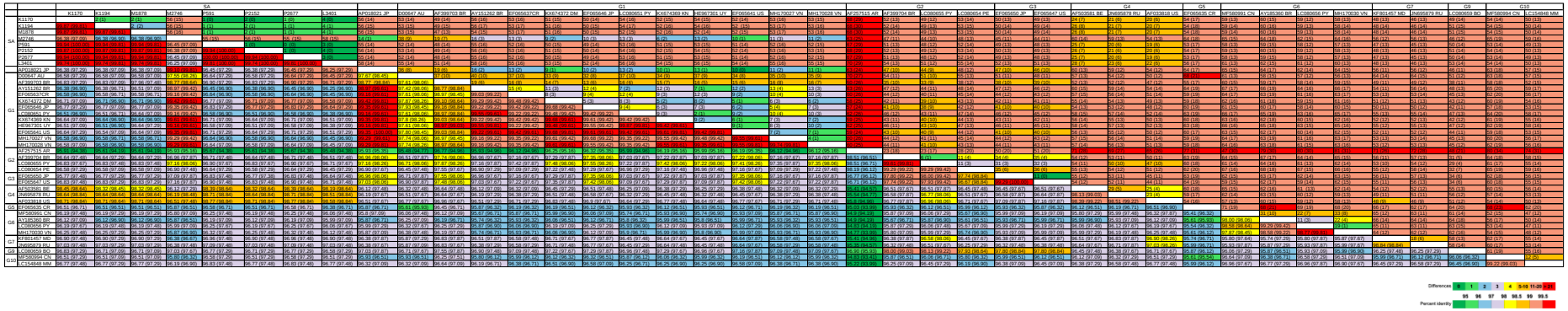


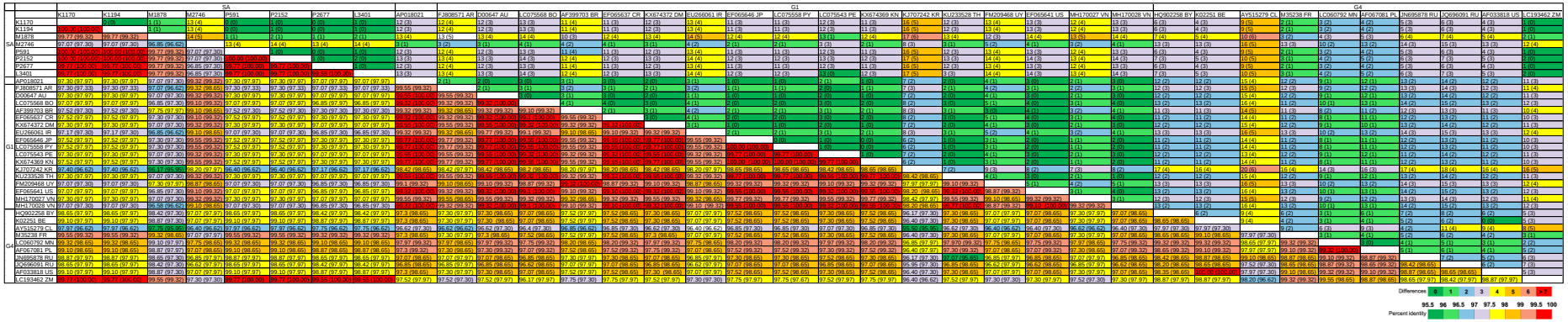

**Figure S2.2: Neighbour-joining phylogenetic tree based on the BLV full-length gag nucleotide sequences from South Africa and other geographic regions worldwide.** BLV strains identified in this study are indicated by filled triangles (▲) followed by their sample ID. The BLV strain used to design recombinant LSDV-BLV-Env-Gag and LSDV-BLV-Env is indicated by a filled square (■). Other strains are shown by genotype followed by GenBank accession number and country of origin. Numbers at the branches denote bootstrap support (1000 iterations). The bar at the bottom of the figure denotes genetic distance.

**Table S2.2: Percent identities and differences of nucleotide and amino acid sequences in the BLV full-length Env sequences from South Africa and other geographic regions worldwide**



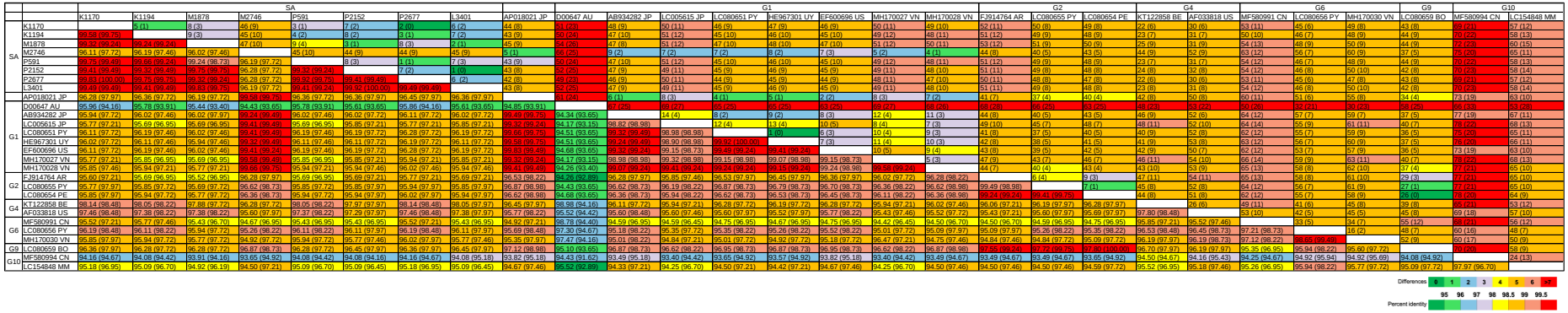
Amino acid sequence identities are shown in brackets. The lower matrix shows the percent identity of nucleotide and amino acid sequences in the BLV full-length Env sequences and the upper matrix shows differences in nucleotide and amino acid sequences in the BLV full-length *env* sequences. Each cell is highlighted according to the amino acid sequence identities and differences. The country of origin is indicated by 2-letter codes. AU, Australia; AR, Argentina; BE, Belgium; BO, Bolivia; BR, Brazil; BY, Belarus; CL, Chile; CR, Costa Rica; DM, Dominica; FR, France; IR, Iran; JP, Japan; KN, Saint Kitts and Nevis; KR, South Korea; MN, Mongolia; PE, Peru; PL, Poland; PY, Paraguay; RU, Russia; TH, Thailand; US, USA; UY, Uruguay; ZM, Zambia.

**Table S2.3: Percent identities and differences of nucleotide and amino acid sequences in the BLV partial Env sequences from South Africa and other geographic regions worldwide**



Amino acid sequence identities are shown in brackets. The lower matrix shows the percent identity of nucleotide and amino acid sequences in the BLV partial Env sequences and the upper matrix shows differences in nucleotide and amino acid sequences in the BLV partial *env* sequences. Each cell is highlighted according to the amino acid sequence identities and differences. The country of origin is indicated by 2-letter codes. AU, Australia; AR, Argentina; BE, Belgium; BO, Bolivia; BR, Brazil; BY, Belarus; CL, Chile; CR, Costa Rica; DM, Dominica; FR, France; IR, Iran; JP, Japan; KN, Saint Kitts and Nevis; KR, South Korea; MN, Mongolia; PE, Peru; PL, Poland; PY, Paraguay; RU, Russia; TH, Thailand; US, USA; UY, Uruguay; ZM, Zambia.

**Table S2.4: Percent identities of nucleotide and amino acid distances in the BLV full-length Gag sequences from South Africa and other geographic regions worldwide**



Amino acid sequence identities are shown in brackets. The lower matrix shows the percent identity of nucleotide and amino acid sequences in the BLV full-length Gag sequences and the upper matrix shows differences in nucleotide and amino acid sequences in the BLV full-length Gag sequences. Each cell is highlighted according to the amino acid sequence identities and differences. The country of origin is indicated by 2-letter codes. AU, Australia; AR, Argentina; BE, Belgium; BO, Bolivia; CN, China; JP, Japan; MM, Myanmar; PE, Peru; PY, Paraguay; US, USA; UY, Uruguay; VN, Vietnam.

# APPENDIX D: SUPPLEMENTAL FIGURES FOR CHAPTER 4

**Table S4.1: GenBank accession numbers of 62 BLV natural isolates used to select consensus Env and Gag sequences**

| | GenBank accession number | References |
|---|---|---|
| 1. | AB934282 | Direct submission by Mekata and Norimine (2014) |
| 2. | AB987702 | [582] |
| 3. | AF033818 | Direct submission by Chappey (1997) |
| 4. | AF257515 | [247] |
| 5. | AP018006 | [347] |
| 6. | AP018007 | [347] |
| 7. | AP018008 | [347] |
| 8. | AP018009 | [347] |
| 9. | AP018010 | [347] |
| 10. | AP018011 | [347] |
| 11. | AP018012 | [347] |
| 12. | AP018013 | [347] |
| 13. | AP018014 | [347] |
| 14. | AP018015 | [347] |
| 15. | AP018016 | [347] |
| 16. | AP018017 | [347] |
| 17. | AP018018 | [347] |
| 18. | AP018019 | [347] |
| 19. | AP018020 | [347] |
| 20. | AP018021 | [347] |
| 21. | AP018022 | [347] |
| 22. | AP018023 | [347] |
| 23. | AP018024 | [347] |
| 24. | AP018025 | [347] |
| 25. | AP018026 | [347] |
| 26. | AP018027 | [347] |
| 27. | AP018028 | [347] |
| 28. | AP018029 | [347] |
| 29. | AP018030 | [347] |
| 30. | AP018031 | [347] |
| 31. | AP018032 | [347] |
| 32. | D00647 | [189] |
| 33. | EF600696 | [219] |
| 34. | FJ914764 | [364] |
| 35. | HE967301 | Direct submission by Cristina (2012) |
| 36. | HE967302 | Direct submission by Cristina (2012) |
| 37. | HE967303 | Direct submission by Cristina (2012) |
| 38. | K02120 | [206] |
| 39. | KP113663 | Direct submission by Kalashnikov and Zinovieva (2014) |

**Table S4.1: GenBank accession numbers of 62 BLV natural isolates used to select consensus Env and Gag sequences (Continued)**

| | GenBank accession number | References |
|---|---|---|
| 40. | KT122858 | Direct submission by Rosewick *et al.* (2015) |
| 41. | LC080656 | [330] |
| 42. | LC080657 | [330] |
| 43. | LC080658 | [330] |
| 44. | LC080659 | [330] |
| 45. | LC080660 | [330] |
| 46. | LC080663 | [330] |
| 47. | LC080666 | [330] |
| 48. | LC080669 | [330] |
| 49. | LC080672 | [330] |
| 50. | LC080675 | [330] |
| 51. | LC154848 | [341] |
| 52. | LC154849 | [341] |
| 53. | LC164083 | [583] |
| 54. | LC164084 | [583] |
| 55. | LC164085 | [583] |
| 56. | LC164086 | [583] |
| 57. | MF580990 | [344] |
| 58. | MF580991 | [344] |
| 59. | MF580992 | [344] |
| 60. | MF580993 | [344] |
| 61. | MF580994 | [344] |
| 62. | MF580995 | [344] |

**Table S4.2: Consensus env (Cons-Env) sequence obtained from BLV env sequences of 62 natural isolates**

| | |
|---|---|
| **Nucleotide sequence** | ATGCCTAAAGAACGACGGTCCCGAAGACGCCCACAACCGATCATCAGATGGGTAAGTCTCACTCTCACT<br>CTCCTCGCTCTCTGTCGGCCCATCCAGACTTGGAGATGCTCCCTGTCCCTAGGAAACCAACAATGGATG<br>ACAGCATATAACCAAGAGGCAAAATTTTCCATCTCCATTGACCAAATACTAGAGGCTCATAATCAGTCA<br>CCTTTCTGTGCCAAGTCTCCCAGATACACCTTGGACTCTGTAAATGGCTATCCTAAGATCTACTGGCCC<br>CCCCCACAAGGGCGGCGCCGGTTTGGAGCCAGGGCCATGGTCACATATGATTGCGAGCCCCGATGCCCT<br>TATGTGGGGGCAGATCGCTTCGACTGCCCCCACTGGGACAATGCCTCCCAGGCCGATCAAGGATCCTTT<br>TATGTCAATCATCAGATTTTATTCCTGCATCTCAAACAATGTCATGGAATTTTCACTCTAACCTGGGAG<br>ATATGGGGATATGATCCCCTGATCACCTTTTCTTTACATAAGATCCCTGATCCCCCTCAACCCGACTTT<br>CCCCAGTTGAACAGTGACTGGGTTCCCTCTGTCAGATCATGGGCCCTGCTTTTAAATCAAACAGCACGG<br>GCCTTCCCAGACTGTGCTATATGTTGGGAACCTTCCCCTCCCTGGGCTCCCGAAATATTAGTATATAAC<br>AAAACCATCTCCAGCTCTGGACCCGGCCTCGCCCTCCCGGACGCCCAAATCTTCTGGGTCAACACGTCC<br>TCGTTTAACACCACCCAAGGATGGCACCACCCTTCCCAGAGGTTGTTGTTCAATGTTTCTCAAGGCAAC<br>GCCTTGTTATTACCTCCTATCTCCCTGGTTAATCTCTCTACGGCTTCCTCCGCCCCTCCTACCCGGGTC<br>AGACGTAGTCCCGTCGCAGCCCTGACCTTAGGCCTAGCCCTGTCAGTGGGGCTCACTGGAATTAATGTG<br>GCCGTGTCTGCCCTTAGCCATCAGAGACTCACCTCCCTGATCCACGTTCTGGAGCAAGATCAGCAACGC<br>TTGATCACAGCAATTAACCAGACCCACTATAATTTGCTTAATGTGGCCTCTGTGGTTGCCCAGAACCGA<br>CGGGGGCTTGATTGGTTGTACATCCGGCTGGGTTTTCAAAGCCTATGTCCCACAATTAATGAGCCTTGC<br>TGTTTCCTGCGCATTCAAAATGACTCCATTATCCGCCTCGGTGATCTCCAGCCTCTCTCGCAAAGAGTC<br>TCTACAGACTGGCAGTGGCCCTGGAATTGGGATCTGGGGCTCACTGCCTGGGTGCGAGAAACCATTCAT<br>TCTGTTCTAAGCCTGTTCCTATTAGCCCTTTTTTTGCTCTTCCTGGCCCCCTGCCTGATAAAATGCTTG<br>ACCTCTCGCCTTTTAAAGCTCCTCCGGCAGGCTCCCCACTTCCCTGAAATCTCCTTAACCCCTAAACCC<br>GATTCTGATTATCAGGCCTTGCTACCATCTGCACCAGAGATCTACTCTCACCTCTCCCCCGTCAAACCC<br>GATTACATCAACCTCCGACCCTGCCCTTGA (1548 nt) |
| **Amino acid sequence** | MPKERRSRRRPQPIIRWVSLTLTLLALCRPIQTWRCSLSLGNQQWMTAYNQEAKFSISIDQILEAHNQS<br>PFCAKSPRYTLDSVNGYPKIYWPPPQGRRRFGARAMVTYDCEPRCPYVGADRFDCPHWDNASQADQGSF<br>YVNHQILFLHLKQCHGIFTLTWEIWGYDPLITFSLHKIPDPPQPDFPQLNSDWVPSVRSWALLLNQTAR<br>AFPDCAICWEPSPPWAPEILVYNKTISSSGPGLALPDAQIFWVNTSSFNTTQGWHHPSQRLLFNVSQGN<br>ALLLPPISLVNLSTASSAPPTRVRRSPVAALTLGLALSVGLTGINVAVSALSHQRLTSLIHVLEQDQQR<br>LITAINQTHYNLLNVASVVAQNRRGLDWLYIRLGFQSLCPTINEPCCFLRIQNDSIIRLGDLQPLSQRV<br>STDWQWPWNWDLGLTAWVRETIHSVLSLFLLALFLLFLAPCLIKCLTSRLLKLLRQAPHFPEISLTPKP<br>DSDYQALLPSAPEIYSHLSPVKPDYINLRPCP* (515 aa) |

Start codon and stop codon are highlighted in gre

**Table S4.3: Consensus gag (Cons-Gag) sequence obtained from BLV gag sequences of 62 natural isolates**

| | |
|---|---|
| **Nucleotide sequence** | ATGGGAAATTCCCCCTCCTATAACCCCCCCGCTGGTATCTCCCCCTCAGACTGGCTCAACCTTCTGCAA<br>AGCGCGCAAAGGCTCAATCCGCGACCCTCTCCTAGCGATTTTACCGATTTAAAGAATTACATCCATTGG<br>TTTCATAAGACCCAGAAAAAACCATGGACTTTCACTTCTGGTGGCCCCACCTCATGTCCACCCGGGAGA<br>TTCGGCCGGGTTCCCCTTGTCTTGGCCACCCTAAACGAAGTGCTCTCAAACGATGGGGGCGCCCCGGGT<br>GCATCGGCCCCAGAAGAACAACCCCCCCCTTATGACCCCCCCGCCGTTTTGCCAATCATATCTGAAGGG<br>AATCGCAACCGCCATCGTGCTTGGGCACTCCGAGAATTACAAGATATCAAAAANGAAATTGAAAATAAG<br>GCACCGGGTTCGCAAGTATGGATACAAACACTACGACTTGCAATCCTGCAGGCCGACCCTACTCCGGCT<br>GACCTAGAACAACTTTGCCAATATATTGCTTCCCCGGTCGACCAAACGGCCCATATGACCAGCCTAACG<br>GCAGCAATAGCCGCCGCTGAAGCGGCCAACACCCTCCAGGGTTTTAACCCCCAAAACGGGACCCTAACC<br>CAACAATCAGCTCAGCCCAACGCCGGGGATCTTAGAAGTCAATATCAAAACCTCTGGCTTCAGGCCTGG<br>AAAAATCTCCCTACTCGTCCTTCAGTACAACCTTGGTCCACCATCGTCCAAGGCCCCGCCGAAAGCTAT<br>GTAGAGTTTGTCAACCGGTTACAAATTTCATTAGCTGACAACCTTCCCGACGGAGTCCCTAAGGAACCC<br>ATTATTGACTCCCTTAGTTATGCAAATGCTAACAAAGAGTGCCAGCAAATTTTGCAGGGGCGAGGCCTA<br>GTGGCCGCCCCGGTGGGGCAAAAACTGCAGGCTTGCGCACATTGGGCCCCCAAGATGAAACAGCCTGCA<br>ATTCTCGTCCACACCCCAGGGCCCAAGATGCCCGGGCCTCGGCAACCGGCCCCCAAAAGGCCTCCCCCA<br>GGACCATGCTATCGATGCCTCAAAGAAGGCCATTGGGCCCGGGATTGTCCTACCAAGGCCACCGGCCCC<br>CCTCCGGGACCTTGCCCCATATGTAAAGATCCTTCCCATTGGAAACGAGACTGTCCAACCCTCAAATCA<br>AAAAACTAA (1182 nt) |
| **Amino acid sequence** | MGNSPSYNPPAGISPSDWLNLLQSAQRLNPRPSPSDFTDLKNYIHWFHKTQKKPWTFTSGGPTSCPPGR<br>FGRVPLVLATLNEVLSNDGGAPGASAPEEQPPPYDPPAVLPIISEGNRNRHRAWALRELQDIKKEIENK<br>APGSQVWIQTLRLAILQADPTPADLEQLCQYIASPVDQTAHMTSLTAAIAAAEAANTLQGFNPQNGTLT<br>QQSAQPNAGDLRSQYQNLWLQAWKNLPTRPSVQPWSTIVQGPAESYVEFVNRLQISLADNLPDGVPKEP<br>IIDSLSYANANKECQQILQGRGLVAAPVGQKLQACAHWAPKMKQPAILVHTPGPKMPGPRQPAPKRPPP<br>GPCYRCLKEGHWARDCPTKATGPPPGPCPICKDPSHWKRDCPTLKSKN* (393 aa) |

Start codon and stop codon are highlighted in grey

**Table S4.4: Nucleotide sequence of the pBLV-Env-Gag transfer vector**

TCGCGCGTTTCGGTGATGACGGTGAAAACCTCTGACACATGCAGCTCCCGGAGACGGTCACAGCTTGTCTGTAAGCGGATG

CCGGGAGCAGACAAGCCCGTCAGGGCGCGTCAGCGGGTGTTGGCGGGTGTCGGGGCTGGCTTAACTATGCGGCATCAGAGC

AGATTGTACTGAGAGTGCACCATATGCGGTGTGAAATACCGCACAGATGCGTAAGGAGAAAATACCGCATCAGGCGCCATT

CGCCATTCAGGCTGCGCAACTGTTGGGAAGGGCGATCGGTGCGGGCCTCTTCGCTATTACGCCAGCTGGCGAAAGGGGGAT

GTGCTGCAAGGCGATTAAGTTGGGTAACGCCAGGGTTTTCCCAGTCACGACGTTGTAAAACGACGGCCAGTGAATTGGAGA

49 flank

TCGGTACTTCGCGAATGCGTCGAGATGAGCTCATATAGACTCATTTTGTATCAGTGATGAAAAATGGACATTTATTATGTC

TTCTTATTATGTTAATATTTTAGAATATGCAAAAATATACTCTAAGGGTGGAAACGATGCATGTGCGTTGGATATTTTTGA

AAGAGAAAATGTTTTAAATGATGATGCCTTTAACGCAATAAAGTTGTTGAATATGAGAGCAAAAATAATAAAGCACAGAAG

ATATAATAAATTATATGTATTAACGTGTAAATTAATGTTTGGTGTATATTTTGGTAAAACATTTAACATTATACATAATCG

mFP promoter

ACCATTATTTGGTTATATTACTATGATAACAGATAGTACTTTTATAACTGATGATTAATTTTTCTGGGCCCAGAAAAATAT

Human tPA leader

CCTAAAATTGAATTGTAATTATCGATAATAAAAGCTTCCACCATGGATGCTATGAAAAGAGGACTGTGCTGCGTGCTGCTG

BLV *env gp51*

CTGTGCGGAGCCGTGTTCGTGTCACCCAGCTGGAGATGTTCCCTGTCACTGGGCAACCAGCAGTGGATGACCGCCTACAAT

CAGGAGGCCAAGTTCAGCATCTCCATCGACCAGATCCTGGAGGCCCACAACCAGAGCCCTTTTTGCGCCAAGTCCCCAAGA

TATACCCTGGATTCCGTGAATGGCTACCCAAAGATCTATTGGCCACCTCCACAGGGCCGGAGAAGGTTCGGCGCCAGAGCT

ATGGTGACATACGACTGCGAGCCTCGCTGTCCATATGTGGGCGCCGACCGGTTTGATTGCCCACACTGGGACAACGCATCT

CAGGCAGAGGAGAAGGTTCGGCGCCAGAGCTATGGTGACATACGACTGCGAGCCTCGCTGTCCATATGTGGGCGCCGACCG

GTTTGATTGCCCACACTGGGACAACGCATCTCAGGCAGATCAGGGCTCCTTCTACGTGAATCACCAGATCCTGTTTCTGCA

CCTGAAGCAGTGTCACGGCATCTTCACCCTGACATGGGAGATCTGGGGCTATGATCCACTGATCACCTTCTCTCTGCACAA

GATCCCCGACCCCCCTCAGCCCGATTTTCCTCAGCTCAACAGCGACTGGGTGCCCTCTGTGCGCTCCTGGGCTCTGCTGCT

GAATCAGACAGCCAGAGCCTTTCCTGATTGCGCCATCTGTTGGGAGCCAAGCCCACCCTGGGCTCCCGAGATCCTGGTGTA

CAACAAGACCATCAGCTCCTCTGGCCCCGGCCTGGCCCTGCCTGACGCACAGATCTTCTGGGTGAACACAAGCTCCTTTAA

TACCACACAGGGATGGCACCACCCTAGCCAGCGCCTGCTGTTCAACGTGTCCCAGGGCAATGCCCTGCTGCTGCCTCCAAT

Gly linker BLV *env gp30*

CTCCCTGGTGAACCTGTCTACCGCCTCTAGCGCCCCCCCTACCGGCGGTGGAGGCTCAGGTGGAGGCGGATCCTCTCCCGT

GGCCGCCCTGACCCTGGGCCTGGCCCTGTCTGTGGGCCTGACAGGCATCAATGTGGCCGTGTCCGCCCTGTCTCACCAGAG

ACTGACCTCTCTGATCCACGTGCTGGAGCAGGACCAGCAGAGGCTGATCACCGCCATCAACCAGACACACTACAACCTGCT

GAATGTGGCAAGCGTGGTGGCACAGAATAGGAGGGGCCTGGATTGGCTGTATATCCGGCTGGGCTTCCAGAGCCTGTGCCC

AACCATCAACGAGCCCTGCTGTTTTCTGAGAATCCAGAATGACTCCATCATCAGGCTGGGCGATCTGCAGCCTCTGTCTCA

GCGCGTGAGCACAGACTGGCAGTGGCCCTGGAATTGGGATCTGGGCCTGACCGCATGGGTGCGGGAGACAATCCACAGCGT

GCTGTCCCTGTTCCTGCTGGCCCTGTTCCTGCTGTTTCTGGCCCCCTGCCTGATCAAGTGTCTGACCTCCAGACTGCTGAA

GCTGCTGAGGCAGGCCCCACACTTTCCCGAGATCTCCCTGACACCAAAGCCCGACTCTGATTATCAGGCCCTGCTGCCTAG

CGCCCCAGAGATCTATTCCCATCTGAGCCCAGTGAAACCTGACTACATCAACCTGAGACCTTGTCCATAATTTTTCTTCTA

*MfeI* mH5 promoter

GACTCGAGCAATTGCCCGGGAAAAATTGAAAATAAATACAAAGGTTCTTGAGGGTTGTGTTAAATTGAAAGCGAGAAATAA

BLV *gag*

TCATAAATAACTAGTACCATGGGCAATAGTCCAAGTTACAATCCACCCGCCGGCATTTCTCCAAGCGACTGGCTGAACCTG

CTGCAGAGCGCACAGAGGCTGAACCCTCGGCCAAGCCCCTCCGACTTCACCGATCTGAAGAACTACATCCACTGGTTTCAC

AAGACACAGAAGAAGCCTTGGACCTTCACATCCGGCGGCCCAACCTCTTGCCCCCCTGGCCGCTTTGGACGGGTGCCCCTG

GTGCTGGCCACACTGAACGAGGTGCTGTCTAATGACGGAGGCGCCCCTGGCGCAAGCGCCCCTGAGGAGCAGCCACCCCCT

TATGATCCACCCGCCGTGCTGCCAATCATCTCCGAGGGAAACCGGAATAGACACAGGGCATGGGCTCTGAGGGAGCTGCAG

GACATCAAGAAGGAGATCGAGAATAAGGCCCCTGGCTCTCAGGTCTGGATTCAGACCCTGAGGCTGGCAATCCTGCAGGCC

GACCCTACACCAGCCGATCTGGAGCAGCTCTGCCAGTACATCGCATCCCCAGTGGACCAGACCGCACACATGACCTCTCTG

ACCGCCGCCATCGCCGCCGCCGAGGCAGCCAACACACTGCAGGGCTTTAACCCACAGAATGGCACCCTGACACAGCAGAGC

GCCCAGCCAAATGCAGGCGATCTGAGATCCCAGTATCAGAACCTGTGGCTGCAGGCCTGGAAGAATCTGCCAACCAGGCCC

AGCGTGCAGCCCTGGTCCACAATCGTGCAGGGACCCGCCGAGTCTTACGTGGAGTTCGTGAACAGACTGCAGATCAGCCTG

GCCGACAATCTGCCAGATGGCGTGCCTAAGGAGCCAATCATCGACTCTCTGAGCTATGCCAACGCCAATAAGGAGTGCCAG

CAGATCCTGCAGGGAAGGGGCCTGGTGGCAGCACCTGTGGGCCAGAAGCTGCAGGCCTGTGCCCACTGGGCTCCCAAGATG

AAGCAGCCAGCAATCCTGGTGCACACCCCCGGCCCTAAGATGCCAGGCCCCAGACAGCCTGCACCAAAGAGGCCTCCACCC

GGCCCTTGCTACAGGTGTCTGAAGGAGGGCCACTGGGCCAGAGACTGCCCAACCAAGGCCACAGGCCCTCCACCCGGCCCC

*EcoRI*

TGTCCTATCTGTAAAGACCCATCTCATTGGAAAAGGGACTGCCCCACTCTGAAAAGCAAGAACTAATTTTTATCTCGAGAA

p7.5

TTCTCCAAACCCACCCGCTTTTTATAGTAAGTTTTTCACCCATAAATAATAAATACAATAATTAATTTCTCGTAAAAGTAG

*egfp*

AAAATATATTCTAATTTATTGCACGGACCATGGTGAGCAAGGGCGAGGAGCTGTTCACCGGAGTGGTGCCCATCCTGGTCG

AGCTGGACGGCGACGTAAACGGCCACAAGTTCAGCGTGTCCGGCGAGGGCGAGGGCGATGCCACCTACGGCAAGCTGACCC

TGAAGTTCATCTGCACCACCGGCAAGCTGCCCGTGCCCTGGCCCACCCTCGTGACCACCCTGACCTACGGCGTGCAGTGCT

TCAGCCGCTACCCTGACCACATGAAGCAGCACGACTTCTTCAAGTCCGCCATGCCCGAAGGCTACGTCCAGGAGCGCACCA

TCTTCTTCAAGGACGACGGCAACTACAAGACCCGCGCCGAGGTGAAGTTCGAGGGCGACACCCTGGTGAACCGCATCGAGC

TGAAGGGCATCGACTTCAAGGAGGACGGCAACATCCTGGGTCACAAGCTGGAGTACAACTACAACAGCCACAACGTCTATA

TCATGGCCGACAAGCAGAAGAACGGCATCAAGGTGAACTTCAAGATCCGCCACAACATCGAGGACGGCAGCGTGCAGCTCG

CCGACCACTACCAGCAGAACACTCCTATCGGCGACGGCCCCGTGCTGCTGCCCGACAACCACTACCTGAGCACCCAGTCCG

CCCTGAGCAAAGACCCCAACGAGAAGCGCGATCACATGGTCCTGCTGGAGTTCGTGACCGCCGCCGGGATCACTCTCGGCA

50 flank

TGGACGAGCTGTACAAGTAATTTTTTCTGATATCAGAAAAATTAAATTAATTTAATTACTATATCTTTTTTCGTCGACGAAA

AATCTTCTACTATTCCTTTTTCTGAAATAACTAAACAATGATTTTTAAAAATATTTTCCGTCGTGATATACTTTACAACAC

TATTTGGATAAATAGTAAACATAAACACGTAATACAGATGTTTGTCTAATTTAGTAAAATCAAGAGAATAAATTAATCCTT

TTCTTTTTAATACCCACATCATATTCGTTAATGATTCATTCGTGATATTAATTCCTTTAACTAAACCACAAACAAAACTGG

AAAATGTTTTTATATGATATTTACTTTTTTTTACCAAACACAATTTTATTTTTAAAATGTTTTACGGGTTTTAAAACAGATT

CCCTATTTAACAGTATATCTTCAATAGAATTTCCACTTACGAAAAAAGATAAAAGTAAGTACTGATACATGGTAGCAGGTT

TTGAGGTACCATCGGATGCCGGGACCGACGAGTGCAGAGGCGTGCAAGCGAGCTTGGCGTAATCATGGTCATAGCTGTTTC

CTGTGTGAAATTGTTATCCGCTCACAATTCCACACAACATACGAGCCGGAAGCATAAAGTGTAAAGCCTGGGGTGCCTAAT

GAGTGAGCTAACTCACATTAATTGCGTTGCGCTCACTGCCCGCTTTCCAGTCGGGAAACCTGTCGTGCCAGCTGCATTAAT

GAATCGGCCAACGCGCGGGGAGAGGCGGTTTGCGTATTGGGCGCTCTTCCGCTTCCTCGCTCACTGACTCGCTGCGCTCGG

TCGTTCGGCTGCGGCGAGCGGTATCAGCTCACTCAAAGGCGGTAATACGGTTATCCACAGAATCAGGGGATAACGCAGGAA

AGAACATGTGAGCAAAAGGCCAGCAAAAGGCCAGGAACCGTAAAAAGGCCGCGTTGCTGGCGTTTTTCCATAGGCTCCGCC

CCCCTGACGAGCATCACAAAAATCGACGCTCAAGTCAGAGGTGGCGAAACCCGACAGGACTATAAAGATACCAGGCGTTTC

CCCCTGGAAGCTCCCTCGTGCGCTCTCCTGTTCCGACCCTGCCGCTTACCGGATACCTGTCCGCCTTTCTCCCTTCGGGAA

GCGTGGCGCTTTCTCATAGCTCACGCTGTAGGTATCTCAGTTCGGTGTAGGTCGTTCGCTCCAAGCTGGGCTGTGTGCACG

AACCCCCCGTTCAGCCCGACCGCTGCGCCTTATCCGGTAACTATCGTCTTGAGTCCAACCCGGTAAGACACGACTTATCGC

CACTGGCAGCAGCCACTGGTAACAGGATTAGCAGAGCGAGGTATGTAGGCGGTGCTACAGAGTTCTTGAAGTGGTGGCCTA

ACTACGGCTACACTAGAAGAACAGTATTTGGTATCTGCGCTCTGCTGAAGCCAGTTACCTTCGGAAAAAGAGTTGGTAGCT

CTTGATCCGGCAAACAAACCACCGCTGGTAGCGGTGGTTTTTTTGTTTGCAAGCAGCAGATTACGCGCAGAAAAAAAGGAT

CTCAAGAAGATCCTTTGATCTTTTCTACGGGGTCTGACGCTCAGTGGAACGAAAACTCACGTTAAGGGATTTTGGTCATGA

GATTATCAAAAAGGATCTTCACCTAGATCCTTTTAAATTAAAAATGAAGTTTTAAATCAATCTAAAGTATATATGAGTAAA

CTTGGTCTGACAGTTACCAATGCTTAATCAGTGAGGCACCTATCTCAGCGATCTGTCTATTTCGTTCATCCATAGTTGCCT

GACTCCCCGTCGTGTAGATAACTACGATACGGGAGGGCTTACCATCTGGCCCCAGTGCTGCAATGATACCGCGAGACCCAC

GCTCACCGGCTCCAGATTTATCAGCAATAAACCAGCCAGCCGGAAGGGCCGAGCGCAGAAGTGGTCCTGCAACTTTATCCG

CCTCCATCCAGTCTATTAATTGTTGCCGGGAAGCTAGAGTAAGTAGTTCGCCAGTTAATAGTTTGCGCAACGTTGTTGCCA

TTGCTACAGGCATCGTGGTGTCACGCTCGTCGTTTGGTATGGCTTCATTCAGCTCCGGTTCCCAACGATCAAGGCGAGTTA

CATGATCCCCCATGTTGTGCAAAAAAGCGGTTAGCTCCTTCGGTCCTCCGATCGTTGTCAGAAGTAAGTTGGCCGCAGTGT

TATCACTCATGGTTATGGCAGCACTGCATAATTCTCTTACTGTCATGCCATCCGTAAGATGCTTTTCTGTGACTGGTGAGT

ACTCAACCAAGTCATTCTGAGAATAGTGTATGCGGCGACCGAGTTGCTCTTGCCCGGCGTCAATACGGGATAATACCGCGC

CACATAGCAGAACTTTAAAAGTGCTCATCATTGGAAAACGTTCTTCGGGGCGAAAACTCTCAAGGATCTTACCGCTGTTGA

GATCCAGTTCGATGTAACCCACTCGTGCACCCAACTGATCTTCAGCATCTTTTACTTTCACCAGCGTTTCTGGGTGAGCAA

AAACAGGAAGGCAAAATGCCGCAAAAAAGGGAATAAGGGCGACACGGAAATGTTGAATACTCATACTCTTCCTTTTTCAAT

ATTATTGAAGCATTTATCAGGGTTATTGTCTCATGAGCGGATACATATTTGAATGTATTTAGAAAAATAAACAAATAGGGG

TTCCGCGCACATTTCCCCGAAAAGTGCCACCTGACGTCTAAGAAACCATTATTATCATGACATTAACCTATAAAAATAGGC

GTATCACGAGGCCCTTTCGTC

Poxvirus sense and antisense promoter sequences are highlighted in yellow. *Mfe*I and *Eco*RI restriction sites are highlighted in cyan.

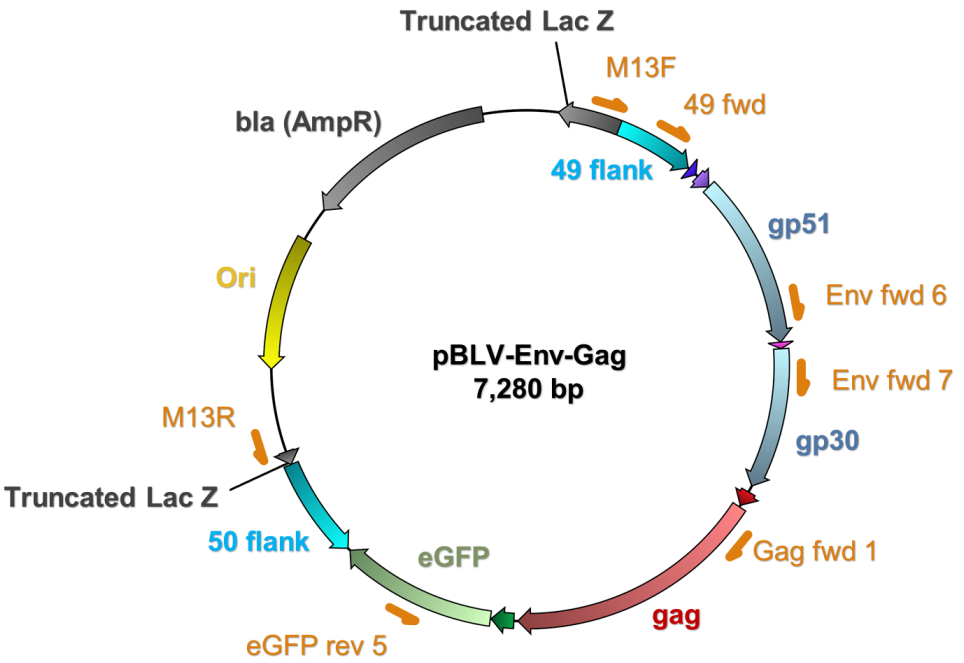



**Figure S4.1: Binding sites of sequencing primers on the pBLV-Env-Gag transfer vector.** Binding sites of five forward primers and two reverse primers on the pBLV-Env-Gag transfer vector plasmid are shown as orange arrows. **Blue triangle**, mFP promoter; **purple arrow**, human tPA signal sequence; **Pink triangle**, Gly linker; **red arrow**, mH5 promoter; **green arrow**, p7.5 promoter; **gp51**, BLV *env gp51*; **gp30**, BLV *env gp30*; **gag**, BLV *gag.* **LacZ**, LacZ alpha fragment of the *beta-galactosidase* gene; **bla (Ap$^R$)**, *beta-lactamase* (*bla*) gene; **Ori**, ColE1 origin of replication.

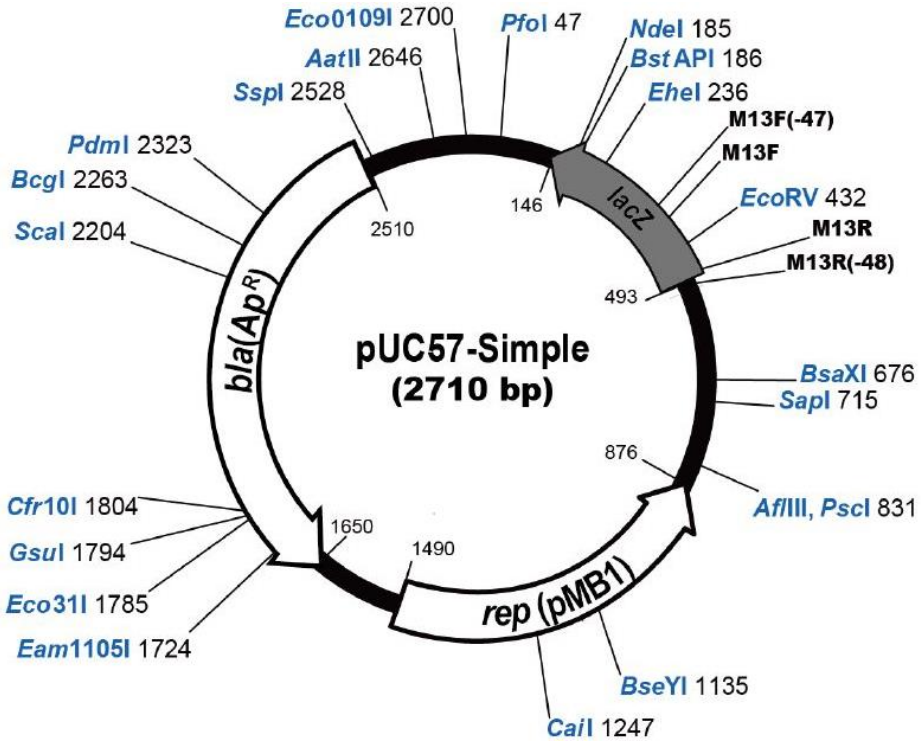



**Figure S4.2: Plasmid map of the pUC57-simple cloning vector used to construct the pBLV-Env-Gag transfer vector.** Plasmid map of pUC57-simple cloning vector (GenScript, China) showing cloning sites and binding sites of M13F and M13R universal primers. **LacZ**, LacZ alpha fragment of the beta-galactosidase gene; **bla (Ap$^R$)**, beta-lactamase (bla) gene; **Ori**, ColE1 origin of replication.

## REFERENCES


1. Agriculture, Forestry and Fisheries, South Africa., *List of controlled and notifiable animal diseases in terms of the animal disease act, 1984 (Act No 35 of 1984)*, F.a.F. Agriculture, Editor. 2016.
2. World Organisation for Animal Health. *WAHIS Interface, Animal Health Situation*. [cited 2019 20.7.2019]; Available from: http://www.oie.int/wahis_2/public/wahid.php/Diseaseinformation/Diseaseoutbreakmaps?disease_type_hidden=&disease_id_hidden=&selected_disease_name_hidden=&disease_type=0&disease_id_terrestrial=7&disease_id_aquatic=-999&speciesselect%5B%5D=3&selected_start_day=1&selected_start_month=1&selected_start_year=2005&selected_end_day=1&selected_end_month=7&selected_end_year=2019&submit2=OK.
3. Ali, A.A., et al., *Clinical and pathological studies of lumpy skin disease in Egypt.* Veterinary Record, 1990. **127**(22): p. 549-550.
4. El-Nahas, E., et al., *Isolation and identification of lumpy skin disease virus from naturally infected buffaloes at Kaluobia, Egypt*. Vol. 7. 2011. 234-237.
5. Carn, V.M. and R.P. Kitching, *An investigation of possible routes of transmission of lumpy skin disease virus (Neethling).* Epidemiol Infect, 1995. **114**(1): p. 219-26.
6. Chihota, C.M., et al., *Attempted mechanical transmission of lumpy skin disease virus by biting insects.* Med Vet Entomol, 2003. **17**(3): p. 294-300.
7. Chihota, C.M., et al., *Mechanical transmission of lumpy skin disease virus by Aedes aegypti (Diptera: Culicidae).* Epidemiology and Infection, 2001. **126**(2): p. 317-321.
8. Davies, F.G., *Lumpy skin disease, an African capripox virus disease of cattle.* Br Vet J, 1991. **147**(6): p. 489-503.
9. Babiuk, S., et al., *Capripoxviruses: an emerging worldwide threat to sheep, goats and cattle.* Transbound Emerg Dis, 2008. **55**(7): p. 263-72.
10. Diesel, A.M. *The epizootiology of lumpy skin disease in South Africa*. in *14th International Veterinary Congress*. 1949. London.
11. Davies, F.G., *Lumpy skin disease of cattle: A growing problem in Africa and the Near East.* World Animal Review, 1991. **68**: p. 37-42.
12. Lubinga, J.C., et al., *Detection of lumpy skin disease virus in saliva of ticks fed on lumpy skin disease virus-infected cattle.* Exp Appl Acarol, 2013. **61**(1): p. 129-38.
13. Tuppurainen, E.S., et al., *Evidence of vertical transmission of lumpy skin disease virus in Rhipicephalus decoloratus ticks.* Ticks Tick Borne Dis, 2013. **4**(4): p. 329-33.
14. Irons, P.C., E.S. Tuppurainen, and E.H. Venter, *Excretion of lumpy skin disease virus in bull semen.* Theriogenology, 2005. **63**(5): p. 1290-7.
15. Tuppurainen, E.S., et al., *A potential role for ixodid (hard) tick vectors in the transmission of lumpy skin disease virus in cattle.* Transbound Emerg Dis, 2011. **58**(2): p. 93-104.
16. Alkhamis, M.A. and K. VanderWaal, *Spatial and temporal epidemiology of lumpy skin disease in the Middle East, 2012-2015.* Front Vet Sci, 2016. **3**: p. 19.
17. EFSA Panel on Animal Health and Welfare (AHAW), *Scientific Opinion on lumpy skin disease.* EFSA Journal, 2015. **13**(1): p. 1-73.
18. Abutarbush, S.M., et al., *Adverse reactions to field vaccination against lumpy skin disease in Jordan.* Transbound Emerg Dis, 2016. **63**(2): p. e213-9.
19. Brenner, J., et al., *Appearance of skin lesions in cattle populations vaccinated against lumpy skin disease: statutory challenge.* Vaccine, 2009. **27**(10): p. 1500-3.
20. Kumar, M., *An outbreak of lumpy skin disease in a Holstein dairy herd in Oman: A clinical report*. Vol. 6. 2011. 851-859.

21. Ayelet, G., et al., *Lumpy skin disease: preliminary vaccine efficacy assessment and overview on outbreak impact in dairy cattle at Debre Zeit, central Ethiopia.* Antiviral Res, 2013. **98**(2): p. 261-5.
22. Yeruham, I., et al., *Adverse reactions in cattle to a capripox vaccine.* Vet Rec, 1994. **135**(14): p. 330-2.
23. Tuppurainen, E.S., et al., *Characterization of sheep pox virus vaccine for cattle against lumpy skin disease virus.* Antiviral Res, 2014. **109**: p. 1-6.
24. Kara, P.D., et al., *Comparative sequence analysis of the South African vaccine strain and two virulent field isolates of Lumpy skin disease virus.* Arch Virol, 2003. **148**(7): p. 1335-56.
25. Burgers, W.A., et al., *The novel capripoxvirus vector lumpy skin disease virus efficiently boosts modified vaccinia Ankara human immunodeficiency virus responses in rhesus macaques.* J Gen Virol, 2014. **95**(Pt 10): p. 2267-72.
26. Shen, Y.-J., et al., *A novel candidate HIV vaccine vector based on the replication deficient Capripoxvirus, Lumpy skin disease virus (LSDV).* Virology Journal, 2011. **8**: p. 265-265.
27. Aspden, K., et al., *Immunogenicity of a recombinant lumpy skin disease virus (neethling vaccine strain) expressing the rabies virus glycoprotein in cattle.* Vaccine, 2002. **20**(21-22): p. 2693-701.
28. Aspden, K., et al., *Evaluation of lumpy skin disease virus, a capripoxvirus, as a replication-deficient vaccine vector.* J Gen Virol, 2003. **84**(Pt 8): p. 1985-96.
29. Wallace, D.B., et al., *Protective immune responses induced by different recombinant vaccine regimes to Rift Valley fever.* Vaccine, 2006. **24**(49-50): p. 7181-9.
30. Soi, R.K., et al., *Protection of sheep against Rift Valley fever virus and sheep poxvirus with a recombinant capripoxvirus vaccine.* Clin Vaccine Immunol, 2010. **17**(12): p. 1842-9.
31. Romero, C.H., et al., *Single capripoxvirus recombinant vaccine for the protection of cattle against rinderpest and lumpy skin disease.* Vaccine, 1993. **11**(7): p. 737-42.
32. Ngichabe, C.K., et al., *Trial of a capripoxvirus-rinderpest recombinant vaccine in African cattle.* Epidemiol Infect, 1997. **118**(1): p. 63-70.
33. Ngichabe, C.K., et al., *Long term immunity in African cattle vaccinated with a recombinant capripox-rinderpest virus vaccine.* Epidemiol Infect, 2002. **128**(2): p. 343-9.
34. Berhe, G., et al., *Development of a dual recombinant vaccine to protect small ruminants against peste-des-petits-ruminants virus and capripoxvirus infections.* J Virol, 2003. **77**(2): p. 1571-7.
35. Weiss, K.E., *Lumpy skin disease virus.* Virology Monographs, 1968. **3**: p. 111-131.
36. Onuma, M., et al., *Studies on the sporadic and enzootic forms of bovine leukosis.* Journal of Comparative Pathology, 1979. **89**(2): p. 159-167.
37. Theilen, G.H. and D.L. Dungworth, *Bovine lymphosarcoma in California. 3. The calf form.* Am J Vet Res, 1965. **26**: p. 696-709.
38. Van Der Maaten, M.J., J.M. Miller, and A.D. Boothe, *Replicating type-C virus particles in monolayer cell cultures of tissues from cattle with lymphosarcoma.* J Natl Cancer Inst, 1974. **52**(2): p. 491-7.
39. Burny, A., et al., *Bovine leukemia virus: Molecular biology and epidemiology*, in *Viral Oncology*, G. Klein, Editor. 1980, Raven Press: New York.
40. Schwartz, I. and D. Levy, *Pathobiology of bovine leukemia virus.* Vet Res, 1994. **25**(6): p. 521-36.
41. Yoon, S.S., et al., *Characteristics of bovine lymphoma caused by bovine leukemia virus infection in Holstein-Friesian dairy cattle in Korea.* Asian-Australas J Anim Sci, 2005. **18**(5): p. 728-733.
42. Ma, J.G., et al., *First report of bovine leukemia virus infection in yaks (bos mutus) in China.* Biomed Res Int, 2016. **2016**: p. 9170167.
43. Meas, S., et al., *Infection of bovine immunodeficiency virus and bovine leukemia virus in water buffalo and cattle populations in Pakistan.* J Vet Med Sci, 2000. **62**(3): p. 329-31.
44. Meas, S., et al., *Seroprevalence of bovine immunodeficiency virus and bovine leukemia virus in draught animals in Cambodia.* J Vet Med Sci, 2000. **62**(7): p. 779-81.

45. Polat, M., et al., *Detection and molecular characterization of bovine leukemia virus in Philippine cattle.* Arch Virol, 2015. **160**(1): p. 285-96.
46. Litwinczuk, Z., et al., *Changes of protein content and its fractions in bovine milk from different breeds subject to somatic cell count.* J Dairy Sci, 2011. **94**(2): p. 684-91.
47. Khan, A., *Basic facts of mastitis in dairy animals: A review*. Vol. 26. 2006.
48. Litwińczuk, Z., J. Król, and A. Brodziak, *Factors determining the susceptibility of cows to mastitis and losses incurred by producers due to the disease – A Review*. Vol. 15. 2015. 1-24.
49. van Knegsel, A.T.M., et al., *Natural antibodies related to energy balance in early lactation dairy cows.* Journal of Dairy Science, 2007. **90**(12): p. 5490-5498.
50. van Knegsel, A.T., et al., *Natural antibodies related to metabolic and mammary health in dairy cows.* Prev Vet Med, 2012. **103**(4): p. 287-97.
51. Zinovieva, N.A., et al., *Prevalence of bovine leukemia virus in black and white cows with the different level of milk productive traits*. 2012. 49-55.
52. Murakami, K., et al., *The recent prevalence of bovine leukemia virus (BLV) infection among Japanese cattle.* Vet Microbiol, 2011. **148**(1): p. 84-8.
53. Hemmatzadeh, F., *Sequencing and phylogenetic analysis of gp51 gene of bovine leukaemia virus in Iranian isolates.* Vet Res Commun, 2007. **31**(6): p. 783-9.
54. Mousavi, S., et al., *Prevalence of bovine leukemia virus (BLV) infection in the northeast of Iran.* Vet Res Forum, 2014. **5**(2): p. 135-9.
55. Xu, A., et al., *Polymorphism in BoLA-DRB3 exon 2 correlates with resistance to persistent lymphocytosis caused by bovine leukemia virus.* J Immunol, 1993. **151**(12): p. 6977-85.
56. Zanotti, M., et al., *Association of BoLA class II haplotypes with subclinical progression of bovine leukemia virus infection in Holstein–Friesian cattle*. Vol. 27. 1996. 337-41.
57. Mirsky, M.L., et al., *Reduced bovine leukaemia virus proviral load in genetically resistant cattle.* Anim Genet, 1998. **29**(4): p. 245-52.
58. Takeshima, S.-n., A. Ohno, and Y. Aida, *Bovine leukemia virus proviral load is more strongly associated with bovine major histocompatibility complex class II DRB3 polymorphism than with DQA1 polymorphism in Holstein cow in Japan.* Retrovirology, 2019. **16**(1): p. 14.
59. Juliarena, M.A., et al., *Association of BLV infection profiles with alleles of the BoLA-DRB3.2 gene.* Anim Genet, 2008. **39**(4): p. 432-8.
60. Panei, C., et al., *Association of BoLA-DRB3.2 alleles with resistance and susceptibility to persistent lymphocytosis in BLV infected cattle in Argentina*. Vol. 4. 2009. 123-128.
61. Nieto Farias, M., et al., *A novel association of BoLA DRB3 alleles in BLV infected cattle with different proviral loads*. Vol. 54. 2017. 215.
62. Hayashi, T., et al., *Cattle with the BoLA class II DRB3*0902 allele have significantly lower bovine leukemia proviral loads.* J Vet Med Sci, 2017. **79**(9): p. 1552-1555.
63. Forletti, A., et al., *Identification of cattle carrying alleles associated with resistance and susceptibility to the Bovine Leukemia Virus progression by real-time PCR.* Res Vet Sci, 2013. **95**(3): p. 991-5.
64. Carignano, H.A., et al., *BOLA-DRB3 gene polymorphisms influence bovine leukaemia virus infection levels in Holstein and Holstein x Jersey crossbreed dairy cattle.* Anim Genet, 2017. **48**(4): p. 420-430.
65. Nikbakht Brujeni, G., R. Ghorbanpour, and A. Esmailnejad, *Association of BoLA-DRB3.2 alleles with BLV infection profiles (persistent lymphocytosis/lymphosarcoma) and lymphocyte subsets in Iranian Holstein cattle.* Biochem Genet, 2016. **54**(2): p. 194-207.
66. Mikko, S. and L. Andersson, *Extensive MHC class II DRB3 diversity in African and European cattle.* Immunogenetics, 1995. **42**(5): p. 408-13.
67. Takeshima, S.N., et al., *The great diversity of major histocompatibility complex class II genes in Philippine native cattle.* Meta Gene, 2014. **2**: p. 176-90.

68. Takeshima, S.N., et al., *Assessment of biodiversity in Chilean cattle using the distribution of major histocompatibility complex class II BoLA-DRB3 allele.* Tissue Antigens, 2015. **85**(1): p. 35-44.
69. Lee, B.Y., et al., *Identification of BoLA-DRB3.2 alleles in Korean native cattle (Hanwoo) and Holstein populations using a next generation sequencer.* Anim Genet, 2012. **43**(4): p. 438-41.
70. Takeshima, S., et al., *The diversity of bovine MHC class II DRB3 genes in Japanese Black, Japanese Shorthorn, Jersey and Holstein cattle in Japan.* Gene, 2003. **316**: p. 111-8.
71. Graves, D.C. and J.F. Ferrer, *In vitro transmission and propagation of the bovine leukemia virus in monolayer cell cultures.* Cancer Res, 1976. **36**(11 Pt 1): p. 4152-9.
72. Altanerova, V., et al., *Infection of rats with bovine leukaemia virus: establishment of a virus-producing rat cell line.* J Gen Virol, 1989. **70 ( Pt 7)**: p. 1929-32.
73. Willems, L., et al., *Attenuation of bovine leukemia virus by deletion of R3 and G4 open reading frames.* Proc Natl Acad Sci U S A, 1994. **91**(24): p. 11532-6.
74. Willems, L., et al., *In vivo infection of sheep by bovine leukemia virus mutants.* J Virol, 1993. **67**(7): p. 4078-85.
75. Mammerickx, M., et al., *Experimental transmission of enzootic bovine leukosis to cattle, sheep and goats: infectious doses of blood and incubation period of the disease.* Leuk Res, 1987. **11**(4): p. 353-8.
76. Gatei, M.H., et al., *Experimental infection of sheep with bovine leukemia virus: infectivity of blood, nasal and saliva secretions.* Zentralbl Veterinarmed B, 1989. **36**(9): p. 652-60.
77. Gatei, M.H., et al., *Lymphosarcoma development in sheep experimentally infected with bovine leukaemia virus.* Zentralbl Veterinarmed B, 1989. **36**(6): p. 424-32.
78. Olson, C. and L.E. Baumgartener, *Pathology of lymphosarcoma in sheep induced with bovine leukemia virus.* Cancer Res, 1976. **36**(7 pt 1): p. 2365-73.
79. Olson, C., et al., *Goat lymphosarcoma from bovine leukemia virus.* J Natl Cancer Inst, 1981. **67**(3): p. 671-5.
80. Altanerova, V., J. Ban, and C. Altaner, *Induction of immune deficiency syndrome in rabbits by bovine leukaemia virus.* Aids, 1989. **3**(11): p. 755-8.
81. Wyatt, C.R., et al., *Persistent infection of rabbits with bovine leukemia virus associated with development of immune dysfunction.* J Virol, 1989. **63**(11): p. 4498-506.
82. Kucerova, L., et al., *Bovine leukemia virus structural gene vectors are immunogenic and lack pathogenicity in a rabbit model.* J Virol, 1999. **73**(10): p. 8160-6.
83. Ferrer, J.F., et al., *Persistent lymphocytosis in cattle: its cause, nature and relation to lymphosarcoma.* Ann Rech Vet, 1978. **9**(4): p. 851-7.
84. Aida, Y., et al., *Further phenotypic characterization of target cells for bovine leukemia virus experimental infection in sheep.* Am J Vet Res, 1989. **50**(11): p. 1946-51.
85. Burny, A., et al., *Biochemical and epidemiological studies on bovine leukemia virus (BLV).* Haematol Blood Transfus, 1979. **23**: p. 445-52.
86. Kenyon, S.J., et al., *Induction of lymphosarcoma in sheep by bovine leukemia virus.* J Natl Cancer Inst, 1981. **67**(5): p. 1157-63.
87. Florins, A., et al., *Emphasis on cell turnover in two hosts infected by bovine leukemia virus: a rationale for host susceptibility to disease.* Vet Immunol Immunopathol, 2008. **125**(1-2): p. 1-7.
88. Kerkhofs, P., et al., *Long-term protection against bovine leukaemia virus replication in cattle and sheep.* J Gen Virol, 2000. **81**(Pt 4): p. 957-63.
89. Dequiedt, F., et al., *Bovine leukemia virus-induced persistent lymphocytosis in cattle does not correlate with increased ex vivo survival of B lymphocytes.* J Virol, 1999. **73**(2): p. 1127-37.
90. Ohishi, K., et al., *Augmentation of bovine leukemia virus (BLV)-specific lymphocyte proliferation responses in ruminants by inoculation with BLV env-recombinant vaccinia virus: their role in the suppression of BLV replication.* Microbiol Immunol, 1992. **36**(12): p. 1317-23.

91. Ohishi, K., et al., *Protective immunity against bovine leukaemia virus (BLV) induced in carrier sheep by inoculation with a vaccinia virus-BLV env recombinant: association with cell-mediated immunity.* J Gen Virol, 1991. **72 ( Pt 8)**: p. 1887-92.
92. Ohishi, K. and Y. Ikawa, *T cell-mediated destruction of bovine leukemia virus-infected peripheral lymphocytes by bovine leukemia virus env-vaccinia recombinant vaccine.* AIDS Res Hum Retroviruses, 1996. **12**(5): p. 393-8.
93. Portetelle, D., et al., *Recombinant vaccinia virus expression of the bovine leukaemia virus envelope gene and protection of immunized sheep against infection.* Vaccine, 1991. **9**(3): p. 194-200.
94. Gatei, M.H., et al., *Protection of sheep against bovine leukemia virus (BLV) infection by vaccination with recombinant vaccinia viruses expressing BLV envelope glycoproteins: correlation of protection with CD4 T-cell response to gp51 peptide 51-70.* J Virol, 1993. **67**(4): p. 1803-10.
95. Okada, K., et al., *Involvement of $CD8^+$ T cells in delayed-type hypersensitivity responses against bovine leukemia virus (BLV) induced in sheep vaccinated with recombinant vaccinia virus expressing BLV envelope glycoprotein.* Vet Pathol, 1993. **30**(2): p. 104-10.
96. Reichert, M., et al., *Protective effects of a live attenuated bovine leukaemia virus vaccine with deletion in the R3 and G4 genes.* J Gen Virol, 2000. **81**(Pt 4): p. 965-9.
97. Cherney, T.M. and R.D. Schultz, *Viral status and antibody response in cattle inoculated with recombinant bovine leukemia virus-vaccinia virus vaccines after challenge exposure with bovine leukemia virus-infected lymphocytes.* Am J Vet Res, 1996. **57**(6): p. 812-8.
98. Buehring, G.C., S.M. Philpott, and K.Y. Choi, *Humans have antibodies reactive with Bovine leukemia virus.* AIDS Res Hum Retroviruses, 2003. **19**(12): p. 1105-13.
99. Buehring, G.C., et al., *Exposure to bovine leukemia virus is associated with breast cancer: A case-control study.* PLoS One, 2015. **10**(9): p. e0134304.
100. Giovanna, M., *Bovine leukemia virus gene segment detected in human breast tissue*. Vol. 03. 2013. 84-90.
101. Burny, A., et al., *Bovine leukaemia virus and enzootic bovine leukosis.* Onderstepoort J Vet Res, 1985. **52**(3): p. 133-44.
102. Juliarena, M.A., S.E. Gutierrez, and C. Ceriani, *Determination of proviral load in bovine leukemia virus-infected cattle with and without lymphocytosis.* Am J Vet Res, 2007. **68**(11): p. 1220-5.
103. Bendixen, H.J., *Bovine enzootic leukosis.* Adv Vet Sci, 1965. **10**: p. 129-204.
104. Kettmann, R., et al., *Bovine leukemia virus*. The Retroviridae. 1994: Plenum Press.
105. DiGiacomo, R.F., R.L. Darlington, and J.F. Evermann, *Natural transmission of bovine leukemia virus in dairy calves by dehorning.* Can J Comp Med, 1985. **49**(3): p. 340-2.
106. Hopkins, S.G. and R.F. DiGiacomo, *Natural Transmission of Bovine Leukemia Virus in Dairy and Beef Cattle.* Veterinary Clinics of North America: Food Animal Practice, 1997. **13**(1): p. 107-128.
107. Lucas, M.H., D.H. Roberts, and G. Wibberley, *Ear tattooing as a method of spread of bovine leukosis virus infection.* Br Vet J, 1985. **141**(6): p. 647-9.
108. Kohara, J., S. Konnai, and M. Onuma, *Experimental transmission of Bovine leukemia virus in cattle via rectal palpation.* The Japanese Journal of Veterinary Science, 2006. **54**(1): p. 25-30.
109. Divers, T.J., et al., *Evidence for transmission of bovine leukemia virus by rectal palpation in a commercial dairy herd.* Preventive Veterinary Medicine, 1995. **23**(3): p. 133-141.
110. Morovati, H., et al., *Seroprevalence of bovine leukemia virus (BLV) infection in dairy cattle in Isfahan Province, Iran.* Trop Anim Health Prod, 2012. **44**(6): p. 1127-9.
111. LaDronka, R.M., et al., *Prevalence of Bovine Leukemia Virus Antibodies in US Dairy Cattle.* Veterinary Medicine International, 2018. **2018**: p. 8.
112. Gutierrez, G., et al., *Dynamics of perinatal bovine leukemia virus infection.* BMC Vet Res, 2014. **10**: p. 82.

113. Gutierrez, G., et al., *Characterization of colostrum from dams of BLV endemic dairy herds.* Vet Microbiol, 2015. **177**(3-4): p. 366-9.
114. Ferrer, J.F., S.J. Kenyon, and P. Gupta, *Milk of dairy cows frequently contains a leukemogenic virus.* Science, 1981. **213**(4511): p. 1014-6.
115. Lassauzet, M.L., et al., *Protection of colostral antibodies against bovine leukemia virus infection in calves on a California dairy.* Can J Vet Res, 1989. **53**(4): p. 424-30.
116. Ferrer, J.F. and C.E. Piper, *Role of colostrum and milk in the natural transmission of the bovine leukemia virus.* Cancer Res, 1981. **41**(12 Pt 1): p. 4906-9.
117. Gutierrez, G., et al., *Natural progression of Bovine Leukemia Virus infection in Argentinean dairy cattle.* Vet Microbiol, 2011. **151**(3-4): p. 255-63.
118. Haghparast, A., et al., *Prevalence of bovine leukemia virus (BLV) antibodies in bulk tank milk of dairy cattle herds of Mashhad Area, North-East of Iran*. Vol. 11. 2012. 276-280.
119. Meas, S., et al., *Vertical transmission of bovine leukemia virus and bovine immunodeficiency virus in dairy cattle herds.* Vet Microbiol, 2002. **84**(3): p. 275-82.
120. Cortese, V.S., *Neonatal immunology.* Vet Clin North Am Food Anim Pract, 2009. **25**(1): p. 221-7.
121. Chase, C.C., D.J. Hurley, and A.J. Reber, *Neonatal immune development in the calf and its impact on vaccine response.* Vet Clin North Am Food Anim Pract, 2008. **24**(1): p. 87-104.
122. Pyeon, D., K.L. O'Reilly, and G.A. Splitter, *Increased interleukin-10 mRNA expression in tumor-bearing or persistently lymphocytotic animals infected with bovine leukemia virus.* J Virol, 1996. **70**(8): p. 5706-10.
123. Amills, M., et al., *Reduced IL-2 and IL-4 mRNA expression in CD4+ T cells from bovine leukemia virus-infected cows with persistent lymphocytosis.* Virology, 2002. **304**(1): p. 1-9.
124. Rodriguez, S.M., et al., *Preventive and therapeutic strategies for bovine leukemia virus: lessons for HTLV.* Viruses, 2011. **3**(7): p. 1210-48.
125. Levy, J.A., *Endogenous C-type viruses in normal and "abnormal" cell development.* Cancer Research, 1977. **37**(8 Part 2): p. 2957.
126. Rous, P., *A transmissible avian neoplasm. (Sarcoma of the common fowl).* J Exp Med, 1910. **12**(5): p. 696-705.
127. Rous, P., *A sarcoma of the fowl transimissible by an agent separable from the tumor cells.* J Exp Med, 1911. **13**(4): p. 397-411.
128. Crittenden, L.B., *The epidemiology of avian lymphoid leukosis.* Cancer Res, 1976. **36**(2 pt 2): p. 570-3.
129. Stephenson, J.R., J.S. Greenberger, and S.A. Aaronson, *Oncogenicity of an endogenous C-type virus chemically activated from mouse cells in culture.* J Virol, 1974. **13**(1): p. 237-40.
130. Tralka, T.S., A.S. Rabson, and C.T. Hansen, *C-Type virus particles in a cell line from a lymphosarcoma of a nude mouse.* J Natl Cancer Inst, 1975. **55**(1): p. 197-8.
131. Gross, L., *"Spontaneous" Leukemia Developing in G3H Mice Following Inoculation, In Infancy, with AK-Emkemic.* 1951. **76**(1): p. 27-32.
132. Levy, J.A. and T. Pincus, *Demonstration of biological activity of a murine leukemia virus of New Zealand black mice.* Science, 1970. **170**(3955): p. 326-7.
133. Jarrett, W.F.H., et al., *Leukaemia in the Cat: A Virus-like Particle associated with Leukaemia (Lymphosarcoma).* Nature, 1964. **202**: p. 567.
134. Jarrett, W.F., et al., *A virus-like particle associated with leukemia (lymphosarcoma)* Nature, 1964. **202**: p. 567-9.
135. Kawakami, T.G., et al., *"C"-type viral particles in plasma of cats with feline leukemia.* Science, 1967. **158**(3804): p. 1049-50.
136. Dutcher, R.M., et al., *Evidence in support of a virus etiology for bovine leukemia.* Cancer, 1967. **20**(5): p. 851-6.
137. Dutcher, R.M., et al., *Etiological studies on bovine lymphosarcoma* Annals of the New York Academy of Sciences, 1963. **108**(3): p. 1149-1162.

138. Dutcher, R.M., et al., *Attempts to demonstrate a Virus for Bovine Lymphosarcoma*.* 1964. **11**(2): p. 93-109.
139. Theilen, G.H., D.L. Dungworth, and T.G. Kawakami, *Leukemia in animals and man.* Calif Med, 1968. **108**(1): p. 14-9.
140. Miller, J.M., et al., *Virus-like particles in phytohemagglutinin-stimulated lymphocyte cultures with reference to bovine lymphosarcoma.* J Natl Cancer Inst, 1969. **43**(6): p. 1297-305.
141. (AHAW)., E.P.o.A.H.a.W., *Enzootic bovine leukosis.* EFSA Journal, 2015. **13**(7): p. 4188.
142. Bollinger, O., *Über Leukämie bei den Haustieren.* Virchows Arch, 1874. **59**: p. 341-349.
143. Siedamgrotzky, O. and V. Hofmeister, *Anleitung zur mikroskopischen und chemischen Diagnostik der Krankheiten der Hausthiere: für Thierärzte und Landwirthe/bearb.* Schönfeld VIII, 1876: p. 192.
144. Schöttler, F. and H. Schöttler, *Über Ätiologie und Therapie der Aleukämischen Lymphadenose des Rindes.* Berliner und Münchener tierärztliche Wochenschrift, 1934. **50**: p. 497-502, 513-517.
145. Zaghawa, A., et al., *An outbreak of enzootic bovine leukosis in upper Egypt: clinical, laboratory and molecular-epidemiological studies.* J Vet Med B Infect Dis Vet Public Health, 2002. **49**(3): p. 123-9.
146. EFSA Panel on Animal Health and Welfare, *Scientific opinion on enzootic bovine leukosis.* EFSA Journal, 2015. **13**(7): p. 1-63.
147. Johson, R. and J.B. Kaneene, *Bovine leukaemia virus and enzootic bovine leukosis.* The Veterinary bulletin 1992. **62**: p. 287-312.
148. Kavanagh, P.J., *Bovine Leukosis is Now Only a Sporadic Problem in Ireland.* Can Vet J, 1981. **22**(12): p. 396-7.
149. Kavanagh, P.J., *Bovine leucosis in Ireland.* Ann Rech Vet, 1978. **9**(4): p. 735-7.
150. Sandev, N., et al., *Prevalence of enzootic bovine leukosis in the Republic of Bulgaria in 1997-2004*. Vol. 76. 2006.
151. Rola-Luszczak, M., et al., *The molecular characterization of bovine leukaemia virus isolates from Eastern Europe and Siberia and its impact on phylogeny.* PLoS One, 2013. **8**(3): p. e58705.
152. Ochirkhuu, N., et al., *Detection of bovine leukemia virus and identification of its genotype in Mongolian cattle.* Arch Virol, 2016. **161**(4): p. 985-91.
153. De Vries, G., *Leucosis in cattle in the Netherlands.* Ann Rech Vet, 1978. **9**(4): p. 903-7.
154. European Commission, *Council Directive 77/391/EEC of 17 May 1977 introducing Community measures for the eradication of brucellosis, tuberculosis and leucosis in cattle.* Official Journal of the European Communities, 1977. **145**: p. 44-47.
155. European Commission, *Summary report on the outcome of EU co-financed animal disease eradication and monitoring programmes: period 2007-2011*. 2013.
156. European Commission, *Report on the outcome of the EU co-financed animal disease eradication and monitoring programmes in the MS and the EU as a whole: Final Report for DG SANCO*. 2011.
157. OIE (World Organisation for Animal Health). *World animal health infromation database-version*. 2018 [cited 2019 Jan, 6]; Available from: http://www.oie.int/wahis_2/public/wahid.php/Diseaseinformation/statuslist.
158. More, S., et al., *Assessment of listing and categorisation of animal diseases within the framework of the Animal Health Law (Regulation (EU) No 2016/429): enzootic bovine leukosis (EBL).* EFSA Journal, 2017. **15**(8).
159. OIE (World Organisation for Animal Health). *World animal health infromation database-version.* 2018 [cited 2019 Jan, 6]; Available from: http://www.oie.int/wahis_2/public/wahid.php/Diseaseinformation/statuslist.
160. European Commission, *Bovine and Swine Diseases Situation 2017*. 2017. p. 1-35.
161. European Commission, *Bovine and Swine Diseases Situation 2016*. 2016. p. 1-39.

162. Polat, M., S.-n. Takeshima, and Y. Aida, *Epidemiology and genetic diversity of bovine leukemia virus.* Virology Journal, 2017. **14**(1).
163. European Commission, *Council Decision 2006/965/EC of 19 December 2006 amending Decision 90/424/ EEC on expenditure in the veterinary field.* Official Journal of the European Union, 2006. **397**: p. 22-27.
164. More, S., et al., *Scientific Opinion on the assessment of listing and categorisation of animal diseases within the framework of the Animal Health Law (Regulation (EU) No 2016/429): enzootic bovine leukosis (EBL)*, in *EFSA Journal 2017*. 2017, EFSA AHAW Panel (EFSA Panel on Animal Health and Welfare),. p. 4956.
165. European Commission, *Council Desion of 25 May 2009 on expenditure in the veterinary field.* Official Journal of the European Union, 2009. **155**: p. 30-44.
166. World Organisation for Animal Health. *WAHIS Interface Animal Health Situation: Disease distribution maps*. 2019 [cited 2019 Jan, 4]; Available from: https://www.oie.int/wahis_2/public/wahid.php/Diseaseinformation/Diseasedistributionmap?disease_type_hidden=&disease_id_hidden=&selected_disease_name_hidden=&disease_type=0&disease_id_terrestrial=35&species_t=0&disease_id_aquatic=-999&species_a=0&sta_method=semesterly&selected_start_year=2016&selected_report_period=1&selected_start_month=12&date_submit=OK.
167. Trono, K.G., et al., *Seroprevalence of bovine leukemia virus in dairy cattle in Argentina: comparison of sensitivity and specificity of different detection methods.* Vet Microbiol, 2001. **83**(3): p. 235-48.
168. Jimenez, C., et al., *Bovine leukaemia-virus infection in Costa Rica.* Zentralbl Veterinarmed B, 1995. **42**(7): p. 385-90.
169. Ndou, R.V., et al., *A serosurvey of the prevalence of enzootic bovine leukosis in the Mafikeng area of the North West Province of South Africa.* Journal of Human Ecology, 2011. **36**(1): p. 53-55.
170. Murakami, K., et al., *Nationwide Survey of Bovine Leukemia Virus Infection among Dairy and Beef Breeding Cattle in Japan from 2009-2011.* Journal of Veterinary Medical Science, 2013. **75**(8): p. 1123-1126.
171. Nekouei, O., et al., *Herd-level risk factors for infection with bovine leukemia virus in Canadian dairy herds.* Prev Vet Med, 2015. **119**(3-4): p. 105-13.
172. Kuczewski, A., et al., *Economic evaluation of 4 bovine leukemia virus control strategies for Alberta dairy farms.* J Dairy Sci, 2019. **102**(3): p. 2578-2592.
173. Erskine, R.J., et al., *Herd-level determinants of bovine leukaemia virus prevalence in dairy farms.* J Dairy Res, 2012. **79**(4): p. 445-50.
174. Kobayashi, S., et al., *Risk factors associated with within-herd transmission of bovine leukemia virus on dairy farms in Japan.* BMC Vet Res, 2010. **6**: p. 1.
175. VanLeeuwen, J.A., et al., *Seroprevalence of infection with Mycobacterium avium subspecies paratuberculosis, bovine leukemia virus, and bovine viral diarrhea virus in maritime Canada dairy cattle.* Can Vet J, 2001. **42**(3): p. 193-8.
176. Scott, H.M., et al., *Seroprevalence of Mycobacterium avium subspecies paratuberculosis, Neospora caninum, Bovine leukemia virus, and Bovine viral diarrhea virus infection among dairy cattle and herds in Alberta and agroecological risk factors associated with seropositivity.* Can Vet J, 2006. **47**(10): p. 981-91.
177. VanLeeuwen, J.A., et al., *Seroprevalences of antibodies against bovine leukemia virus, bovine viral diarrhea virus, Mycobacterium avium subspecies paratuberculosis, and Neospora caninum in beef and dairy cattle in Manitoba.* Can Vet J, 2006. **47**(8): p. 783-6.
178. VanLeeuwen, J.A., et al., *Seroprevalence of antibodies against bovine leukemia virus, bovine viral diarrhea virus, Mycobacterium avium subspecies paratuberculosis, and Neospora caninum in dairy cattle in Saskatchewan.* Can Vet J, 2005. **46**(1): p. 56-8.

179. Nekouei, O., et al., *Predicting within-herd prevalence of infection with bovine leukemia virus using bulk-tank milk antibody levels.* Prev Vet Med, 2015. **122**(1-2): p. 53-60.
180. Ott, S.L., R. Johnson, and S.J. Wells, *Association between bovine-leukosis virus seroprevalence and herd-level productivity on US dairy farms.* Prev Vet Med, 2003. **61**(4): p. 249-62.
181. United States Department of Agriculture Animal and Plant Health Inspection Service, *Bovine Leukosis Virus (BLV) in U.S. Beef Cattle* 1999. p. 1-2.
182. Gnad, D.P., et al., *Prevalence of Bovine Leukemia Virus in Young, Purebred Beef Bulls for Sale in Kansas.* The International Journal of Applied Research in Veterinary Medicine, 2004. **2**(3): p. 215-219.
183. United States Department of Agriculture Animal and Plant Health Inspection Service, *Bovine Leukosis Virus (BLV) on U.S. Dairy Operations, 2007*. 2008. p. 1-2.
184. Bauermann, F.V., J.F. Ridpath, and D.A. Dargatz, *Bovine leukemia virus seroprevalence among cattle presented for slaughter in the United States.* J Vet Diagn Invest, 2017. **29**(5): p. 704-706.
185. Monti, G., R. Schrijver, and D. Beier, *Genetic diversity and spread of Bovine leukaemia virus isolates in Argentine dairy cattle.* Arch Virol, 2005. **150**(3): p. 443-58.
186. Sandev, N., et al., *Prevalence of enzootic bovine leukosis in Bulgaria.* Bulletin UASVM Veterinary Medicine, 2015. **72**(1): p. 43-46.
187. European Commission Health and Consumers Directorate-General, *Eradication programme of enzootic bovine leucosis in Malta: Approved for 2010 by Commission Decision 2009/883/EC*, in *Programmes for the eradication, control and monitoring of certain animal diseases and zoonoses*. 2010, European Commission. p. 1-66.
188. EFSA AHAW Panel (EFSA Panel on Animal Health and Welfare), *Scientific Opinion on the assessment of listing and categorisation of animal diseases within the framework of the Animal Health Law (Regulation (EU) No 2016/429): enzootic bovine leukosis (EBL).* EFSA Journal, 2017. **15**(8): p. 4956.
189. Coulston, J., et al., *Molecular cloning and sequencing of an Australian isolate of proviral bovine leukaemia virus DNA: comparison with other isolates.* J Gen Virol, 1990. **71** (( Pt 8)): p. 1737-46.
190. EFSA Panel on Animal Health and Welfare (AHAW), *Enzootic bovine leukosis.* EFSA Journal, 2015. **13**(7): p. 1-63.
191. Australian Government, *Animal Health Australia 2017*, D.o.A.F.a. Forestry, Editor. 2010. p. 1-256.
192. Schoepf, K.C., et al., *Serological evidence of the occurrence of enzootic bovine leukosis (EBL) virus infection in cattle in Tanzania.* Trop Anim Health Prod, 1997. **29**(1): p. 15-9.
193. Mushi, E.Z., G. Wibberley, and D.C. Kupe, *Antibodies to enzootic bovine viral leukosis in Botswana.* Trop Anim Health Prod, 1990. **22**(2): p. 126.
194. Kiugu, E.K., *Seroprevalence of bovine leukosis infection in selected farming systems in Kenya*, in *Publich Health, Pharmacology and Toxicology*. 2018, University of Nairobi. p. 1-68.
195. Ghanem, Y.M., M.S. Ahmed, and A.H. Abdelkader, *Prevalence of enzootic bovine leukosis (EBL) in the Northern Somalia. .* Bulletin of Animal Health and Production in Africa, 2011. **59**(2): p. 190-198.
196. Morris, S.D., et al., *Serological survey to determine the prevalence of bovine leukaemia virus antibodies in dairy cattle on selected farms in the Gauteng and Mpumalanga provinces.* J S Afr Vet Assoc, 1996. **67**(3): p. 146-7.
197. Meas, S., et al., *Evidence for bovine immunodeficiency virus infection in cattle in Zambia.* Jpn J Vet Res, 2004. **52**(1): p. 3-8.
198. Zhang, L.L., et al., *Human T-cell lymphotropic virus type 1 and its oncogenesis.* Acta Pharmacol Sin, 2017. **38**(8): p. 1093-1103.
199. Franchini, G., *Molecular mechanisms of human T-cell leukemia/lymphotropic virus type I infection.* Blood, 1995. **86**(10): p. 3619-39.

200. Gillet, N., et al., *Mechanisms of leukemogenesis induced by bovine leukemia virus: prospects for novel anti-retroviral therapies in human.* Retrovirology, 2007. **4**: p. 18.
201. Camargos, M.F., J.K.P. Reis, and R.C. Leite, *Bovine Leukemia Virus.* Journal of the Brazilian Society for Virology, 2004. **9**(1): p. 44-59.
202. Lamb, D., et al., *Charge-surrounded pockets and electrostatic interactions with small ions modulate the activity of retroviral fusion proteins.* PLoS Pathog, 2011. **7**(2): p. e1001268.
203. Obal, G., et al., *STRUCTURAL VIROLOGY. Conformational plasticity of a native retroviral capsid revealed by x-ray crystallography.* Science, 2015. **349**(6243): p. 95-8.
204. Barez, P.Y., et al., *Recent Advances in BLV Research.* Viruses, 2015. **7**(11): p. 6080-8.
205. Polat, M., S.N. Takeshima, and Y. Aida, *Epidemiology and genetic diversity of bovine leukemia virus.* Virol J, 2017. **14**(1): p. 209.
206. Sagata, N., et al., *Complete nucleotide sequence of the genome of bovine leukemia virus: its evolutionary relationship to other retroviruses.* Proc Natl Acad Sci U S A, 1985. **82**(3): p. 677-81.
207. Sagata, N., et al., *Identification and some biochemical properties of the major $X_{BL}$ gene product of bovine leukemia virus.* Proceedings of the National Academy of Sciences of the United States of America, 1985. **82**(23): p. 7879-7883.
208. Rice, N.R., et al., *Expression of the bovine leukemia virus X region in virus-infected cells.* J Virol, 1987. **61**(5): p. 1577-85.
209. Alexandersen, S., et al., *Identification of alternatively spliced mRNAs encoding potential new regulatory proteins in cattle infected with bovine leukemia virus.* Journal of Virology, 1993. **67**(1): p. 39-52.
210. Kerkhofs, P., et al., *In vitro and in vivo oncogenic potential of bovine leukemia virus G4 protein.* J Virol, 1998. **72**(3): p. 2554-9.
211. Rice, N.R., et al., *The gag and pol genes of bovine leukemia virus: nucleotide sequence and analysis.* Virology, 1985. **142**(2): p. 357-77.
212. Nisole, S. and A. Saïb, *Early steps of retrovirus replicative cycle.* Retrovirology, 2004. **1**: p. 9-9.
213. Weiss, R.A., *Retrovirus classification and cell interactions.* Journal of Antimicrobial Chemotherapy, 1996. **37**(suppl_B): p. 1-11.
214. Kettmann, R., et al., *Genomic integration of bovine leukemia provirus: comparison of persistent lymphocytosis with lymph node tumor form of enzootic bovine leukosis.* Proc Natl Acad Sci U S A, 1980. **77**(5): p. 2577-81.
215. Kettmann, R., et al., *Experimental infection of sheep and goat with bovine leukemia virus: localization of proviral information on the target cells.* Leuk Res, 1984. **8**(6): p. 937-44.
216. Kettmann, R., et al., *Leukemogenesis by bovine leukemia virus: proviral DNA integration and lack of RNA expression of viral long terminal repeat and 3' proximate cellular sequences.* Proc Natl Acad Sci U S A, 1982. **79**(8): p. 2465-9.
217. Kettmann, R., et al., *Chromosome integration domain for bovine leukemia provirus in tumors.* J Virol, 1983. **47**(1): p. 146-50.
218. Derse, D., S.J. Caradonna, and J.W. Casey, *Bovine leukemia virus long terminal repeat: a cell type-specific promoter.* Science, 1985. **227**(4684): p. 317-20.
219. Derse, D., et al., *Nucleotide sequence and structure of integrated bovine leukemia virus long terminal repeats.* Virology, 1985. **141**(1): p. 162-6.
220. Derse, D., *trans-acting regulation of bovine leukemia virus mRNA processing.* J Virol, 1988. **62**(4): p. 1115-9.
221. Sperka, T., et al., *Bovine leukemia virus protease: comparison with human T-lymphotropic virus and human immunodeficiency virus proteases.* J Gen Virol, 2007. **88**(Pt 7): p. 2052-63.
222. Mansky, L.M. and H.M. Temin, *Lower mutation rate of bovine leukemia virus relative to that of spleen necrosis virus.* J Virol, 1994. **68**(1): p. 494-9.

223. Mansky, L.M. and H.M. Temin, *Lower in vivo mutation rate of human immunodeficiency virus type 1 than that predicted from the fidelity of purified reverse transcriptase.* J Virol, 1995. **69**(8): p. 5087-94.
224. Abram, M.E., et al., *Nature, position, and frequency of mutations made in a single cycle of HIV-1 replication.* Journal of Virology, 2010. **84**(19): p. 9864.
225. Mamoun, R.Z., et al., *The pX region of the bovine leukemia virus is transcribed as a 2.1-kilobase mRNA.* J Virol, 1985. **54**(2): p. 625-9.
226. Rosen, C.A., et al., *The 3' region of bovine leukemia virus genome encodes a transactivator protein.* The EMBO Journal, 1986. **5**(10): p. 2585-2589.
227. Derse, D., *Bovine leukemia virus transcription is controlled by a virus-encoded trans-acting factor and by cis-acting response elements.* J Virol, 1987. **61**(8): p. 2462-71.
228. Willems, L., et al., *The bovine leukemia virus p34 is a transactivator protein.* Embo j, 1987. **6**(11): p. 3385-9.
229. Willems, L., et al., *Cooperation between bovine leukaemia virus transactivator protein and Ha-ras oncogene product in cellular transformation.* Embo j, 1990. **9**(5): p. 1577-81.
230. Kincaid, R.P., J.M. Burke, and C.S. Sullivan, *RNA virus microRNA that mimics a B-cell oncomiR.* Proc Natl Acad Sci U S A, 2012. **109**(8): p. 3077-82.
231. Cullen, B.R., *MicroRNA expression by an oncogenic retrovirus.* Proc Natl Acad Sci U S A, 2012. **109**(8): p. 2695-6.
232. Gillet, N.A., et al., *Bovine leukemia virus small noncoding RNAs are functional elements that regulate replication and contribute to oncogenesis in vivo.* PLoS Pathog, 2016. **12**(4): p. e1005588.
233. Rosewick, N., et al., *Deep sequencing reveals abundant noncanonical retroviral microRNAs in B-cell leukemia/lymphoma.* Proc Natl Acad Sci U S A, 2013. **110**(6): p. 2306-11.
234. Zarkik, S., et al., *Comparative processing of bovine leukemia virus envelope glycoprotein gp72 by subtilisin/kexin-like mammalian convertases.* FEBS Letters, 1997. **406**(1-2): p. 205-210.
235. Johnston, E.R. and K. Radke, *The SU and TM envelope protein subunits of bovine leukemia virus are linked by disulfide bonds, both in cells and in virions.* J Virol, 2000. **74**(6): p. 2930-5.
236. Johnston, E.R., L.M. Albritton, and K. Radke, *Envelope proteins containing single amino acid substitutions support a structural model of the receptor-binding domain of bovine leukemia virus surface protein.* Journal of Virology, 2002. **76**(21): p. 10861-10872.
237. Gatot, J.S., et al., *Bovine leukemia virus SU protein interacts with zinc, and mutations within two interacting regions differently affect viral fusion and infectivity in vivo.* Journal of Virology, 2002. **76**(16): p. 7956-7967.
238. Bruck, C., et al., *Topographical analysis by monoclonal antibodies of BLV-gp51 epitopes involved in viral functions.* Virology, 1982. **122**(2): p. 353-362.
239. Bruck, C., et al., *Monoclonal antibodies define eight independent antigenic regions on the bovine leukemia virus (BLV) envelope glycoprotein gp51.* Virology, 1982. **122**(2): p. 342-52.
240. Bruck, C., et al., *Epitopes of bovine leukemia virus glycoprotein gp51 recognized by sera of infected cattle and sheep.* Leuk Res, 1984. **8**(3): p. 315-21.
241. Bruck, C., et al., *Biologically active epitopes of bovine leukemia virus glycoprotein gp51: their dependence on protein glycosylation and genetic variability.* Virology, 1984. **136**(1): p. 20-31.
242. Ban, J., et al., *Mapping of sequential epitopes recognized by monoclonal antibodies on the bovine leukaemia virus external glycoproteins expressed in Escherichia coli by means of antipeptide antibodies.* J Gen Virol, 1992. **73 ( Pt 9)**: p. 2457-61.
243. Callebaut, I., et al., *Use of synthetic peptides to map sequential epitopes recognized by monoclonal antibodies on the bovine leukemia virus external glycoprotein.* Virology, 1991. **185**(1): p. 48-55.
244. Bai, L., et al., *Novel CD8(+) cytotoxic T cell epitopes in bovine leukemia virus with cattle.* Vaccine, 2015. **33**(51): p. 7194-7202.

245. de Brogniez, A., J. Mast, and L. Willems, *Determinants of the bovine leukemia virus envelope glycoproteins involved in infectivity, replication and pathogenesis.* Viruses, 2016. **8**(4): p. 88.
246. Callebaut, I., et al., *Mapping of B-neutralizing and T-helper cell epitopes on the bovine leukemia virus external glycoprotein gp51.* J Virol, 1993. **67**(9): p. 5321-7.
247. Dube, S., et al., *The complete genomic sequence of a BLV strain from a Holstein cow from Argentina.* Virology, 2000. **277**(2): p. 379-86.
248. Moratorio, G., et al., *A detailed molecular analysis of complete bovine leukemia virus genomes isolated from B-cell lymphosarcomas.* Vet Res, 2013. **44**: p. 19.
249. Zhao, X. and G.C. Buehring, *Natural genetic variations in bovine leukemia virus envelope gene: possible effects of selection and escape.* Virology, 2007. **366**(1): p. 150-65.
250. Gatot, J.S., et al., *Conservative mutations in the immunosuppressive region of the bovine leukemia virus transmembrane protein affect fusion but not infectivity in vivo.* J Biol Chem, 1998. **273**(21): p. 12870-80.
251. Voneche, V., et al., *Fusogenic segments of bovine leukemia virus and simian immunodeficiency virus are interchangeable and mediate fusion by means of oblique insertion in the lipid bilayer of their target cells.* Proc Natl Acad Sci U S A, 1992. **89**(9): p. 3810-4.
252. Willems, L., et al., *In vivo infection of sheep by bovine leukemia virus mutants.* Journal of Virology, 1993. **67**(7): p. 4078-4085.
253. Willems, L., et al., *The YXXL signalling motifs of the bovine leukemia virus transmembrane protein are required for in vivo infection and maintenance of high viral loads.* J Virol, 1995. **69**(7): p. 4137-41.
254. Cianciolo, G.J., et al., *Inhibition of lymphocyte proliferation by a synthetic peptide homologous to retroviral envelope proteins.* Science, 1985. **230**(4724): p. 453-5.
255. Blinov, V.M., et al., *Immunosuppressive domains of retroviruses: Cell mechanisms of the effect on the human immune system.* Molecular Biology, 2013. **47**(5): p. 613-621.
256. Rice, N.R., et al., *The gag and pol genes of bovine leukemia virus: Nucleotide sequence and analysis.* Virology, 1985. **142**(2): p. 357-377.
257. Llames, L., et al., *Cellular distribution of bovine leukemia virus proteins gp51SU, Pr72(env), and Pr66(gag-pro) in persistently infected cells.* Virus Res, 2001. **79**(1-2): p. 47-57.
258. Darlix, J.L., et al., *First glimpses at structure-function relationships of the nucleocapsid protein of retroviruses.* J Mol Biol, 1995. **254**(4): p. 523-37.
259. Morcock, D.R., et al., *Fluorescence and nucleic acid binding properties of bovine leukemia virus nucleocapsid protein.* Biophys Chem, 2002. **97**(2-3): p. 203-12.
260. Wang, H., K.M. Norris, and L.M. Mansky, *Involvement of the matrix and nucleocapsid domains of the bovine leukemia virus Gag polyprotein precursor in viral RNA packaging.* J Virol, 2003. **77**(17): p. 9431-8.
261. Mager, A., et al., *T cell proliferative response to bovine leukaemia virus (BLV): identification of T cell epitopes on the major core protein (p24) in BLV-infected cattle with normal haematological values.* J Gen Virol, 1994. **75 ( Pt 9)**: p. 2223-31.
262. Willems, L., et al., *The major homology region of bovine leukaemia virus p24gag is required for virus infectivity in vivo.* J Gen Virol, 1997. **78 ( Pt 3)**: p. 637-40.
263. Wills, J.W. and R.C. Craven, *Form, function, and use of retroviral gag proteins.* Aids, 1991. **5**(6): p. 639-54.
264. Wang, H., K.M. Norris, and L.M. Mansky, *Analysis of bovine leukemia virus Gag membrane targeting and late domain function.* Journal of Virology, 2002. **76**(16): p. 8485-8493.
265. Li, H., et al., *Myristoylation is required for human immunodeficiency virus type 1 Gag-Gag multimerization in mammalian cells.* J Virol, 2007. **81**(23): p. 12899-910.
266. Resh, M.D., *A myristoyl switch regulates membrane binding of HIV-1 Gag.* Proc Natl Acad Sci U S A, 2004. **101**(2): p. 417-8.
267. Farazi, T.A., G. Waksman, and J.I. Gordon, *The biology and enzymology of protein N-myristoylation.* J Biol Chem, 2001. **276**(43): p. 39501-4.

268. McLaughlin, S. and A. Aderem, *The myristoyl-electrostatic switch: a modulator of reversible protein-membrane interactions.* Trends Biochem Sci, 1995. **20**(7): p. 272-6.
269. Zhou, W., et al., *Identification of a membrane-binding domain within the amino-terminal region of human immunodeficiency virus type 1 Gag protein which interacts with acidic phospholipids.* J Virol, 1994. **68**(4): p. 2556-69.
270. Zhou, W. and M.D. Resh, *Differential membrane binding of the human immunodeficiency virus type 1 matrix protein.* J Virol, 1996. **70**(12): p. 8540-8.
271. Wang, H., K.M. Norris, and L.M. Mansky, *Involvement of the Matrix and Nucleocapsid Domains of the Bovine Leukemia Virus Gag Polyprotein Precursor in Viral RNA Packaging.* Journal of Virology, 2003. **77**(17): p. 9431-9438.
272. Ellenberger, D., et al., *Comparative immunogenicity in rhesus monkeys of multi-protein HIV-1 (CRF02_AG) DNA/MVA vaccines expressing mature and immature VLPs.* Virology, 2005. **340**(1): p. 21-32.
273. Thrane, S., et al., *A novel virus-like particle based vaccine platform displaying the placental malaria antigen VAR2CSA.* PLOS ONE, 2015. **10**(11): p. e0143071.
274. Pinto, L.A., et al., *Cellular immune responses to human papillomavirus (HPV)-16 L1 in healthy volunteers immunized with recombinant HPV-16 L1 virus-like particles.* The Journal of Infectious Diseases, 2003. **188**(2): p. 327-338.
275. Guu, T.S., et al., *Structure of the hepatitis E virus-like particle suggests mechanisms for virus assembly and receptor binding.* Proc Natl Acad Sci U S A, 2009. **106**(31): p. 12992-7.
276. Chackerian, B., *Virus-like particles: flexible platforms for vaccine development.* Expert Rev Vaccines, 2007. **6**(3): p. 381-90.
277. Sedlik, C., et al., *Recombinant parvovirus-like particles as an antigen carrier: a novel nonreplicative exogenous antigen to elicit protective antiviral cytotoxic T cells.* Proc Natl Acad Sci U S A, 1997. **94**(14): p. 7503-8.
278. Cubas, R., et al., *Virus-like particle (VLP) lymphatic trafficking and immune response generation after immunization by different routes.* J Immunother, 2009. **32**(2): p. 118-28.
279. Harper, D.M., et al., *Sustained efficacy up to 4.5 years of a bivalent L1 virus-like particle vaccine against human papillomavirus types 16 and 18: follow-up from a randomised control trial.* Lancet, 2006. **367**(9518): p. 1247-55.
280. Braciale, T.J. and Y.S. Hahn, *Immunity to viruses.* Immunol Rev, 2013. **255**(1): p. 5-12.
281. Romanowski, B., et al., *Sustained efficacy and immunogenicity of the human papillomavirus (HPV)-16/18 AS04-adjuvanted vaccine: analysis of a randomised placebo-controlled trial up to 6.4 years.* Lancet, 2009. **374**(9706): p. 1975-85.
282. Onuma, M., et al., *Cell fusion activity of bovine leukaemia virus.* J Gen Virol, 1980. **48**(Pt 2): p. 421-4.
283. Miller, J.M. and M.J. Van Der Maaten, *Evaluation of an inactivated bovine leukemia virus preparetion as an immunogen in cattle.* Annales de Recherches Veterinaires, 1978. **9**(4): p. 871-877.
284. Onuma, M., et al., *Protection by vaccination against bovine leukemia virus infection in sheep.* Am J Vet Res, 1984. **45**(6): p. 1212-5.
285. Mammerickx, M., et al., *Detection by immunodiffusion- and radioimmunoassay-tests of antibodies to bovine leukemia virus antigens in sera of experimentally infected sheep and cattle.* Zentralbl Veterinarmed B, 1980. **27**(4): p. 291-303.
286. Kono, Y., et al., *Protection against bovine leukemia virus infection in sheep by active and passive immunization.* Nihon Juigaku Zasshi, 1986. **48**(1): p. 117-25.
287. Kettmann, R., et al., *Bovine leukemia virus: an exogenous RNA oncogenic virus.* Proc Natl Acad Sci U S A, 1976. **73**(4): p. 1014-8.
288. Kettmann, R., et al., *Integration of bovine leukemia virus DNA in the bovine genome.* Proc Natl Acad Sci U S A, 1979. **76**(10): p. 4822-6.

289. Wattel, E., et al., *Clonal expansion of infected cells: a way of life for HTLV-I.* J Acquir Immune Defic Syndr Hum Retrovirol, 1996. **13 Suppl 1**: p. S92-9.
290. Mortreux, F., et al., *Somatic mutation in human T-cell leukemia virus type 1 provirus and flanking cellular sequences during clonal expansion in vivo.* J Natl Cancer Inst, 2001. **93**(5): p. 367-77.
291. Sibon, D., et al., *HTLV-1 propels untransformed CD4 lymphocytes into the cell cycle while protecting CD8 cells from death.* J Clin Invest, 2006. **116**(4): p. 974-83.
292. Gillet, N.A., et al., *Massive depletion of bovine leukemia virus proviral clones located in genomic transcriptionally active sites during primary infection.* PLoS Pathog, 2013. **9**(10): p. e1003687.
293. Parfanovich, M.I., et al., *The possibility of specific protection against bovine leukaemia virus infection and bovine leukaemia with inactivated BLV.* British Veterinary Journal, 1983. **139**(2): p. 137-146.
294. Fukuyama, S., et al., *Protection against bovine leukemia virus infection by use of inactivated vaccines in cattle.* J Vet Med Sci, 1993. **55**(1): p. 99-106.
295. Altaner, C., et al., *Bovine leukemia virus: isolation and characterization of nonproducer cell clones.* Neoplasma, 1987. **34**(6): p. 641-52.
296. Altaner, C., et al., *Protective vaccination against bovine leukaemia virus infection by means of cell-derived vaccine.* Vaccine, 1991. **9**(12): p. 889-895.
297. Altaner, C., et al., *Cell‐derived vaccine against bovine leukaemia virus infection.* Journal of Veterinary Medicine, Series B, 1988. **35**(1-10): p. 736-746.
298. Boris-Lawrie, K. and H.M. Temin, *Genetically simpler bovine leukemia virus derivatives can replicate independently of Tax and Rex.* J Virol, 1995. **69**(3): p. 1920-4.
299. Boris-Lawrie, K., et al., *In vivo study of genetically simplified bovine leukemia virus derivatives that lack tax and rex.* J Virol, 1997. **71**(2): p. 1514-20.
300. Altanerova, V., et al., *Long-term infection with retroviral structural gene vector provides protection against bovine leukemia virus disease in rabbits.* Virology, 2004. **329**(2): p. 434-9.
301. Willems, L., et al., *Bovine leukemia virus, an animal model for the study of intrastrain variability.* J Virol, 1993. **67**(2): p. 1086-9.
302. Boris-Lawrie, K. and M. Temin Howard, *The retroviral vector: Replication cycle and safety considerations for retrovirus-mediated gene therapy.* Annals of the New York Academy of Sciences, 1994. **716**(1): p. 59-71.
303. Temin, H.M., *A proposal for a new approach to a preventive vaccine against human immunodeficiency virus type 1.* Proceedings of the National Academy of Sciences, 1993. **90**(10): p. 4419.
304. Hanley, K.A., *The double-edged sword: How evolution can make or break a live-attenuated virus vaccine.* Evolution (N Y), 2011. **4**(4): p. 635-643.
305. Khetsuriani, N., et al., *Persistence of vaccine-derived polioviruses among immunodeficient persons with vaccine-associated paralytic poliomyelitis.* The Journal of Infectious Diseases, 2003. **188**(12): p. 1845-1852.
306. Shahmahmoodi, S., et al., *Vaccine-associated paralytic poliomyelitis in immunodeficient children, Iran, 1995-2008.* Emerg Infect Dis, 2010. **16**(7): p. 1133-6.
307. Odoom, J.K., et al., *Changes in population dynamics during long-term evolution of sabin type 1 poliovirus in an immunodeficient patient.* J Virol, 2008. **82**(18): p. 9179-90.
308. Guillot, S., et al., *Point mutations involved in the attenuation/neurovirulence alternation in type 1 and 2 oral polio vaccine strains detected by site-specific polymerase chain reaction.* Vaccine, 1994. **12**(6): p. 503-7.
309. Zhou, B., et al., *Reversion of cold-adapted live attenuated influenza vaccine into a pathogenic virus.* J Virol, 2016. **90**(19): p. 8454-63.
310. Yoshida, H., et al., *Prevalence of vaccine-derived polioviruses in the environment.* J Gen Virol, 2002. **83**(Pt 5): p. 1107-11.

311. Trainin, Z., et al., *Detrimental effect of bovine leukemia virus (BLV) on the immunological state of cattle.* Vet Immunol Immunopathol, 1996. **54**(1-4): p. 293-302.
312. Blagitz, M.G., et al., *Immunological implications of bovine leukemia virus infection.* Res Vet Sci, 2017. **114**: p. 109-116.
313. Frie, M.C., et al., *Dairy cows naturally infected with bovine leukemia virus exhibit abnormal B- and T-cell phenotypes after primary and secondary exposures to keyhole limpet hemocyanin.* Front Vet Sci, 2017. **4**: p. 112.
314. He, C.Q., et al., *Homologous recombination is apparent in infectious bursal disease virus.* Virology, 2009. **384**(1): p. 51-8.
315. Mochizuki, M., et al., *Recombination between vaccine and field strains of canine parvovirus is revealed by isolation of virus in canine and feline cell cultures.* J Vet Med Sci, 2008. **70**(12): p. 1305-14.
316. Thiry, E., et al., *Recombination in the alphaherpesvirus bovine herpesvirus 1.* Vet Microbiol, 2006. **113**(3-4): p. 171-7.
317. Estevez, C., P. Villegas, and J. El-Attrache, *A recombination event, induced in ovo, between a low passage infectious bronchitis virus field isolate and a highly embryo adaptedvaccine strain.* Avian Dis, 2003. **47**(4): p. 1282-90.
318. Weaver, S.C., et al., *Recombinational history and molecular evolution of western equine encephalomyelitis complex alphaviruses.* J Virol, 1997. **71**(1): p. 613-23.
319. Hahn, C.S., et al., *Western equine encephalitis virus is a recombinant virus.* Proc Natl Acad Sci U S A, 1988. **85**(16): p. 5997-6001.
320. Ohishi, K., et al., *Induction of bovine leukaemia virus Env-specific Th-1 type immunity in mice by vaccination with short synthesized peptide-liposome.* Vaccine, 1996. **14**(12): p. 1143-1148.
321. Kabeya, H., et al., *An effective peptide vaccine to eliminate bovine leukaemia virus (BLV) infected cells in carrier sheep.* Vaccine, 1996. **14**(12): p. 1118-22.
322. Hislop, A.D., et al., *Vaccine-induced cytotoxic T lymphocytes protect against retroviral challenge.* Nat Med, 1998. **4**(10): p. 1193-6.
323. Mateo, L., J. Gardner, and A. Suhrbier, *Delayed emergence of bovine leukemia virus after vaccination with a protective cytotoxic T cell-based vaccine.* AIDS Res Hum Retroviruses, 2001. **17**(15): p. 1447-53.
324. Takeshima, S.-n., et al., *A novel bovine leukemia virus peptide vaccine targeting susceptible cattle-Estimating vaccine effectiveness using susceptible cattle constructed by fertilized ovum transplantation.* Retrovirology, 2015. **12**(Suppl 1): p. P50-P50.
325. Aida, Y., et al., *A novel bovine leukemia virus peptide vaccine targeting susceptible cattle-Production by 3-D modelling and nanotechnology.* Retrovirology, 2015. **12**(Suppl 1): p. P48-P48.
326. Ohishi, K., et al., *Induction of neutralizing antibodies against bovine leukosis virus in rabbits by vaccination with recombinant vaccinia virus expressing bovine leukosis virus envelope glycoprotein*. Vol. 51. 1990. 1170-3.
327. Kumar, S., et al., *Expression of bovine leukaemia virus envelope gene by recombinant vaccinia viruses.* Virus Res, 1990. **17**(2): p. 131-42.
328. Rodriguez, S.M., et al., *Bovine leukemia virus can be classified into seven genotypes: evidence for the existence of two novel clades.* J Gen Virol, 2009. **90**(Pt 11): p. 2788-97.
329. Lee, E., et al., *Molecular epidemiological and serological studies of bovine leukemia virus (BLV) infection in Thailand cattle.* Infect Genet Evol, 2016. **41**: p. 245-254.
330. Polat, M., et al., *A new genotype of bovine leukemia virus in South America identified by NGS-based whole genome sequencing and molecular evolutionary genetic analysis.* Retrovirology, 2016. **13**: p. 4.
331. Portetelle, D., et al., *Antigenic variants of bovine leukemia virus (BLV) are defined by amino acid substitutions in the NH2 part of the envelope glycoprotein gp51.* Virology, 1989. **169**(1): p. 27-33.

332. Portetelle, D., et al., *Synthetic peptides approach to identification of epitopes on bovine leukemia virus envelope glycoprotein gp51.* Virology, 1989. **169**(1): p. 34-41.
333. Pluta, A., et al., *Computational analysis of envelope glycoproteins from diverse geographical isolates of bovine leukemia virus identifies highly conserved peptide motifs.* Retrovirology, 2018. **15**(1): p. 2.
334. Pluta, A., et al., *Molecular characterization of bovine leukemia virus from Moldovan dairy cattle.* Arch Virol, 2017. **162**(6): p. 1563-1576.
335. Licursi, M., et al., *Genetic heterogeneity among bovine leukemia virus genotypes and its relation to humoral responses in hosts.* Virus Res, 2002. **86**(1-2): p. 101-10.
336. Fechner, H., et al., *Provirus variants of the bovine leukemia virus and their relation to the serological status of naturally infected cattle.* Virology, 1997. **237**(2): p. 261-9.
337. Asfaw, Y., et al., *Distribution and superinfection of bovine leukemia virus genotypes in Japan.* Arch Virol, 2005. **150**(3): p. 493-505.
338. Felmer, R., et al., *Molecular analysis of a 444 bp fragment of the bovine leukaemia virus gp51 env gene reveals a high frequency of non-silent point mutations and suggests the presence of two subgroups of BLV in Chile.* Vet Microbiol, 2005. **108**(1-2): p. 39-47.
339. Camargos, M.F., et al., *Molecular characterization of the env gene from Brazilian field isolates of Bovine leukemia virus.* Virus Genes, 2007. **34**(3): p. 343-50.
340. Licursi, M., et al., *Provirus variants of bovine leukemia virus in naturally infected cattle from Argentina and Japan.* Veterinary Microbiology, 2003. **96**(1): p. 17-23.
341. Polat, M., et al., *The molecular epidemiological study of bovine leukemia virus infection in Myanmar cattle.* Arch Virol, 2017. **162**(2): p. 425-437.
342. Dao, T.D., et al., *Application of the SureSelect target enrichment system for next-generation sequencing to obtain the complete genome sequence of bovine leukemia virus.* Arch Virol, 2018. **163**(11): p. 3155-3159.
343. Yang, Y., et al., *First molecular characterization of bovine leukemia virus infections in the caribbean.* PLoS One, 2016. **11**(12): p. e0168379.
344. Wang, M., et al., *Molecular epidemiology and characterization of bovine leukemia virus in domestic yaks (Bos grunniens) on the Qinghai-Tibet Plateau, China.* Arch Virol, 2018. **163**(3): p. 659-670.
345. Pandey, G.S., et al., *Clinical and subclinical bovine leukemia virus infection in a dairy cattle herd in Zambia.* Arch Virol, 2017. **162**(4): p. 1051-1056.
346. Lee, E., et al., *Sequencing and phylogenetic analysis of the gp51 gene from Korean bovine leukemia virus isolates.* Virol J, 2015. **12**: p. 64.
347. Murakami, H., et al., *Variations in the viral genome and biological properties of bovine leukemia virus wild-type strains.* Virus Res, 2018. **253**: p. 103-111.
348. Mamoun, R.Z., et al., *Sequence variability of bovine leukemia virus env gene and its relevance to the structure and antigenicity of the glycoproteins.* J Virol, 1990. **64**(9): p. 4180-8.
349. Balic, D., et al., *Identification of a new genotype of bovine leukemia virus.* Arch Virol, 2012. **157**(7): p. 1281-90.
350. European Commission, *Bovine and Swine Diseases Situation 2016*. 2016. p. 1-38.
351. Molteni, E., et al., *Molecular characterization of a variant of proviral bovine leukaemia virus (BLV).* Zentralbl Veterinarmed B, 1996. **43**(4): p. 201-11.
352. Bazzucchi, M., et al., *Molecular characterization of Italian bovine leukemia virus isolates reveals the presence of distinct phylogenetic clusters.* Arch Virol, 2019.
353. Moratorio, G., et al., *Phylogenetic analysis of bovine leukemia viruses isolated in South America reveals diversification in seven distinct genotypes.* Arch Virol, 2010. **155**(4): p. 481-9.
354. Vafin, R.R., et al., *Genotypic identification of the bovine leukemia virus.* Molecular Genetics, Microbiology and Virology, 2015. **29**(4): p. 195-203.
355. Donnik, I.M., et al., *Genetic identification of bovine leukaemia virus.* Foods and Raw Materials, 2018. **6**(2): p. 1-12.

356. Ababneh, M.M., et al., *Detection and molecular characterization of bovine leukemia viruses from Jordan.* Arch Virol, 2012. **157**(12): p. 2343-8.
357. Alkan, F., et al., *Characterisation of env and gag gene fragments of bovine leukemia viruses (BLVs) from cattle in Turkey.* Arch Virol, 2011. **156**(10): p. 1891-6.
358. Sevik, M., O. Avci, and O.B. Ince, *An 8-year longitudinal sero-epidemiological study of bovine leukaemia virus (BLV) infection in dairy cattle in Turkey and analysis of risk factors associated with BLV seropositivity.* Trop Anim Health Prod, 2015. **47**(4): p. 715-20.
359. Gautam, S., et al., *Molecular characterization of bovine leukaemia virus (BLV) strains reveals existence of genotype 6 in cattle in India with evidence of a new subgenotype.* Transbound Emerg Dis, 2018. **65**(6): p. 1968-1978.
360. Mekata, H., et al., *Horizontal transmission and phylogenetic analysis of bovine leukemia virus in two districts of Miyazaki, Japan.* J Vet Med Sci, 2015. **77**(9): p. 1115-20.
361. Kim, Y.-H., et al., *Genetic analysis of env and gag gene fragments of bovine leukemia virus identified in cattle from Korea.* Korean Journal of Veterinary Research, 2015. **55**(1): p. 53-56.
362. Heinecke, N., et al., *Detection and genotyping of bovine leukemia virus in Mexican cattle.* Arch Virol, 2017. **162**(10): p. 3191-3196.
363. Heinecke, N., et al., *Detection and genotyping of bovine leukemia virus in Mexican cattle.* Archives of Virology, 2017. **162**.
364. Dube, S., et al., *The complete genomic sequence of an in vivo low replicating BLV strain.* Virol J, 2009. **6**: p. 120.
365. Camargos, M.F., et al., *Partial sequencing of env gene of bovine leukaemia virus from Brazilian samples and phylogenetic analysis.* J Vet Med B Infect Dis Vet Public Health, 2002. **49**(7): p. 325-31.
366. D'Angelino, R.H., et al., *Detection of bovine leukemia virus in brains of cattle with a neurological syndrome: pathological and molecular studies.* Biomed Res Int, 2013. **2013**: p. 425646.
367. Gregory, L., et al., *Bovine leukaemia virus genotypes 5 and 6 are circulating in cattle from the state of Sao Paulo, Brazil.* J Med Microbiol, 2017. **66**(12): p. 1790-1797.
368. Úsuga-Monroy, C., et al., *Presebce of bvine leukemia virus genotype 1 and 3 in Antioquia, Colombia* 2018. **21**: p. 119-126.
369. USAD Foreign Agricultural Service, *Russian Federation livestock and products annual Russia 2018 livestock and products annual*. 2018. p. 1-30.
370. Licursi, M., et al., *Provirus variants of bovine leukemia virus in naturally infected cattle from Argentina and Japan.* Vet Microbiol, 2003. **96**(1): p. 17-23.
371. Úsuga-Monroy, C., et al., *Presence of bovine leukemia virus genotypes 1 and 3 in Antioquia, Colombia* Revista U.D.C.A Actualidad & Divulgación 2018. **21**: p. 119-126.
372. European Commission, *Commission implementing decision (EU) 2017/1910 of 17 October 2017.* Official Journal of the European Union, 2017. **269**: p. 46-52.
373. Feliziani, F., et al., *Italy finally complies with the requirements to be declared as free from enzootic bovine leukosis*. Vol. 24. 2018. 3-9.
374. Altschul, S.F., et al., *Basic local alignment search tool.* J Mol Biol, 1990. **215**(3): p. 403-10.
375. Kumar, S., G. Stecher, and K. Tamura, *MEGA7: Molecular evolutionary genetics analysis version 7.0 for bigger datasets.* Mol Biol Evol, 2016. **33**(7): p. 1870-4.
376. Schwarz, G., *Estimating the dimension of a model.* The Annals of Statistics, 1978. **6**(2): p. 461-464.
377. Tamura, K. and M. Nei, *Estimation of the number of nucleotide substitutions in the control region of mitochondrial DNA in humans and chimpanzees.* Mol Biol Evol, 1993. **10**(3): p. 512-26.
378. Kimura, M., *A simple method for estimating evolutionary rates of base substitutions through comparative studies of nucleotide sequences.* Journal of Molecular Evolution, 1980. **16**(2): p. 111-120.

379. Hasegawa, M., H. Kishino, and T. Yano, *Dating of the human-ape splitting by a molecular clock of mitochondrial DNA.* J Mol Evol, 1985. **22**(2): p. 160-74.
380. Felsenstein, J., *Confidence limits on phylogenies: an approach using the bootstrap.* Evolution, 1985. **39**(4): p. 783-791.
381. Geer, L.Y., et al., *The NCBI BioSystems database.* Nucleic Acids Res, 2010. **38**(Database issue): p. D492-6.
382. Jones, D.T., W.R. Taylor, and J.M. Thornton, *The rapid generation of mutation data matrices from protein sequences.* Comput Appl Biosci, 1992. **8**(3): p. 275-82.
383. Trifinopoulos, J., et al., *W-IQ-TREE: a fast online phylogenetic tool for maximum likelihood analysis.* Nucleic Acids Research, 2016. **44**(W1): p. W232-W235.
384. Bicka, L., et al., *Detection of genetic diversity among bovine leukemia virus population by single-strand conformational polymorphism analysis* in *Bulletin of the Veterinary Institute in Pulawy*. 2002. p. 205-212.
385. Matsumura, K., et al., *Molecular epidemiology of bovine leukemia virus associated with enzootic bovine leukosis in Japan.* Virus Res, 2011. **155**(1): p. 343-8.
386. Michener, C.D. and R.R. Sokal, *A quantitative appproach to a problem in classification.* Evolution, 1957. **11**(2): p. 130-162.
387. Blazhko, N., S. Vyshegurov, and K. Shatokhin. *Application of omix technologies in studying of BLV biological diversity by gag gene*. in *International Scientific and Practical Conference "Digitization of Agriculture - Development Strategy" (ISPC 2019)*. 2019. Russia: Atlantis Press.
388. Teto, G., et al., *Gag P2/NC and pol genetic diversity, polymorphism, and drug resistance mutations in HIV-1 CRF02_AG- and non-CRF02_AG-infected patients in Yaoundé, Cameroon.* Scientific Reports, 2017. **7**(1): p. 14136.
389. Kletenkov, K., et al., *Role of Gag mutations in PI resistance in the Swiss HIV cohort study: bystanders or contributors?* Journal of Antimicrobial Chemotherapy, 2016. **72**(3): p. 866-875.
390. Pillay, S.K., et al., *Gag drug resistance mutations in HIV-1 subtype C patients, failing a protease inhibitor inclusive treatment regimen, with detectable lopinavir levels.* J Int AIDS Soc, 2014. **17**(4 Suppl 3): p. 19784.
391. Dam, E., et al., *Gag mutations strongly contribute to HIV-1 resistance to protease inhibitors in highly drug-experienced patients besides compensating for fitness loss.* PLoS Pathog, 2009. **5**(3): p. e1000345.
392. Fun, A., et al., *Human Immunodeficiency Virus Gag and protease: partners in resistance.* Retrovirology, 2012. **9**: p. 63.
393. Auerbach, M.R., et al., *Functional characterization of a portion of the Moloney murine leukemia virus gag gene by genetic footprinting.* Proc Natl Acad Sci U S A, 2003. **100**(20): p. 11678-83.
394. Fu, W. and W.S. Hu, *Functional replacement of nucleocapsid flanking regions by heterologous counterparts with divergent primary sequences: effects of chimeric nucleocapsid on the retroviral replication cycle.* J Virol, 2003. **77**(1): p. 754-61.
395. Gorelick, R.J., et al., *Point mutants of Moloney murine leukemia virus that fail to package viral RNA: evidence for specific RNA recognition by a "zinc finger-like" protein sequence.* Proc Natl Acad Sci U S A, 1988. **85**(22): p. 8420-4.
396. Schwartz, M.D., D. Fiore, and A.T. Panganiban, *Distinct functions and requirements for the Cys-His boxes of the human immunodeficiency virus type 1 nucleocapsid protein during RNA encapsidation and replication.* J Virol, 1997. **71**(12): p. 9295-305.
397. Bowzard, J.B., et al., *Importance of basic residues in the nucleocapsid sequence for retrovirus Gag assembly and complementation rescue.* J Virol, 1998. **72**(11): p. 9034-44.
398. Poon, D.T., J. Wu, and A. Aldovini, *Charged amino acid residues of human immunodeficiency virus type 1 nucleocapsid p7 protein involved in RNA packaging and infectivity.* J Virol, 1996. **70**(10): p. 6607-16.

399. Schmalzbauer, E., et al., *Mutations of basic amino acids of NCp7 of human immunodeficiency virus type 1 affect RNA binding in vitro.* J Virol, 1996. **70**(2): p. 771-7.
400. Dannull, J., et al., *Specific binding of HIV-1 nucleocapsid protein to PSI RNA in vitro requires N-terminal zinc finger and flanking basic amino acid residues.* Embo j, 1994. **13**(7): p. 1525-33.
401. Jewell, N.A. and L.M. Mansky, *In the beginning: genome recognition, RNA encapsidation and the initiation of complex retrovirus assembly.* J Gen Virol, 2000. **81**(Pt 8): p. 1889-99.
402. Rerks-Ngarm, S., et al., *Vaccination with ALVAC and AIDSVAX to Prevent HIV-1 Infection in Thailand.* New England of Journal of Medicine, 2009. **361**: p. 2209–2220.
403. Haynes, B., et al., *Immune-Correlates Analysis of an HIV-1 Vaccine Efficacy Trial.* New England of Journal of Medicine, 2012. **366**: p. 1275–1286.
404. Kutinova, L., et al., *Influence of the parental virus strain on the virulence and immunogenicity of recombinant vaccinia viruses expressing HBV preS2-S protein or VZV glycoprotein I.* Vaccine, 1996. **14**: p. 1045–1052.
405. Grigorieva, I.M., et al., *Immunogenicity of recombinant vaccinia viruses expressing hepatitis B virus surface antigen in mice.* Immunol. Lett., 1993. **36**: p. 267–271.
406. Ogwang, C., et al., *Safety and immunogenicity of heterologous prime-boost immunisation with Plasmodium falciparum malaria candidate vaccines, ChAd63 ME-TRAP and MVA ME-TRAP, in healthy Gambian and Kenyan adults.* PLOS ONE, 2013. **8**: p. e57726.
407. Hodgson, S.H., et al., *Evaluation of the Efficacy of ChAd63-MVA Vectored Vaccines Expressing CS & ME-TRAP Against Controlled Human Malaria Infection in Malaria Naive Individuals.* J. Infect. Dis., 2015. **211**: p. 1076–1086.
408. Blancou, J., et al., *Oral vaccination of the fox against rabies using a live recombinant vaccinia virus.* Nature, 1986. **322**(6077): p. 373-5.
409. Brochier, B., et al., *Towards rabies elimination in Belgium by fox vaccination using a vaccinia-rabies glycoprotein recombinant virus.* Vaccine, 1994. **12**(15): p. 1368-71.
410. Maki, J., et al., *Oral vaccination of wildlife using a vaccinia–rabies-glycoprotein recombinant virus vaccine (RABORAL V-RG®): a global review.* Veterinary Research, 2017. **48**(1): p. 57.
411. Breitbach, C.J., et al., *Pexa-Vec double agent engineered vaccinia: oncolytic and active immunotherapeutic.* Curr Opin Virol, 2015. **13**: p. 49-54.
412. Downs-Canner, S., et al., *Phase 1 Study of Intravenous Oncolytic Poxvirus (vvDD) in Patients With Advanced Solid Cancers.* Mol Ther, 2016. **24**(8): p. 1492-501.
413. Zeh, H.J., et al., *First-in-man study of western reserve strain oncolytic vaccinia virus: safety, systemic spread, and antitumor activity.* Mol Ther, 2015. **23**(1): p. 202-14.
414. Heo, J., et al., *Randomized dose-finding clinical trial of oncolytic immunotherapeutic vaccinia JX-594 in liver cancer.* Nature Medicine, 2013. **19**: p. 329.
415. Sanchez-Sampedro, L., et al., *The evolution of poxvirus vaccines.* Viruses, 2015. **7**(4): p. 1726-803.
416. McFadden, G., *Poxvirus tropism.* Nat Rev Microbiol, 2005. **3**(3): p. 201-13.
417. Sutter, G. and C. Staib, *Vaccinia vectors as candidate vaccines: the development of modified vaccinia virus Ankara for antigen delivery.* Curr Drug Targets Infect Disord, 2003. **3**(3): p. 263-71.
418. Broyles, S.S., *Vaccinia virus transcription.* Journal of General Virology, 2003. **84**(9): p. 2293-2303.
419. Cochran, M.A., C. Puckett, and B. Moss, *In vitro mutagenesis of the promoter region for a vaccinia virus gene: evidence for tandem early and late regulatory signals.* J Virol, 1985. **54**(1): p. 30-7.
420. Venkatesan, S., B.M. Baroudy, and B. Moss, *Distinctive nucleotide sequences adjacent to multiple initiation and termination sites of an early vaccinia virus gene.* Cell, 1981. **25**(3): p. 805-13.
421. Gorse, G.J., et al., *DNA and modified vaccinia virus Ankara vaccines encoding multiple cytotoxic and helper T-lymphocyte epitopes of human immunodeficiency virus type 1 (HIV-1) are safe*

*but weakly immunogenic in HIV-1-uninfected, vaccinia virus-naive adults.* Clin Vaccine Immunol, 2012. **19**(5): p. 649-58.
422. Hanke, T., et al., *Clinical experience with plasmid DNA- and modified vaccinia virus Ankara-vectored human immunodeficiency virus type 1 clade A vaccine focusing on T-cell induction.* J Gen Virol, 2007. **88**(Pt 1): p. 1-12.
423. Staib, C., et al., *Transient host range selection for genetic engineering of modified vaccinia virus Ankara.* Biotechniques, 2000. **28**(6): p. 1137-42, 1144-6, 1148.
424. Von Beust, B.R., et al., *Development and in vitro characterization of recombinant vaccinia viruses expressing bovine leukemia virus gp51 in combination with bovine IL4 or IL12.* Vaccine, 1999. **17**(4): p. 384-95.
425. Garces, J., et al., *Reactivation of transcription from a vaccinia virus early promoter late in infection.* J Virol, 1993. **67**(9): p. 5394-401.
426. Chakrabarti, S., J.R. Sisler, and B. Moss, *Compact, synthetic, vaccinia virus early/late promoter for protein expression.* Biotechniques, 1997. **23**(6): p. 1094-7.
427. Kidokoro, M., et al., *Large-scale preparation of biologically active measles virus haemagglutinin expressed by attenuated vaccinia virus vectors.* Microbes and Infection, 2002. **4**(10): p. 1035-1044.
428. Coupar, B.E., et al., *Temporal regulation of influenza hemagglutinin expression in vaccinia virus recombinants and effects on the immune response.* Eur J Immunol, 1986. **16**(12): p. 1479-87.
429. Baur, K., et al., *Immediate-early expression of a recombinant antigen by modified vaccinia virus ankara breaks the immunodominance of strong vector-specific B8R antigen in acute and memory CD8 T-cell responses.* J Virol, 2010. **84**(17): p. 8743-52.
430. Isshiki, M., et al., *Effects of different promoters on the virulence and immunogenicity of a HIV-1 Env-expressing recombinant vaccinia vaccine.* Vaccine, 2014. **32**(7): p. 839-45.
431. Orubu, T., et al., *Expression and cellular immunogenicity of a transgenic antigen driven by endogenous poxviral early promoters at their authentic loci in MVA.* PLoS One, 2012. **7**(6): p. e40167.
432. Moutaftsi, M., et al., *Vaccinia virus-specific $CD4^+$ T cell responses target a set of antigens largely distinct from those targeted by $CD8^+$ T cell responses.* J Immunol, 2007. **178**(11): p. 6814-20.
433. Yang, Z., et al., *Expression profiling of the intermediate and late stages of poxvirus replication.* J Virol, 2011. **85**(19): p. 9899-908.
434. Moutaftsi, M., et al., *Uncovering the interplay between CD8, CD4 and antibody responses to complex pathogens.* Future Microbiol, 2010. **5**(2): p. 221-39.
435. Bronte, V., et al., *Antigen expression by dendritic cells correlates with the therapeutic effectiveness of a model recombinant poxvirus tumor vaccine.* Proc Natl Acad Sci U S A, 1997. **94**(7): p. 3183-8.
436. Di Pilato, M., et al., *New vaccinia virus promoter as a potential candidate for future vaccines.* J Gen Virol, 2013. **94**(Pt 12): p. 2771-6.
437. Di Pilato, M., et al., *Modification of promoter spacer length in vaccinia virus as a strategy to control the antigen expression.* J Gen Virol, 2015. **96**(8): p. 2360-71.
438. Omar, R., *Comparison of the two lumpy skin disease virus vaccines, Neethling and Herbivac, and construction of a recombinant Herbivac-Rift Valley fever virus vaccine*, in *Clinical Laboratory Sciences*. 2015, University of Cape Town: Cape Town. p. 1-128.
439. Kumar, S. and D.B. Boyle, *A poxvirus bidirectional promoter element with early/late and late functions.* Virology, 1990. **179**(1): p. 151-8.
440. Wyatt, L.S., et al., *Development of a replication-deficient recombinant vaccinia virus vaccine effective against parainfluenza virus 3 infection in an animal model.* Vaccine, 1996. **14**(15): p. 1451-8.
441. Munyanduki, H.M., *Improvement of the capripoxvirus, lumpy skin disease virus for use as a vaccine vector.* 2018, University of Cape Town. p. 194.

442. Douglass, N., *The complete genome sequence of the lumpy skin disease virus vaccine, Herbivac LS, reveals a mutation in the superoxide dismutase gene homolog*, University of Cape Town, Editor. 2019.
443. Phelan, M.C., *Basic techniques in mammalian cell tissue culture.* Curr Protoc Cell Biol, 2007. **Chapter 1**: p. Unit 1.1.
444. Cotter, C.A., et al., *Preparation of cell cultures and vaccinia virus stocks.* Curr Protoc Protein Sci, 2017. **89**: p. 5.12.1-5.12.18.
445. Tran, S.L., et al., *Trypan blue dye enters viable cells incubated with the pore-forming toxin HlyII of Bacillus cereus.* PLoS One, 2011. **6**(9): p. e22876.
446. Boyle, D.B., *Quantitative assessment of poxvirus promoters in fowlpox and vaccinia virus recombinants.* Virus Genes, 1992. **6**(3): p. 281-90.
447. Spriggs, M.K., et al., *Expression of the F and HN glycoproteins of human parainfluenza virus type 3 by recombinant vaccinia viruses: contributions of the individual proteins to host immunity.* J Virol, 1987. **61**(11): p. 3416-23.
448. Funahashi, S., et al., *Increased expression in vivo and in vitro of foreign genes directed by A-type inclusion body hybrid promoters in recombinant vaccinia viruses.* J Virol, 1991. **65**(10): p. 5584-8.
449. Jin, N.Y., S. Funahashi, and H. Shida, *Constructions of vaccinia virus A-type inclusion body protein, tandemly repeated mutant 7.5 kDa protein, and hemagglutinin gene promoters support high levels of expression.* Arch Virol, 1994. **138**(3-4): p. 315-30.
450. Gammon, D.B. and D.H. Evans, *The 3'-to-5' exonuclease activity of vaccinia virus DNA polymerase is essential and plays a role in promoting virus genetic recombination.* J Virol, 2009. **83**(9): p. 4236-50.
451. Wennier, S.T., et al., *A novel naturally occurring tandem promoter in modified vaccinia virus Ankara drives very early gene expression and potent immune responses.* PLOS ONE, 2013. **8**(8): p. e73511.
452. Howley, P.M., D. Spehner, and R. Drillien, *A vaccinia virus transfer vector using a GUS reporter gene inserted into the I4L locus.* Gene, 1996. **172**(2): p. 233-7.
453. Sato, H., et al., *Immunogenicity and safety of the vaccinia virus LC16m8Delta vector expressing SIV Gag under a strong or moderate promoter in a recombinant BCG prime-recombinant vaccinia virus boost protocol.* Vaccine, 2013. **31**(35): p. 3549-57.
454. Cormack, B.P., R.H. Valdivia, and S. Falkow, *FACS-optimized mutants of the green fluorescent protein (GFP).* Gene, 1996. **173**(1 Spec No): p. 33-8.
455. Macdonald, P.J., Y. Chen, and J.D. Mueller, *Chromophore maturation and fluorescence fluctuation spectroscopy of fluorescent proteins in a cell-free expression system.* Anal Biochem, 2012. **421**(1): p. 291-8.
456. Patterson, G.H., et al., *Use of the green fluorescent protein and its mutants in quantitative fluorescence microscopy.* Biophys J, 1997. **73**(5): p. 2782-90.
457. Swaminathan, R., C.P. Hoang, and A.S. Verkman, *Photobleaching recovery and anisotropy decay of green fluorescent protein GFP-S65T in solution and cells: cytoplasmic viscosity probed by green fluorescent protein translational and rotational diffusion.* Biophys J, 1997. **72**(4): p. 1900-7.
458. Cubitt, A.B., et al., *Understanding, improving and using green fluorescent proteins.* Trends Biochem Sci, 1995. **20**(11): p. 448-55.
459. Olenych, S.G., et al., *The fluorescent protein color palette.* Curr Protoc Cell Biol, 2007. **Chapter 21**: p. Unit 21.5.
460. Dixit, R. and R. Cyr, *Cell damage and reactive oxygen species production induced by fluorescence microscopy: effect on mitosis and guidelines for non-invasive fluorescence microscopy.* Plant J, 2003. **36**(2): p. 280-90.

461. Niswender, K.D., et al., *Quantitative imaging of green fluorescent protein in cultured cells: comparison of microscopic techniques, use in fusion proteins and detection limits.* J Microsc, 1995. **180**(Pt 2): p. 109-16.
462. Yang, Z., et al., *Cascade regulation of vaccinia virus gene expression is modulated by multistage promoters.* Virology, 2013. **447**(1): p. 213-220.
463. Koksharov, M.I. and N.N. Ugarova, *Thermostabilization of firefly luciferase by in vivo directed evolution.* Protein Eng Des Sel, 2011. **24**(11): p. 835-44.
464. de Wet, J.R., et al., *Firefly luciferase gene: structure and expression in mammalian cells.* Mol Cell Biol, 1987. **7**(2): p. 725-37.
465. White, E.H., et al., *The chemi- and bioluminescence of firefly luciferin: An efficient chemical production of electronically excited states.* Bioorganic Chemistry, 1971. **1**(1): p. 92-122.
466. Fan, F. and K.V. Wood, *Bioluminescent assays for high-throughput screening.* Assay Drug Dev Technol, 2007. **5**(1): p. 127-36.
467. Jenner, E., *An inquiry into the causes and effects of the variolae vaccinae: a disease discovered in some of the western counties of England, particularly Gloucestershire, and known by the name of the cow pox*. 1798.
468. Pead, P.J., *Benjamin Jesty: new light in the dawn of vaccination.* Lancet, 2003. **362**(9401): p. 2104-9.
469. Hodgson, S.H., et al., *Evaluation of the efficacy of ChAd63-MVA vectored vaccines expressing circumsporozoite protein and ME-TRAP against controlled human malaria infection in malaria-naive individuals.* J Infect Dis, 2015. **211**(7): p. 1076-86.
470. Tulman, E.R., et al., *The genome of canarypox virus.* J Virol, 2004. **78**(1): p. 353-66.
471. Moyer, R.W. and R.L. Graves, *The mechanism of cytoplasmic orthopoxvirus DNA replication.* Cell, 1981. **27**(2 Pt 1): p. 391-401.
472. Smith, G.L. and B. Moss, *Infectious poxvirus vectors have capacity for at least 25 000 base pairs of foreign DNA.* Gene, 1983. **25**(1): p. 21-8.
473. Panicali, D., et al., *Two major DNA variants present in serially propagated stocks of the WR strain of vaccinia virus.* J Virol, 1981. **37**(3): p. 1000-10.
474. Tine, J.A., et al., *NYVAC-Pf7: a poxvirus-vectored, multiantigen, multistage vaccine candidate for Plasmodium falciparum malaria.* Infect Immun, 1996. **64**(9): p. 3833-44.
475. Coupar, B.E., P.G. Oke, and M.E. Andrew, *Insertion sites for recombinant vaccinia virus construction: effects on expression of a foreign protein.* J Gen Virol, 2000. **81**(Pt 2): p. 431-9.
476. Byrd, C.M. and D.E. Hruby, *Construction of recombinant vaccinia virus: cloning into the thymidine kinase locus.* Methods Mol Biol, 2004. **269**: p. 31-40.
477. Sutter, G. and B. Moss, *Nonreplicating vaccinia vector efficiently expresses recombinant genes.* Proc Natl Acad Sci U S A, 1992. **89**(22): p. 10847-51.
478. Ishii, K., et al., *Structural analysis of vaccinia virus DIs strain: application as a new replication-deficient viral vector.* Virology, 2002. **302**(2): p. 433-44.
479. Burgers, W.A., et al., *Construction, characterization, and immunogenicity of a multigene modified vaccinia Ankara (MVA) vaccine based on HIV type 1 subtype C.* AIDS Res Hum Retroviruses, 2008. **24**(2): p. 195-206.
480. Wyatt, L.S., et al., *Elucidating and minimizing the loss by recombinant vaccinia virus of human immunodeficiency virus gene expression resulting from spontaneous mutations and positive selection.* J Virol, 2009. **83**(14): p. 7176-84.
481. Manuel, E.R., et al., *Intergenic region 3 of modified vaccinia ankara is a functional site for insert gene expression and allows for potent antigen-specific immune responses.* Virology, 2010. **403**(2): p. 155-62.
482. Wallace, D.B. and G.J. Viljoen, *Immune responses to recombinants of the South African vaccine strain of lumpy skin disease virus generated by using thymidine kinase gene insertion.* Vaccine, 2005. **23**(23): p. 3061-7.

483. Giavedoni, L., et al., *A vaccinia virus double recombinant expressing the F and H genes of rinderpest virus protects cattle against rinderpest and causes no pock lesions.* Proc Natl Acad Sci U S A, 1991. **88**(18): p. 8011-5.
484. Jones, L., et al., *Protection of goats against peste des petits ruminants with a vaccinia virus double recombinant expressing the F and H genes of rinderpest virus.* Vaccine, 1993. **11**(9): p. 961-4.
485. Mackett, M. and L.C. Archard, *Conservation and Variation in Orthopoxvirus Genome Structure.* Journal of General Virology, 1979. **45**(3): p. 683-701.
486. Kumar, S. and D.B. Boyle, *Activity of a fowlpox virus late gene promoter in vaccinia and fowlpox virus recombinants.* Arch Virol, 1990. **112**(3-4): p. 139-48.
487. Fick, W.C. and G.J. Viljoen, *Identification and characterisation of an early/late bi-directional promoter of the capripoxvirus, lumpy skin disease virus.* Arch Virol, 1999. **144**(6): p. 1229-39.
488. Bertholet, C., R. Drillien, and R. Wittek, *One hundred base pairs of 5' flanking sequence of a vaccinia virus late gene are sufficient to temporally regulate late transcription.* Proc Natl Acad Sci U S A, 1985. **82**(7): p. 2096-100.
489. Meng, X., J. Chao, and Y. Xiang, *Identification from diverse mammalian poxviruses of host-range regulatory genes functioning equivalently to vaccinia virus C7L.* Virology, 2008. **372**(2): p. 372-383.
490. Perkus, M.E., et al., *Vaccinia virus host range genes.* Virology, 1990. **179**(1): p. 276-86.
491. Seet, B.T., et al., *Poxviruses and immune evasion.* Annu Rev Immunol, 2003. **21**: p. 377-423.
492. Sancho, M.C., et al., *The block in assembly of modified vaccinia virus Ankara in HeLa cells reveals new insights into vaccinia virus morphogenesis.* J Virol, 2002. **76**(16): p. 8318-34.
493. de Bruyn, G., et al., *Safety profile of recombinant canarypox HIV vaccines.* Vaccine, 2004. **22**(5-6): p. 704-13.
494. Plotkin, S.A., et al., *The safety and use of canarypox vectored vaccines.* Dev Biol Stand, 1995. **84**: p. 165-70.
495. Garcia-Arriaza, J. and M. Esteban, *Enhancing poxvirus vectors vaccine immunogenicity.* Hum Vaccin Immunother, 2014. **10**(8): p. 2235-44.
496. Tartaglia, J., et al., *Highly attenuated poxvirus vectors.* AIDS Res Hum Retroviruses, 1992. **8**(8): p. 1445-7.
497. Tartaglia, J., et al., *Safety and immunogenicity of recombinants based on the genetically-engineered vaccinia strain, NYVAC.* Dev Biol Stand, 1994. **82**: p. 125-9.
498. Smith, G.L., et al., *Vaccinia virus immune evasion: mechanisms, virulence and immunogenicity.* J Gen Virol, 2013. **94**(Pt 11): p. 2367-92.
499. Dai, K., et al., *Pathogenicity and immunogenicity of recombinant Tiantan Vaccinia Virus with deleted C12L and A53R genes.* Vaccine, 2008. **26**(39): p. 5062-71.
500. Garber, D.A., et al., *Deletion of Specific Immune-Modulatory Genes from Modified Vaccinia Virus Ankara-Based HIV Vaccines Engenders Improved Immunogenicity in Rhesus Macaques.* Journal of Virology, 2012. **86**(23): p. 12605.
501. Garber, D.A., et al., *Expanding the repertoire of Modified Vaccinia Ankara-based vaccine vectors via genetic complementation strategies.* PLoS One, 2009. **4**(5): p. e5445.
502. Fuerst, T.R., P.L. Earl, and B. Moss, *Use of a hybrid vaccinia virus-T7 RNA polymerase system for expression of target genes.* Mol Cell Biol, 1987. **7**(7): p. 2538-44.
503. Ward, G.A., et al., *Stringent chemical and thermal regulation of recombinant gene expression by vaccinia virus vectors in mammalian cells.* Proc Natl Acad Sci U S A, 1995. **92**(15): p. 6773-7.
504. Oh, S., et al., *Coadministration of HIV vaccine vectors with vaccinia viruses expressing IL-15 but not IL-2 induces long-lasting cellular immunity.* Proc Natl Acad Sci U S A, 2003. **100**(6): p. 3392-7.
505. Rao, J.B., et al., *IL-12 is an effective adjuvant to recombinant vaccinia virus-based tumor vaccines: enhancement by simultaneous B7-1 expression.* J Immunol, 1996. **156**(9): p. 3357-65.

506. Legrand, F.A., et al., *Vaccinia viruses with a serpin gene deletion and expressing IFN-γ induce potent immune responses without detectable replication in vivo.* 2005. **102**(8): p. 2940-2945.
507. Nakano, E., D. Panicali, and E. Paoletti, *Molecular genetics of vaccinia virus: demonstration of marker rescue.* Proc Natl Acad Sci U S A, 1982. **79**(5): p. 1593-6.
508. Weir, J.P., G. Bajszar, and B. Moss, *Mapping of the vaccinia virus thymidine kinase gene by marker rescue and by cell-free translation of selected mRNA.* Proc Natl Acad Sci U S A, 1982. **79**(4): p. 1210-4.
509. Moss, B., *Genetically engineered poxviruses for recombinant gene expression, vaccination, and safety.* Proc Natl Acad Sci U S A, 1996. **93**(21): p. 11341-8.
510. Mackett, M., G.L. Smith, and B. Moss, *General method for production and selection of infectious vaccinia virus recombinants expressing foreign genes.* J Virol, 1984. **49**(3): p. 857-64.
511. Moss, B. and C. Flexner, *Vaccinia virus expression vectors.* Ann N Y Acad Sci, 1989. **569**: p. 86-103.
512. Hertig, C., et al., *Retrovirus-like particles produced by vaccinia viruses expressing gag-pro-pol region genes of bovine leukaemia virus.* J Gen Virol, 1994. **75 ( Pt 9)**: p. 2213-21.
513. Kakker, N.K., et al., *Bovine leukemia virus Gag particle assembly in insect cells: formation of chimeric particles by domain-switched leukemia/lentivirus Gag polyprotein.* Virology, 1999. **265**(2): p. 308-18.
514. Deml, L., et al., *Recombinant HIV-1 Pr55gag virus-like particles: potent stimulators of innate and acquired immune responses.* Mol Immunol, 2005. **42**(2): p. 259-77.
515. Rice, P., I. Longden, and A. Bleasby, *EMBOSS: The European Molecular Biology Open Software Suite.* Trends in Genetics, 2000. **16**(6): p. 276-277.
516. Futatsumori-Sugai, M. and K. Tsumoto, *Signal peptide design for improving recombinant protein secretion in the baculovirus expression vector system.* Biochem Biophys Res Commun, 2010. **391**(1): p. 931-5.
517. Wen, B., et al., *Signal peptide replacements enhance expression and secretion of hepatitis C virus envelope glycoproteins.* Acta Biochimica et Biophysica Sinica, 2010. **43**(2): p. 96-102.
518. Wang, J.Y., et al., *Improved expression of secretory and trimeric proteins in mammalian cells via the introduction of a new trimer motif and a mutant of the tPA signal sequence.* Appl Microbiol Biotechnol, 2011. **91**(3): p. 731-40.
519. Romano, M., et al., *Priming but not boosting with plasmid DNA encoding mycolyl-transferase Ag85A from Mycobacterium tuberculosis increases the survival time of Mycobacterium bovis BCG vaccinated mice against low dose intravenous challenge with M. tuberculosis H37Rv.* Vaccine, 2006. **24**(16): p. 3353-64.
520. Cai, H., et al., *A combined DNA vaccine-prime, BCG-boost strategy results in better protection against Mycobacterium bovis challenge.* DNA Cell Biol, 2006. **25**(8): p. 438-47.
521. Zhang, Y., et al., *Effects of the fusion design and immunization route on the immunogenicity of Ag85A-Mtb32 in adenoviral vectored tuberculosis vaccine.* Hum Vaccin Immunother, 2015. **11**(7): p. 1803-13.
522. Kou, Y., et al., *Tissue plasminogen activator (tPA) signal sequence enhances immunogenicity of MVA-based vaccine against tuberculosis.* Immunol Lett, 2017. **190**: p. 51-57.
523. Zhang, L., Q. Leng, and A.J. Mixson, *Alteration in the IL-2 signal peptide affects secretion of proteins in vitro and in vivo.* The Journal of Gene Medicine, 2005. **7**(3): p. 354-365.
524. Li, Y., et al., *Control of expression, glycosylation, and secretion of HIV-1 gp120 by homologous and heterologous signal sequences.* Virology, 1994. **204**(1): p. 266-278.
525. Li, Y., et al., *The HIV-1 Env protein signal sequence retards its cleavage and down-regulates the glycoprotein folding.* Virology, 2000. **272**(2): p. 417-28.
526. Herrera, A.M., et al., *Efficiency of erythropoietin's signal peptide for HIV(MN)-1 gp 120 expression.* Biochemical and Biophysical Research Communications, 2000. **273**(2): p. 557-559.

527. Upadhyay, C., et al., *Alterations of HIV-1 envelope phenotype and antibody-mediated neutralization by signal peptide mutations.* PLOS Pathogens, 2018. **14**(1): p. e1006812.
528. Yolitz, J., et al., *Signal peptide of HIV envelope protein impacts glycosylation and antigenicity of gp120.* Proceedings of the National Academy of Sciences, 2018.
529. Megati, S., et al., *Modifying the HIV-1 env gp160 gene to improve pDNA vaccine-elicited cell-mediated immune responses.* Vaccine, 2008. **26**(40): p. 5083-94.
530. van Diepen, M.T., et al., *The adjuvant AlhydroGel elicits higher antibody titres than AddaVax when combined with HIV-1 subtype C gp140 from CAP256.* PLoS One, 2018. **13**(12): p. e0208310.
531. van Diepen, M.T., et al., *Prime-boost immunizations with DNA, modified vaccinia virus Ankara, and protein-based vaccines elicit robust HIV-1 tier 2 neutralizing antibodies against the CAP256 superinfecting virus.* J Virol, 2019. **93**(8).
532. Tulman, E.R., et al., *Genome of lumpy skin disease virus.* Journal of Virology, 2001. **75**(15): p. 7122.
533. Smith, G.L. and M. Law, *The exit of vaccinia virus from infected cells.* Virus Res, 2004. **106**(2): p. 189-97.
534. Vossen, M.T., et al., *Viral immune evasion: a masterpiece of evolution.* Immunogenetics, 2002. **54**(8): p. 527-42.
535. Ringe, R.P., et al., *Closing and opening holes in the glycan shield of HIV-1 envelope glycoprotein SOSIP trimers can redirect the neutralizing antibody response to the newly unmasked epitopes.* J Virol, 2019. **93**(4).
536. Richard, M., et al., *Influenza A virus reassortment is limited by anatomical compartmentalization following coinfection via distinct routes.* Journal of Virology, 2018. **92**(5): p. e02063-17.
537. Korber, B.T., N.L. Letvin, and B.F. Haynes, *T-cell caccine strategies for human immunodeficiency virus, the virus with a thousand faces.* Journal of Virology, 2009. **83**(17): p. 8300.
538. Rosel, J.L., et al., *Conserved TAAATG sequence at the transcriptional and translational initiation sites of vaccinia virus late genes deduced by structural and functional analysis of the HindIII H genome fragment.* J Virol, 1986. **60**(2): p. 436-49.
539. Shchelkunov, S.N., et al., *Comparison of the genetic maps of variola and vaccinia viruses.* FEBS Lett, 1993. **327**(3): p. 321-4.
540. Shida, H., et al., *Effect of the recombinant vaccinia viruses that express HTLV-I envelope gene on HTLV-I infection.* Embo j, 1987. **6**(11): p. 3379-84.
541. Shida, H., et al., *Effects and virulences of recombinant vaccinia viruses derived from attenuated strains that express the human T-cell leukemia virus type I envelope gene.* Journal of Virology, 1988. **62**(12): p. 4474-4480.
542. Parks, R.J., et al., *DNA ligase gene disruptions can depress viral growth and replication in poxvirus-infected cells.* Virus Res, 1998. **56**(2): p. 135-47.
543. Rempel, R.E. and P. Traktman, *Vaccinia virus B1 kinase: phenotypic analysis of temperature-sensitive mutants and enzymatic characterization of recombinant proteins.* J Virol, 1992. **66**(7): p. 4413-26.
544. Bayliss, C.D. and G.L. Smith, *Vaccinia virion protein I8R has both DNA and RNA helicase activities: implications for vaccinia virus transcription.* J Virol, 1996. **70**(2): p. 794-800.
545. Hedengren-Olcott, M., et al., *The vaccinia virus G1L putative metalloproteinase is essential for viral replication in vivo.* Journal of virology, 2004. **78**(18): p. 9947-9953.
546. Ansarah-Sobrinho, C. and B. Moss, *Vaccinia virus G1 protein, a predicted metalloprotease, is essential for morphogenesis of infectious virions but not for cleavage of major core proteins.* J Virol, 2004. **78**(13): p. 6855-63.

547. Wille-Reece, U., et al., *Immunization with HIV-1 Gag protein conjugated to a TLR7/8 agonist results in the generation of HIV-1 Gag-specific Th1 and CD8+ T cell responses.* J Immunol, 2005. **174**(12): p. 7676-83.
548. Stephenson, K.E., et al., *Gag-specific cellular immunity determines in vitro viral inhibition and in vivo virologic control following simian immunodeficiency virus challenges of vaccinated rhesus monkeys.* J Virol, 2012. **86**(18): p. 9583-9.
549. Buseyne, F., et al., *Gag-specific cytotoxic T lymphocytes from human immunodeficiency virus type 1-infected individuals: Gag epitopes are clustered in three regions of the p24gag protein.* J Virol, 1993. **67**(2): p. 694-702.
550. Turk, G., et al., *Early Gag immunodominance of the HIV-specific T-cell response during acute/early infection is associated with higher $CD8^+$ T-cell antiviral activity and correlates with preservation of the $CD4^+$ T-cell compartment.* J Virol, 2013. **87**(13): p. 7445-62.
551. Jia, M., et al., *Preferential CTL targeting of Gag is associated with relative viral control in long-term surviving HIV-1 infected former plasma donors from China.* Cell Res, 2012. **22**(5): p. 903-14.
552. Lülf, A.-T., et al., *Non-plaque-forming virions of modified vaccinia virus Ankara express viral genes.* Virology, 2016. **499**: p. 322-330.
553. Perkus, M.E., K. Limbach, and E. Paoletti, *Cloning and expression of foreign genes in vaccinia virus, using a host range selection system.* Journal of virology, 1989. **63**(9): p. 3829-3836.
554. Liu, L., et al., *Transient dominant host-range selection using Chinese hamster ovary cells to generate marker-free recombinant viral vectors from vaccinia virus.* Biotechniques, 2017. **62**(4): p. 183-187.
555. Ricci, P.S., et al., *Selection of recombinant MVA by rescue of the essential D4R gene.* Virol J, 2011. **8**: p. 529.
556. Holzer, G.W., et al., *Dominant host range selection of vaccinia recombinants by rescue of an essential gene.* Virology, 1998. **249**(1): p. 160-6.
557. Lorenzo, M.M., I. Galindo, and R. Blasco, *Construction and isolation of recombinant vaccinia virus using genetic markers.* Methods Mol Biol, 2004. **269**: p. 15-30.
558. Murata, H., et al., *Plaque purification as a method to mitigate the risk of adventitious-agent contamination in influenza vaccine virus seeds.* Vaccine, 2011. **29**(17): p. 3155-61.
559. Srikanth Reddy, M., et al., *Plaque purification of bluetongue virus serotype-4 (BTV-4).* International Journal of Current Microbiology and Applied Sciences, 2018. **7**: p. 2837-2844.
560. Moss, B., *Vaccinia virus: a tool for research and vaccine development.* Science, 1991. **252**(5013): p. 1662.
561. Rice, A.D., et al., *An efficient method for generating poxvirus recombinants in the absence of selection.* Viruses, 2011. **3**(3): p. 217-32.
562. Hruby, D.E., *Vaccinia virus vectors: new strategies for producing recombinant vaccines.* Clin Microbiol Rev, 1990. **3**(2): p. 153-70.
563. Yao, X.D. and D.H. Evans, *Effects of DNA structure and homology length on vaccinia virus recombination.* Journal of virology, 2001. **75**(15): p. 6923-6932.
564. Cannan, W.J. and D.S. Pederson, *Mechanisms and consequences of double-strand DNA break formation in chromatin.* J Cell Physiol, 2016. **231**(1): p. 3-14.
565. Belfort, M. and R.P. Bonocora, *Homing endonucleases: from genetic anomalies to programmable genomic clippers.* Methods Mol Biol, 2014. **1123**: p. 1-26.
566. Ma, A.C., et al., *TALEN-mediated mutagenesis and genome editing.* Methods Mol Biol, 2016. **1451**: p. 17-30.
567. Carroll, D., *Genome engineering with zinc-finger nucleases.* Genetics, 2011. **188**(4): p. 773-82.
568. Sargent, R.G., M.A. Brenneman, and J.H. Wilson, *Repair of site-specific double-strand breaks in a mammalian chromosome by homologous and illegitimate recombination.* Mol Cell Biol, 1997. **17**(1): p. 267-77.

569. Gomez-Cabello, D., et al., *New tools to study DNA double-strand break repair pathway choice.* PLoS One, 2013. **8**(10): p. e77206.
570. Shrivastav, M., L.P. De Haro, and J.A. Nickoloff, *Regulation of DNA double-strand break repair pathway choice.* Cell Res, 2008. **18**(1): p. 134-47.
571. Hu, Z., et al., *Ligase IV inhibitor SCR7 enhances gene editing directed by CRISPR-Cas9 and ssODN in human cancer cells.* Cell Biosci, 2018. **8**: p. 12.
572. Maruyama, T., et al., *Increasing the efficiency of precise genome editing with CRISPR-Cas9 by inhibition of nonhomologous end joining.* Nat Biotechnol, 2015. **33**(5): p. 538-42.
573. Vartak, S.V. and S.C. Raghavan, *Inhibition of nonhomologous end joining to increase the specificity of CRISPR/Cas9 genome editing.* Febs j, 2015. **282**(22): p. 4289-94.
574. Ma, Y., et al., *Increasing the efficiency of CRISPR/Cas9-mediated precise genome editing in rats by inhibiting NHEJ and using Cas9 protein.* RNA Biol, 2016. **13**(7): p. 605-12.
575. Lin, C., et al., *Increasing the efficiency of CRISPR/Cas9-mediated precise genome editing of HSV-1 virus in human cells.* Sci Rep, 2016. **6**: p. 34531.
576. Chu, V.T., et al., *Increasing the efficiency of homology-directed repair for CRISPR-Cas9-induced precise gene editing in mammalian cells.* Nat Biotechnol, 2015. **33**(5): p. 543-8.
577. Kuwayama, H., T. Yanagida, and M. Ueda, *DNA oligonucleotide-assisted genetic manipulation increases transformation and homologous recombination efficiencies: Evidence from gene targeting of Dictyostelium discoideum.* J Biotechnol, 2008. **133**(4): p. 418-23.
578. Chen, F., et al., *High-frequency genome editing using ssDNA oligonucleotides with zinc-finger nucleases.* Nat Methods, 2011. **8**(9): p. 753-5.
579. Katsafanas, G.C. and B. Moss, *Colocalization of transcription and translation within cytoplasmic poxvirus factories coordinates viral expression and subjugates host functions.* Cell Host Microbe, 2007. **2**(4): p. 221-8.
580. Luteijn, R.D., et al., *Mutagenic repair of double-stranded DNA breaks in vaccinia virus genomes requires cellular DNA ligase IV activity in the cytosol.* J Gen Virol, 2018. **99**(6): p. 790-804.
581. Rice, N.R., et al., *The nucleotide sequence of the env gene and post-env region of bovine leukemia virus.* Virology, 1984. **138**(1): p. 82-93.
582. Oguma, K., M. Suzuki, and H. Sentsui, *Enzootic bovine leukosis in a two-month-old calf.* Virus Res, 2017. **233**: p. 120-124.
583. Murakami, H., et al., *Inefficient viral replication of bovine leukemia virus induced by spontaneous deletion mutation in the G4 gene.* J Gen Virol, 2016. **97**(10): p. 2753-2762.