Generation of Complex Recombinant Fowlpox Virus 9 (FP9) Encoding Simian Immunodeficiency Virus (SIVmac239) Sequences as a Model HIV Vaccine Candidate

A thesis submitted to The University of Manchester for the degree of Doctor of Philosophy in the Faculty of Medical and Human Sciences

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List of Abbreviations

ADCC: Antibody dependent cell mediated cytotoxicity

AdV: Adenovirus

AIDS: Acquired immune deficiency syndrome

ALVAC: Attenuated canarypox virus vector

AP: Alkaline phosphatase

APCs: Antigen presenting cells

ASPA: Animal Scientific Procedures Act

bp: Base pairs

BSA: Bovine Serum Albumin

BSC-1: Monkey kidney epithelial cell line

CEFs: Chicken embryo fibroblasts

CEA: carcinoembryonic antigen

CD4: Cluster of differentiation 4

CD8: Cluster of differentiation 8

CIP: Calf Intestinal-Alkaline Phosphatase

CO_2: Carbon dioxide

CPD: Cyclobutane pyrimidine dimer

CPE: Cytopathic effects

CTL: Cytotoxic T cell

CNPV: Canarypox virus

CMV: Cytomegalovirus

cm: Centimetre

CV-1: Monkey kidney fibroblast cell line

DAB: 3,3'-Diaminobenzidine

DC: Dendritic cells

DNA: Deoxyribose nucleic acid

DMEM: Dulbecco's modified eagle medium

DMSO: Dimethyl Sulphoxide

dH_2O: deionised water

DSIV: Defective SIV genome

EEV: Extracellular enveloped virus

E.coli: *Escherichia coli*

EDTA: Ethylenediaminetetraacetic acid

ELISA: Enzyme-linked immunosorbent assay

ELISpot: Enzyme-linked immune spot assay

EMCV: Encephalitis myocarditis virus

Env: Envelope

FBS: Fetal bovine serum

FITC: Fluorescein isothiocyanate

FP9: Fowlpox virus 9

Gag: Group specific antigen

GFP: Green-flourescent protein

gp: Glycoprotein

g: gram

HCV: Hepatitis C virus

HCl: Hydrochloric acid

HF: High-Fidelity

HI: Hemagglutination-Inhibition

HR: Homologous recombination

HRP: Horseradish peroxidase

HS: Homologous sequence

HIV: Human immunodeficiency virus

H&E: Hematoxylin and Eosin

hpi: Hours post-infection

h: Hours

H9: Human embryonic stem cell line

H_2So_4: Sulfuric acid

ICC: Immunocytochemical

ICS: Intracellular cytokine staining

IgA: Immunoglobulin A

IgG: Immunoglobulin G

IgM: Immunoglobulin M

IEV: Intracellular enveloped virus

IFN: Interferon

IL: Interleukin

Int: Integrase

IMV: Intracellular mature virus

IRES: Internal ribosome entry sites

ITR: Inverted terminal repeat

Kb: Kilobase

Kbp: Kilobase pair

KDa: Kilodalton

KCl: Potassium Chloride

L: Litre

LB: Luria Bertani

LF: Left fragment

L-Glut: L-glutamine

LHS: Left homologous sequence

LTR: Long terminal repeat

LTNPs: Long term non-progressors

LMP: Low melting point

M: Mole

mA: Milliampere

ml: Millilitre

mm: Millimetre

Mm: Millimolar

MACs: Membrane attack complexes

MEM: Modified eagles medium

mDCs: Myeloid dendritic cells

MOI: Multiplicity of infection

$MgCl_2$: Magnesium Chloride

$MgSo_4$: Magnesium Sulfate

min: Minutes

MHC: Major histocompatibility complex

MPER: Membrane proximal external region

mRNA: Messenger ribose nucleic acid

MRC-5: Normal human lung cell line

MVA: Modified vaccinia Ankara

Nabs: Neutralising antibodies

NaCl: Sodium Chloride

NaOH: Sodium Hydroxide

ng: nanogram

NEB: New England Biolabs

NF: Negative factor

ND: Nanodrop

NDV: Newcastle disease virus

NK: Natural killer

NIBSC: National Institute for Biological Standards and Controls

NSI: Non Syncytia inducing strains

NS: No significant

NYVA: New York Vaccinia virus

ORFs: Open reading frames

OD: Optical density

pCEF: Primary chick embryo fibroblast

PBMCs: Peripheral blood mononuclear cells

PbCS: Plasmodium berghei circumsporozoite protein

pBR322: Low copy number plasmid DNA

PCP: Pneumocystis carinii pneumonia

PCR: Polymerase chain reaction

PfCS: Plasmodium falciparum circumsporozoite protein

pDCs: Plasmacytoid dendritic cells

pfu: Plaque-forming unit

pCO: Plasmid containing human codon-optimised sequences

pol: Polymerase

PND: Principal neutralising domain

pUC: High copy number plasmid DNA

PVDF: Polyvinylidene difluoride

P-value: Probability of obtaining the observed result

RANTES: Regulated on activation normal T cell expressed and secreted

RE: Restriction enzyme

RF: Right fragment

Rev: Regulatory of virus

rFP9: Recombinant fowlpox virus 9

RNA: Ribose nucleic acid

rpm: Round per minute

RHS: Right homologous sequence

RT: Reverse transcription

RIPA: Radio immune precipitation assay

REV: Reticuloendotheliosis virus

RFP: Red fluorescence protein

rVACV: Recombinant vaccinia virus

rMVA: Recombinant modified vaccinia Ankara

Sec: Seconds

SD: Standard deviation

SDS: Sodium dodecyl sulphate

SDS-PAGE: Sodium dodecyl sulphate polyacrylamide gel electrophoresis

SIV: Simian immunodeficiency virus

SIVmac239: Simian immunodeficiency virus of macaques 239 strain

SHIV: Simian human immunodeficiency virus

SI: Syncytia inducing strains

SOC: Super optimal broth with catabolite repression

SPF: Specific pathogen free

ssRNA: Single stranded ribose nucleic acid

tat: transactivation

TAE: Tris-Acetate-EDTA buffer

TAR: Transactivation response element

TBS: Tris-buffered saline

TCS: Transient colour selection

Tcm: Central memory T cell

TE: Tris-Acetate

Tem: Effector memory T cell

Th: T helper cell

TMB: 3,3’,5,5’ Tetramethylbenzidine

™: Trade Mark

TNFs: Tumour necrosis factor

TK: Thymidine kinase

T5NT: Transcription termination signals

UK: United Kingdom

USA: United States of America

UV: Ultraviolet

VERO: African green monkey kidney cell line

Vif: Viral infectivity

VACV: Vaccinia virus

V: Volts

VLPs: Virus-like-particles

V: Variable loop

Vpr: Viral protein R

Vpu: Viral protein U

Vpx: Viral protein X

WB: Western blot

WVs: Wrapped virions

w/v%: Weight per volume percentage

X-Gal: 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside

α: Alpha

β: Beta

β-Gal: Beta-galctosidase

γ: Gamma

γδ: Gamma delta T cells

°C: Degree Celsius

µg: Microgram

µL: Microlitre

µm: Micrometer

-ve: Negative

+ve: Positive

Abstract

A thesis submitted to The University of Manchester by Radi Alsafi for the degree of Doctor of Philosophy entitled

''Generation of Complex Recombinant Fowlpox Virus 9 (FP9) Encoding Simian Immunodeficiency Virus (SIVmac239) Sequences as a Model HIV Vaccine Candidate''

March 2016

The development of a safe and effective HIV vaccine remains challenging due to its high antigenic variability. Poxviruses are large, stable, and have a track record of use as human vaccine candidates. Recombinant fowlpox virus 9 (rFP9), a highly attenuated host range-restricted poxvirus strain, has been safely administered to humans with no ill effects, and is known to be immunogenic. This thesis describes the construction of complex rFP9 encoding various sequences of SIVmac239. The SIVmac239/macaque model is widely used for HIV vaccine development. The ultimate aim of this work was to combine the advantages of FP9 with those of live attenuated SIV to produce a safe yet hopefully effective model HIV vaccine candidate.

Transfer plasmids for five different insertion sites within the FP9 genome were designed and constructed. Homologous recombination (HR) of adjacent FP9 sequences was employed to facilitate the integration of SIVmac239 sequences into the FP9 genome. Positive rFP9 were identified by blue colouration in presence of X-gal using a transient colour selection (TCS) technique, and the final markerless pure recombinants were confirmed by PCR. Expression of the target SIV proteins in the presence of T7 polymerase has been demonstrated by immunocytochemical (ICC) staining and Western blotting (WB) assays. Expression was also quantified by enzyme-linked immunosorbent assay (ELISA) in various cell lines at multiple time points.

Five different unique rFP9 have been constructed through this project. All SIVmac239 open reading frames (ORFs) save nef have been integrated into the FP9 genome, and protein expression demonstrated where possible. Moreover, a single rFP9 vector expressing the defective SIVmac239 genome driven by T7 RNA polymerase has been successfully constructed and validated using a green fluorescent protein marker.

rFP9 showed appropriate transgene expression in both avian and mammalian cells, although at different levels. The expression efficiency of rFP9 was finally compared to another attenuated poxvirus vector, modified vaccinia Ankara (MVA). Comparing the protein expression levels between rFP9 and rMVA was quite difficult because different poxvirus promoters (early/late in rFP9; intermediate in rMVA) were used to direct the transcription of the T7 RNA gene. Given this limitation, although generally higher levels of expression were seen with rFP9, this cannot be attributed to the FP9 with any certainty.

Declaration

I declare that no portion of the work referred to in the thesis has been submitted in support of an application for another degree or qualification of this or any other university or other institute of learning.

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I am deeply indebted to my parents for encouragement and constant support during my whole life. I would also like to show my appreciation and gratefulness to my wife and children for their love, patience and support.

Dedication

I would like to dedicate this thesis to my beloved Father and Mother, who support and encourage me throughout my whole life. I would also like to dedicate this work to my lovely wife and children for their ongoing encouragement.

1 Introduction

1.1 Vaccines

Vaccines are considered the second most important preventive measures that can be used to limit the spread of infectious diseases and improve human health. Despite the variety of prevention and control measures that are applied in both developed and developing countries, the spread of communicable diseases remains high (Gold, 2002). Only effective vaccination programmes have successfully led to the eradication of some of these infectious diseases worldwide, such as in the case of smallpox (Fenner et al., 1988; Riedel, 2005). However, the development of effective vaccines remains complex, extremely expensive and time consuming (Gold, 2002).

1.1.1 Brief history of vaccination

There has been a great deal of progress over the last two hundred years. In 1796, Edward Jenner discovered the first smallpox vaccine when he used the secreted materials from a fresh cowpox (a mild skin disease in humans) lesion to vaccinate an 8 years old boy against smallpox. Around two months later, Jenner used a live smallpox virus to infect the boy. He realised that the boy was fully protected and immunised against smallpox (Willis, 1997; Riedel, 2005). In the next century, in 1881, Louis Pasteur was the first to conclude that microbes could be modified and attenuated through physical and/or chemical processes in order to be used as a vaccine. Pasteur's hypothesis was confirmed with regard to rabies and anthrax (Pasteur, 1880; Pasteur and Chamberland, 1881; Plotkin, 2005). Calmette and Guerin also used this hypothesis to attenuate *Mycobacterium bovis* by serial passage of the pathogen onto different artificial media (Calmette et al., 1907; Plotkin, 2005). In addition, in the late 1930s, Theiler attenuated the yellow fever virus strain through serial passage of the virus in chick

embryos and mice (Theiler and Smith, 1937; Silverstein, 2001; Plotkin, 2005). In 1884, Ilya Metchnikoff developed the theory of stimulating the cellular immune response as another form of vaccination. In 1890, Alexandre Yersin and Emile Roux reported that an extracellular bacterial toxin could be extracted from diphtheria cultures and used as toxoids (inactivated toxins) to protect against diphtheria and tetanus. These toxoids have the ability to generate specific antibodies that are capable of destroying the bacterial toxins. However, the concept of antibody formation through an injection of a specific antigen was developed by Paul Ehrlich in 1897 (Silverstein, 2001).

In the twentieth century, it was realised that cell culture can be used as another means of attenuating viruses through the passage of the organism onto different cell cultures. In the middle of that century, cell culture was successfully modified to permit the growth of viruses, and the development of attenuated viruses was numerous, such as in the case of polio (Sabin oral), varicella and rubella (Weller, 1949; Plotkin, 2005). At the end of the twentieth century, it was found that a conjugation of protein-polysaccharide could be used as a vaccine to protect against encapsulated bacteria, such as *Haemophilus influenza* type b (Schneerson et al., 1980; Plotkin, 2005). In the same century, genetic engineering had a significant impact on the development of vaccines. The first achievement of this technique was the formation of a Hepatitis B subunit vaccine using a recombinant yeast culture (McAleer et al., 1984; Plotkin, 2005). Since then, the concept of vectored vaccines has been widely used for live recombinant vaccines (Plotkin, 2005).

1.1.2 Approaches to the development of effective vaccines against human immunodeficiency virus (HIV)

1.1.2.1 Initial approaches

The majority of the initial approaches attempted to generate neutralising antibodies against viral envelope proteins in order to prevent or control viral replication. These immunisations were able to induce serum antibodies that only conferred protection against laboratory-adapted strains of HIV (Belshe et al., 1994; Connor et al., 1998; Geise and Duerr, 2009). However, natural HIV strains were resistant to these generated antibodies (Moore et al., 1995). There are a number of fundamental approaches that are being used to develop an effective HIV vaccine: live attenuated vaccines, inactivated or killed vaccines, and subunit vaccines (Table1.1).

1.1.2.1.1 Live attenuated vaccines

Live attenuated vaccines rely on the attenuation of the virus in order to reduce its pathogenicity. This allows it to simulate the natural infection without causing serious illness. In 1992, Daniel et al. proved the protective effects of a live attenuated simian immunodeficiency virus (SIV) vaccine in animal models. The study showed that vaccinated monkeys (rhesus macaques) with a nef-deleted live attenuated SIV of macaques 239 (SIVmac239) were fully protected from the naturally occurring SIV (Daniel et al., 1992). However, this type of vaccine candidates has not been tested in clinical trials due to the possibility that the attenuated virus might revert spontaneously into its pathogenic state and cause serious disease (Klein and J.Y. Ho, 2000). In 1998, Hulskotte et al. reported that some of the vaccinated neonatal monkeys with a nef-deleted live attenuated SIV vaccine developed an infection leading to a fatal progression of the acquired immune deficiency syndrome (AIDS). They found that immune

responses induced by the live attenuated virus (nef-deleted SIV) were insufficient to prevent progression to AIDS (Hulskotte et al., 1998).

1.1.2.1.2 Inactivated or killed vaccines

Inactivated or killed vaccines are created by inactivating the whole virus particle using heat, or chemicals such as formaldehyde or formalin. This type of vaccines prevents the virus from replicating, but keeps the viral capsid and envelope proteins intact, in order to stimulate the protective immune response (Klein and J.Y. Ho, 2000). Johnson et al. (1992) tested the ability of inactivated whole SIV vaccines to confer protection in animal models. They demonstrated that inactivated whole SIV vaccines have the ability to protect the immunised macaques (juvenile pig-tailed macaques) against either a homologous or heterologous pathogenic SIV infection that was administered intravenously (Johnson et al., 1992). However, it has been reported that inactivated vaccines tend to provide weak cytotoxic T cell (CTL) responses. For this reason, the main course of inactivated vaccination should consist of two to three doses, with a booster injection, in order to elicit high levels of antibody response (Mackett and Williamson, 1995).

1.1.2.1.3 Recombinant subunit vaccines

Subunit vaccines are produced by isolating specific proteins or the subunits that are expressed on the viral envelope. These proteins can then be used as specific antigens to stimulate the host's immune response, leading to the production of specific antibodies against the virulent form of the virus (Geise and Duerr, 2009). This type of HIV vaccines was the first candidate that has been advanced to human trials. In contrast, both inactivated and attenuated vaccine approaches were only tested in primate models but have not proceeded to clinical trials (Cohen and Dolin, 2013). Despite the fact that

recombinant subunit vaccines are characterised by their high safety profile and production cost, the immune responses induced by these vaccines alone are weak (Nascimento and Leite, 2012). In 1998, Connor et al. used a recombinant gp120 subunit vaccine, which encodes different HIV envelope proteins (MN and SF-2 gp120), to immunise 16 individuals against HIV type 1 (HIV-1). The vaccinated individuals developed weak specific CTL immune responses against HIV envelope antigens, and there were no significant differences in the antibody titre between the vaccinated and the control groups. The study concluded that there was no obvious beneficial effect of using these recombinant subunit vaccines among vaccinated individuals (Connor et al., 1998). In 2003, Pitisuttithum et al. evaluated the safety and efficacy of two recombinant subunit HIV vaccines (HIV envelope gp120 CM-235 and SF-2 proteins) in Thai individuals. The study found that more than 80% of the vaccinated individuals developed neutralising antibodies against HIV SF-2 and NP03. In addition, binding antibodies were observed in all the immunised volunteers who received both recombinant vaccines. However, low levels of binding antibodies were observed in those volunteers who received only one HIV recombinant protein (gp120 CM-235). At least two doses of recombinant vaccines were required to be able to detect specific antibodies. This phase II clinical study proved the safety and immunogenicity of these two combined recombinant vaccines (Pitisuttithum et al., 2003).

There were two main efficacy trials that evaluated the concept of recombinant subunit HIV vaccine: VAX004 and VAX003 (Cohen and Dolin, 2013). The VAX004 trial was the first phase III human trial that has been conducted among 5095 HIV-1 uninfected homosexual men and 308 women at high risk for HIV-1 infection. In this trial, the safety and immunogenicity of a recombinant HIV (subtype B) gp120 vaccine, which encodes two HIV envelope gp120, MN and GNE8 proteins, have been evaluated. The

study reported that the vaccine was safe, however, it was ineffective at providing protection against HIV-1 infection (Flynn et al., 2005). The second phase III VAX003 trial, which was conducted among 2546 injection drug users in Thailand, used a recombinant HIV (subtype B/E) gp120 vaccine encoding two HIV envelope gp120, MN and A244 proteins. This trial also reported the failure of this vaccine candidate to prevent HIV-1 infection or even slow disease progression (Pitisuttithum et al., 2006). In both trials (VAX004 and VAX003), the recombinant subunit HIV vaccines were able to elicit only limited neutralising antibodies that had little activity against a broad spectrum of HIV-1 strains (Gilbert et al., 2010; Montefiori et al., 2012; Cohen and Dolin, 2013).

It has been reported that the genetic diversity of HIV, particularly in the envelope antigens, makes it difficult to induce specific neutralising antibodies (Pitisuttithum et al., 2003; Geise and Duerr, 2009). The genetic variation of the HIV envelope antigens between the different HIV subtypes accounts for around 20–30% (Pitisuttithum et al., 2003). In 1995, Moore et al. found that some natural strains of HIV-1 were resistant to specific neutralizing antibodies that target gp120 viral envelope proteins (Moore et al., 1995). It seems likely that the failure of eliciting protective neutralising antibodies may be attributed to the quaternary structure of the HIV envelope gp120 that reduces the exposure of the target neutralising epitopes (Center et al., 2000).

1.1.2.2 Current approaches and future directions

Unfortunately, traditional vaccines have many limitations and raise different issues concerning human safety. These limitations prompted the scientists to design novel types of vaccine candidates, such as viral vectors, DNA plasmid vaccines, heterologous

prime-boost recombinant strategy, and antibody gene transfer immunoprophylaxis (Table1.1) (Geise and Duerr, 2009; Balazs and West, 2013).

1.1.2.2.1 Live recombinant viral vector vaccines

Since the twentieth century, the concept of viral vectors has been widely used for live recombinant vaccines. This approach uses a non-pathogenic live microorganism (often a virus) that has the ability to carry the genetic materials and subsequently express the target genes of the pathogen without causing illness (Plotkin, 2005; Stanberry and Strugnell, 2011). These genes are processed and expressed on the surface of the host cell in order to induce a high level of immunogenic foreign proteins. The expressed proteins are then recognised by specific types of the host immune cells that eliminate all the infected cells, resulting in a long-term immunity against these foreign proteins (Stanberry and Strugnell, 2011). The key feature of this approach is to mimic the natural infection by presenting the pathogenic antigen to the host immune system without the existence of the infectious pathogen itself (Holman et al., 2009). It has been found that vectored vaccines are a good way to elicit both cellular and humoral immune responses (Draper and Heeney, 2010).

The successful utilization of this approach was reported in 1984, when Wiktor et al. used vaccinia virus as an expression vector for rabies virus glycoproteins in non-human primates (rabbits and mice). The rabbits and mice successfully developed an effective long-lasting immunity against the pathogenic rabies virus (Wiktor et al., 1984). Since then, the gene delivery system approach has been widely used for the development of both prophylactic and therapeutic vaccine candidates. There are a number of viral vectors that have been commonly used for vaccine development, including

adenoviruses, poxviruses, herpesviruses, alphaviruses, adeno-associated viruses, measles viruses, and vesicular stomatitis viruses (Robert-Guroff, 2007).

1.1.2.2.2 DNA plasmid vaccines

The DNA plasmid vaccine approach is one of the novel techniques that have been used for vaccine development against HIV (Geise and Duerr, 2009). The concept of these vaccines is relying on the direct injection of a naked DNA plasmid that contains the target's antigens into the host tissues. This naked DNA plasmid is then taken up by the host cells and processed to produce foreign proteins, in order to induce an immune response against the target pathogen (Holman et al., 2009). These vaccine candidates differ from vectored vaccines in that they use a circular plasmid DNA vector to express particular genes rather than utilising another organism. Therefore, there is no possibility for the DNA vaccines to produce anti-vector immune responses, which could interfere with the main purpose of these vaccines (Bråve et al., 2006).

The efficacy and immunogenicity of DNA plasmid vaccines were assessed in various studies. Boyer et al. (1997), Robinson et al. (1999), and Cherpelis et al. (2001) used the DNA vector in a heterologous prime-boost vaccination strategy. They were able to prove the protective effects of these DNA vaccines against HIV/SIV infection in rhesus macaques (Robinson et al., 1999; Cherpelis et al., 2001) and against HIV-1 in chimpanzee model system (Boyer et al., 1997). For example, in 2001, Cherpelis et al. used a DNA plasmid vector as a prime vaccine followed by protein-boosting immunisations. These vaccines were used to deliver a V2 (variable loop 2) deleted HIV-1 gp140 envelope gene into rhesus macaques, in order to immunise them against homologous HIV/SIV infection (SF162P4 isolate). The study reported that the vaccinated macaques developed an effective immune response (anti-HIV/SIV envelope

antibodies) against the HIV/SIV infection. During the first seven days after challenge, the immunised macaques had low-level viremia and were able to control the viral infection effectively (Cherpelis et al., 2001).

On the other hand, Akhata et al. (2000), Akahata et al. (2003), and Horiuchi et al. (2006) used a DNA plasmid vector alone without employing any boosting vaccines. For instance, in 2000, Akahata et al. used a DNA vaccine, which encodes a full-size HIV-1 genome with a mutant in the nucleocapsid protein, to immunise rhesus macaques against homologous HIV/SIV infection. The study demonstrated that all the immunised macaques developed immunological responses against HIV-1 and they had lower levels of viraemia than those of the unimmunised macaques. However, the protection induced by these vaccines was partial and only two out of four vaccinated macaques developed HIV-1 env-specific antibodies (Akhata et al., 2000). It has been found that the immune responses elicited by the DNA vaccines could protect the immunised animal from those viruses that are replicating poorly (Boyer et al., 1997; Cherpelis et al., 2001). However, when the immunised macaques are challenged with viruses (such as SIVmac) that are replicating efficiently, the DNA vaccination-induced responses only reduced the viral load but did not prevent viral infection (Boyer et al., 1996; Cherpelis et al., 2001).

1.1.2.2.3 Heterologous prime-boost recombinant vaccines

The heterologous prime-boost vaccination strategy relies on a combination of two different types of vaccines against a target pathogen. This approach uses one type of vaccine (such as a DNA plasmid) as a prime, which is then followed by the administration of another type of vaccine (such as a live vectored vaccine) as a booster. This technique aims to induce a range of host defences in order to increase the overall

host immune response protection against the target pathogen (Stanberry and Strugnell, 2011).

The majority of heterologous prime-boost immunisation strategies against HIV/SIV infection have been used in animal models. It has been found that using a prime DNA plasmid vaccine followed by a recombinant live vectored vaccine as a booster can induce potent cellular immune responses against HIV/SIV infection in non-human primates. A few of these trials have demonstrated the effect of this regimen on the induction of humoral immunity in animal models (Shahin and Proll, 2006).

The concept of heterologous prime-boost vaccination has also been used in humans. In 2004, Mwau et al. assessed the effect of this approach against HIV infection in uninfected individuals. They reported that using prime DNA vaccines followed by live vector (modified vaccinia Ankara [MVA]) booster vaccines, which expressed HIV-1 gag p24/p17 proteins, can elicit HIV-specific CTL responses in the majority of healthy individuals. However, only one out of 35 vaccinated individuals developed gag-specific antibodies (Mwau et al., 2004). Moreover, in 2012, the most recent phase III clinical trial of an HIV-1 vaccine (RV 144 trial) was performed in Thailand by De Souza et al. This clinical trial was performed using the heterologous prime-boost vaccination strategy. De Souza et al. used a recombinant canarypox vector (ALVAC-HIV vCP1521) as prime vaccines to express three different genes of HIV-1 subtype B (gag, env, and protease). Then, a recombinant subunit vaccine (AIDSVAX B/E) was used as a booster to deliver the HIV gp120. The RV 144 trial has proved the safety and the modest efficacy (about 31%) of using the prime-boost vaccination strategy in preventing the acquisition of HIV infection. However, it has been found that the cellular T cell immune responses elicited by the regimen vaccine was not as robust as the humoral immune responses (De Souza et al., 2012).

1.1.2.2.4 Antibody gene transfer for HIV immunoprophylaxis using adeno-associated virus (AAV)

Gene therapy is one of the most novel techniques that have been used recently to prevent the spread of HIV infection. This type of therapy utilises viral vectors for introducing the target genes into the host cell. Adeno-associated virus (AAV), which is a member of the *Parvoviridae* family, is one of the promising gene delivery candidates. This virus, which has been safely administered to humans, has the ability to infect both human and some primate species but induces only a mild inflammatory response. It has been found that this gene delivery vector can achieve long-term expression without integrating into the host genome (Aalbers et al., 2011). Thus, AAV was used as a viral vector to deliver DNA that codes for broad and potent neutralizing HIV antibodies obtained from HIV-infected patients into non-hematopoietic tissues, such as muscle. The concept of this therapy (antibody gene transfer) is based on the formation of episomal target DNA within the host cell and this programmes the cell to produce HIV-specific antibodies. Unlike previous attempts at developing HIV vaccines, this novel strategy bypasses the natural defence system and forces the muscle cell to express the target antibodies without the participation of the immune system (Balazs and West, 2013).

Approaches to the development of effective vaccines against Human Immunodeficiency Virus (HIV)	
A) Initial approaches	**B) Current approaches and future directions**
1- Live attenuated vaccines 2- Inactivated or killed vaccines 3- Recombinant subunit vaccines	1- Live, recombinant viral vector vaccines 2- DNA plasmid vaccines 3- Heterologous prime-boost recombinant vaccines 4- Antibody gene transfer immunoprophylaxis

Table 1.1: Summarises the different approaches for the development of HIV vaccines.

1.2 Recombinant poxviruses in vaccine research

Since 1796 when Edward Jenner discovered the use of cowpox virus as the first vaccine against smallpox, poxviruses have had a significant impact in the field of vaccination. These viruses have drawn much attention due to their proven advantages in gene transfer applications. They have been extensively studied as recombinant vectors for the induction of specific immune responses against HIV (Willis, 1997; Pastoret and Vanderplasschen, 2003).

Poxviruses belong to the poxviridae family that is characterised by a large, complex, linear double stranded DNA genome, which ranges from 130 to 300 kbp, with covalently closed ends (hairpin loops). There are two main subfamilies of viruses within the poxviridae family: entomopoxvirinae (insect poxviruses) and chordopoxvirinae (vertebrate poxviruses). The latter subfamily is the main one that is being used as viral vectors in vaccine research. The chordopoxvirinae subfamily is composed of eight different genera: orthopoxviruses, capripoxvirus, molluscipoxvirus, avipoxvirus, yatapoxvirus, parapoxvirus, leporipoxvirus, and suipoxvirus (Figure 1.1-A). Basically, there are a number of specific poxviruses that have been principally used as vectors for vaccine development, including vaccinia virus (VACV), fowlpox virus (FPV), fowlpox virus 9 (FP9), modified vaccinia Ankara (MVA), and canarypox virus (CNPV) (Figure 1.1-B) (Pastoret and Vanderplasschen, 2003).

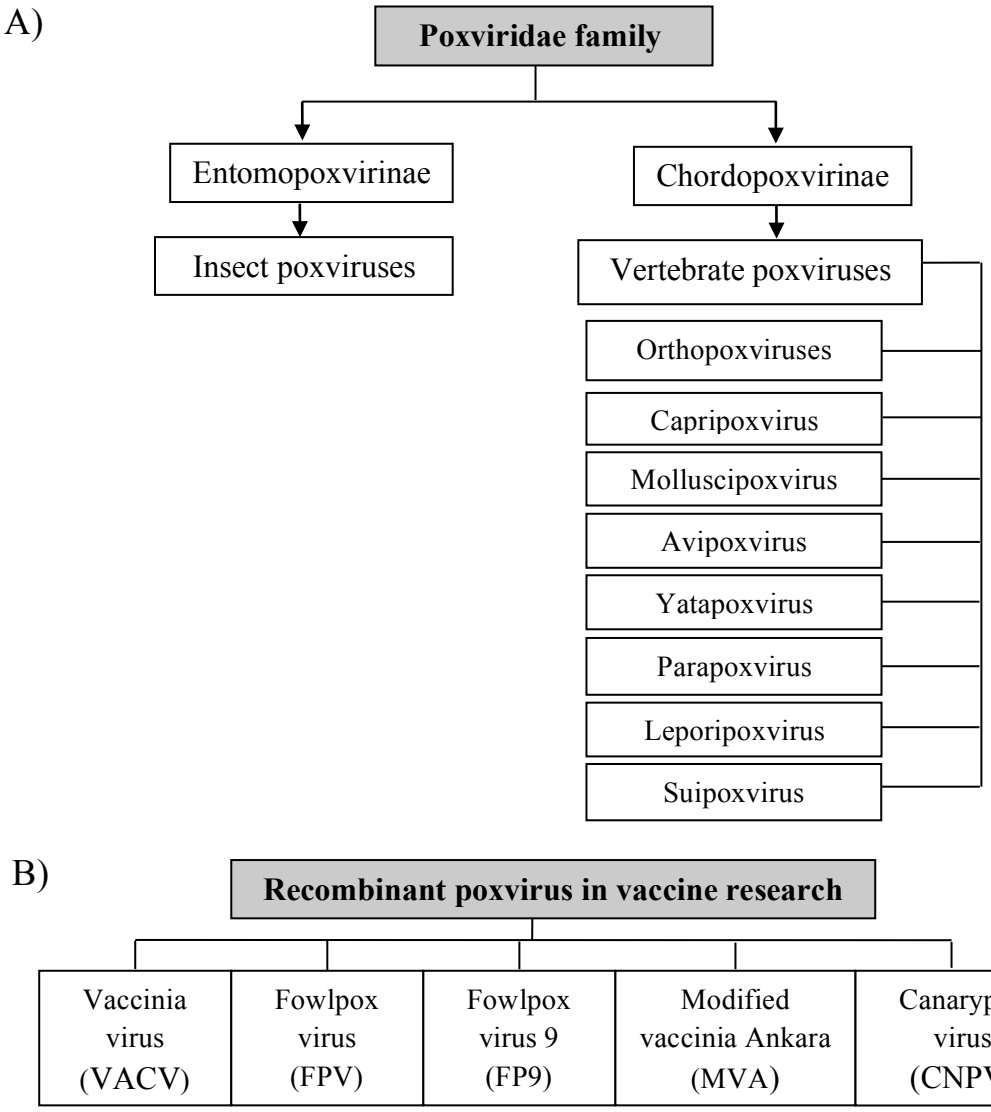

Figure 1.1: Shows the main classification of family *Poxviridae* (A) and the most common recombinant poxviruses that involve in vaccine research (B).

1.2.1 Vaccinia virus (VACV)

VACV is a member of the orthopoxvirus genus, which includes many species that are antigenically and genetically related to each other, such as variola virus and cowpox virus. The VACV was the first virus to be successfully used as a vaccine to eradicate smallpox (Fenner et al., 1988; Pastoret and Vanderplasschen, 2003).

1.2.1.1 Molecular biology

The VACV has a complex virion structure and a single linear double stranded DNA genome (about 200 Kbp) that encodes around 200 different genes (Figure 1.2). There are three main types of genes that have been identified in the viral genome, including early (expressed prior to DNA replication), intermediate and late genes (expressed after genome replication). The VACV differs from most of the DNA viruses, which replicate in the nucleus, by its ability to replicate in the cytoplasm of the host cell (Rosales et al., 1994; Carroll and Kovacs, 2003). This virus contains the early genes and different transcriptional enzymes (such as DNA-dependent RNA polymerase) that are necessary for early mRNA synthesis and translation. Some studies found that viral transcription is mainly regulated by virus encoded proteins. However, other studies have shown that host cell nuclear factors may have a significant role to play in viral transcription (Rosales et al., 1994; Bråve et al., 2006; Carroll and Kovacs, 2003).

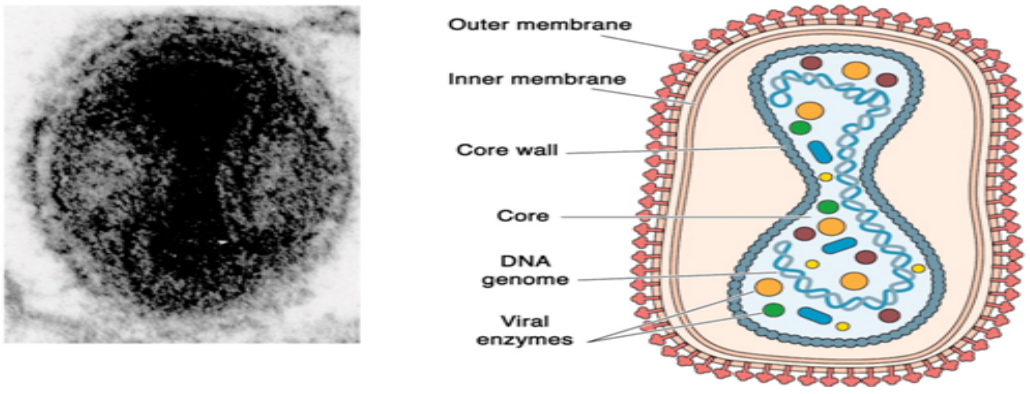

Figure 1.2: **Schematic representation of vaccinia virus virion structure.** This figure is adopted from Flint et al., 2003.

The VACV has simple promoters (approximately 30 bp), which are usually functional across the different genera. Most recombinant VACV (rVACV) vectors have early and/or late gene promoters. Generally speaking, the late promoters are stronger than the early ones due to their ability to express the foreign genes after the viral DNA replication starts. However, it is sometimes recommended to use early promoters for gene expression in order to avoid cytotoxicity of the target genes (Carroll and Kovacs, 2003).

1.2.1.2 rVACV vectors

The first use of the rVACV as a cloning vector was in the early 1980s. It was used for the expression of a specific gene (thymidine kinase) of herpes simplex virus into the host cell. Since that time, there has been a dramatic increase in the use of the VACV vectors as a gene delivery system (Smith, 1987; Panicali and Paoletti, 1982). The success of using this virus as an effective recombinant expression system has been due to many factors, including stability, flexibility, and the ability of its genome to accommodate more than 25 Kbp of target genes. It is also easy to manipulate and administer (Smith and Moss, 1983; Carroll and Kovacs, 2003).

1.2.1.2.1 Construction of rVACV

The complete procedure for the construction of rVACV vector was reviewed by Smith et al. (1984). The most common way for generating these recombinants is through homologous recombination (HR) (Figure 1.3). Briefly, foreign genes (expression cassette) should be firstly linked to suitable VACV promoters before they are inserted into the viral genome. These target genes have to be inserted into non-essential loci on the viral genome in order to avoid affecting the essential genes of the virus (Smith et al., 1984). HR occurs between viral sequences in the transfer plasmid DNA flanking the

target genes, and the homologous sequences (HS) of the VACV genome (Figure 1.3). There are several sites where recombination can be targeted. The most commonly used is the thymidine kinase (TK) gene of the VACV (Carroll and Kovacs, 2003).

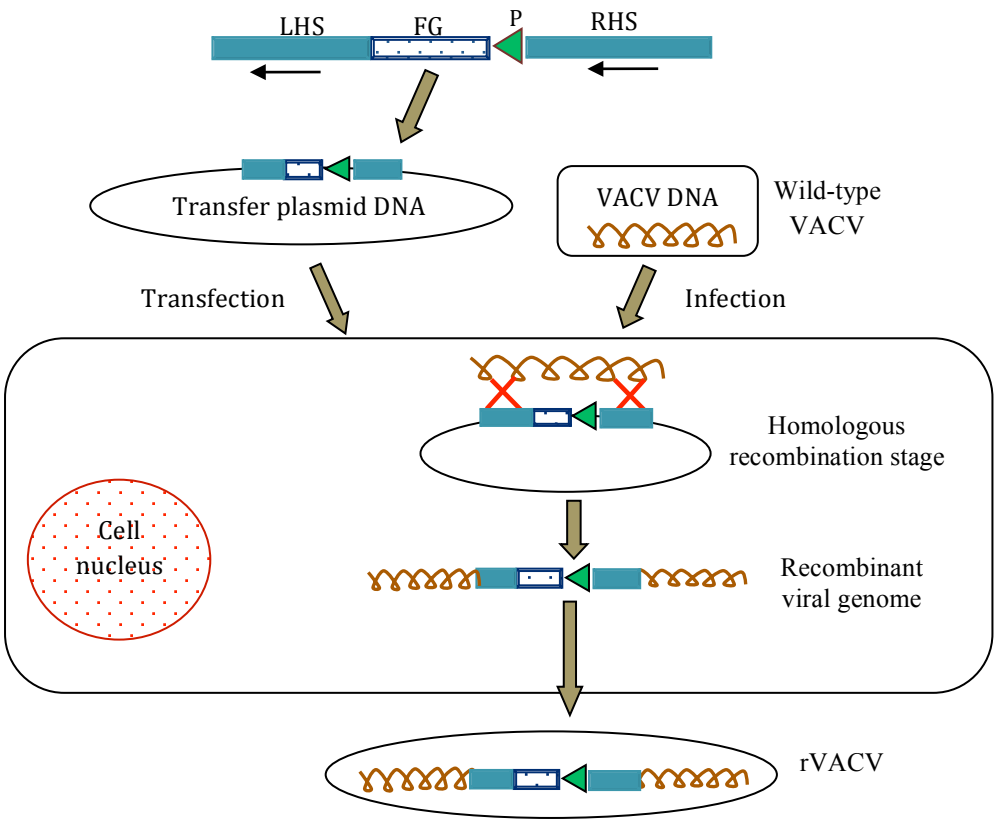

Figure 1.3: Schematic representation of construction of rVACV vector using HR.

(RHS: Right HS of VACV, LHS: Left HS of VACV, FG: Foreign gene sequences, P: VACV promoter). This figure is adopted from Carroll and Kovacs, 2003.

Identification of rVACV is usually based on insertion of a reporter expression cassette (such as *Escherichia coli* [*E.coli*] lacZ gene) into the plasmid DNA to facilitate screening of the recombinant viruses. The lacZ gene encodes a beta-galactosidase enzyme that cleaves the X-gal chemical substrate (5-bromo-4-chloro-indolyl-β-D-galactopyranoside) to form a blue colour compound. Generally, this marker gene (LacZ) has been widely used as the basis for a rapid screening strategy called blue/colourless plaque screening (Chakrabarti et al., 1985).

Transient colour selection (TCS) is one of the methods that can be used for the construction of recombinant viruses (Figure 1.4) (Parks et al., 1994). This technique is based on the insertion of the marker gene outside the genomic flanking regions within the plasmid, and hence it will not be stably integrated into the viral genome during the single crossover recombination event. Therefore, following several rounds of plaque purification, the marker gene will be eliminated from the recombinant genome due to intragenomic HR between the repeated sequences, which is facilitated by colourless plaque screening (Figure 1.4) (Falkner and Moss, 1990; Parks et al., 1994).

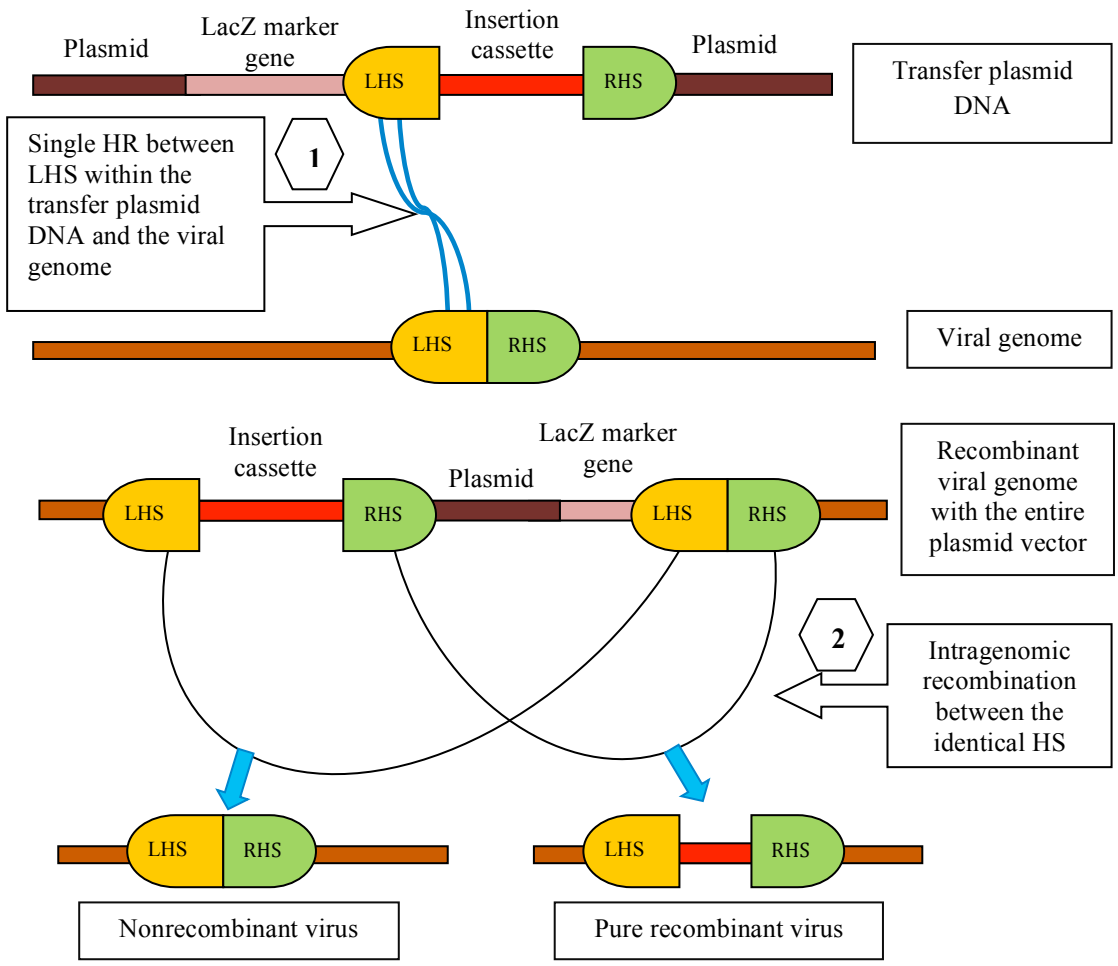

Figure 1.4: **Schematic representation of construction of recombinant virus using TCS method.**

Step one (1) shows single crossover HR between the HS in the plasmid DNA flanking the target genes, and the HS of the viral genome. This step results in full-length plasmid integration with the marker gene. Step two (2) shows intragenomic HR between the identical HS in the viral genome, resulting in elimination of the marker gene (LacZ). There is a chance that all foreign DNA will be eliminated, resulting in reversion to nonrecombinant virus. The recombinant and nonrecombinant viruses at this final stage have to be distinguished by polymerase chain reaction (PCR). (RHS: right HS, LHS: left HS).

1.2.1.2.2 rVACV in vaccine research

Several studies have demonstrated the ability of rVACV vectors for the expression of exogenous antigens. In 1996, Pastoret and Brochier used a rVACV to express the surface glycoprotein (F protein) of rabies virus in foxes. They proved the potent effect of rVACV vaccine in controlling the rabies infection among wild animals (Pastoret and Brochier, 1996). Moreover, Cooney et al. (1991) examined the effect of rVACV vectors for the expression of the envelope glycoprotein (gp160) of HIV in mammalian hosts. The study proved the safety and the efficacy of the rVACV vaccine in inducing T cell responses and specific HIV antibodies in healthy individuals. However, they found that the responses induced by the boosting doses of the recombinant vaccine were weaker than those responses that were induced by the prime doses. In addition, they realised that the priming effect of the rVACV vaccine on HIV in individuals with pre-existing immunity to the VACV was variable (Cooney et al., 1991). Moreover, it has been reported that individuals without pre-existing immunity to the VACV are at risk of developing serious post-vaccinial complications from rVACV vector vaccines (Smith, 1987). Furthermore, Vazquez-Blomquist et al. (2002) stated the possibility that the recombinant vaccinia vector vaccine might cause diseases in immunocompromised patients (Vazquez-Blomquist et al., 2002). Fatal diseases (such as encephalitis) and progressive infection were reported to be caused by VACV in patients with HIV (Redfield et al., 1987). Despite the potential negative impacts of using the rVACV vaccine in humans, most previous studies have proved the efficacy of using this recombinant vector in inducing both cellular and humoral immune responses against different types of recombinant antigens.

1.2.1.3 Attenuated VACV strains

MVA and NYVAC are the most promising attenuated vectors that have been derived from the wild-type VACV. NYVAC vector was derived from the Copenhagen strain of VACV by the deletion of 18 OFRs from the original wild-type genome. As a result of this substantial loss of viral genes, this highly attenuated strain has a restricted host range, and an enhanced ability to induce stronger antigen-specific immune responses compared to the wild-type VACV (Tartaglia et al., 1992). Despite the limited replication of the NYVAC strain in mammalian hosts, several studies have demonstrated the immunogenicity and the ability of this vector to express high levels of exogenous antigens in macaques (Hel et al., 2002; Mooij et al., 2008) and humans (Najera et al., 2010; Quakkelaar et al., 2011).

1.2.2 MVA

1.2.2.1 Molecular biology

MVA is a highly attenuated strain of the wild-type VACV that has been successfully used in the eradication of smallpox in the human population. MVA was derived by many serial passages (more than 570 times) of the wild-type VACV in CEF cultures (Hochstein-Mintzel et al., 1975). It is characterised by its inability to replicate efficiently in mammalian cell lines, due to the fact that the MVA genome has lost substantial part (about 15%) of the cellular host range genes of the VACV during tissue culture passages (Blanchard et al., 1998; Webster et al., 2006). It has been found that MVA has limited ability to replicate in HeLa cells (human cell line) and transformed human cells such as TK-143B. This attenuated strain was also reported to be a good inducer of type one interferon (IFN-α and β) from primary human cells (Blanchard et al., 1998).

The whole genome of the MVA strain was fully sequenced and characterised by Antoine et al. (1998). The MVA has a large double stranded DNA genome (178 Kbp) which is shorter than the wild-type VACV Copenhagen (CPN) genome (192 Kbp). The viral genome has 9.8 kpb ITR regions that consist of six different sequence elements, and has also around 193 ORFs. The left terminal part of the MVA genome possesses four major deletion mutations, while there are only three major genomic deletions present in the right terminal region of the viral genome (Antoine et al., 1998). It has been reported that the MVA genome harbours six major deletion mutations, which account for 30 kbp, compared with the parental wild-type virus. These numerous mutations among the viral genome affect most of the host range genes, such as K1L and C7L (Meyer et al., 1991). It has also been found that the MVA genome lacks several functional receptors that act as immunomodulators in the host cells, such as IFN-α/β, IFN-γ, tumour necrosis factor (TNFs), and CC chemokine receptors (beta chemokine receptors). However, the MVA genome possesses an interleukin 1β (IL-1β) receptor that has a significant role in reducing the virulence of the vector. Indeed, MVA was found to be more immunogenic than other strains of VACV, such as the Western Reserve (WR) and Wyeth strains (Blanchard et al., 1998). In addition, MVA produces IL-18 binding protein that has the ability to stimulate the production of type two IFN (IFN-γ) and other inflammatory responses (Webster et al., 2006).

1.2.2.2 Recombinant MVA (rMVA) vectors

MVA is a highly attenuated strain that has lost several members of the host cellular genes of the wild-type VACV during tissue culture passages. These broad host cellular genes (particularly K1L and C7L genes) play an important role in regulating the productive replication cycle of the VACV in mammalian cell lines by modulating host immune responses (Backes et al., 2010). It appears, therefore, that loss of these genes

gave the MVA strain an enhanced ability to induce stronger cellular immune responses compared to the VACV. Moreover, MVA strain is considered apathogenic in most animal models particularly immunosuppressed macaques, neonatal, and irradiated mice. It has also a restricted host range, and has a good safety profile among human studies. All of these characteristics of MVA described above were essential factors in the success of this vector as a safe and effective human vaccine candidate (Blanchard et al., 1998; Webster et al., 2006).

Several studies have demonstrated the potential effect of the rMVA vectors for the expression of foreign genes. In 1994, Sutter et al. used a rMVA vaccine candidate encoding nucleoprotein and haemagglutinin genes of influenza virus to induce protective immune responses in mice against a lethal strain of influenza virus. They found that vaccinated mice developed specific antibodies and CTL immune responses to both influenza virus proteins, resulting in complete protection against the lethal challenge with influenza virus strain (Sutter et al., 1994). In 2000, Seth et al. assessed the protective efficacy of a rMVA vector encoding gag and pol SIVsmH4 genes against the pathogenic SIV strain in rhesus macaques. They found that the rMVA was able to induce specific CTL immune responses and confer protection in the vaccinated macaques against challenge with the pathogenic SIVsmE660 strain (Seth et al., 2000). Likewise, rMVA vector vaccines were used either alone or in combination with other viral vectors for the expression of different malarial antigens (such as ME-TRAP, PfCS, and PbCS). The rMVA vector was able to induce potent and protective immune responses against malaria in mice (Anderson et al., 2004; Reyes-Sandoval et al., 2010), macaques (Draper et al., 2010), and humans (Webster et al., 2005; Imoukhuede et al., 2006; McConkey et al., 2003). The safety profile of this vector vaccine was also

evaluated in several studies (Imoukhuede et al., 2006; Webster et al., 2006; Bejon et al., 2006).

In 2004, Mwau et al. demonstrated the efficacy of using the rMVA vector either alone or in a prime-boost immunisation regimen with a DNA priming vaccine against HIV-1 in HIV-1/2 uninfected individuals. This phase I clinical trial reported that the rMVA, which encodes HIV-1 clade A gag P17 and P24 antigens, was able to elicit HIV-1 specific CTL immune responses in the majority of the vaccinated individuals. However, gag-specific antibodies were only observed in one out of 35 vaccinated individuals (Mwau et al., 2004). Moreover, Cebere et al. (2006) proved the safety of using this recombinant vector (MVA/HIV-1 clade A gag P17 and P24 antigens) against HIV-1 in humans. They reported that the vector vaccine was safe (causes only minor acceptable side effects) in vaccinated individuals (Cebere et al., 2006).

1.2.3 Fowlpox virus

1.2.3.1 Molecular biology

Fowlpox virus (FPV) is a member of the avipoxvirus genus that belongs to the chordopoxvirinae subfamily, which contains the poxviruses of vertebrates. FPV is a cytoplasmic DNA virus that has a single linear double stranded DNA genome (about 288 Kbp), which encodes for more than 250 different genes (Swenson et al., 2008). This genome has large and multiple families of genes (around 10 multigene families) in comparison with other chordopoxvirinae genomes that have small numbers or sometimes single-gene families. The presence of these multigene families, together with the cellular homologous genes, is considered the reason behind the large size of the FPV genome (Afonso et al., 2000). In 2000, the whole genome of the FPV was sequenced by Afonso et al. who reported that both strands of the viral DNA genome have many

protein encoding genes, and there is no clear evidence for the presence of any introns (Afonso et al., 2000). One of these protein-coding genes is the photolyase gene that is expressed late in the viral replication cycle. The photolyase gene plays an important role in the persistence of the FPV in the poultry environment for a long time. This gene encodes an enzyme known as cyclobutane pyrimidine dimer (CPD) photolyase that acts as a DNA repair enzyme to protect and repair the FPV genome from the UV light-induced DNA damage during the period of exposure to sunlight (Swenson et al., 2008; Srinivasan, et al., 2001). However, Srinivasan et al. (2001) demonstrated that the photolyase gene is one of the non-essential genes for viral growth in cell cultures (Table 1.2) (Srinivasan et al., 2001).

It has been found that the FPV genome comprises about 65 conserved genes that are homologous to those of other chordopoxvirinae genomes. These conserved regions include most of the gene families that are responsible for mRNA synthesis, processing of viral proteins, nucleotide metabolic process, virion structural proteins, and DNA replication and repair genes (such as DNA polymerase, DNA ligase, DNA topoisomerase, and ATP-GTP binding proteins) (Afonso et al., 2000). The FPV genome has two similar inverted terminal repeat (ITR) regions (about 9.5 kpb) that are bound to the central coding region of the viral genome, and has also around 260 open reading frames (ORFs). About 101 of these ORFs show homology to those genes of known function. Moreover, each of the ITR regions has about ten ORFs and 42 copies of a 31–32 bp tandem repeat (Afonso et al., 2000).

1.2.3.2 Fowlpox diseases of poultry

The FPV affects domestic poultry, especially chicken and turkeys, and causes a viral infection (fowlpox) that is characterised by a slow spread rate. The signs of this disease

are the development of various lesions that can be classified into two types: dry pox lesions (cutaneous form) and wet pox lesions (diphtheritic form). The cutaneous form, which is the most common type, can be identified by the appearance of proliferative skin lesions that occur following the infection. However, the diphtheritic form is characterised by the development of various nodules on the mucous membranes of different parts of the body, such as mouth, larynx, oesophagus, and occasionally the trachea. This form of lesion is associated with a high mortality rate (about 50%) due to their poor prognosis that can be lead to death by asphyxiation. Indeed, the development of the fowlpox disease can lead to reduced egg production and growth rate of young birds (Swenson et al., 2008; Afonso et al., 2000). It has been reported that vaccination with non-attenuated viruses (such as the pigeonpox virus) and live attenuated viruses (such as fowlpox and canarypox viruses) can confer complete protection against fowlpox diseases in birds (Afonso et al., 2000).

1.2.3.3 Recombinant fowlpox virus vectors

The FPV is a member of the genus avipoxvirus, which is characterised by the inability to productively replicate in non-avian species (Somogyi et al., 1993). Although FPV does not replicate efficiently in mammalian cell lines, it still has the ability to bind and enter into the target cells, express the encoded proteins, and induce protective immunity (Brown et al., 1999). Therefore, it has been suggested that FPV be used as a recombinant expression vector for the expression of specific foreign genes in human or other mammalian cells (Skinner et al., 2005; Pacchioni et al., 2010). The FPV has also a host range that is restricted to avian species and a large genome size that allows the accommodation of a significant amount of foreign DNA (Webster et al., 2006). Moreover, it has been found that the FPV vector does not cross-react immunologically with the VACV in those individuals with pre-existing immunity to the VACV (Irvine et

al., 1997; Pacchioni et al., 2010). All of these characteristics of FPV described above are essential factors in the success of this vector as an effective gene expression system.

Several different insertion sites (non-essential genes) among the FPV genome have been identified (Table 1.2). It has been found that non-essential genes of the FPV genome are considered the suitable loci for the insertion of heterologous target genes (Boulanger et al., 2002). The thymidine kinase (TK) gene, which is located in a highly conserved region among the FPV genome, is considered one of these non-essential genes (Boyle et al., 1987). However, Scheiflinger et al. (1997) reported that the TK gene can be considered as one of the essential genes for viral growth, particularly in the highly attenuated poxviruses such as MVA and FPV strain HP1.441 (Scheiflinger et al., 1997). In contrast to Scheiflinger et al.'s findings, in fact the TK gene has been very successfully used for recombination in MVA (Dorrell et al., 2001). In 2002, Boulanger et al. identified another non-essential locus that can be used as a useful insertion site for foreign genes among the FPV genome. They found that the FPV F11L orthologue is a non-essential gene for viral replication, and can be used for the generation of recombinant FPV (rFPV) vector (Table 1.2). In addition, they reported that the F11L gene is a highly conserved sequence between the FPV and pigeonpox viruses (Boulanger et al., 2002).

It has been found that the use of rFPV vector vaccine can effectively induce both cellular and humoral immune responses. However, these protective responses can be further improved by using rFPV vectors as a booster after a prime vaccine (such as a DNA plasmid vaccine) in a heterologous prime-boost vaccination strategy (Pacchioni et al., 2010).

Name of gene	Number of ORF	Length	Function
Thymidine Kinase (TK)	86	552 bp	The encoded enzyme (TK) is non-essential for viral replication in cell culture
F11L orthologue	110	1365 bp	No known function
Photolyase	158	603 bp	The encoded enzyme (CPD) acts as a DNA repair enzyme to protect and repair the FPV genome from the UV light-induced DNA damage

Table 1.2: Non-essential genes for FPV replication in tissue culture.

Several studies have demonstrated the efficiency of rFPV vectors for the expression of foreign genes. Indeed, this recombinant vector was initially used for the development of poultry vaccines against various diseases (Skinner et al., 2005). In 1990, Taylor et al. demonstrated the efficacy of the rFPV (FPV-1 strain), which encodes the fusion protein of the Newcastle disease virus (NDV), in eliciting protective immunity in chickens against the NDV. The study reported that the vaccinated chickens were fully protected against the challenge virulent strain of the NDV (Taylor et al., 1990). Subsequently, Wild et al. (1990) proved the efficacy of these recombinant vectors (FPV3113 strain) encoding the fusion proteins of the measles virus in inducing a strong protective immune response in mice against fatal measles encephalitis (Wild et al., 1990). In 1991, Beard et al. used the rFPV vector, which encodes the H5 hemagglutinin (HA-5) gene of the avian influenza, to induce a protective immune response in chickens against the highly pathogenic strain of avian influenza virus (H5N2). They found that vaccinated chickens developed high levels of hemagglutination-inhibition (HI) antibodies following the challenge with the H5N2 strain of avian influenza virus (Beard et al., 1991). Since 1995, the avian influenza H5 rFPV (rFPV-H5) vaccine has been used as

one of the licensed vaccines by the United States Department of Agriculture (USDA) against avian influenza viruses (Myers and Morgan, 2003; Swayne et al., 2000).

The approach of the rFPV vector vaccines has also been used in macaques against HIV-1. In 1998, Kent et al. evaluated the efficacy of the rFPV vector (FPV-M3 strain) that involved in a heterologous prime-boost immunisation strategy as a booster vaccine (encoding gag, pol, and env HIV-1 genes) after a DNA prime vaccine (encoding only gag and env HIV-1 genes). They found that this vaccination regimen was safe and able to induce enhanced T cell immunity responses in juvenile macaques against a non-pathogenic HIV-1 (Kent et al., 1998). In 2000, Kent et al. used the rFPV vector (FPV-M3 strain) for the expression of human interferon gamma (IFN-γ), and two types of HIV-1 antigens (gag and pol genes). This vector was used as a therapeutic vaccine in HIV-1-infected macaques (*Macaca nemestrina*). They found that the rFPV vector (FPV gag/pol-IFN-γ) has the ability to induce both HIV-specific CTL (CD8) and T helper cell (CD4) responses in macaques, with no systemic side effects. This study has also proved the safety of using these types of vectors in non-human primates (Kent et al., 2000). In 2005, De Rose et al. evaluated one of the immunisation regimens (DNA priming/rFPV boosting vaccine) that encode five different HIV-1 subtype AE genes (env, gag, pol, rev, and tat). They proved the safety and the efficacy of this regimen in eliciting broad T-cell immunity responses in pigtail macaques against HIV-1. They also suggested that this vaccination regimen is suitable for progression into human/clinical studies (De Rose et al., 2005).

The concept of the rFPV vector vaccines was also used in human cancers. In 2005, Marshall et al. evaluated the efficacy of FPV and VACV recombinant vectors in inducing specific immune responses against carcinoembryonic antigen (CEA). This phase I clinical trial was the first study that proved the safety and the efficacy of these

vector vaccines (either alone or in combination) in inducing potent CEA-specific immune responses without causing systemic toxicity in patients with CEA-expressing cancers (Marshall et al., 2005).

1.2.4 Fowlpox virus 9 (FP9)

1.2.4.1 Molecular biology

Fowlpox virus 9 (FP9) is a highly attenuated strain of the wild-type FPV that does not replicate efficiently in mammalian cell lines and has been safely administered to humans with no ill effects (Mayr and Malicki, 1966). The FP9 strain was derived by extensive serial passages (438 times) of the wild-type virus (HP-1 Munich) in chicken embryo fibroblast (CEF) cultures, and is then plaque-purified three times. The entire genome of the FP9 strain was fully sequenced and characterised by Laidlaw and Skinner in 2004. It has been found that the FP9 genome has numerous insertion and deletion mutations that account for about 8.5% of the total genome (25 kb), in comparison with the sequenced genome of the wild-type FPV (Table 1.3) (Laidlaw and Skinner, 2004; Cottingham et al., 2006).

The FP9 strain has a double stranded DNA genome (266.145 Kbp), which is shorter than the wild-type FPV genome (288.539 Kbp). However, the FP9 has longer ITR regions (10.158 Kbp) than those that have been reported for the wild-type virus (9.520 Kbp). It appears, therefore, that each terminus of the FP9 genome has 94-bp extra nucleotides. In addition, it has been found that the ITRs of the FP9 genome has a repeat region of 3.753 Kbp, which is longer than the equivalent repeat regions (1.675 Kbp) that are found in the ITRs of the FPV genome (Table 1.3) (Laidlaw and Skinner, 2004).

Laidlaw and Skinner (2004) found that the FP9 has a partial copy of the reticuloendotheliosis virus (REV) long terminal repeat (LTR), which is totally identical in sequence to the one that is found in the FPV M (Australian strain) and US strains. Moreover, they found that there is an orthologue of the VACV between ORFs 179 and 180 in both the FP9 and the wild-type virus (FPV). This gene encodes an intracellular mature virus (IMV) membrane protein, which is highly conserved and may have a role in the viral virulence, but is non-essential (Laidlaw and Skinner, 2004). When the FP9 genome was compared to the wild-type virus genome, it has been realised that there are only 118 differences between the two genomes. These differences occur as a result of the various mutations that take place within the FP9 genome during the tissue culture passage. These mutations include 15 insertions, 26 deletions, and 77 single base pair substitutions (Table 1.3). Forty-five of these substitutions cause amino acid substitutions (Laidlaw and Skinner, 2004).

	FPV (wild-type virus)	**FP9 (attenuated strain)**
Size of genome	288.539 Kbp	266.145 Kbp
Number of ORFs	260	244
Length of each ITRs	9.520 Kbp	10.158 Kbp
Length of repeated regions within each ITRs	1.675 Kbp	3.753 Kbp
Presence of mutations	----------	118 various mutations: • 26 deletions • 15 insertions • 77 single base pair substitutions

Table 1.3: Shows the major genomic differences between the FPV (wild-type) and the FP9 (highly attenuated strain).

Out of the 260 ORFs that have been reported for the FPV genome, the FP9 genome carries 244 and about 189 of these ORFs were found to be identical (Table 1.3). Some of these ORFs (19) were shorter in the FP9 than in the FPV, as a result of the potential effect of substitutions (non-sense and mis-sense mutations) that occur in ORFs. However, it has been found that only 5 ORFs were longer in the FP9 than in the wild-type FPV, due to the effect of insertion or deletion mutations. Generally, it has been realised that about 71 ORFs of the FP9 genome were mutated, and subsequently affected the products of 71 genes (Laidlaw and Skinner, 2004). There are several gene families that have been affected by the passage-specific mutations that take place within the FP9 genome, including FPV EFc family genes (1 out of 3 members were affected), ankyrin repeat-containing gene family (12 out of 31 members were affected), G protein coupled receptor gene family (1 out of 3 members were affected), C-type lectin-like genes family (3 out of 8 members were affected), Rabbit fibroma virus (N1R)/Ectromelia virus p28 (N1R/p28) gene family (2 out of 10 members were affected), VACV C4L/C10L-like genes family (2 out of 3 members were affected), Variola virus strain Bangladesh B22R (B22R) gene family (2 out of 6 members were affected), and V type immunoglobulin domain genes family (1 out of 5 members were affected). In addition, it has been found that many single copy genes such as photolyase, mouse T10-like, and VACV A47L-like genes were also affected by the passage-specific mutations. It appears, therefore, that several members of the multigene families, which are immunomodulators, have been lost or disturbed as a consequence of the attenuation mechanism of the FPV by tissue culture passage (Laidlaw and Skinner, 2004). However, the FP9 strain still retains some of the immunomodulators, such as chemokine like proteins, interferon gamma (IFN-γ) binding proteins, and interleukin 18 (IL18) binding proteins (Cottingham et al., 2006).

1.2.4.2 FP9 recombinant technology

The FP9 is a highly attenuated strain that has lost several members of multigene families of the wild-type FPV during the tissue culture passage (Laidlaw and Skinner, 2004). This attenuated strain harbours many deletions and modifications that may account for the enhanced ability of this vector to induce stronger cellular immune responses than the FPV (Anderson et al., 2004). In prime-boost approaches, recombinant FP9 (rFP9) has been shown to be more immunogenic (a two-fold increase) than the wild-type virus (Cottingham et al., 2006). The FP9 is one of the poxviruses that have a large and complex DNA genome into which a considerable number of multiple genes can be stably inserted and successfully expressed with the advantage that each single gene remains immunogenic (Webster et al., 2006). It has therefore been suggested that rFP9 could be used as one of the recombinant vectors to express specific foreign genes inside human or other mammalian cells (Anderson et al., 2004; Webster et al., 2006).

Construction of a rFP9 vector requires knowledge of the viral genome, in order to identify the regions of nonessential genes that can be used as insertion sites for foreign (target) genes. It has been found that the FPV shuttle pEFL29 transfer vector has regions of homology to the 6-kb BamHI fragment of the terminal region of the FP9 genome. This genomic fragment contains three ORFs (ORF 1, 2, and 3), which are considered nonessential genes for virus replication, and so can be used as insertion sites for exogenous antigens (Qingzhong et al., 1994; Boursnell et al., 1990).

1.2.4.3 Application of rFP9 as human vaccines

Several human clinical trials have used the rFP9 vector vaccines for eliciting protective immune responses against malaria in humans. This type of recombinant is capable of

expressing different antigens in humans without causing ill effects. It appears, therefore, that the rFP9 vector is an attractive vaccine candidate that can be used in humans (Anderson et al., 2004).

In 2005, Webster et al. demonstrated the efficacy of using a heterologous prime-boost vaccination regimen (priming FP9 vaccine/MVA boosting vaccine) encoding the pre-erythrocytic malaria antigen thrombospondin-related adhesion protein (ME-TRAP) in humans against malaria. They found that administration of two doses of priming FP9 vaccine followed by a single dose of MVA boosting vaccine was able to induce both CD4 and CD8 T cell immune responses. However, the protection conferred by this vaccination regimen was variable. Partial and sometimes complete protection levels, which can last for twenty months, were observed in humans against experimental malaria challenge (Webster et al., 2005). The prime-boost vaccination strategy (priming FP9 vaccine/MVA boosting vaccine), which encodes the *Plasmodium falciparum* circumsporozoite (PfCS) protein, was also assessed against malaria in semi-immune African individuals. It has been found that these viral vectors (FP9 and MVA) were safe and able to induce potent immune responses (particularly CD4) with no detectable antibody response to the target PfCS protein (Imoukhuede et al., 2006).

In 2006, Webster et al. assessed the safety of using the FP9 and MVA recombinant vectors in a prime-boost vaccination strategy. They found that this vaccination regimen, which encodes the ME-TRAP antigen, had the ability to induce protective malaria-specific CTL immune responses in humans with some acceptable side effects (no systemic side effects). In addition, they demonstrated that vector-specific immune responses were very low to moderate. This study (phase I clinical trial) was the first report to prove the safe performance of the rFP9 vaccine against malaria in non-immune individuals (Webster et al., 2006). However, in 2007, Bejon et al. evaluated the same

vaccination regimen (priming FP9 vaccine/MVA boosting vaccine), which encodes the ME-TRAP antigen, against malaria among children in Kenya. They found that these recombinant vectors were not able to confer protection against malaria, and the incidence of the infection was somewhat higher among the vaccinated children (Bejon et al., 2007).

1.2.4.4 Application of rFP9 as veterinary vaccines

In 2002, Vazquez-Blomquist et al. used a rFP9 vector to elicit specific CD8 CTL responses in mice against HIV-1. This viral vector (HP-438 strain) was genetically engineered with a multi-epitope polypeptide (TAB9) that contains six epitopes of the variable loop 3 (V3) sequences (gp120) from different isolates of HIV-1. This study has proved the ability of the rFP9 vaccine to produce potent HIV-specific CTL responses in mice (Vazquez-Blomquist et al., 2002). In addition, in 2003, Vázquez-Blomquist et al. used the pEFL29 transfer vector to clone HIV-1 chimeric protein (CR3) composed of multiple CTL and Th epitopes from different HIV-1 antigens, including gag, pol, nef, vpr, RT, gp41, and gp120, and then generated a rFP9 vector using HR approach. They demonstrated that the target genes (HIV-1 multiepitope protein) were successfully inserted into the FP9, and subsequently expressed in mice under the control of VACV p7.5K and FP9 virus 4b promoters. They proved the efficacy of the rFP9 vector in inducing simultaneous CD8 CTL protective responses against HIV-1 in mice (Vázquez-Blomquist et al., 2003).

In 2004, Anderson et al. used a rFP9 vector to express the *Plasmodium berghei* circumsporozoite protein (PbCS Ag) in mice. This study was the first to examine the ability of the rFP9 vector to induce cellular protective immunity in a mouse malaria model. They found that the rFP9 vector was highly immunogenic and had the ability to

elicit stronger malaria-specific CTL immune responses than the wild-type FPV vaccine (Anderson et al., 2004). In addition, when the rFP9 vector was used in a heterologous prime-boost immunisation strategy as a booster after a prime MVA vaccine (both vectors encoding the same malarial antigens [PbCS]), the malaria-specific CTL immune responses were further improved, and a significant level of protection against malaria was observed among the immunised mice (Anderson et al., 2004). In 2008, Alvarez-Lajonchere et al. used a rFP9 vector to express hepatitis C virus (HCV) antigens (core-E1 polyprotein) in mice and African green monkeys (*Chlorocebus aethiops sabaeus*). They found that this vaccine candidate was able to induce IFN-γ and anti-HCV immune responses that effectively control viraemia in the immunised animals (mice and monkeys). However, the induced humoral immune responses were very weak in both species (Alvarez-Lajonchere et al., 2008).

In 2011, Bridge et al. assessed the efficacy of using the rFP9 vector in a prime-boost vaccination regimen with the MVA booster vaccine against HIV-1 in Chinese cynomolgus macaques. They found that these vaccine candidates, which encode different clades (A, B, C, and D) of the HIV-1 envelope (env) and capsid proteins, could be safely administered and induced modest T cell immune responses in the vaccinated macaques. Only one of the three immunised macaques developed HIV-specific antibody responses. However, these antibodies did not neutralise HIV *in vitro*. The study concluded that these viral vectors were poorly immunogenic in the cynomolgus macaque model (Bridge et al., 2011).

1.2.5 Canarypox virus

1.2.5.1 Molecular biology

Canarypox virus (CNPV) is a member of the avipoxvirus genus that belongs to the chordopoxvirinae subfamily of poxviruses in vertebrates. CNPV is a cytoplasmic DNA virus that has a large double stranded DNA genome (about 360 Kbp), which encodes 328 different genes. Indeed, the CNPV genome is considered the largest of the genomes that have been completely sequenced in the chordopoxvirinae subfamily. This viral genome is composed of many gene families and several cellular homologous genes that may account for the very large size of the genome (Tulman et al., 2004).

In 2004, the whole genome of the CNPV was sequenced by Tulman et al. who reported that the CNPV genome was composed of about 106 conserved genes that were homologous to those of the FPV genome. These conserved genes are implicated in the main mechanisms of viral replication, including viral transcription (mRNA synthesis), DNA replication, and intracellular and extracellular virion structural proteins. Like FPV, the CNPV genome has two similar ITR regions (about 6.5 kpb) that are bound to the central coding region of the viral genome. Each one of the ITR regions has six ORFs. Generally, the CNPV genome has about 328 ORFs that are encoding proteins of more than 1900 amino acids. The genomic arrangement of genes and the gene complements reveal that the CNPV genome is more closely related to the FPV genome than other members of the subfamily chordopoxvirinae (Tulman et al., 2004).

1.2.5.2 Recombinant canarypox virus (rCNPV) vector

CNPV is one of the naturally attenuated avian poxviruses that have been used as a promising candidate vector for vaccine development (Kibler et al., 2011). CNPV, like other members of the avipoxviruses, is characterised by its inability to undergo a full

replication cycle in the cytoplasm of mammalian cells. Nevertheless, the CNPV vector is still able to express exogenous antigens and induce protective immune responses against the target proteins. Due to the fact that CNPV replication is restricted to avian species, these recombinant vectors are unlikely to cross-react immunologically with the exogenous antigens expressed in mammalian cells. Specific-vector immune responses induced by CNPV in mammalian hosts are very low to moderate, which cannot interfere with the main purpose of these recombinant vectors (Vázquez-Blomquist et al., 2003). All the features described above suggest that CNPV is a good and safe candidate for human and veterinary vaccine development.

The recombinant vector of CNPV is named ALVAC, which is a single plaque-cloned isolated from the attenuated CNPV (KANAPOX strain). Briefly, the CNPV was first attenuated by extensive serial passages (200 times) in CEF cultures, and then the resultant KANAPOX strain was further attenuated through four rounds of plaque purification. One of the isolated plaques was propagated and designated ALVAC (Tartaglia et al., 1992). Several studies have demonstrated the protective effect of the ALVAC vectors. These recombinants have been successfully used for the induction of protective immune responses against several diseases, such as rabies, Japanese encephalitis B, and cytomegalovirus (CMV) (Webster et al., 2006). In 1996, Fries et al. used ALVAC vector to express a specific rabies glycoprotein gene (ERA strain) in humans. They reported that the vector vaccine was safe and able to induce rabies-specific cellular immune responses in humans (Fries et al., 1996). Subsequently, the use of these types of vectors has been extensively studied in the development of HIV vaccines (Bruyn et al., 2004). In 1997, Ferrari et al. evaluated the potential impact of two ALVAC vectors encoding various HIV-1 genes among HIV-infected patients. The study found that both vaccine candidates were able to enhance and expand HIV-specific

CTL (CD8) immune responses that already existed in those HIV-infected patients due to the infection (Ferrari et al., 1997). Moreover, in 2003, Cao et al. demonstrated the safety and efficacy of ALVAC vector encoding different antigens (env-gp120/gp41, gag, and protease) of HIV-1 clade A, B, and D against HIV-1 among HIV-uninfected individuals in Ugandan. This phase I clinical trial concluded that the vector was safe, but weakly immunogenic. Only 10-15% of the vaccinated volunteers developed neutralising antibodies against the target HIV-1 clade B strains. However, no immune responses were detected against clades A and D. They also found that there was cross-clade reactivity against the two A and D clade antigens in the vaccinated individuals (Cao et al., 2003).

In 2004, Bruyn et al. also investigated the safety and the reactogenicity of different ALVAC constructs against HIV in humans. They reported that the vector was safe and the reactogenicity induced by the different CNPV vectors against HIV was similar. The authors discussed the fact that greater reactogenicity is associated with greater immune responses in pneumococcal vaccines, but this relationship has not been explored for canarypox vaccines (Bruyn et al., 2004). Recently, the ALVAC vector was used by De Souza et al. (2012) in a phase III clinical trial of an HIV-1 vaccine (RV144 trial). The ALVAC vector, which encodes three different genes of HIV-1 subtype B (env-gp120/gp41, gag, and protease), was used as a prime vaccine in a heterologous prime-boost vaccination strategy with a subunit booster vaccine (HIV gp120) to induce protective immunity against HIV in humans. This trial has proved the safety and the modest efficacy (about 31%) of using this vaccination strategy in preventing HIV infection. The trial also reported that the induced cellular immune responses were not as robust as the humoral immune responses. However, this trial did not include a control arm with canarypox control (not containing HIV sequences) alone which would be

necessary to prove that the protective effect seen was due to specific adaptive immunity to HIV rather than a non-specific temporary antiviral state induced by innate responses to the vector alone (De Souza et al., 2012).

1.2.6 Life cycle of poxviruses

Unlike other several nuclear DNA viruses, poxviruses encode a unique transcription system that gives them the ability to replicate entirely in the cytoplasm of host cells (Minnigan and Moyer, 1985; Broyles, 2003). Poxviruses are characterised by their independence from the host cells, in which the processes involved in viral genome replication and mRNA synthesis are controlled by the virus-encoded transcriptional proteins. This level of independence allows poxviruses to synthesise their mRNA immediately after virus entry (Yang et al., 2011).

Poxviruses have two distinct forms of infectious virions that can initiate the replication cycle: intracellular mature virion (IMV) and extracellular enveloped virion (EEV) (McFadden, 2005). The IMV has one wrapping membrane with twenty proteins, while the EEV has a double membrane with six proteins that facilitate virus attachment and entry. Attachment of the IMV was found to be promoted by four membrane proteins (A26, A27, D8 and H3) that bind to glycosaminoglycans or laminin on surface of the host cell (Moss, 2012). However, the mechanism of the EEV attachment has never been identified due to the fragile nature of its outer membrane (Zeh and Bartlett, 2002). Cellular-binding receptors for poxviruses are still not known. It seems likely that there are multiple receptors on different cell types. In terms of virus entry, the IMV enters cells by fusion with the plasma membrane, while the EEV enters through either endocytosis process or fusing with the cell membrane (Moss, 2012). Following virus entry, there are two phases of uncoating that take place. The initial stage involves

releasing the viral core particle, which contains the viral genome and virus-encoded enzymes that are essential for early gene transcription, into the cytoplasm. Expression of the early viral genes (early mRNA) starts immediately after releasing the virus core under the control of viral early promoters (McFadden, 2005). This early expression occurs before genome replication, while intermediate and late genes are expressed after genome replication (McFadden, 2005; Moss, 2012). The early viral genes represent around 50% of the total genome, and are considered non-structural genes that promote viral DNA replication (Oda and Joklik, 1967; Eaton et al., 2008). After synthesising early mRNA, the second stage of core uncoating takes place leading to release of the viral DNA genome that serves as a template for DNA replication and for the synthesis of intermediate and late viral mRNA (McFadden, 2005; Moss, 2012). The late viral genes encode several structural proteins and enzymes that are essential for assembly of mature virions (Eaton et al., 2008). It has been reported that expression of the intermediate and late viral genes occurs under the control of both host-derived and virus-encoded transcription factors (McFadden, 2005). Following viral DNA replication, DNA concatomeric structures are formed. These concatemers are then resolved into linear double-stranded DNA genome that assembles with the late synthesised proteins into IMV (Zeh and Bartlett, 2002). Some of these IMVs gain another double membrane layer from trans-Golgi membrane or endosomal membrane, resulting in particles with three membranes called wrapped virions (WVs). The outer membrane layer of these WVs will then fuse with the cell membrane to release EEV with a double membrane. Generally, releasing of the infectious virions from the infected cells occurs either by cell lysis (IMV) or membrane fusion (EEV) (Moss, 2012) (Figure 1.5).

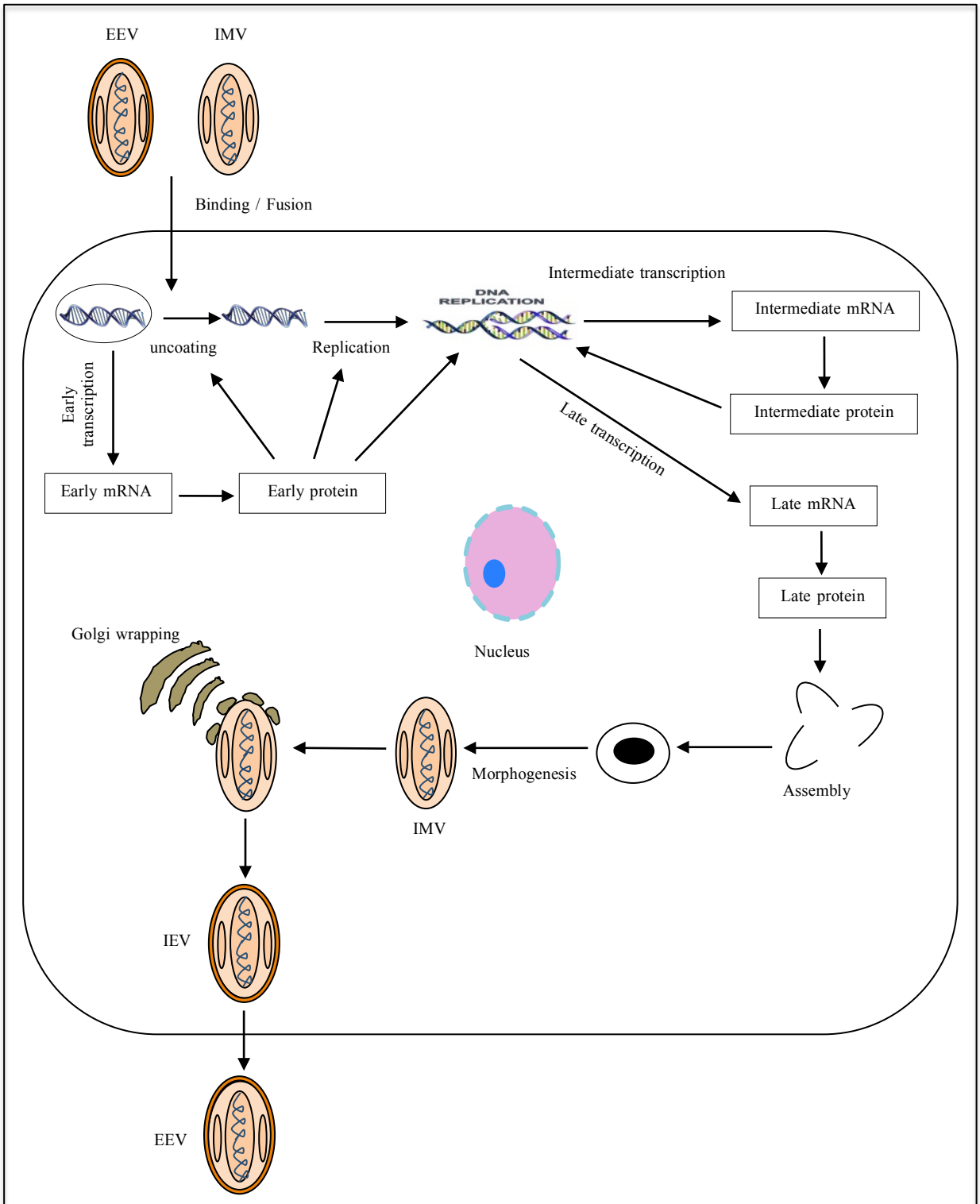

Figure 1.5: Life cycle of poxviruses.

This figure illustrates the different stages of poxvirus replication that takes place in the cytoplasm of the infected cells. It shows the sequential expression of early, intermediate, and late viral mRNA that are essential for assembly of mature virions. The figure is adopted from McFadden, 2005.

1.2.7 Poxviral promoters

Poxviral promoters are characterised by their unique consensus sequences that are different from other eukaryotic or prokaryotic sequences (Venkatesan et al., 1981). They are divided into three different types based on the transcriptional gene classes: early, intermediate, and late. Each promoter type controls the transcription of one gene class. However, some genes have two classes of promoters (early and intermediate or early and late) in tandem (Assarsson et al., 2008). For construction of recombinant poxviruses, it is essential to use poxviral promoters for transgene expression due to the fact that non-poxviral promoters are not recognized by the poxviral transcription machinery (Moss and Earl, 2001; Broyles, 2003). It has been shown that poxviral promoters are usually functional and active across different poxvirus genera (Carroll and Kovacs, 2003).

The choice of suitable promoter for optimal transgene expression is crucial. This choice is usually based on the intended use of the recombinant vector and the potential induction of immune responses (Carroll and Kovacs, 2003). Early poxvirus promoters (such as P_F) are usually used to express target proteins in the early phase of infection before the appearance of major cytopathic effects (CPE) caused by the viral vector. This type of promoter can also be used to direct transgene expression in those cell lines that restrict the replication of recombinant viral vector and thereby prevent late expression (Kriajevska et al., 1993; Moss and Earl, 2001; Carroll and Kovacs, 2003). It has been reported that using an early promoter in rMVA ensures persistent and high-level transgene expression in infected human macrophage-derived dendritic cells (Kastenmuller et al., 2006). In 2010, Baur et al. demonstrated that early transgene expression is associated with the induction of robust specific CTL responses against the encoded target antigens (Baur et al., 2010). The sequence of early poxvirus promoters

contains transcription termination site TTTTTNT, which should be avoided in sequences encoding recombinant antigens (Carroll and Kovacs, 2003).

Intermediate poxviral promoters (such as I1L, A1L, A2L and G8R) were shown to have a critical role in regulating the life cycle of poxviruses (Hirschmann et al., 1990; Baldick Jr et al., 1992). The I1L is an example of intermediate poxviral promoter that has stronger activity (over ten times) in directing selective transgene transcription than other classes of poxviral promoters. It has been shown that the I1L promoter rivals the expression efficiency of the T7 promoter in the rVACV-T7 expression system. Among all members of poxviruses, the intermediate I1L promoter sequence was found to be highly conserved (Liu et al., 2004).

Late poxvirus promoters (such as P11) are usually used to direct higher levels of transgene expression than early and intermediate promoters. Indeed, there could be several factors behind the strong activity of these promoters, such as viral genome synthesis (high copy number of DNA), intrinsic strength factors, and time of expression (more than 20 hours) (Moss and Earl, 2001). The initiator element for transcription of late promoters is characterized by the highly conserved region, TAAAT (Davison and Moss, 1989). Interestingly, the downstream sequences of this conserved region were found to have no role in gene transcription (Broyles, 2003). P11 is an example of a strong late poxviral promoter that has been used in recombinant poxviruses for increasing transgene expression and inducing potent immune responses (Mackett and Smith, 1986). Wittek et al. (1984) demonstrated that the late p11 promoter was able to direct the transcription of an 11K structural protein (Wittek et al., 1984).

Combined early and late poxvirus promoters (such as p7.5) have been extensively used in the construction of recombinant poxviruses (Mackett et al., 1984; Cochran et al.,

1985). This type of promoter has the ability to direct the transgene expression throughout the whole viral replication cycle, in order to ensure presence of adequate levels of expression to induce potent immune responses (Moss and Earl, 2001; Wennier et al., 2013). There are two types of early/late promoters: native (such as p7.5 and modified H5 [H5m]) and synthetic promoters (such as PrS and pHyp) (Wennier et al., 2013).

1.2.8 FPV- and MVA-T7 RNA expression system

The bacteriophage T7 RNA polymerase gene (T7 gene 1) is considered one of the gene expression systems that is widely used to direct high levels of transgene expression in different cell types. This RNA polymerase has the ability to direct a selective transcription of the cloned genes under the control of its own strong promoters (T7 promoter). The T7 polymerase is characterised by its high specific activity and ability to generate large amounts of RNA transcripts that are comparable with those complete transcripts obtained in a normal cell (Studier and Moffatt, 1986; Britton et al., 1996).

VACV was the first viral vector encoding the T7 RNA polymerase gene. This recombinant vector (vTF7-3) was used widely for the expression of heterologous genes in a variety of vertebrate cells, due to the fact that VACV has wide host range (Fuerst et al., 1986). In 1987, Fuerst et al. developed a VACV expression system encoding the T7 RNA polymerase gene. Development of this system was based on simultaneous infection of cells with two types of rVACV vectors. One of them encoded the target genes (HBsAg and HIV gp160) flanked by T7 promoters, and the second one encoded the T7 polymerase gene flanked by poxvirus promoters. This combination of vectors provided higher levels of protein expression compared to the previously existed rVACV vector (Fuerst et al., 1987). However, it has been reported that VACV produces a

variety of CPE that can interfere with the expressed proteins. Moreover, this viral vector is considered a pathogenic in mammalian cells. For these reasons, rMVA and rFPV encoding the bacteriophage T7 polymerase gene (rMVA-T7 and rFPV-T7) have been developed, and are considered alternative expression systems to the previously existing rVACV-T7 (Wyatt et al., 1995; Britton et al., 1996). The safety and efficacy of using rMVA-T7 and rFPV-T7 for the expression of cloned genes in mammalian cells have been demonstrated in several studies. It has been found that these recombinants were able to express high levels of heterologous antigens in mammalian cell lines under the control of the T7 promoters (Hebben et al., 2007; Wyatt et al., 1995; Britton et al., 1996; Das et al., 2000).

In 2000, Das et al. compared the efficacy and cytotoxicity of the rMVA-T7 and rFPV-T7 in different cell lines, such as ovine, caprine, and bovine primary cell lines. They found that both of them (rMVA-T7 and rFPV-T7) were able to express similar amounts of the T7 polymerase. However, the rMVA-T7 produced higher levels of toxicity than the rFPV-T7 in primary cell lines (ovine, caprine and bovine). In addition, the rMVA-T7 was found to be capable of replicating rapidly in these cells, leading to kill them within 3-5 days after infection, while the rFPV-T7 was safe and did not kill the cells. The study was concluded that the rFPV-T7 can be used as an effective alternative for the MVA-T7 in the expression of the T7 polymerase gene in these primary cell lines (Das et al., 2000).

1.3 HIV infection

1.3.1 Natural history of HIV type 1 and 2

HIV infection is an important pandemic disease. At the end of 2014, it was estimated that approximately 36.9 million people were living with HIV infection worldwide. The majority of these HIV cases (about 70%) have been reported in sub-Saharan Africa. Since the first recognised cases of HIV infection in 1981, around 39 million HIV-infected patients have died as a result of the disease (Granich et al., 2012; Siliciano, 2006; WHO, 2015).

Two major viral types of HIV have been recognised as causative agents of acquired immune deficiency syndrome (AIDS): HIV type 1 (HIV-1) and HIV type 2 (HIV-2). HIV-1 is considered more pathogenic and it is the predominant type of HIV. Most cases of HIV-1 infection were reported in North America, Europe and Africa. However, high prevalence rates of HIV-2 infection have been found principally in the West African nations (Knipe et al., 2007).

It is clear that the origins of HIV-1 and HIV-2 were in non-human primates (*Pan troglodytes troglodytes* chimpanzees for HIV-1; sooty mangabeys for HIV-2). These two types of HIV have evolved from animal viruses that were able to cross the animal species barrier and infect (zoonotic infection) human hosts in western equatorial Africa. Chimpanzee hunters were likely the first human hosts for HIV infection acquired from the consumption of infected chimpanzees (Seale, 1989; Siliciano, 2006; Collier and Oxford, 2006).

1.3.1.1 HIV type 1 (HIV-1)

In 1981, epidemiologists noticed that some healthy homosexual individuals were infected with *Pneumocystis carinii* pneumonia (PCP), which is a type of opportunistic

infection (severe pneumonia) usually apparent in immunocompromised patients. They therefore concluded that these healthy individuals might be suffering from an acquired immune deficiency problem. Subsequently a link was found between the immune deficiency and the decrease in CD4+ T cell count. By 1983, scientists were able to isolate the infectious agent, which targets the CD4+ T cells, from those infected patients. This pathogenic agent was a virus, which is now called HIV-1 (Montagnier et al., 1984; Gottlieb et al., 1981; Siliciano, 2006).

HIV-1 targets specific types of cells of the host immune system, T helper lymphocytes (CD4+ T cells). Macrophages and dendritic cells were also found to be targeted by HIV. These types of immune cells have CD4 protein receptors that interact efficiently (high affinity) with the viral binding sites (HIV envelope protein) and permits viral attachment to the CD4 T cells (Maddon et al., 1986). The binding reaction between the viral envelope glycoprotein (gp120) and CD4 molecule receptor is considered the main critical step in HIV infection. It has been found that HIV-1 utilises another co-receptor (CXCR4 and/or CCR5) for viral entry into target cells (Deng et al., 1996). Indeed, following the binding reaction between gp120 and CD4 receptor, a region of the gp120 is exposed and attaches to one of the co-receptors. Binding to these co-receptors (chemokine receptors), which are members of the G protein-coupled receptor family, triggers a conformational change in the other viral envelope glycoprotein (gp41). This facilitates the membrane fusion mechanism between the virus and the target cells, so that the viral genetic material can be transferred into the host cells. CXCR4 was found to be the human co-receptor for the T cell tropic HIV-1 isolates (syncytia-inducing strains [SI]), while CCR5 is considered the major co-receptor used by macrophage tropic HIV-1 isolates (M-tropic or non-syncytia-inducing strains [NSI]) (Berger et al., 1999; Siliciano, 2006; Collier and Oxford, 2006). Reverse transcription (RT) of the

viral genomic RNA into double-stranded DNA is considered the next step after viral entry into the host cell cytoplasm. This step is controlled by the viral RT enzyme that is encoded in the virion. The RT enzyme is characterised by its lack of proof reading mechanism, which gives rise to a great many mutations at this stage (Baltimore, 1970; Temin and Mizutani, 1970). It has been reported that the viral genome produces about one new mutation during each viral replication cycle. Accumulation of these mutations plays a critical role in allowing the viruses to evade the host immune responses. The third step of the HIV life cycle is integration of the transcribed copy of the viral DNA into the DNA genome of the infected cell, where the virus starts to utilize the host cell's machinery to synthesise large amounts of viral proteins and genetic materials (Siliciano, 2006). Thereafter, the final stage of HIV replication starts with the assembly of all essential viral components to generate immature HIV particles. This stage of packaging takes place at the inner surface of the plasma membrane. Once new HIV viruses are assembled, they acquire their lipid envelope by budding through the cell membrane of the host cell. After releasing of these non-infectious HIV viruses, the maturation process begins with the production of viral protease enzyme that is responsible for processing other viral structural proteins, which are required for generating mature infectious virion (Sundquist and Kräusslich, 2012).

Three phases of HIV infection have been identified among HIV-infected patients: primary (acute) infection phase, chronic asymptomatic phase, and AIDS, which is the last stage of HIV infection (Siliciano, 2006). In the primary infection stage, the virus starts to replicate and produces a low level of viraemia that can be controlled by the host immune responses. About 50–70% of HIV-infected patients experience mild flu-like symptoms during the primary phase, such as fever, muscle and joint pain, headache, enlarged lymph nodes, and lethargy. During the symptomatic acute stage, the virus

replicates rapidly, leading to an increase in the plasma viral RNA load to reach a level of up to more than 10^6 copies per ml. This stage may last for approximately 2–3 weeks after the initial viral infection (Shors, 2009; Siliciano, 2006; Kumwenda et al., 2008). The chronic asymptomatic phase (second stage of HIV infection) is usually the longest phase of the infection, which is characterised by the absence of any signs or symptoms for about eight to ten years. During this stage, the virus continues to replicate leading to a gradual loss of CD4 T cells. It has been found that excessive loss of CD4 T cells (<200 cells/µl) is the main reason for the development of serious opportunistic infections, such as *Pneumocystis jirovecii* pneumonia. When the CD4 cell count falls to a level of less than 200 cells/µl, the HIV-infected patient will become susceptible to progress to the third and last stage of the HIV infection (AIDS), which is characterised by the development of severe diseases and some types of malignancy (Shors, 2009; Siliciano, 2006).

1.3.1.2 HIV type 2 (HIV-2)

In 1985, scientists recognised another HIV related to but distinct from HIV-1. This pathogenic virus was isolated from African infected patients in Senegal, and is now known as HIV-2 (Clavel et al., 1986). HIV-2 acts in the same way as HIV-1 in targeting the primary CD4+ T cells of the immune system, and was also found to be capable of causing AIDS. In addition, HIV-2 utilises the same cellular CD4 molecule receptor and may also use the same chemokine co-receptors (CXCR4 and/or CCR5) for viral entry into target cells. Generally, HIV-1 and HIV-2 share some similarity in terms of their natural infection, and also in the clinical features of HIV/AIDS infection. However, HIV-2 replicates slowly and is a less pathogenic strain, taking longer (more than 20 years) to cause AIDS than HIV-1. Many studies reported that HIV-2-infected individuals tend to have low levels of plasma viral load and many of them do not

progress to the last stage of the HIV infection (AIDS). Most cases of HIV infection have occurred as a result of the pandemic HIV-1 strains, while cases of HIV-2 infection are a minority, being endemic in the West African countries, such as Guinea-Bissau (Siliciano, 2006; Dalgleish and Weiss, 1999; Reeves and Doms, 2002). Initially, HIV-2 infection was reported to confer protection in human hosts against HIV-1 infection (Travers et al., 1995). However, HIV-1 and HIV-2 co-infection has subsequently been reported, and this was associated with accelerated progression to AIDS (Nkengasong et al., 2000; Gilleece et al., 2010).

1.3.2 Biology and genomic organisation of HIV-1 and 2

HIV-1 and 2 are members of the genus lentivirus that belongs to the retroviridae family, which consists of three main subfamilies: oncovirinae (Type C), spumavirinae, and lentivirinae. Both types of HIV are enveloped RNA viruses that have two copies of a positive sense, single stranded RNA genome (+ssRNA), which is 7–10 Kb in length. The genetic material (RNA) is surrounded and protected by the viral nucleocapsid protein (p24) that is enclosed by a matrix protein layer (p17). Several copies of the reverse transcriptase (RT), protease (pro), and integrase (int) enzymes are bound to the viral RNA genome within the core (Figure 1.6). The complete HIV particle is almost spherical in shape with a diameter of about 100–120 nm. This particle is surrounded by a viral envelope (lipid bilayer) that is composed of a surface envelope glycoprotein (env). The env protein consists of two main types of glycoprotein spikes that are associated noncovalently: transmembrane envelope glycoproteins (gp41 for HIV-1 and gp36 for HIV-2) and the outer envelope (surface) glycoproteins (gp120 for HIV-1 and gp125 for HIV-2) (Figure 1.6). For both HIV types, the surface glycoproteins consist of several sites of N-linked glycosylation, five constant regions (C1–C5), conserved disulphide bonds, and five variable loops (V1–V5). It has been found that there is a high

degree of similarity in the transmembrane glycoproteins of HIV (type 1 and 2) and SIV, which are composed of two major domains, intracellular (C-terminal) domains, and extracellular (N-terminal) domains with two amphipathic alpha helical regions (Shors, 2009; Fields et al., 1991; Reeves and Doms, 2002).

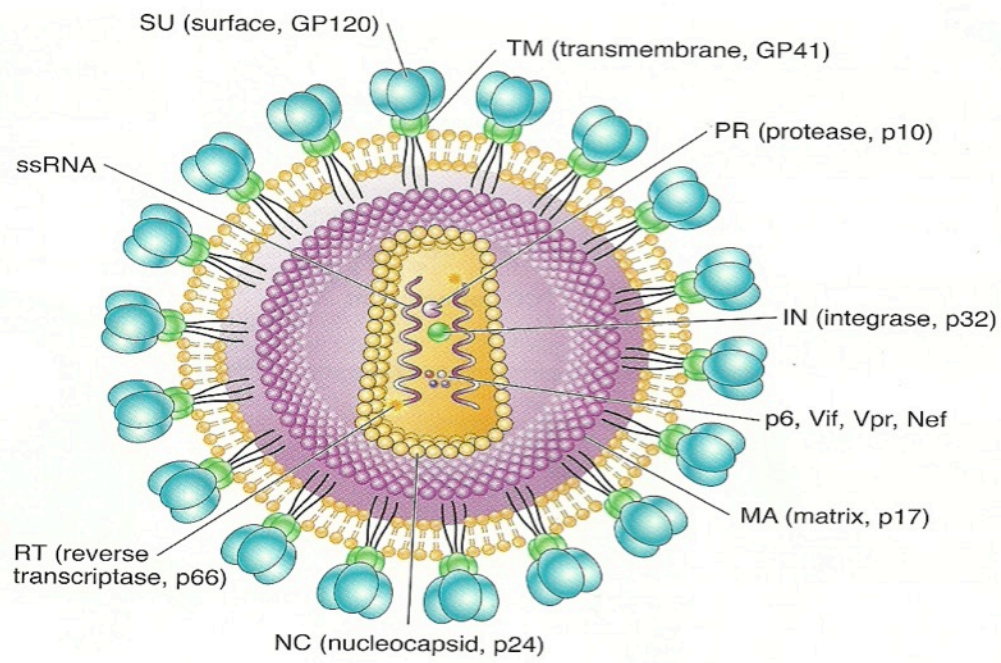

Figure 1.6: Schematic representation of HIV virion structure.
This figure is adopted from Shors, 2009.

As with all retroviruses, the HIV-1 and 2 genomes have two identical LTR regions at each terminus and several genes: gag (group specific antigen), pol (polymerase), env (envelope), vif (viral infectivity), tat (transactivation), rev (regulatory of virus), nef (negative factor), and vpr (viral protein R). HIV-1 and HIV-2 differ in some accessory genes: vpu (viral protein U) gene for HIV-1, and vpx (viral protein X) gene for HIV-2 (Figure 1.7). In both HIV-1 and 2, gag, pol, and env are considered the main genes that

encode for all the structural and regulatory proteins for virus replication (Table 1.4). The gag gene encodes three main structural proteins (nucleocapsid proteins [p6 and p7], matrix protein [p17], and the capsid protein [p24]) that are essential for virus particle (virion) formation. In addition, there are three other essential proteins encoded by the pol gene: RT, pro, and int enzymes. The env gene gives rise to the outer glycoproteins precursor 160 (gp160) for HIV-1, and the precursor 140 (gp140) for HIV-2. These precursors are then cleaved by the host protease into two mature glycoprotein spikes, gp120/gp41 for HIV-1 and gp125/gp36 for HIV-2. However, both gag and pol genes are cleaved by the viral pro enzyme during the maturation stage (Fields et al., 1991; Shors, 2009; Siliciano, 2006; Collier and Oxford, 2006). The remaining genes are considered non-essential (accessory) genes for virus replication (Table 1.4). These genes encode several non-structural proteins that can be used to regulate the different stages of the HIV life cycle (Siliciano, 2006; Levy, 2007). For example, the HIV-1 vpu (p16) gene plays a role in reducing the surface expression of cellular CD4, preventing the formation of gp160/CD4 complexes in the endoplasmic reticulum, and so enhancing release of the virus from infected cell. The tat (p14) gene enhances the process of viral RNA transcription through the binding interaction with the transactivation response element (TAR) (Levy, 2007). However, the rev (p19) gene acts as a post-transcriptional regulator for the expression of viral mRNA. Indeed, this gene facilitates the export of unspliced mRNA into the host cell cytoplasm for translation (Cheng et al., 1992; Karn and Stoltzfus, 2012; Levy, 2007). Nef (p27), a pleiotropic gene, has various effects that can promote viral replication. This gene also has the ability to down regulate the cellular expression of CD4 and major histocompatibility complex class I (MHC I) (Levy, 2007). The vpr and HIV-2 vpx (p15) genes have almost the same structure and functions (Tristem et al., 1992). These genes play a role in virus entry and may affect the

apoptotic process (either enhance or reduce) within infected cells. They also can enhance the transcription levels of LTR regions (Levy, 2007). The vif (p23) gene is responsible for increasing the virus infectivity and supporting the synthesis of proviral DNA. Vif is also found to interact with the APOBEC system (particularly APOBEC3G protein) to down regulate it and prevent cytidine deamination (Table 1.4) (Levy, 2007).

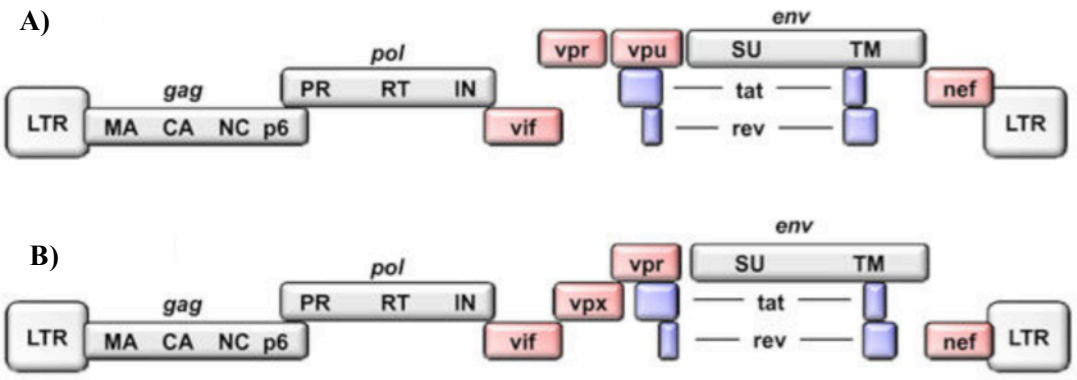

Figure 1.7: Genomic organisation of HIV.

A) HIV-1 genome, B) HIV-2 and SIV genome. This figure is adopted from Ayinde et al., 2010.

In general, the genomic organisation of both HIV types is similar but with some genetic diversity (about 25%) among their gag, pol, and env genes. Moreover, it has been found that HIV-1 genome encodes a unique gene (vpu), which is not present in HIV-2. Likewise, the HIV-2 genome has vpx gene, which is absent in HIV-1 (Fields et al., 1991; Reeves and Doms, 2002).

Type of genes	Name of genes	Encoded proteins	Function
Structural genes	Gag	• Nucleocapsid proteins (p6 and p7). • Matrix protein (p17). • Capsid protein (p24).	Essential for the virus particle (virion) formation.
	Pol	• RT (p64). • Pro (p10). • Int (p32).	- For reverse transcription of the viral RNA (includes RNase activity). - For cleaving both gag and pol polyproteins into their individual proteins. - For viral genome (cDNA) integration.
	Env	Precursor for the outer glycoprotein spikes (gp160 for HIV-1 and gp140 for HIV-2).	Essential for the outer envelope (surface) glycoproteins formation.
Non-structural (accessory) genes	Vpu (only in HIV-1)	p16	Play a role in reducing the surface expression of cellular CD4.
	Nef	p27	Promotes viral replication and down regulates the cellular expression of CD4 and MHC I.
	Tat	p14	Enhances the process of viral RNA transcription.
	Rev	p19	Acts as a post-transcriptional regulator for the expression of viral mRNA.
	Vpr and Vpx (vpx only in HIV-2)	p15	Play a role in virus entry, affect the apoptotic process and enhance the LTR transcription.
	Vif	p23	Increase the virus infectivity and support the synthesis of proviral DNA.

Table 1.4: Different types of HIV genes and their functions.

1.3.3 A brief overview of immunological response to HIV infection

There are two different patterns of host immune responses that can arise during HIV infection: innate and adaptive immunity.

1.3.3.1 Innate immune responses to HIV infection

The innate immune system represents the first defence barrier that starts immediately during the early stages of HIV infection. Many cellular factors and different types of phagocytic and antigen presenting cells (APCs) are involved during this stage of host immune responses, such as natural killer (NK) cells, macrophages, dendritic cells (DCs), and gamma delta (γδ) T cells. In addition, cytokines (such as IFN-α), chemokines, and complement constitute a major part of the innate immunity. Of these earlier immune cells, NKs and DCs were found to play a pivotal role in directing the establishment of the adaptive immune responses (Quaranta et al., 2012). DCs are one of the initial HIV targets that are mainly involved in priming and activating HIV-specific T cell immune responses (CD4+ and CD8+ T cells). Indeed, there are two major types of blood DCs that constitute the importance of the innate immune responses: Myeloid DCs (mDCs) and plasmacytoid DCs (pDCs). Both of them express CD4 and act as APCs. However, the pDCs has a vital role in the production of type one IFN (IFN-α and β), whereas the mDCs are the main source of interleukin-12 (IL-12) and are considered one of the professional APCs. The mDCs exist in various areas of the body such as mucosal tissue, blood, and skin, while the other types of DCs (pDCs) are found only in secondary lymphoid organs and blood (Quaranta et al., 2012; Levy, 2007).

Type one IFNs have critical roles to play in antiviral immunity through direct interference with the HIV replication in T cells. They have the ability to increase the antiviral activity of NKs and CD8+ T cells through enhancing the cellular expression of

MHC I molecules. In addition, type one IFNs are able to increase the production of type two IFNs (IFN-γ), particularly by CD4+ T cells. IFN-α has a direct effect on activating the mDCs and enhancing their activity as APCs through up-regulating the expression of MHC molecules (Levy, 2007).

NK cells constitute one of the major components of the innate immune system. These cells, which comprise about 15% of the human peripheral blood mononuclear cells (PBMCs), have the ability to recognise and kill certain HIV-infected cells that are no longer recognised by CD8+ T cells due to down-regulation of MHC I. It has been found that some of HIV proteins (such as nef, tat, and vpu) are implicated in the protection of these virus-infected cells via down-regulation of MHC I molecules (Levy, 2007). These types of NK cells lack T cell antigen receptors (TCR) that are responsible for recognising foreign antigen. However, they still have the ability to lyse the virus-infected cells. This occurs either through the direct release of perforin and granzyme that can trigger apoptosis of the target cells, or by the antibody-dependent cell mediated cytotoxicity (ADCC) strategy (Quaranta et al., 2012; Levy, 2007), thereby interacting with adaptive immunity. The ADCC strategy is based on the binding between the Fc region of the host immunoglobulin (primarily IgG1 isotypes) and the Fc receptor (FcR) on the effector NKs. This binding can then promote the cytotoxic responses of the NKs against the virus-infected cells (Martin, 2004; Levy, 2007). Moreover, NKs have been shown to have an important role in promoting the activation of the adaptive immunity, particularly the CD8+ CTLs through the production of a variety of cytokines and soluble factors, such as IFN-γ, β-chemokines, IL-5, IL-12, and tumour necrosis factors (TNFs) (Levy, 2007).

γδ T cells are another type of the innate immune cells that involved in the antiviral immunity. These cells do not require APCs as they express TCR that recognises foreign

antigens independently of MHC molecules. Once γδ T cells become activated, they can express chemokine receptor type 7 (CCR7), and then display cytotoxic activities against HIV in the infected lymph nodes (Levy, 2007). Moreover, γδ T cells produce several antiviral factors such as RANTES (CCL-5), MIP-1α (CCL3), and MIP-1β (CCL-4). These chemokines have the ability to suppress HIV replication through down regulating the expression of the CCR5 co-receptor on the target CD4+ T cells (Quaranta et al., 2012). γδ T cells also produce a variety of effector cytokines (such as Th1, Th2, IFN-γ, and TNF-α) that can affect the viral pathogenesis (Quaranta et al., 2012; Levy, 2007).

The complement system is one of the main components of the innate immunity that is involved in controlling HIV replication. This system is composed of various complement components (about 30 plasma proteins and different regulatory and cell bound proteins) that can be activated through three different pathways: classical, alternative, and lectin pathways. These distinct pathways occur in sequential multistep processes leading to the formation of membrane attack complexes (MACs) that have the ability to clear the HIV-infected cells (Chen et al., 2009). Activation of the complement system plays a key role in controlling the viral replication through different antibody-independent mechanisms, including the direct interaction with either the virion or gp120. These interactions can lead to complement activation and then destruction of the virus-infected cells (Levy, 2007).

To sum up, innate immunity responds rapidly and can act independently of the adaptive immune response. This type of immunity is non-specific and lacks memory. It causes early restriction of the virus, allowing the adaptive immune responses to mount a more comprehensive defence (Quaranta et al., 2012; Levy, 2007).

1.3.3.2 Adaptive immune responses to HIV infection

The adaptive (acquired) immune system represents the antigen-specific immune responses that are classified into two main classes: cellular and humoral immunity. The cell-mediated (cellular) immunity is mediated by the T lymphocytes that are responsible for the production of antigen-specific CD8+ CTL and $CD4^+$ (helper) T cells. However, the antibody-mediated (humoral) immunity is mediated by the B-lymphocytes that are involved in the production of antigen-specific antibodies (Quaranta et al., 2012).

1.3.3.2.1 Cell-mediated (cellular) immunity

The T cell-mediated immune responses are considered to be the most effective type of adaptive immune responses. Each T cell type has two major categories: antigen-naïve T cell and an antigen-experienced T cell that can also be divided into two main classes, central memory T cell (Tcm) and effector memory T cell (Tem). The Tcm express two different chemokine receptors (CCR7 and CD62L) that allow them to recirculate through the peripheral lymphoid organs (Blood and lymph tissues). However, the Tem, which do not express these receptors, only has the ability to migrate along the peripheral lymphatics to the extra-lymphoid tissues (such as the intestine and epithelium) (Quaranta et al., 2012; Levy, 2007).

1.3.3.2.1.1 $CD4^+$ T cell immune response to HIV

$CD4^+$ T helper (Th) cells are one of the main types of the T lymphocytes that are involved in cellular immunity. $CD4^+$ T cells recognise specific peptides presented in the context of MHC class II (MHC II) molecules. These cells (naïve CD4+ T helper cell [Th0]) can be divided into two major subsets (Th1 and Th2) according to their profiles of cytokine production (Table 1.5). Th1 (type 1) cells produce a variety of cytokines (IL-2, TNF-α, and IFN-γ) that are mainly promoting cellular immune anti-HIV

responses. However, Th2 (type 2) cells secrete different types of cytokines (IL-4, IL-5, IL-6, IL-10, and IL-13) that primarily activate B-lymphocytes and enhance their production of antibodies. Although type 2 cytokines have ability to suppress the HIV replication, they can lead to reduction of CTL immune responses. Another main difference between the two Th subtypes (1 and 2) is that Th1 cells express the IL-12β receptor, which is not present in Th2 (Table 1.5). It has been found that IFN-α and IFN-γ promote and regulate the expression of the IL-12β receptor, which can be inhibited by IL-4 (Levy, 2007). Indeed, both types of cytokines (type 1 and 2) are considered critical for the development of effective CTL immune responses and immunoglobulin production (Levy, 2007; Porichis and Kaufmann, 2011).

Development and differentiation of the naïve T helper cells (TH0) into different Th cell subsets is dependent upon various cytokines that can enhance and direct their development. For example, it has been found that IL-12 promotes the development of Th1 cells, while inhibits the cytokine production by Th2 cells. In contrast, IL-4 and IL-10 induce Th2 cell development, while down regulating the cytokine expression by Th1 cells (Table 1.5). Therefore, these cytokines, which are expressed by Th1 and 2 cells, antagonise each other's function (Levy, 2007).

Activation of $CD4^+$ T cell responses, during an HIV infection, occurs through the recognition of processed viral antigenic peptide on APCs (MHC II). Once activated, they produce various types of cytokines that can promote the cellular immunity and induce humoral immunity against the invading pathogen (Quaranta et al., 2012). These cytokines, which are produced by Th1 and Th2 cells, also have an immune regulatory function that may affect HIV disease progression (Levy, 2007).

Different Markers	Subsets of CD4+ T cells	
	Th1	**Th2**
Secreted cytokines	IL-2, TNF-α, and IFN-γ	IL-4, IL-5, IL-6, IL-10, and IL-13
Cytokines that promote their development	IL-12	IL-4 and IL-10
Expression of IL-12β receptor	Yes	No
Function	Promoting cellular immunity	Activating the B lymphocytes and enhancing production of antibodies

Table 1.5: Different subsets of CD4 T helper cells and their functions.

Most of the HIV-specific Th1 responses are observed primarily during the asymptomatic stage of HIV infection, whereas the HIV-specific Th2 responses are found during the symptomatic infection stage (Levy, 2007). Generally, the activated HIV-specific $CD4^+$ T cell responses are observed during the early stages of infection; subsequently, they become difficult to detect. However, HIV-infected long-term nonprogressors (LTNPs) patients, who are capable of controlling their infection, have an exceptional situation where the $CD4^+$ T cell immune responses can also be detected during the later (chronic) stages of HIV infection (Quaranta et al., 2012).

1.3.3.2.1.2 $CD8^+$ T cell immune response to HIV

$CD8^+$ CTL are considered the major T cell types that are involved in the cellular immune responses against virus-infected cells. These T cells react specifically, and their responses are MHC restricted and require cell-to-cell contact (Levy, 2007). Activation of $CD8^+$ T cell responses occurs through the recognition of processed viral antigenic peptide on the MHC I molecule (Quaranta et al., 2012). Once activated, they can combat and kill HIV-infected cells by the induction of apoptosis either through the direct release of perforin and granzyme, or by Fas/Fas ligand interaction. It has been

found that one CD8+ T cell could have the ability to kill around two to three virus-infected cells. This strong lytic activity of the CD8+ CTLs plays a key role in control of HIV infection (Levy, 2007).

Two different subsets of HIV-specific CD8+ T cells have been observed among HIV-infected individuals: CD8+ T cells that express CD28 molecule (CD8+/CD28+ T cells), and those CD8+ T cells that lack the CD28 molecule (CD8+/CD28- T cells). The latter subset, which has reached to the non-proliferative final stage of the replicative cycle, has a strong cytotoxic (effector) anti-HIV activity, whereas the CD8+/CD28+ T cells have a non-cytotoxic anti-HIV response. Another main difference between the two subsets of CD8+ T cells is that CD8+/CD28- T cells express CD57 protein, which is not expressed on the CD8+/CD28+ T cell subset. However, both of them produce IL-2 and IFN-γ (Levy, 2007; Gamberg et al., 2004). In addition, these CTLs produce RANTES protein that can enhance the cytotoxic activity of the HIV-specific CD8+ T cells through the direct interaction with the chemokine receptor type 3 (CCR3) (Levy, 2007).

HIV-specific CTL immune responses are directed against peptide epitopes within several different HIV antigens, such as gag, vif, env, nef, and RT. CTLs react with multiple epitopes in gag, but only with a restricted number of epitopes on the HIV env protein (Levy, 2007). This is because the HIV env protein has the ability to down regulate the CD40 ligand (CD40L/CD154) expression on the activated CD4+ T cells, disturbing the process of antigen presentation via DCs that express CD40 (Levy, 2007; Lin et al., 2009). HIV-specific CTL responses are also directed against the smaller nef and tat proteins (Levy, 2007). Generally, during the acute stage of HIV infection, CTL responses are produced firstly against the HIV proteins that expressed early in the viral life cycle, such as rev, tat, and nef accessory proteins. It has been found that gag-specific CTL responses may decline gradually with disease progression, whereas

specific CTL responses against viral env protein are not so affected by disease progression (Levy, 2007).

1.3.3.2.2 Antibody-mediated (humoral) immunity

Humoral immunity is mediated by the B-lymphocytes that are responsible for the production of specific antibodies in response to the foreign antigen (Quaranta et al., 2012). During HIV infection, the development of humoral immune responses usually starts with the production of specific antibodies against gag antigen followed by nef, rev, and env proteins (Levy, 2007).

Several subclasses of antibodies, which are targeting HIV gag protein, have been observed among infected individuals. However, there is only one main antibody subclass, IgG1, has been found against viral env protein. In fact, the IgG1 is considered the predominant IgG subclass that can be detected during all stages of HIV infection with almost similar concentration. Development of IgG responses is usually followed by the production of IgA antibodies that can be detected during the seroconversion phase and more frequently during the last stage (AIDS) of the infection than the asymptomatic phase. The titre of these antibody classes (such as IgA, IgM, and IgG4) is unstable and differs depending on the stage of infection (Levy, 2007).

Initial HIV-specific antibody responses are normally produced within a few weeks after the exposure to the virus. More recently, these specific antibodies have been shown to be released after approximately 13 days of viremia, targetting the gp41 subunits of the virus envelope glycoproteins. These types are considered non-neutralising antibodies that cannot effectively control the HIV replication (Liao et al., 2011). It has been found that most of the humoral immune responses, which are targeting the HIV envelope antigen, have little effect on neutralising the virus. Neutralising antibodies (Nabs) are

those that have the ability to bind to the virus and neutralise it (Quaranta et al., 2012; Levy, 2007). Development of Nabs generally occurs about three months or later after virus transmission. This delayed response is due to the massive destruction of T helper cells during the acute stage of infection (Euler et al., 2011). These Nabs generally target specific regions within the variable loops of the viral surface glycoproteins (gp120). The membrane proximal external region (MPER) of gp41, which is highly conserved, was also found to be a region that can be targeted by NAbs (Quaranta et al., 2012; Levy, 2007; Ringe and Bhattacharya, 2012; Zwick, 2005). Indeed, there are five main neutralising sites that have been identified on the HIV envelope. Four of these distinct neutralising epitopes exist on the gp120, including the third variable (V3) loop, the CD4-binding domain, the first variable (V1) loop, and finally the second variable (V2) loop. Only one neutralising epitope was found on the MPER of the gp41 (Table 1.6) (Li-B et al., 2011; Walker et al., 2010; Levy, 2007).

The central region of the V3 loop is considered one of the critical neutralising regions of gp120. This region is named the principal neutralising domain (PND) that was found conserved among various strains of HIV-1 with only a slight difference in the amino acid structure. Therefore, this PND is an ideal target for vaccine-elicited Nab responses. Both conformational and linear epitopes have been identified on the V3 loop. Unfortunately, several studies have shown that V3-specific antibodies are not always able to neutralise the homologous strains of HIV, which has the ability to escape from these neutralising antibodies (Broliden et al., 1991; Levy, 2007). The CD4-binding domain is the second main site for eliciting Nabs to HIV. This domain, which is located within the fourth conserved (C4) region on the gp120, can present only conformational determinants. CD4-specific Nabs were found capable of inhibiting the attachment of HIV onto cells. They have the ability to attach to the CD4-binding domain and

effectively block the binding reaction between the viral gp120 and CD4 cellular receptor (Levy, 2007). The V1 and V2 loops are considered the third and fourth important neutralising regions for HIV. Deletion of any of these regions (V1/V2) can have a major impact on the neutralisation susceptibility of the virus (Levy, 2007). It was found that the deletion of the V1 region could lead the virus to develop resistance to Nabs. However, the susceptibility of HIV-1 to neutralisation may be increased by the deletion of the V2 region (Saunders et al., 2005).

Viral envelope glycoproteins (gp)	Specific regions
gp120	Central region of the V3 loop
	CD4-binding domain (C4)
	V1 loop
	V2 loop
gp41	Membrane proximal external region (MPER)

Table 1.6: Neutralising epitopes of the HIV env antigen.

1.3.4 Primate models of HIV infection

Non-human primate models have been widely used for evaluating HIV vaccine candidates prior to human trials. These primate models give an opportunity to study HIV pathogenesis and vaccines (Morgan et al., 2008). It has been found that HIV-2 is closely related to the SIV that has been found in sooty mangabey macaques (SIVsm), while HIV-1 is related to the SIV that infects chimpanzees (SIVcpz) (Sharp et al., 1995; Siliciano, 2006). In 2002, Reeves and Doms reported that SIVsm, SIVcpz, and SIVagm (from African monkeys) do not cause any serious diseases in their natural hosts (sooty mangabey [*Cercocebus atys*], chimpanzees, and African green monkeys, respectively). However infection may occur by cross-species transmission of these non-human primate viruses from their natural hosts to other hosts, such as Asian rhesus macaques

and humans. SIVsm and SIVcpz were found to be capable of causing an AIDS-like disease in rhesus macaques. Generally, SIVsm is considered the progenitor of HIV-2 infection in human hosts, while SIVcpz represents the common progenitor of HIV-1 infection in humans (Sharp et al., 1995; Reeves and Doms, 2002).

1.3.4.1 SIV of chimpanzee (SIVcpz)

SIV is one of the primate lentiviruses that infect a wide range of African non-human primate species (about 36 different species). SIVs were found to be non-pathogenic in their natural hosts, in spite of their strong activity (high viral loads) in those hosts. SIVcpz is one of these viruses that have evolved and were found to be capable of causing HIV-1 infection and AIDS in humans (Reeves and Doms, 2002; Sharp et al., 2005). Four distinct subspecies of chimpanzee have been recognised: *Pan troglodytes troglodytes*, *Pan troglodytes schweinfurthii*, *Pan troglodytes verus*, and *Pan troglodytes vellerosus*. Two of these (*Pan troglodytes troglodytes* and *Pan troglodytes schweinfurthii*) were found to harbour SIVcpz strains naturally (Sharp et al., 2005).

SIVcpz (*P. t. troglodytes* and *P. t. schweinfurthii* chimpanzees) exhibits a high degree of molecular (genomic organisation) similarity to HIV-1. In addition, it has been found that both viruses (SIVcpz and HIV-1) harbour a unique gene (vpu) that is not present in all lentiviruses. Both utilise the same cellular receptors (CD4 and CCR5) for viral entry into host cells. The strong similarity between the two viruses makes SIVcpz a promising model for the study of HIV-1 pathogenesis and also for HIV-1 vaccine development, but it has practical and theoretical drawbacks compared to SIVmac239 (Heeney et al., 2006; Sharp et al., 2005).

The natural history of SIV infection in chimpanzee is characterised by the apparent lack of pathogenicity. SIVcpz infection appears similar to other non-pathogenic lentiviral

infections that do not cause serious illnesses in their natural SIV hosts. It is thought that the cellular restriction of CD4 and CCR5 expression in the natural SIV hosts (such as chimpanzee) might serve to limit SIVcpz replication. Interestingly, SIVcpz was found to be capable of establishing persistent chronic infections in its natural hosts without causing successive decline of CD4 T cells (Sharp et al., 2005; Silvestri-A, 2008). Indeed, the exact underlying mechanisms by which these natural SIV hosts might resist SIV infection are still not understood. Most naturally SIV-infected hosts (85-90%) tend to have normal levels of CD4 T cell count (more than 500/cm³), and are likely to remain healthy. Only 10-15% of those naturally SIV-infected hosts may experience a moderate to severe decrease in CD4 T cell count. Lower levels of immune system activation as well as low CCR5 expression on CD4 T cells were observed in the naturally SIV-infected hosts (such as chimpanzees, sooty mangabey, and African green monkeys), compared to the non-natural SIV-infected host (such as rhesus macaques). These low levels of immune activation (attenuated immune system) are thought to be responsible for protecting the natural hosts from serious diseases such as AIDS. Interestingly, a similar level of specific CD8 T cell immune response against SIV was observed in both natural and non-natural SIV-infected hosts (Silvestri-B, 2008).

1.3.4.2 SIVmac239 and variants in rhesus macaques

SIVmac239 was identified as a pathogenic HIV-like virus that is able to cause AIDS in non-natural hosts, such as rhesus (*Macaca mulatta*) and cynomolgus macaques. SIVmac239-infected rhesus macaques develop chronic, persistent, and even fatal infections that closely resemble HIV/AIDS in humans. This pathogenic clone (SIVmac239) has therefore been widely used as a model system for studying HIV pathogenesis and HIV vaccine development (Regier and Desrosiers, 1990; Silvestri-B, 2008). In 1990, the whole proviral genome (DNA) of the SIVmac239 strain was fully

sequenced and characterised by Regier and Desrosiers (Regier and Desrosiers, 1990). It has been reported that there is a high degree of similarity (98%) at the nucleotide level between SIVmac251 and SIVmac239, which was derived from the SIVmac251 strain through serial animal passages. SIVmac239 was also found to be closely related (96.3%) to SIVmac142 strain. Despite these similarities between SIVmac239, SIVmac251, and SIVmac142, the infectious SIVmac239 strain differed from the rest of SIV macaque strains in its pathogenic potential (Regier and Desrosiers, 1990).

The proviral genome of SIVmac239 has two LTR regions (10.3 kpb) at each terminus, and nine different genes: gag, pol, env, vpx, rev, vif, tat, vpr, and nef. Generally, three of these genes (gag, pol, and env) were found to be conserved among all three strains of SIV macaque (SIVmac239, SIVmac251, and SIVmac142). Another three highly conserved genes (vpr, vif, and vpx) were also identified among the SIVmac and HIV-2 strains (Regier and Desrosiers, 1990). Some genes of the SIVmac239 genome were identified as non-essential genes for virus replication in cell culture, such as vpr, vif, vpx, and nef. Although the nef gene is considered non-essential for virus replication *in vitro*, it was found to be critical for the maintenance of high viral loads *in vivo*, and also for AIDS development (Kestler et al., 1991; Regier and Desrosiers, 1990).

Two different phases of SIVmac239 infection have been identified among infected rhesus macaques: primary (acute) infection and chronic phase of infection. In the acute infection stage, high levels of viral replication occurred during the first two weeks of infection. This peak of replication is then followed by a rapid decline to reach a level of 10^5–10^7 RNA copies/ml. It has been reported that specific neutralising antibodies against SIVmac239 appear within 20 weeks after infection (Kaur et al., 1998; Silvestri-B, 2008). During the chronic phase of SIVmac239 infection, a steady state level of virus replication was observed in infected animals. However, the majority of SIV-infected

rhesus macaques (non-natural SIV host) developed persistent infection with a progressive decline in CD4 T cell count (≤200 cells/ml) during this phase. These SIVmac239-infected rhesus macaques were more likely to progress to AIDS and die within 11–13 months after the onset of infection (Kaur et al., 1998; Silvestri-B, 2008). Generally, higher levels of viraemia with less sustained SIV-specific CTL immune responses were observed in the infected non-natural hosts (such as Indian rhesus macaques) compared with the natural hosts (such as sooty mangabey). The production of IFN-γ and IL-2 were also found to be higher in the infected rhesus macaques than those naturally infected hosts (Silvestri-B, 2008).

1.3.4.3 SIVmac239 and variants in cynomolgus macaques

The cynomolgus macaque (*Macaca fascicularis*) is one of the primate models used for studying HIV/AIDS pathogenesis. The pathogenesis of the infectious clone SIVmac239 in cynomolgus macaques was found almost identical to that described previously in rhesus macaques. However it has been found that the plasma viral loads of SIVmac239 are lower in cynomolgus macaques than in rhesus macaques (Antony and MacDonald, 2015). The primary SIVmac239 infection in cynomolgus macaques became established within the first two weeks after the onset of the infection, reaching a level of 10^5–10^7 RNA copies/ml (Willer et al., 2010). Generally, SIV-infected cynomolgus macaques develop severe immunodeficiency with a rapid progression to fatal diseases. These macaques have a limited diversity of MHC alleles. This heterogeneity of MHC classes appears to play a major role in influencing the susceptibility of the cynomolgus macaque hosts to SIVmac239 infection and subsequent disease progression (Willer et al., 2010; Connor et al., 2010). Interestingly, HIV-2 (SBL-K135 strain) infection was reported to confer protection in cynomolgus macaques against SIVsm-induced immunodeficiency (Putkonen et al., 1990). Putkonen et al. (1990) showed that the HIV-

2-infected cynomolgus macaques had normal CD4 T cell count for 9 months (as a follow up) following SIVsm pathogenic strain challenge (Putkonen et al., 1990).

1.3.4.4 Reasons for choosing SIVmac239 instead of SIVcpz strain in this project

HIV-1 is closely related to the SIV that infects chimpanzees (SIVcpz), while HIV-2 is similar to the SIV found in sooty mangabey macaques (SIVmac). Although SIVcpz is considered a promising animal model for studying HIV-1 pathogenesis and also for HIV-1 vaccine research, it has practical and theoretical drawbacks compared to the pathogenic SIVmac239 strain (Reeves and Doms, 2002; Siliciano, 2006; Heeney et al., 2006; Sharp et al., 2005).

For this project, there are several reasons for choosing the SIVmac239 rather than SIVcpz strain for HIV vaccine development. Firstly, the natural history of SIVcpz infection is characterised by an apparent lack of pathogenicity (Silvestri-A, 2008). It has been found that SIVcpz-infected macaques have a very long incubation period (17–18 years), a period that exceeded the normal lifespan of the primate species (Pandrea et al., 2009; Ling et al., 2004). However, SIVmac239 strain was found to be capable of replicating rapidly and causes a fatal AIDS-like disease in rhesus macaques within a suitable time frame (Desrosiers et al., 1998). Secondly, housing and maintaining chimpanzees in research laboratories for studying the natural history of SIV infection are extremely expensive and difficult (Pandrea et al., 2009; Altevogt et al., 2011). Thirdly, SIVmac 239 strains have been extensively studied for the development of HIV vaccines (Regier and Desrosiers, 1990; Silvestri-B, 2008). There are several analytical tools that have been developed for identifying SIVmac239 strains, such as specific T cell epitopes and MHC typing. Finally, the opportunity has arisen to use the SIVmac239 strain that was fully sequenced and characterised. In addition, our collaborative partner

(Neil Almond) has worked extensively with attenuated retroviruses and cynomolgus macaques (Li-A et al., 2011). Therefore, we plan to combine this well characterised macaque model with the well characterised SIVmac239 virus.

1.3.5 Simian human immunodeficiency viruses (SHIV) macaque model

This type of primate model employs recombinant SIV combined with various portions of HIV-1. SHIV viruses provide an opportunity to infect a macaque model with SIVs expressing HIV-1 genes, in order to generate specific Nabs against HIV-1 in macaques. The main uses of these chimeric SHIV viruses are to study HIV-1/AIDS pathogenesis, and also to test and evaluate HIV-1 envelope-based vaccine candidates in non-human primates (Nath et al., 2000; Ozkaya Sahin et al., 2010). However, there may be no specific advantages in using the SHIV model for evaluating the protective immune responses induced against gag and pol SIV proteins (Seth et al., 2000).

Most SHIV viruses have the ability to replicate in macaques and represent a promising model for the evaluation of HIV vaccines (Kim et al., 2001). However, it is necessary to passage non-pathogenic and poorly replicating SHIV in macaques, in order to generate an efficient replicating virus that is able to cause a progressive decrease in CD4 T cells and subsequently an AIDS-like disease (Cayabyab et al., 1999). For this reason, chimeric SHIV viruses need to be firstly tested for their ability to infect and replicate efficiently in macaque PBMCs *in vitro*, before they get involved *in vivo* studies (Nath et al., 2000).

Several studies have used SHIV viruses for studying the pathogenesis of HIV-1, and for evaluating the efficacy of new therapies against HIV-1 in primate models. In 1995, Uberla et al. developed an animal model for demonstrating the potential effect of non-nucleoside RT inhibitors as a treatment of HIV-1 infection. They constructed a SHIV

virus encoding the HIV-1 RT gene by replacing the RT gene of SIVmac239 with the HIV-1 (HxB2 clone) RT gene. They reported that using RT inhibitors could delay the onset of the viraemic phase in the SHIV-infected macaques. They were able to prove the efficacy of using this primate model (SIV/HIV-1 in rhesus macaques) for evaluating the protective effect of non-nucleoside RT inhibitors against HIV-1 infection in macaques (Uberla et al., 1995).

Moreover, in 1996, Reimann et al. used a SHIV virus encoding the HIV-1 env gene for evaluating HIV envelope-based vaccines in rhesus macaques. These macaques were infected with a SHIV construct (SHIV-89.6P) that was composed of the SIVmac239 pathogenic strain expressing the env protein and some of the non-essential genes of HIV-1, including rev, tat, and vpu. They found that this type of SHIV-89.6P, which was generated after two serial *in vivo* passages of SHIV-89.6, was able to cause AIDS-like illness in the infected rhesus macaques. This study also proved the efficacy of the SHIV model for evaluating HIV-1 vaccines in non-human primates (Reimann et al., 1996). In yet another study in 1998, Joag et al. demonstrated the protective effect of the SHIV model in preventing the sexual transmission of HIV-1 infection between macaques. The study showed that the majority (10 out of 12) of vaccinated macaques (pig-tailed macaques) with nef/vpu-deleted SHIV-KU-1 strain were protected from acquiring HIV-1 by the intravaginal route (Joag et al., 1998).

1.4 Poxvirus/retrovirus recombinants

1.4.1 Existing poxvirus recombinants expressing SIV proteins

Several attenuated poxvirus recombinants have been developed as viral vectors for the expression of various SIV proteins. There are a number of studies that have demonstrated the potential effect of these recombinant vectors for the induction of

specific immune responses against SIV infection. In 1996, Hirsch et al. used a rMVA vector vaccine encoding gag, pol, and env SIVsmH4 genes on rhesus macaques. The study reported that the protection conferred against the virulent SIV strain by this recombinant vaccine was variable. Indeed, the rMVA vaccine was capable of modulating viraemia levels and disease progression (Hirsch et al., 1996). Likewise, in 2000, Seth et al. used the same viral vector (MVA) for the expression of gag and pol SIVsmH4 genes in rhesus macaques. They found that this recombinant vector was capable of inducing gag-specific CTL immune responses, and reducing the viral load set point in the vaccinated macaques following SIVsmE660 strain challenge (Seth et al., 2000). Radaelli et al. (2004) used a rFPV vector vaccine in a heterologous prime-boost immunisation strategy with a DNA prime vaccine for the expression of gag and pol SIVmac239 genes in SIVmac251-infected rhesus macaques. They found that this vaccination regimen was capable of inducing higher gag-specific CTL immune responses than would be elicited by the FPV vector alone (Radaelli et al., 2004). In 2005, Van Rompay et al. used two different types of poxvirus vectors (CNPV and MVA) for the expression of several SIV proteins [(CNPV/SIVmac142 gag, pol, and env) and (MVA/SIVmac239 gag, pol, and env)] in newborn rhesus macaques and juvenile macaques. The vaccinated animals were orally challenged with the highly pathogenic SIVmac251 strain. The study demonstrated that these poxvirus-based vector vaccines were able to reduce the SIV infection rates (30–50%) in the vaccinated macaques compared with the control group (Van Rompay et al., 2005). To summarise these studies, the SIV proteins expressed in poxvirus recombinant vectors have included gag, pol, env, and nef genes. The following table shows some examples of existing recombinant poxvirus-based vector vaccines against SIV in macaques (Table 1.7). To our knowledge, FP9 has not been used before as a viral vector encoding SIV proteins.

Viral vector	Exogenous antigens	Target pathogen	Host model	Reference
FPV	Gag and pol of SIVmac239	SIVmac251	Rhesus macaques	Radaelli et al., 2004
FPV	Gag and pol of SIVmac239/ human IFN-γ	SHIVmm229	Juvenile (*Macaca nemestrina*) monkeys	Dale et al., 2004
MVA	Gag, pol, and env of SIVsmH4	SIVsmE660	Rhesus macaques	Hirsch et al., 1996
MVA	Gag and pol of SIVsmH4	SIVsmE660	Rhesus macaques	Seth et al., 2000
MVA	Gag, pol, and env of SIVmac239	SIVmac251	Newborn rhesus macaques and juvenile macaques	Van Rompay et al., 2005
MVA	Gag, pol, and nef of SIVmac239/ env of SHIV89.6P	SHIV89.6P	Rhesus macaques	Mooij et al., 2008
CNPV	Gag, pol, and env of SIVmac142	SIVmac251	Newborn rhesus macaques and juvenile macaques	Van Rompay et al., 2005
NYVAC	Gag, pol, and env of SIVk6w	SIVmac251	Rhesus macaques	Hel et al., 2002
NYVAC	Gag, pol, and nef of SIVmac239/ env of SHIV89.6P	SHIV89.6P	Rhesus macaques	Mooij et al., 2008

Table 1.7: Examples of recombinant poxvirus-based vector vaccines against SIV in macaques.

1.4.2 Existing poxvirus recombinants expressing HIV proteins

Several recombinant poxvirus vectors have been developed as HIV vaccine candidates. These viral vectors have been used for the expression of different HIV proteins in various hosts, such as mice, macaques, and humans. In 1991, Cooney et al. used a rVACV vector for the expression of HIV (HIVAC-1e) env glycoprotein (gp160) in humans. This study demonstrated the safety and efficacy of the rVACV vaccine that was capable of inducing strong T cell responses in most individuals. However, only 16.6% (3 out of 18) of the vaccinated individuals developed specific HIV antibodies (Cooney et al., 1991). In 1998, Kent et al. proved the efficacy of a rFPV vector encoding three HIV-1 antigens (gag, pol, and env) as a booster vaccine, in inducing T cell immune responses in juvenile macaques (Kent et al., 1998). In addition, Kent et al. (2000) used the same viral vector (FPV) for the expression of IFN-γ and two HIV-1 genes (gag and pol) in HIV-1-infected macaques (*Macaca nemestrina*). This viral vector had the ability to generate both HIV-specific CTL (CD8) and T helper cell (CD4) responses in the immunised macaques (Kent et al., 2000). In yet another study in 2002, Vazquez-Blomquist et al. also proved the ability of a rFPV vector encoding six epitopes of the V3 loop sequences (gp120) from different isolates of HIV-1, in eliciting specific CD8 CTL responses in mice against HIV-1 (Vazquez-Blomquist et al., 2002). In 2003, Vázquez-Blomquist et al. generated a rFP9 vector encoding HIV-1 CR3 protein (multiepitope protein) composed of multiple CTL and Th epitopes from different HIV-1 proteins, including gag, pol, nef, vpr, RT, gp41, and gp120. This viral vector was able to elicit simultaneous CD8 CTL responses against HIV-1 in mice (Vázquez-Blomquist et al., 2003). In 2005, De Rose et al. constructed a rFPV as a booster vector vaccine for the expression of five different HIV-1 subtype AE genes (env, gag, pol, rev, and tat) in pigtail macaques. This study also demonstrated the potency of these poxvirus vectors in

producing broad T-cell immunity responses in macaques (De Rose et al., 2005). In 2011, Bridge et al. used two different types of poxvirus vectors (rFP9 and rMVA) in a prime-boost vaccination regimen for the expression of different clades of the HIV-1 env (A, C, and D) and capsid proteins in Chinese cynomolgus macaques. These two viral vectors induced modest T cell immune responses but no neutralising antibodies, and were poorly immunogenic in the cynomolgus macaque model (Bridge et al., 2011). The following tables show some examples of existing recombinant poxvirus-based vector vaccines against HIV in humans, mice, and macaques (Tables 1.8, 1.9, and 1.10).

Viral vector	Exogenous antigens	Target pathogen	Host model	Reference
NYVAC	Env, gag, pol, and nef of HIV-1 (clade B)	HIV-1	Humans	Najera et al., 2010
NYVAC	Env, gag, pol, and nef of HIV-1 (clade C)	HIV-1	Humans	Quakkelaar et al., 2011
ALVAC	Env (gp120 and gp41), gag, pol, nef, pro, and CD40L (MN and LA1 HIV-1 strains)	HIV-1	Mice	Liu et al., 2008
ALVAC	Env, gag, human CTL epitopes from nef, and pol of HIV-1	HIV-1	Humans	Angel et al., 2011
ALVAC	Env (gp120 and gp41), gag, protease, and human CTL epitopes from nef and pol of HIV-1	HIV-1	Humans	Frey et al, 2014

Table 1.8: Examples of attenuated poxvirus-based vector vaccines against HIV in mice and humans.

Viral vector	Exogenous antigens	Target pathogen	Host model	Reference
FP9	Gag, pol, nef, vpr, RT, gp41, and gp120 of HIV-1	HIV-1	Humans	Vázquez-Blomquist et al., 2003
FP9	Env and gag of HIV-1 (clade A, C, and D)	HIV-1	Chinese cynomolgus macaques	Bridge et al., 2011
MVA	Env, gag, pol, nef, and tat of HIV-1 (clade B/C)	HIV-1	Humans	Vasan et al., 2010
MVA	Env and gag of HIV-1 (clade A, C, and D)	HIV-1	Chinese cynomolgus macaques	Bridge et al., 2011
MVA	Gag, env (gp160), rev, tat, RT, and nef of HIV-1 (clade C)	HIV-1	Humans	Mehendale et al., 2013
MVA	Gag p24 and p17 of HIV-1 (clade A)/overlapping CD8 T cells	HIV-1	Gambian infants	Afolabi et al., 2013
MVA	Env, gag, pro, and RT of HIV-1 (clade B)	HIV-1	Humans	Goepfert et al., 2014
MVA	Gag, pol (clade A), and env (clade E) of HIV-1	HIV-1	Swedish individuals	Nilsson et al., 2014

Table 1.9: Examples of attenuated poxvirus-based vector vaccines against HIV in macaques and humans.

Viral vector	Exogenous antigens	Target pathogen	Host model	Reference
VACV	Env (gp160) of HIV-1	HIV-1	Humans	Cooney et al., 1991
FPV	Gag, pol, and env of HIV-1	HIV-1	Mice	Kent et al., 1998
FPV	Gag and pol of HIV-1/ human IFN-γ	HIV-1	Pigtail macaques (*Macaca nemestrina*)	Kent et al., 2000
FPV	Six epitopes of the V3 loop (gp120) of HIV-1	HIV-1	Mice	Vazquez-Blomquist et al., 2002
FPV	Env, gag, pol, rev, and tat of HIV-1	HIV-1	Pigtail macaques (*Macaca nemestrina*)	De Rose et al., 2005
FPV	Multiepitopic polypeptide (TAB9) of HIV-1	HIV-1	Mice	Quintana-Vazquez et al., 2005
FPV	Gag and pol of HIV-1/ human IFN-γ	HIV-1	Humans	Emery et al., 2005
FPV	Gag and pol of HIV-1 (clade B)	HIV-1	Humans	Kelleher et al., 2006
FPV	Gag, pol, env, rev, and tat of HIV-1 (clade A/E)	HIV-1	Humans	Hemachandra et al., 2010
FPV	Env, gag, rev, tat, nef, and RT of HIV-1 (clade B)	HIV-1	Humans	Keefer et al., 2011

Table 1.10: Examples of recombinant poxvirus-based vector vaccines against HIV in mice, macaques, and humans.

1.5 Aims and objectives

HIV infection is an important pandemic disease, and its incidence remains high. It was estimated that approximately 36.9 million people were living with HIV infection worldwide in 2014. To date no preventive HIV vaccine is available, and hence this study is aimed to develop an HIV vaccine, but by studying a related SIVmac239 strain. The SIVmac239/macaque represents a promising animal model for studying HIV pathogenesis, and also for HIV vaccine development (Regier and Desrosiers, 1990; WHO, 2015).

This PhD project aims to construct rFP9 vectors encoding all the antigenic components of SIVmac239 (env, gag, pol, and accessory proteins except nef), and a defective SIVmac239 genome, to make complex rFP9 employing TCS. SIV protein expression is regulated by a phage T7 RNA polymerase to give an added level of safety and hopefully immunogenicity. The overall aim of this project is to generate a safe and effective live-attenuated FP9 based retroviral vaccine for later use in the SIVmac239 model of HIV infection, which (when adapted with HIV sequences) may go forward to human trial.

The following is a brief list of the specific objectives that have been set for this PhD project:

- To design five different insertion sites within the FP9 genome, then flanking regions to these insertion sites to be synthesised with appropriate restriction sites.
- To generate five FP9 recombination vectors (transfer plasmid DNA) that will permit insertion of various retroviral (SIVmac239) sequences at multiple sites in the FP9 genome.
- To employ a TCS strategy that will facilitate insertion of multiple SIV insertion cassettes within the same rFP9 virus.

- To construct a genomic rFP9 that encodes defective SIVmac239 genome and the bacteriophage T7 RNA polymerase gene.
- To construct a packaging rFP9 that encodes all SIVmac239 structural (env, gag, and pol) and key accessory genes (except nef) individually driven by T7 promoters.
- To compare the expression efficiency between rFP9 and rMVA (highly attenuated poxvirus vector) in producing SIV proteins in different types of cells.

1.6 Hypotheses

1). Is it possible to construct a single rFP9 vector expressing a defective SIVmac239 (DSIV) genome driven by a T7 RNA polymerase.

2). Is it possible to generate a complex rFP9 encoding various genomic components (env, rev, tat, gag-pro, vpx, RT, int, vpr, and vif) of SIVmac239 driven by a T7 RNA polymerase.

3). Compare protein expression levels from various cell types (avian and mammalian cells) infected with rFP9; with the null hypothesis being that there will be no difference.

4). Compare protein expression levels between different recombinants (rFP9 and rMVA) and combinations thereof; with null hypothesis being that there will be no difference.

2 Materials and Methods

2.1 Materials

2.1.1 Viruses

- FP9 was kindly provided by Dr. Mike Skinner, at University College London. The FP9 stocks were stored at -80˚C for long-term storage.
- Two pure rMVAs were constructed and provided by Dr. Gowda CPC. One recombinant virus encoding env/rev SIVmac239 sequences flanked by bacteriophage T7 promoters (rMVA.env.rev), and the other recombinant containing the T7 RNA polymerase gene flanked by the VACV intermediate 3L (I3L) promoter (rMVA.T7pol).

2.1.2 DNA plasmids

Two types of DNA plasmids were used in this project:

- pBR322 (4361 bp), which is a low copy number plasmid expressing an ampicillin resistance marker gene, was used to clone large DNA sequences (SIVmac239 sequences).
- pUC minus multiple cloning site (3305 bp), which is a high copy number plasmid expressing a kanamycin resistance marker gene, was provided by BlueHeron^{TM} Biotechnology, USA, and used to clone small DNA sequences (FP9 HS).

2.1.3 MVA transfer plasmid carrying various SIVmac239 sequences

Five MVA transfer plasmids (pCO-Vpx.T7pol, pCO-Env.Rev, pCO-Tat.Gag-pro, pCO-RT.RNase.Int.Vpr.Vif, and pCO-DSIV genome) encoding various SIVmac239 sequences, MVA HS, and reporter expression cassette (LacZ gene [β-galactosidase]) were constructed by Dr. Thomas Blanchard and Dr. Gowda CPC, The University of

Manchester. The DNA sequences were synthesised by BlueHeron™ Biotechnology, and cloned into a pBR322 plasmid vector for propagation. Some encoded SIVmac239 DNA sequences were human-codon optimised to mimic the mammalian codon usage, and to enhance the expression of these proteins in mammalian cell lines. MVA transfer plasmids are driven under the control of VACV promoters and T7 RNA polymerase promoter. They contain some regulatory elements including Kozak consensus sequence, internal ribosome entry sites (IRES), and separately transcription termination signals (such as T5NT-poxvirus). Moreover, appropriate and unique restriction enzyme sites were incorporated into multiple locations within the MVA plasmids in order to allow any future modifications (such as cloning of FP9 HS). These MVA transfer plasmids were used for cloning the newly synthesised FP9 HS, and generating FP9 transfer plasmids carrying SIVmac239 sequences (See CD-ROM for annotated sequence details of MVA recombination transfer plasmids).

2.1.4 Specific pathogen free (SPF) eggs

SPF eggs were supplied in batches by Institute of Animal Health Compton Laboratories, UK (contact Jackie Goddard–Tel. 01635578411/ext. 2245). These eggs were transported by Citysprint (contact David Potter–Tel. 01618779348–email: dpotter@citysprint.co.uk).

2.1.5 Commercial kits

Name	Manufacturer	Catalogue number
EndoFree® Plasmid Maxi Kit	Qiagen	12362
PureLink™ Genomic DNA Mini Kit	Invitrogen	K1820-01
QIAquick PCR Purification Kit	Qiagen	28104
QIAEX™ II Gel Extraction Kit	Qiagen	20021
Qiagen Plasmid Maxi Kit	Qiagen	12163
QIAprep® Spin Miniprep Kit	Qiagen	27106
Quick Ligation™ Kit	BioLabs	M22005

Table 2.1: List of commercial kits.

2.1.6 Chemical reagents

Name	Manufacturer	Catalogue number
Absolute ethanol	Fisher Scientific, UK	10610813
Agarose, low gelling temperature (for molecular biology)	Sigma Life Science	A9414-100g
Agarose, low gelling temperature Ultrapure ™ (for cell culture)	Invitrogen	16520-100
Agarose, low gelling temperature (for cell culture)	Sigma Life Science	A9045-100g
Alkaline phoshatase calf intestinal (CIP)	BioLabs	M029S
Amersham™ ECL Western blotting detection reagents	GE Healthcare, UK	RPN2106
Ampicillin	SIGMA-ALDRICH, UK	A5354
Bovine serum albumin (powder)	SIGMA-ALDRICH	A7906-100g
BupH ™ Carbonate-bicarbonate buffer	Thermo Scientific	28382
Chromatography paper (3mm, 46X57cm)	Whatman ™	3030917
Dimethyl sulphoxide (DMSO)	Sigma Life Science	D2438
DNase, RNase free distal water	SIGMA-ALDRICH	W4502
Dulbecco's phosphate buffered saline (500ml)	Sigma Life Science	D8537
3,3'-Diaminobenzidine (DAB) tablets	Sigma	D4293
1kb DNA ladder	NEB	N3232L
100bp DNA ladder	NEB	N3231L
EthyleneDiamine-tetra-acetic acid (EDTA)	SIGMA-ALDRICH	1000984973
Fetal bovine serum albumin	Biosera (Australia)	S1700-500
GelRed™ nucleic acid stain	NEB	41003
Gel loading dye blue 6X	New England Biolabs (NEB), UK	B7021S
Glycerol ($C_3H_6O_3$)	AnalaR/BDH	101184K
Haematoxylin Harris stain	VWR Prolabo Chemicals	351945S
Hydrogen peroxide solution 30-31%	SIGMA-ALDRICH	18312-500ml
Immobilon-P PVDF transfer membrane (0.45um)	EMD Millipore	IPVH00010
Industrial methylated spirit	Fisher Scientific, UK	10552904
Isopropanol	Fisher Scientific, UK	BP2618-212
Kanamycin monosulphate	MELFORD, UK	K0126
L-Glutamine	Sigma	G7513
Methanol	Fisher Scientific, UK	10284580
N,N-Dimethylformamide	SIGMA-ALDRICH	319937-1L

Novex 4-12% Tris-glycine gel (1.5mm X 15 well)	Novex (life technologies ™)	EC60385
Nupage® MES SDS running buffer (20X)	Invitrogen (life technologies ™)	NP0002
Nupage® transfer buffer (20X)	Invitrogen (life technologies ™)	NP0006
NuPAGE 4-12% Bis-tris gel (1.0mm X 10 well)	Novex (life technologies ™)	NP0321
Precision plus protein™ dual colour standards marker	BIO-RAD	161-0394
Protease inhibitor cocktail	SIGMA-ALDRICH	P8340
Radioimmunoprecipitation assay buffer (RIPA buffer)	Sigma	R0278
Sample buffer, Laemmli 2X consentrate	Sigma Life Science	S3401-1VL
Skim milk powder	SIGMA-ALDRICH	70166
Stop Reagent for TMB substrate (powder)	SIGMA-ALDRICH	S5814-100ml
SuperFect® transfection reagent	Qiagen	301305
TAE buffer 10X (Ultra pure)	Invitrogen, USA	15558-026
Taq 5X master mix	NEB	M0285S
Trigene/distel high level enviromental disinfectant	Scientific Laboratory Supplies Ltd., UK	TRI1312
Triton™ X-100	SIGMA-ALDRICH	101536228
Trizma base ($C_4H_{11}NO_3$)	Sigma Life Science	93362-250g
Trypan blue solution 0.4%	SIGMA-ALDRICH	93595-50ml
TrypZean solution 1X	Sigma	T3449
Tween® 20	SIGMA-ALDRICH	P2287-100ml
3,3',5,5' Tetramethylbenzidine (TMB) liquid substrate for ELISA	SIGMA-ALDRICH	T4444
Ultra pure agarose	Invitrogen	16500-500
X-Gal	Ambion	AM9944

Table 2.2: List of chemical reagents.

2.1.7 Media

Name	Manufacturer	Catalogue number
Double strength (2X) MEM	Gibco (life technologies ™)	21935-028
Dulbecco's modified eagle medium (DMEM)	SIGMA-ALDRICH	D6429
Luria Bertani (LB) agar	SIGMA-ALDRICH	L2897
LB broth	SIGMA-ALDRICH	L3022
S.O.C Medium	Prepared centrally	-

Table 2.3: List of media.

2.1.8 Cell lines and competent cells

Name	Type	Provider
African green monkey kidney (VERO)	Continuous cell line	American Type Culture Collection (ATCC)
Human fetal lung tissue (MRC-5)	Cell line	ATCC
Primary Chick Embryo Fibroblast (pCEF)	Primary cell line	Prepared from SPF eggs (see methods)
SCS1 Supercompetent cells	Competent cells	Agilent technologies, USA, Cat no. 200231

Table 2.4: List of cell lines and competent cells.

2.1.9 Primer pairs

All the following primer sets were designed using primer design software (Primer Select, LaserGene), and were purchased from eurofins genomics, UK.

Primer name	Product length	Annealing temp.	Sequences (5'–3')
pBR322 plasmid	479 bp	62.1˚C	**F**) CGAAGCGCTGGCATTGACCCTGAGT
			R) ACACCCGCCAACACCCGCTGACG
LacZ sequences	469 bp	60.7˚C	**F**) CGTTTACAGGGCGGCTTCGTCT
			R) AGGCGCTGATGTGTCCGGCTTCTGA
FP9-CoEnv	515 bp	63.2˚C	**F**) GAAGCCCAGGAAGCCCAGCACGAAC
			R) ACCCCAGATACACCGGCACCAACAA
FP9-CoRev	458 bp	63.0˚C	**F**) CAGGGTCAGGCAAAGCGTGGAGAGC
			R) GGCCGGTGGGGTAGGGGTTGGTC
FP9-CoVpx	439 bp	59.5˚C	**F**) GCTCATGCCCTGCTCGTCGTG
			R) GGTCGGCCAGGGTGTTGAAGG
FP9-CoT7pol	404 bp	62.4˚C	**F**) CCATCGGCAAGGAGGGCTACTACT
			R) GTCGGCCTGCAGGATCTCGTTCAC
FP9-CoGag	577 bp	62.5˚C	**F**) TGAGACCCAACGGCAAGAAGAAGTA
			R) TCGGCGGCCTCCTCGTTGATGATGT
FP9-Co.Tat	477 bp	61.8˚C	**F**) TGCTGGCGCTGCTGGTGTTGG
			R) GGCGGGCGCGTGAAGGAGAG

FP9-CoRT.Int.Pro	568 bp	63.8˚C	**F**) TGTCGGTCAGGGCCATCAGGAAGG
			R) GCCGAGGCCGAGTACGAGGAGAACA
FP9-CoVif	452 bp	62.0˚C	**F**) AGCCTCTAATCCGCGGGGGTCTATC
			R) GCCGGCGGTGAAGCAGGGGAAGTA
FP9-DSIVgenER	406 bp	55.5˚C	**F**) ATGGCCAAATGCAAGTCTAACACC
			R) ACCGCCTTCTCCACCGTCTCTTTCT
FP9-DSIVgenGP	500 bp	57.2˚C	**F**) ACTGGGGTTGCAAAAATGTGTCA
			R) AAAAACCCGCCTGTCTGTCTGG

Table 2.5: List of all primer sets.

(F: Forward primer/ R: Reverse primer/ Co: Codon-optimised/ DSIVgen: Defective SIVmac239 genome/ ER: Env and rev regions/ GP: Gag and pol regions/ temp: Temperature).

2.1.10 Recombinant proteins

Name	Provider	Reference number
Recombinant env SIV gp130 protein	National Institute for Biological Standards and Controls (NIBSC)	EVA670
Recombinant gag p27 SIVmac251	NIBSC	EVA643
Recombinant Rev protein from SIVmac clone J5	NIBSC	ARP684
Recombinant SIV GST-tat-Pk.His (*E.coli*)	NIBSC	EVA681.1

Table 2.6: List of standard recombinant proteins.

2.1.11 Primary and secondary antibodies

Name	Provider	Reference number	Host
Primary monoclonal antibodies			
Monoclonal antibody to SIV env gp120	NIBSC	ARP3045 (KK9)	Mouse
Monoclonal antibody to SIV env gp 160/32	NIBSC	ARP3044 (KK41)	Mouse
Monoclonal antibody to SIV gag p27	NIBSC	ARP396/397 (SIV 27e/27f)	Mouse
Monoclonal antibody to SIV rev	NIBSC	EVA3072.4	Mouse
Monoclonal antibody to SIV rev	NIBSC	EVA3072.2	Mouse
Monoclonal antibody to SIV tat	NIBSC	ARP3248 (MH6)	Mouse
Primary polyclonal antibodies			
Antiserum to SIV env C-terminal peptide (env gp120)	NIBSC	ARP415 (S210)	Sheep
Antiserum to SIVmac251 (32H) (for the whole proteins, env, rev, gag, and pol)	NIBSC	ARP416	Cynomolgus macaques
Antiserum to SIV p27 (gag p27)	NIBSC	ARP414 (S108)	Sheep
Rabbit anti-SIVmac tat	NIBSC	ARP4006	Rabbit
Secondary antibodies			
Anti-mouse IgG, HRP-linked Secondary Antibody	Cell Signaling	7076S	Mouse
Donkey Anti-Sheep IgG, Horseradish peroxidase (HRP) Conjugate	Novex (life technologies ^{TM})	A16041	Sheep
Goat Anti-Rabbit IgG, Horseradish peroxidase (HRP) Conjugate	Novex (life technologies ^{TM})	A16096	Rabbit
Goat Anti-Monkey IgG, IgA, IgM, Peroxidase (HRP) antibody	SIGMA-ALDRICH	SAB3700770	Monkey

Table 2.7: List of primary and secondary antibodies.

2.1.12 Restriction enzymes (RE)

All the following type II RE, reaction NEBuffer, and bovine serum albumin (BSA) (Cat no. B9001S) were supplied by New England Biolabs Inc. (NEB Inc., UK), and used for RE digestion analyses.

Restriction enzymes	Reaction buffer (1X)	Optimum incubation temp.	Recognition site	Requirement of BSA 100X
Acc651 (Cat no. R0599S)	NEBuffer 3	37ºC	5’ G▾GTACC 3’ 3’ CCATG▴G 5’	Yes
AgeI-HF (Cat no. R3552S)	NEBuffer 4	37ºC	5’ A▾CCGGT 3’ 3’TGGCC▴A 5’	Yes
AscI (Cat no. R0558S)	NEBuffer 4	37ºC	5’ GG▾CGCGCC 3’ 3’ CCGCGC▴GG 5’	No
AsiSI (Cat no. R0630S)	NEBuffer 4	37ºC	5’ GCGAT▾CGC 3’ 3’ CGC▴TAGCG 5’	No
Bgl II (Cat no. R0144S)	NEBuffer 3	37ºC	5’ A▾GATCT 3’ 3’ TCTAG▴A 5’	No
BsiWI (Cat no. R0553S)	NEBuffer 3 (Cat no. B7003S): 50 mM Tris-HCl, 100 mM NaCl, 10 mM MgCl, 1 mM Dithiothreitol, pH 7.9	55ºC	5’ C▾GTACG 3’ 3’ GCATG▴C5’	No
KpnI-HF (Cat no. R3142S)	NEBuffer 4	37ºC	5’ GGTAC▾C 3’ 3’ C▴CATGG 5’	No
NotI-HF (Cat no. R3189S)	NEBuffer 4 (Cat no. B7004S): 20 mM Tris-acetate, 50 mM potassium acetate,10 mM Magnesium Acetate, 1 mM Dithiothr-eitol, pH 7.9	37ºC	5’ GC▾GGCCGC 3’ 3’ CGCCGG▴CG 5’	Yes

PacI (Cat no. R0547S)	NEBuffer 1 (Cat no. B7001S): 10 mM Bis-Tris-Propane-HCl, 10 mM $MgCl_2$, 1 mM Dithiothreitol, pH 7.0 AND NEBuffer 4	37ºC	5' TTAAT▾TAA 3' 3' AAT▴TAATT 5'	Yes
SacII (Cat no. R0157S)	NEBuffer 4	37ºC	5' CCGC▾GG 3' 3' GG▴CGCC 5'	No
SpeI-HF (Cat no. R3133S)	NEBuffer 4	37ºC	5' A▾CTAGT 3' 3' TGATC▴A 5'	Yes
XhoI (Cat no. R0146S)	NEBuffer 4	37ºC	5' C▾TCGAG 3' 3' GAGCT▴C 5'	Yes

Table 2.8: List of type II RE (endonucleases).

2.1.13 Equipment and experimental apparatuses

Name	Provider
Alpha lmager™ 2200	Alpha Innotech, USA
Automated ELISA plate washer (ELX50)	BioTek Northstar Scientific Ltd., USA
Bacteria incubator	WTC binder, USA
Benchtop microcentrifuge	Eppendorf, UK
Blotting roller	BIO-RAD, UK
Bio class II safety cabinet	Contained Air Solution (CAS) ltd, Manchester, UK
Brinsea egg incubator (Octagon 40 Advance model)	Brinsea Products Ltd., Sandford, UK
ChemiDoc™ XRS molecular imager	BIO-RAD Laboratories Ltd., UK
Colour squid magnetic stirrer	IKA, Germany
Dymax 5 vaccum pump	Charles Austen Pumps Ltd., Surrey, UK
Electrophoresis power supply (EV231)	PeqLab Ltd., UK
Electrophoresis power supply EPS 500/400	Pharmacia Fine Chemicals, UK
ELISA plate reader (ELX800)	BioTek Northstar Scientific Ltd., USA
Gel electrophoresis tank	Scie-Plas, UK
Harrier 18/80 refrigerated centrifuge	MSE Ltd., London, UK
Heat block	Eppendorf, UK
Integra pipetman (micropipettes)	Gilson S.A.S., Villiers-le-Bel, France
Integra pipetboy	Integra Biosciences, USA
Inverted fluorescent microscope (Optika-XDS-3FL)	Optika, Ponteranica, Italy

Lab dancer	IKA®, UK
Liquid nitrogen storage (Biorack 750)	Statebourne cryogenics Ltd., Washington, UK
Microwave oven	DAEWOO, Korea
Misonix sonicator 3000 ultrasonic cell disruptor	Misonix, New York, USA
M-Pact precision balance	Sartorius Ltd., Surrey, UK
Mr.Frosty freezing container (Nalgene)	Thermo Scientific, UK
Nanodrop ND-1000 spectrophotometer	Thermo Scientific Lab tech., USA
Novex semi-dry blotter	Invitrogen, UK
Orbital shaker	Heidolph, UK
Shaker incubator	Gerhardt, Germany
Thermal cycler PCR machine (2720)	Applied Biosystems, Warrington, UK
Tissue culture CO_2 incubator (safe cell UV)	SANYO, Osaka, Japan
UltraSlim-LED transilluminator	Syngene, Cambridge, UK
Unstirred water bath (SUB Aqua 18 plus)	Grant, Cambridge, UK
Vortex-Genie 2	Scientific Industries, Inc., New York, USA
XCell SureLock™ mini-cell electrophoresis system	Invitrogen, UK

Table 2.9: List of all equipment and experimental apparatuses.

2.1.14 Consumables and glassware

Name	Provider
Bijou tubes with cap (7 ml)	SIGMA-ALDRICH, UK (Cat No. Z645338)
Cryogenic vial (1.8 ml), internal thread and starfoot	Thermo Scientific (Nunc®), Denmark (Cat No. 375418)
Cell scraper (25 cm)	BD Falcon, Mexico (Cat no. 353086)
Cell strainer (70 μm)	BD Falcon, Mexico (Cat no. 352350)
Disbosable haemocytometer counting chamber (FastRead 102)	Immune Systems Ltd., UK (Cat No. BVS100)
Disposable bacterial petri dishes (9 cm)	Scientific Laboratory Supplies, UK (Cat No. SLS2002)
EasySeal plate sealer, Transparent	Greiner bio-one, UK (Cat No. 676001)
Eppendorf microcentrifuge tubes (0.5 ml/1.5 ml and 2 ml)	Start Lab Group, UK
Fast PES filter unit (500 ml)	Nalgene®, Mexico (Cat No. 566-0020)
Fine point micro extended, single wrapped sterile Pasteur pipettes	Elkay Laboratory Products Ltd., UK
Glass reagent bottles (250 ml/500 ml)	Schott Duran, Germany
Hypodermic syringe without needle (50 ml)	Terumo, USA (Cat No. BS-50LG)

Inoculation loops (1 µl)	Greiner bio-one, Germany (Cat No. 731161)
Individual PCR tubes with attached flat-top cap (0.2 ml)	BIO-RAD, UK (Cat No. TF10201)
Lab waste Trap glass flask (5L)	Pyrex SciLabware, UK
Polypropylene conical centrifuge tubes (15 ml)	BD Falcon®, UK (Cat No. 352097)
Polypropylene conical centrifuge tubes (50 ml)	BD Falcon®, UK (Cat No. 352070)
Sterile filter pipette tips (0.1-10 µl/ 2-30 µl/ 200 µl/ 1000 µl)	Gilson, France
Sterile disposable scalpels	Swann-Morton Ltd., UK (Cat No.05XX)
Serological pipette with plug (5 ml/10 ml/25 ml and 50 ml)	BD Falcon®, UK
Tissue culture filter cap flasks (T75 cm^2/ T175 cm^2)	Thermo Scientific (Nunc®), Denmark
Tissue-culture petri dishes (100mm X 20mm)	Corning Incorporated, USA (Cat No. 430167)
6-well cell culture flat bottom plate	Corning Incorporated (Costar), USA (Cat No. 3516)
96-well microplate, F-bottom and crystal-clear	Greiner bio-one, UK (Cat No. 655101)

Table 2.10: List of consumables and glassware.

2.1.15 Computer software

- **Lasergene™ Core Suite software:**

Lasergene™ Core Suite (DNASTAR Inc., Madison, USA), which is a comprehensive sequence analysis software, was used to analyse and design different potential insertion sites within the FP9 genome. It is also used to create sequence maps for FP9 HS plasmids, and for designing primer sets.

- **GraphPad prism software**
- **Image lab™ software (BIO RAD)**
- **Optika vision® software**
- **Microsoft office®**

2.2 Methods

2.2.1 Preparation of buffers, solutions, and media

2.2.1.1 Preparation of bacteriological media

- **Luria Bertani (LB) broth:** 20 g of LB broth powder (10 g/L Tryptone, 5 g/L yeast extract and 5 g/L of NaCl) was dissolved in 1 L of deionised water (dH_2O). The mixture was mixed thoroughly and autoclaved for 15 minutes (min) at 121°C.
- **LB agar:** 35 g of LB agar powder (10 g/L Tryptone, 5 g/L yeast extract, 5 g/L of NaCl and 15 g/L agar) was added to 1 L of dH_2O and autoclaved for 15 min at 121°C. The autoclaved media was then cooled to 50°C and the appropriate antibiotic were added just before pouring the plates.
- **Super optimal broth with catabolite repression (SOC) medium:** SOC medium was pre-prepared centrally. A total of 20 g of Tryptone, 5 g of yeast extract, 0.5 g of 10 mM NaCl, 2.5 ml of 2.5 M KCl, 10 ml of 10 mM $MgCl_2$ and 10 ml of 10 mM $MgSo_4$ was added to 950 ml of dH_2O. The pH was then adjusted to 7.2 and the mixture was autoclaved. The autoclaved media was then cooled to <50 °C and 2 ml of filter sterilised 20 mM glucose was added to the mixture.

2.2.1.2 Preparation of ELISA buffers

- **ELISA coating buffer:** One full pack of BupH ^{TM} Carbonate-bicarbonate buffer powder was dissolved in 500 ml of dH_2O to give a final concentration of 0.2 M sodium carbonate-bicarbonate, pH 9.4. This buffer was used for coating ELISA plates with capture antibodies.
- **ELISA blocking buffer:** 6 g of BSA powder was dissolved in 200 ml of 1X Dulbecco's phosphate buffered saline (1X PBS) to give a final concentration of 3% BSA blocking buffer.

- **ELISA washing buffer (PBS-T):** 0.5 ml of tween® 20 was added to 1 L of 1X PBS to give a final concentration of PBS-0.05% v/v tween 20 (0.05% PBS-T).

2.2.1.3 Preparation of Western blot (WB) buffers

- **1 M Tris solution:** 121.1 g of Tris (Trizma) base was dissolved in 700 ml of dH_2O. The pH was then adjusted to 8, and the mixture filled up with dH_2O to a final volume of 1 L.
- **5 M NaCl:** 292.2 g of NaCl was dissolved in 1 L of dH_2O to give a final concentration of 5 M NaCl.
- **10X Tris-buffered saline (TBS) solution:** 100 ml of 1 M Tris buffer and 300 ml of 5 M NaCl were added to 600 ml of dH_2O.
- **WB 1X running buffer:** 50 ml of 20X Nupage® MES SDS running buffer was added to 950 ml of dH_2O.
- **WB transfer (transblotting) buffer:** 50 ml of 20X Nupage® transfer buffer and 100 ml of absolute methanol were added to 850 ml of dH_2O.
- **WB washing buffer (TBS-T):** 0.5 ml of tween® 20 and 100 ml of 10X TBS were added to 900 ml of dH_2O to give a final concentration of TBS-0.05% v/v tween 20 (0.05% TBS-T).
- **WB blocking buffer:** 10 g of BSA powder was dissolved in 200 ml of 0.05% TBS-T to give a final concentration of 5% BSA blocking buffer.

2.2.1.4 Preparation of immunocytochemical (ICC) buffers

- **Permeabilization buffer:** 0.05 ml of Triton™ X-100 was added to 50 ml of PBS to give a final concentration of 0.1 Triton X-100.
- **ICC washing buffer:** 0.5 ml of tween® 20 was added to 500 ml of PBS to give a final concentration of PBS-0.1% v/v tween 20 (0.1% PBS-T).

- **ICC blocking buffer:** 0.5 g of BSA powder was dissolved in 50 ml of 0.1% PBS-T to give a final concentration of 1% BSA blocking buffer.

2.2.1.5 Preparation of other solutions and reagents

- **10% growth DMEM for maintaining cell growth:** 50 ml (10%) of fetal bovine serum (FBS) and 5 ml (10 mM) of L-glutamine were added to 500 ml of DMEM.
- **Agarose overlay:** agarose overlay contains a 1:1 mixture solution of: a) double-strength (2X) MEM with 5% of FBS, and b) 2% low-melting point (LMP) agarose.
- **X-gal overlay:** X-gal overlay contains a 1:1 mixture solution of: a) 2X MEM with 60 mg/ml of X-gal substrate, and b) 2% LMP agarose.
- **1X TAE buffer:** 1 L of UltraPure™ 10X TAE buffer (400 mM Tris-acetate and 10 mM EDTA at pH 8.3) was added to 9 L of dH_2O.
- **10 mM Tris-EDTA buffer (pH 7.5):** 500 µl of 1 M Tris-HCl (pH 7.5) and 100 µl of 0.5 M EDTA (pH 7.5) were added to 49.4 ml of dH_2O. The mixture was mixed thoroughly and autoclaved for 15 min at 121°C.
- **40% glycerol solution:** 40 ml of glycerol ($C_3H_8O_3$= 92.10 g/mol) was added to 60 ml of dH_2O, and then autoclaved for 15 min at 121°C.

2.2.2 Molecular biology methods and DNA cloning techniques

2.2.2.1 Design and synthesis of FP9 insertion sites

The whole FP9 genome (GenBank accession no. AJ581527) was analysed, and the non-essential ORFs were determined using Lasergene™ Core Suite software, SeqBuilder programme. Briefly, five potential insertion sites within the FP9 genome were chosen based on their non-essential function for virus replication (Figure 2.1).

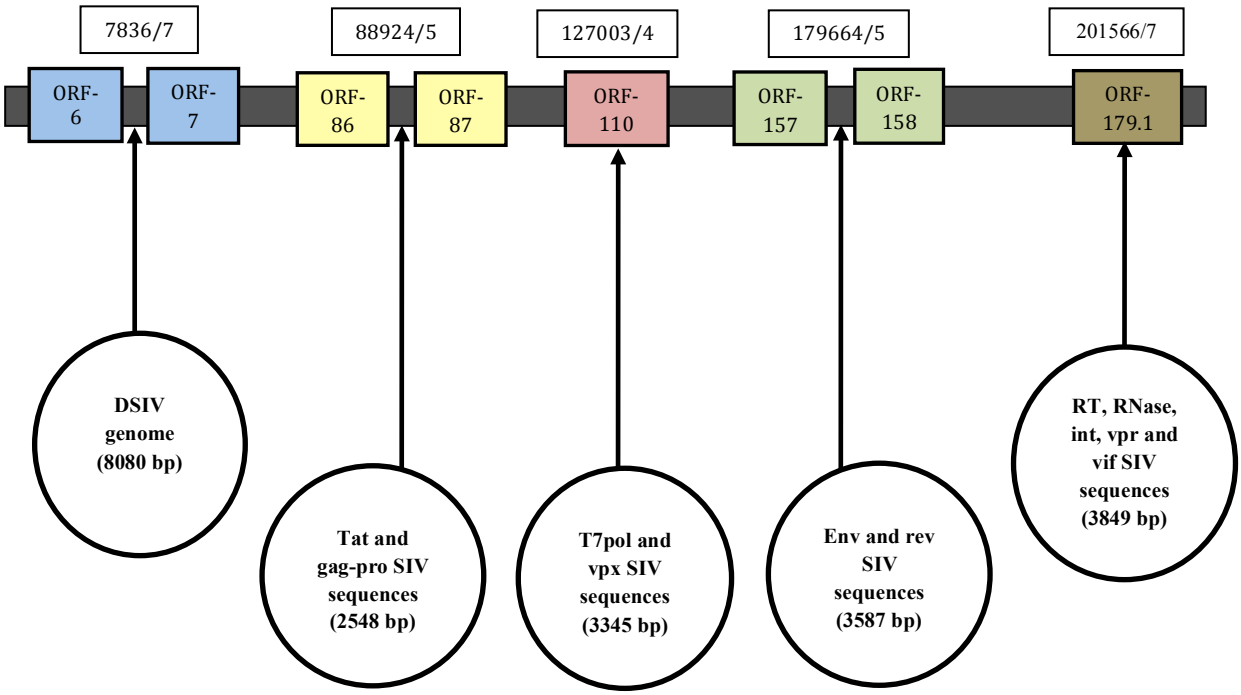

Figure 2.1: Schematic representation of the five different insertion sites within the FP9 genome.

All these five insertion sites were chosen based on their non-essential function for virus replication. It was decided to use three intergenic (non-essential) regions and two intragenic sites for insertion of SIVmac239 sequences.

Homologous flanking regions (each fragment around 500 bp) to these insertion sites were designed. Moreover, a few extra non-coding sequences were added to separate between the two right and left HSs. The FP9 HSs were flanked on both sides with the same unique RE sites, which were incorporated in MVA transfer plasmids, in order to allow cloning of these FP9 HSs into the previously constructed MVA transfer plasmids. The designed DNA sequences were submitted to BlueHeron^{TM} Biotechnology for synthesis. They were subcloned into a variant of pUC plasmid vector, a high copy number plasmid vector lacking a multiple cloning site and encoding kanamycin resistance gene. It is important to note that promoter sequences (TATA box), start and

stop codons have been avoided as insertion sites, since these regions play critical roles in vector replication and expression of target genes (See CD-ROM for annotated sequence details of FP9 HS plasmids).

2.2.2.2 Isolation of bacteria (transformed *E.coli*) containing the synthesised FP9 HS from agar stabs

Synthesised DNA sequences were delivered in two forms by BlueHeron™ Biotechnology: purified plasmid DNA and bacterial stab cultures containing transformed *E.coli* strain (either GC10 or EC100) with the plasmid DNA. The agar stab of each construct was then plated out on LB agar plates containing kanamycin monosulphate (50 µg/ml), and incubated in an inverted position in a 37˚C incubator overnight. For each construct, four isolated colonies were picked up individually using different sterile inoculation loops. Each single colony was inoculated into 5 ml LB broth containing 5 µl of kanamycin monosulphate, and incubated overnight at 37˚C with shaking at 125 rounds per minute (rpm).

2.2.2.3 Preparation and purification of plasmid DNA

Two different kits from Qiagen were used to extract and purify plasmid DNA from bacterial cells: small- and large-scale plasmid DNA preparation.

- **Small-scale plasmid DNA preparation (mini-prep kit):**

Small-scale plasmid DNA purification was performed using the QIAprep® spin miniprep kit following the manufacturer's instructions. A single bacterial colony was inoculated into 5 ml LB broth containing the appropriate selective antibiotic (either ampicillin [100 µg/ml] or kanamycin monosulphate [50 µg/ml]), and incubated overnight at 37˚C with shaking at 125 rpm. Overnight bacterial cells were pelleted by centrifugation at 3000 rpm for 10 min, and then resuspended in 250 µl of resuspension

buffer P1 that contains RNase A. The resuspended cells were transferred into a 2 ml microcentrifuge tube, and 250 µl of lysis buffer P2 (contains sodium hydroxide [NaOH] and sodium dodecyl sulfate [SDS]) were added and mixed gently by inverting the tubes several times. The mixture was then neutralised by the addition of 350 µl of neutralisation buffer N3 (contains guanidine hydrochloride and acetic acid), and mixed immediately by inverting the tubes several times. The tubes were then centrifuged at 13,000 rpm for 10 min, and the resultant supernatant was transferred into the QIAprep spin column and centrifuged at 13,000 rpm for a minute, in order to allow the plasmid DNA to attach to the silica membrane. The flow-through was discarded, and the QIAprep spin column was then washed by the addition of 750 µl of wash buffer PE (contains ethanol) and centrifuged at 13,000 rpm for one min. The flow-throw was discarded again, and the column was centrifuged at 13,000 rpm for another minute to remove residual wash buffer (PE). The column was then placed in a clean 1.5 ml microcentrifuge tube, and the plasmid DNA was eluted from the membrane by adding 50 µl of elution buffer EB (10 mM Tris-HCl, pH 8.5) to the centre of the column. After one min incubation, the tube was centrifuged at 13,000 rpm for another one min, and the resultant plasmid DNA was stored at 4°C until further use.

- **Large-scale plasmid DNA preparation (maxi-prep kit):**

Large-scale plasmid DNA purification was performed using the EndoFree® plasmid maxi kit following the manufacturer's instructions. A single bacterial colony was inoculated into 5 ml LB broth containing the appropriate selective antibiotic, and incubated at 37˚C with shaking at 125 rpm for 6 hours (h). The starter bacterial culture (5 ml) was then used to inoculate either 100 ml (for high copy number plasmid) or 250 ml (for low copy number plasmid) of LB broth containing the appropriate selective antibiotic, and incubated overnight at 37˚C with shaking at 125 rpm. Overnight bacterial

cells were harvested and pelleted by centrifugation at 5500 rpm for 20 min at 4˚C, and then resuspended in 10 ml of resuspension buffer P1 that contains RNase A. A 10 ml of lysis buffer P2 (contains 200 mM NaOH and 1% SDS [w/v]) was added and mixed vigorously by inverting the tubes several times, and incubated at room temperature (15–25˚C) for 5 min. The mixture was then neutralised by adding 10 ml of chilled neutralisation buffer P3 (contains 3.0 M potassium acetate, pH 5.5) and mixed immediately and vigorously by inverting the tubes several times. The lysate mixture was then poured into the QIAfilter cartridge, and incubated at room temperature for 10 min. After incubation, the cell lysate was filtered into a 50 ml Falcon tube using the provided plunger. A 2.5 ml of buffer ER (contains isopropanol and polyethylene glycol octylphenyl ether) was added to the filtered lysate and mixed by inverting the tube several times, and then incubated on ice for 30 min. The filtered lysate was applied to the equilibrated QIAGEN-tip 500, which was equilibrated by applying 10 ml of equilibration buffer QBT (contains 750 mM NaCl and 50 mM MOPS [pH 7], 15% isopropanol, and 0.5% Triton), and allowed to enter by gravity flow. The QIAGEN-tip 500 was washed twice by the addition of 30 ml of wash buffer QC (contains 1 M NaCl, 50 mM MOPS [pH 7], and 15% isopropanol [v/v]). The column was then placed in a clean 50 ml Falcon tube, and the plasmid DNA was eluted by adding 15 ml of elution buffer QN (contains 1.6 M NaCl, 50 mM MOPS [pH 7], and 15% isopropanol [v/v]). The DNA was precipitated by adding 10.5 ml (0.7 volumes) of isopropanol, and centrifuged at 12,000 rpm for 30 min at 4˚C. The resultant DNA pellet was then washed with 5 ml of 70% ethanol, and centrifuged at 12,000 rpm for 10 min. The pellet was air-dried for 5–10 min and redissolved in 1.5 ml of endotoxin free buffer TE (contains 10 mM Tris-HCl [pH 8] and 1 mM EDTA). The eluted plasmid DNA was stored at 4°C until further use.

2.2.2.4 Quantitation of plasmid DNA

The concentration of plasmid DNA was measured using the NanoDrop™ ND-1000 spectrophotometer. This instrument can measure 1 µl samples with high accuracy. The absorbance (optical density [OD]) of each sample was measured at 260 and 280 nm (1 OD_{260}= 50 µg/ml of DNA). This ratio (A_{260}/ A_{280}) was also used to assess the purity of the samples. Pure DNA samples usually give a value of 1.8, while pure RNA samples give a value of 2.0. Lower ratio values (either <1.8 or <2.0) indicate the presence of contaminants in the samples.

2.2.2.5 RE digestion analysis of plasmid DNA

Restriction digestion analyses were performed to analyse and confirm the previously constructed MVA transfer vectors and the synthesised FP9 HSs. Both plasmids (pBR322 and pUC) were double digested to remove the HSs of MVA and FP9. Briefly, the plasmid DNA was digested using 5–10 units of RE per 1 µg of DNA for 2–4 h at 37˚C (55˚C for only BsiWI enzyme [Table 2.8]). A volume of 25 µl of reaction mixture was prepared separately for each digested sample by adding 2.5 µl of the recommended NEB buffer (10X), 0.5 µl of BSA (100X) [if required], 1 µl of RE, 0.5–1 µg of DNA sample, and made up to a final reaction volume of 25 µl with dH_2O. The reaction mixture was then mixed thoroughly, spun briefly, and incubated for 2–4 h at 37˚C using a water bath.

2.2.2.6 Confirmation of DNA fragments (digested products) by agarose gel electrophoresis

Uncut and digested DNA samples were analysed using gel electrophoresis. The target sequences were resolved and separated through an agarose gel containing 0.5–1% UltraPure™ agarose and 5–10 µl of GelRed™ nucleic acid stain in 50–100 ml of 10X

TAE buffer. The GelRed stain, which is very stable and safe (non-toxic), was used instead of the highly toxic ethidium bromide stain. Briefly, each digested sample (15 µl) was mixed with 5 µl of 6X gel loading dye (containing 2.5% Ficoll-400, 11 mM EDTA, 3.3 mM Tris-HCl, 0.017% SDS, and 0.015% bromophenol blue [pH 8.0]). However, 2 µl of uncut plasmid DNA (control sample) was mixed with 5 µl of 6X gel loading dye and 8 µl of 10X TAE buffer. 1 kb and 100 bp DNA ladders were used as calibration markers to estimate the size of the digested products. All samples (uncut and digested) and DNA ladders were loaded into the wells of the gel, and electrophoresis was performed in 10X TAE buffer at 70–80 volts (V) for 1–2 h. The DNA bands were then visualised under ultra-violet (UV) light in a range of 490–620 nm using UV transilluminator AlphaImager^{TM} 2200.

2.2.2.7 Isolation and purification of DNA fragments from agarose gel

DNA fragments were isolated by electrophoresis through a low-melting point (LMP) agarose (2-Hydroxyethyl agarose) containing 1–2% LMP agarose (1% for the vector and 2% for the insert) and 10 µl of GelRed^{TM} stain in 100 ml of 10X TAE buffer. The samples were run at low voltage (60–70 V) for 1–2 h, in order to avoid melting the gel. DNA fragments were then visualised under a blue light (LED) transilluminator using UltraSlim-LED transilluminator to reduce DNA damage.

The target fragments were excised from the gel as a thin slice using a fresh sterile scalpel. These DNA fragments were purified using the QIAEX^{TM} II gel extraction kit following the manufacturer's instructions. Briefly, the gel slices were weighed and 3 volumes of binding buffer QX1 plus 2 volumes of dH_2O were added to 1 volume of a gel slice (e.g. 300 µl of QX1 and 200 µl of dH_2O for 100 mg of a gel). The mixture was mixed thoroughly for 30 seconds (sec) using the Vortex-Genie mixer. A 10 µl of

suspension buffer QIAEX II was added, resuspended by vortexing for 30 sec, and incubated at 50˚C for 10 min in a heating block, to allow the DNA fragment to attach to the silica particles. It was necessary to vortex the tubes every 2 min to keep the QIAEX II buffer in suspension. The mixture was then centrifuged at 13,000 rpm for 30 sec, and the supernatant was discarded. The pellet was washed by the addition of 500 µl of binding buffer QX1 and centrifuged at 13,000 rpm for 30 sec. Again, the pellet was washed twice by adding 500 µl of wash buffer PE and centrifuged to remove all traces of agarose. Thereafter, the pellet was air-dried for 10–15 min and redissolved in 20 µl of endotoxin free water. The sample was incubated at 50˚C for 5 min, and then centrifuged at 13,000 rpm for 30 sec. The supernatant was transferred into a clean tube and stored at 4°C until further use.

2.2.2.8 Ligation of DNA fragments

Ligation of DNA fragments was performed using Quick Ligation™ kit following the manufacturer's instructions. However, some of these protocols were optimised in order to increase the ligation efficiency. Briefly, the vector (plasmid DNA) to insert (DNA fragment) molar ratio (vector:insert) was increased from 1:3 to 1:5 and 1:10. The concentration of the linearized plasmid vector was also increased from 50 ng to 200 ng. Specifically, a volume of 21 µl of ligation mixture was prepared by adding 200 ng (4 µl) of the vector, 40 ng (5 µl) of the insert, 1 µl of endotoxin free water (to adjust the volume to 10 µl), 10 µl of 2X Quick Ligation Reaction Buffer (contains 132 mM Tris-HCl, 20 mM $MgCl_2$, 2 mM ATP, 2 mM dithiothreitol, and 15% polyethylene glycol [pH7.6]), and 1 µl of Quick T4 DNA ligase. The reaction mixture was then mixed thoroughly, spun briefly, and incubated at room temperature for 20 min. After incubation, the mixture was either chilled on ice for transformation or stored at -20°C until further use.

2.2.2.9 Transformation of SCS1 supercompetent *E.coli* cells

SCS1 supercompetent cells were used in transformation experiment according to the manufacturer's protocols. Briefly, the competent cells and the ligation mixture were thawed on ice and gently mixed. When thawed, a volume of 4 µl (10 ng) of the ligation mixture and 1.7 µl of β-mercaptoethanol (1.42 M) were added to 100 µl of the competent cells, mixed thoroughly and incubated on ice for 30 min. The mixture was heat-pulsed in a 42°C water bath for 50 sec, and then incubated on ice for 2 min. A volume of 900 µl of pre-heated (at 42°C) SOC medium was added to the mixture, and incubated for 1 h at 37˚C with shaking at 125 rpm. Following incubation, 150–200 µl of the transformation mixture was spread out on LB agar plates containing the appropriate selective antibiotic, and incubated in an inverted position in a 37˚C incubator overnight. After 24 h, 10 isolated transformed colonies were picked up individually using different sterile inoculation loops. Each single colony was inoculated into 5 ml LB broth containing 5 µl of ampicillin, and incubated overnight at 37˚C with shaking at 125 rpm. Recombinant plasmids DNA were extracted and purified from these overnight bacterial cultures, digested, and then analysed by gel electrophoresis as previously described.

In this experiment, positive and negative control plates were included to ensure the viability and the validity of the competent cells. 100 µl of the competent cells only (without the recombinant plasmid) was treated in the same way as the experimental cells. 50 µl of these cells was spread out on LB agar medium without antibiotic added (positive control plate), and the other 50 µl was plated on a selective LB agar medium (negative control plate). In addition, the linearized plasmid alone (MVA vector) was transformed into the SCS1 supercompetent cells, and then plated on a selective LB agar medium. This plate was used as another negative control plate to confirm that the vector was completely digested.

2.2.2.10 Preparation of bacterial glycerol stocks

A single bacterial colony was inoculated into 5 ml LB broth containing the appropriate selective antibiotic, and incubated overnight at 37˚C with shaking at 125 rpm. A volume of 500 µl of the overnight bacterial cells, which were grown in a log phase, was mixed thoroughly with 500 µl of 40% sterile glycerol solution in a sterile cryovial tube. These bacterial glycerol stocks were cooled gradually from room temperature to -80˚C using Mr. Frosty freezing container that contained 200 ml of isopropanol. All the cryovial tubes were placed in the Mr. Frosty container, which was then placed at -80˚C for 4–6 h. Thereafter, the tubes were removed from the container and immediately placed at -80˚C.

2.2.3 Tissue culture techniques and virology methods

All tissue culture procedures, which were used in this project, were performed in class II (Bio-Class 2) microbiological safety cabinet (CAS Ltd., UK).

2.2.3.1 Preparation of primary chick embryo fibroblasts (pCEFs)

pCEFs were prepared from SPF eggs. The SPF eggs were incubated for 10 days at 37.5–37.6˚C under humid conditions (64–65%) using a rocking egg incubator. After 9 days of incubation, the eggs were candled to mark out the air sac, and to determine the viability of the embryos. Eggs with viable embryos were carefully cleaned with 70 % ethanol, and the shells were pierced along the marked line using sterile sharp-pointed scissors. The embryos were taken out of the eggs using a sterile set of forceps, decapitated, and transferred into a 50 ml Falcon tube containing 12 ml of serum-free dulbecco's modified eagle medium (DMEM), in order to wash the cells and remove large amount of the blood. The medium was discarded and the embryos were poured into a sterile 50 ml syringe. By using the plunger, the embryos were forced through the

syringe into a conical flask containing magnetic beads and a mixture of 200 ml of 1X phosphate buffered saline (1X PBS) and 15 ml of trypsin/EDTA (TrypZean Solution). The mixture was then stirred at 150 rpm for 30 min using a color squid magnetic stirrer. This step (harvesting stage) was an important to harvest the embryos' tissues and separate the cells. After 30 min of stirring, the mixture was left to stand for 5–10 min, and then the supernatant was transferred into 50 ml Falcon tubes containing 5 ml of FBS, which was used to inactivate the digestive activity of trypsin. The tubes were mixed thoroughly and centrifuged at 1400 rpm for 10 min at 22˚C using Harrier 18/80 refrigerated centrifuge. The supernatant was discarded, and the cell pellets were resuspended in 5 ml of 10 % growth DMEM. Further 4–5 rounds of harvesting stage were performed depending on the yield of the embryo digest.

The final cell suspension was used to count the number of viable cells using a disposable haemocytometer counting chamber. Briefly, a volume of 50 µl of cell suspension was diluted in 450 µl (1:10) of trypan blue solution, and only 10 µl of this mixture was loaded into the chamber for counting. The numbers of viable (unstained) cells were counted per 1 mm square, and were adjusted to a cell concentration of 3.5 X 10^7 cells/ml, using the following formula: (number of viable cells X dilution factor [10] X 10^4). After cell counting, the cell suspension was centrifuged at 1400 rpm for 10 min, and the supernatant was discarded. The cell pellets were resuspended in 40 ml (at 3.5 X 10^7 cells/cryovial tube) of cryopreservation (freezing) medium (containing 3.75 ml of dimethyl sulphoxide [DMSO], 5 ml of FBS, and 41.25 ml of 10 % growth DMEM). The mixture was then aliquot into 40 sterile cryovial tubes, and stored in the Mr. Frosty container at -80˚C overnight. Next day, the tubes were transferred to liquid nitrogen (-196˚C) for long-term storage.

To check the cell viability, a volume of 500 µl of fresh cells was used to seed a T175 cm^2 tissue culture flask containing 25 ml of 10 % growth medium. The flask was incubated horizontally at 37˚C in 5% CO_2 incubator for 2–3 days until the cells had formed confluent CEF monolayers. The pCEFs were also checked for sterility in the following day. Briefly, a frozen vial of pCEFs was thawed completely at 37˚C, resupended in 30 ml of 10 % growth DMEM, and transferred to seed a T175 cm^2 tissue culture flask. The flask was then incubated horizontally at 37˚C in 5% CO_2 incubator for a maximum of 12 days, in order to ensure that there was no bacterial or fungal overgrowth.

2.2.3.2 Culturing of pCEFs

A frozen vial of pCEFs (containing 3.5 X 10^7 cells/ml of cryopreservation medium) was thawed completely at 37˚C. The cells were mixed thoroughly by pipetting up and down several times to break up cell clumps. A volume of 1 ml of these cells was used to seed a T175 cm^2 tissue culture flask containing 30 ml of 10 % growth DMEM. The flask was gently agitated, and then incubated horizontally at 37˚C in 5% CO_2 incubator for 1–2 days, until the cells had formed confluent monolayers.

2.2.3.3 Splitting of pCEFs

Confluent or near confluent cell monolayers were harvested by trypsinisation using TrypZean solution. Briefly, the culture medium was carefully aspirated using Dymax-5 vaccum pump, and the cell monolayers were washed twice with 12 ml of pre-warmed 1X PBS. A volume of 4–5 ml of pre-warmed trypsin solution was added to the washed cells, and incubated at 37˚C for 3–5 min, until all the cells became detached from the wall of the flask. The cells were then transferred into a 50 ml Falcon tube containing 5 ml of fresh 10% growth DMEM. The tube was mixed and centrifuged at 1200 rpm for

10 min at 4˚C. The supernatant was discarded, and the cell pellet was resuspended in 5–6 ml of fresh 10 % growth DMEM. The cells were then counted as described previously, and reseeded into either a 6-well cell culture plate, 100 mm petri dish or T175 cm^2 tissue culture flask at an appropriate density.

2.2.3.4 FP9 infection of pCEF monolayers for small-scale expansion

A T175 cm^2 tissue culture flask containing near confluent (80–90%) pCEF monolayers (8 X 10^6 cells) was used to expand FP9. The culture medium was aspirated from the flask, and the cell monolayers were washed with 12 ml of pre-warmed 1X PBS. A volume of 500 µl of the FP9 was thawed in a 37˚C water bath, and then diluted in 10 ml of serum-free DMEM. The virus inoculum was inoculated onto the pCEF monolayers, and incubated horizontally at 37˚C in 5% CO_2 incubator for 6 h with gentle agitation every 15–30 min. After 6 h, a volume of 20 ml of 10% growth DMEM was added to the culture flask and incubated horizontally at 37˚C in 5% CO_2 incubator for 2–3 days, until a maximal yield (a good CPE) is obtained.

2.2.3.5 Harvesting of FP9 small-scale preparation

At 48–72 h after infection, a good CPE was observed, and the FP9-infected cells were harvested by scraping the cell monolayers into the culture medium using a 25 cm cell scraper. The entire medium was transferred into a 50 ml Falcon tube and centrifuged at 1500 rpm for 10 min. The supernatant was discarded, and the cell pellet was incubated at -20˚C for 30-60 min. The pellet was then resuspended in 1 ml of 10 mM Tris-EDTA (pH 7.5) and transferred into a sterile cryovial tube. The cell-virus suspension was subjected to three freeze-thaw cycles using liquid nitrogen (-196˚C) and water bath at 37˚C. It was necessary to vortex vigorously for 30 sec after each freeze-thaw cycle. After the third cycle, the FP9 suspension was sonicated for 10 sec at 0.5 pulses using

sonicator 3000-Ultrasonic liquid processor, in order to lyse the cells completely and disaggregate the virus. The virus suspensions were then either titrated or stored at -80˚C for further use.

2.2.3.6 Plaque assay for virus titration

Plaque assay was used to titrate nonrecombinant and recombinant viruses using 6-well tissue culture plates containing near confluent (80–85%) pCEF monolayers (5 X 10^5 cells/well). Briefly, the stock virus suspension was serially diluted (10-fold) in serum-free DMEM (starting from 10^{-3} to 10^{-8}). The cell culture medium was carefully aspirated from the plate, and 750 µl of each diluted virus inoculum was used to infect each well. All dilution panels were performed in triplicate to enhance accuracy. A negative control well (containing only 750 µl of serum-free DMEM) was also included in each plate to monitor the contamination. The 6-well tissue culture plate was then incubated at 37˚C in 5% CO_2 incubator for 3 h with gentle mixing for every 15 min. Following the incubation, the virus inoculum was aspirated, and 2 ml of agarose overlay was added into each well. The plate was left to sit for 10–15 min at room temperature, and then incubated at 37˚C in 5% CO_2 incubator for 5–6 days, until clear and large plaques are obtained.

The titre of a virus stock was calculated in plaque-forming unit (pfu)/ml. In brief, the highest virus dilutions, which had clear and countable plaques, were used to count the number of plaques that were visible to the naked eye. The plaques were counted, and then multiplied by the serial dilution factor. The formula presented below was used to calculate the virus titre:

[Average number of plaques X serial dilution factor = virus titre (pfu/ml)]

2.2.4 rFP9 construction

2.2.4.1 Generation of FP9 transfer plasmid encoding various SIVmac239 sequences

Five FP9 transfer plasmids encoding various genomic components of SIVmac239 (GenBank accession no. M33262) were generated using five previously constructed MVA plasmids DNA. All MVA HSs were cleaved out and replaced with the newly synthesised FP9 HSs (Figure 2.2). In brief, HS of both MVA (vector) and FP9 (insert) were double digested (as described previously) for 6 h using specific RE sites. About 8 µg of both plasmids DNA was digested with 25 units of enzyme in an overall volume of 150 µL, in order to get single, clear, and completely digested bands for both plasmids. Digested DNA fragments were then isolated by electrophoresis through a LMP agarose at 60–70V for 1–2 h. Both target fragments (MVA vector and FP9 insert) were excised from the gel, purified, ligated, and then transformed into SCS1 supercompetent *E.coli* cells as described previously.

These FP9 transfer plasmids employing a TCS technique were constructed by insertion the LacZ marker gene (β-gal) outside the FP9 genomic flanking regions. The expression of this marker gene was under the control of VACV late promoter (p11). However, the expression of the encoded SIVmac239 DNA sequences was under the control of T7 promoters that were inserted back-to-back adjacent to each other. These inserted SIV sequences were flanked by two FP9 sequences homologous to the intergenic insertion site in the FP9 genome (See CD-ROM for annotated sequence details of FP9 recombination transfer plasmids).

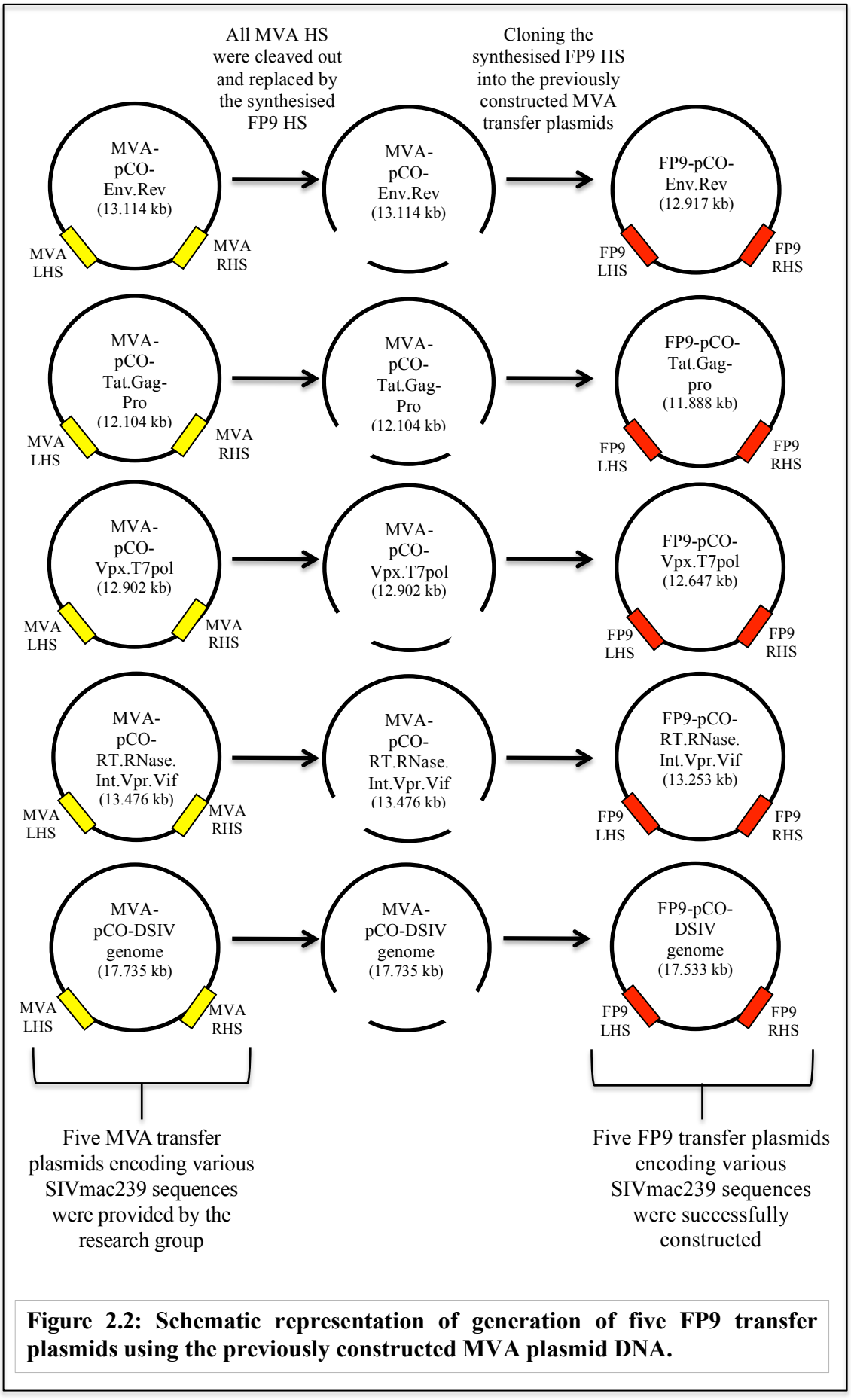

Figure 2.2: Schematic representation of generation of five FP9 transfer plasmids using the previously constructed MVA plasmid DNA.

2.2.4.2 Construction of rFP9 using HR

rFP9 were generated by *in vitro* HR in pCEFs (Smith et al., 1984). Monolayers of pCEFs were grown in a 6-well tissue culture plate until 80–85% confluency, with a seeding density of 5 X 10^5 cells per well. The monolayers were first infected with the FP9 at 0.05 pfu/cell in a volume of 1 ml serum-free DMEM. The plate was incubated at 37˚C in 5% CO_2 incubator for 3 h, with gentle agitation every 15–20 min. Thereafter, the infected cells were transfected with FP9 transfer plasmid DNA encoding the target SIVmac239 sequences using SuperFect® transfection reagent according to the manufacturer's protocols. Briefly, around 3–5 µg of the FP9 plasmid DNA was diluted in 100 µl of serum-free DMEM in a sterile 2 ml eppendorf tube, and 15–20 µl of the transfection reagent was added to the DNA solution. For positive transfection control, only 2 µg of purified red fluorescent protein (RFP) was diluted in 100 µl of serum-free DMEM, and 20 µl of the transfection reagent was added to the DNA solution. The transfection mixture was then vortexed for 10 sec, and incubated for 10 min at room temperature. After incubation, a volume of 600 µl of 10% growth medium was added to the mixture and mix gently by pipetting up and down 2–3 times. The mixture was then applied gently (dropping) to the infected cells (with the exception of transfection control that was applied to un-infected cells), and incubated at 37ºC in 5% CO_2 incubator for 3 h with gentle agitation every 15–20 min. Following incubation, the mixture was carefully aspirated from the well and replaced with 2 ml of 10% growth medium, and incubated at 37ºC in 5% CO_2 incubator. At 48–72 h after infection, good CPE was observed and the infected pCEFs were harvested using a 25 cm cell scraper, and pelleted by centrifugation at 1400 rpm for 10 min. The supernatant was discarded and the cell pellet was resuspended in 500 µl of 10 mM Tris-EDTA buffer. The recombinant virus lysate was subjected to three freeze-thaw cycles, and then sonicated at 0.5 pulses

for 10–15 sec using sonicator 3000-Ultrasonic liquid processor with a cup-horn attachment. The lysate was then either used immediately for screening of recombinant plaques or stored at -20˚C for further use.

2.2.4.3 Screening of rFP9 plaques

Positive rFP9 were identified by β-gal expression in the presence of X-gal overlay. Plaque assay was performed in a 6-well tissue culture plate using 80–85% confluent monolayers of pCEFs (5 X 10^5 cells/well). The monolayers were infected with different dilutions of rFP9 lysate that was recovered from infection/transfection mixture. The plate was incubated at 37˚C in 5% CO_2 incubator for 3 h with gentle agitation every 15–20 min. Thereafter, the virus inoculum was removed from each well, and replaced with 2 ml of agarose overlay. The plate was left to sit for 10–15 min at room temperature, and then incubated for 5–6 days at 37ºC in 5% CO_2 incubator until clear CPE were visible. A second overlay containing X-gal substrate was carefully applied onto the primary overlay, and incubated for 24–48 h at 37ºC in 5% CO_2 incubator. Usually after 18–24 h, positive recombinant plaques developed blue colour, indicating successful integration of plasmid DNA into the FP9 genome. Only individual and well-isolated blue plaques were marked on the bottom of the plate, and then carefully picked using a sterile fine point Pasteur pipette. Each plaque was dispersed in 1 ml of serum-free DMEM, and vortexed vigorously for few seconds to release the virus-infected cells from the agar. These plaques were subjected to three freeze-thaw cycles, and then underwent several rounds of plaque purification until a pure recombinant virus was obtained.

2.2.4.4 Elimination of the marker gene

Several rounds of plaque purification were performed on pCEFs to isolate pure recombinants with subsequent elimination of the marker gene. After 4–5 sequential rounds of blue plaque purification, markerless recombinant plaques were identified by loss of blue staining under X-gal, with confirmation of target sequence retention by polymerase chain reaction (PCR). Individual and well-isolated colourless plaques were picked and purified (as described previously) for further rounds until all the picked colourless plaques (12–14 plaques) gave positive PCR results for the insertion cassette.

2.2.4.5 Plaque expansion and total genomic DNA extraction

Recombinant plaques (blue/colourless) were expanded in 6-well tissue culture plates using 70–80% confluent monolayers of pCEFs (4 X 10^5 cells/well). A volume of 200 µl of the 1 ml of each plaque material was diluted in 800 µl of serum-free DMEM, and expanded in one well of the plate. In brief, a total volume of 1 ml of virus inoculum was applied into one well, and then incubated at 37˚C in 5% CO_2 incubator for 3 h with gentle mixing every 15–20 min. Thereafter, 1 ml of 10 % growth DMEM was added into each well and mixed gently. The plate was incubated at 37˚C in 5% CO_2 incubator for 5–6 days until clear CPE was visible. The infected cell monolayers were then harvested using a 25 cm cell scraper, and pelleted by centrifugation at 10,000 rpm for 10 min. The supernatant was discarded, and the cell pellet was either processed immediately for genomic DNA extraction or stored at -20˚C for further use.

Total genomic DNA was extracted and purified from recombinant plaques (virus-infected cells) using the PureLink™ genomic DNA mini kit according to the manufacturer's instructions. This kit is based on a spin-column procedure for the purification of genomic DNA, and is composed of 4 key steps: cell lysis, selective DNA

binding, washing step, and finally DNA elution. Briefly, the cell pellet was resuspended in 200 µl of 1X PBS with gentle vortex for few seconds until the pellet is completely suspended. A volume of 20 µl of Proteinase K and 20 µl of RNase A were then added to the cell suspension, mixed well by brief vortexing, and incubated for 2 min at room temperature. Thereafter, 200 µl of PureLink genomic lysis/binding buffer was added and mix vigorously with the mixture to obtain homogenous solution, which was then incubated at 55˚C for 10 min. Next, a volume of 200 µl of absolute (96–100%) ethanol was mixed thoroughly with the lysate to obtain homogenous lysate. By the end of this step, the cell lysate was ready for the selective DNA binding using silica-based membrane particles. The cell lysate was transferred immediately into a PureLink spin column with a collection tube, and centrifuged at 9,000 rpm for a minute, to allow the DNA to attach to the silica membrane. A fresh collection tube was used, and the column was washed by 500 µl of wash buffer-1, and centrifuged at 9,000 rpm for a minute. Another fresh collection tube was used, and the column was washed again by 500 µl of wash buffer-2, and centrifuged at 13,000 rpm for 3 min to remove residual wash buffers. The column was then placed in a clean 1.5 ml microcentrifuge eppendorf tube, and the genomic DNA was eluted from the membrane using 100 µl of low salt elution buffer (10 mM Tris-HCl, pH 9, 0.1 mM EDTA). After one min incubation, the tube was centrifuged at 13,000 rpm for a minute. The resultant DNA was quantified using the NanoDrop^{TM} ND-1000 spectrophotometer as describe previously, and then stored at 4°C until further use.

2.2.4.6 Confirmation of blue and markerless recombinant plaques by PCR

rFP9 genome was amplified using Thermal Cycler PCR machine (Applied Biosystems 2720). Different sets of specific primer pairs targeting the insertion cassettes (SIVmac239 sequences), the marker gene (β-gal), and the plasmid sequence were

designed using the primer design software (Primer Select, LaserGene) (Table 2.5).

The PCR master mix for the amplification step was prepared in 0.2 ml PCR tubes using Taq 5X master mix according to the manufacturer's instructions. For each sample, a volume of 25 µl of the PCR mixture was prepared by adding 5 µl of Taq 5X, 1 µl of each primer (forward and reverse), and 18 µl of DNase/RNase free water. A concentration of 50–200 ng of each genomic DNA sample was added to the PCR mixture, and mixed thoroughly by pipetting up and down. The thermal cycling profile of the PCR reaction was 95ºC for 5 min initial denaturation, then cycled for 35 cycles: 30 sec at 95ºC, 30 sec for primer annealing (based on primer annealing temperature), and 30 sec at 68ºC. The final extension was at 68ºC for 7 min. FP9 genomic DNA and pCEFs DNA were used as negative controls, while the FP9 transfer plasmid DNA was used as a positive control.

2.2.4.7 Construction of double (complex) rFP9

Single rFP9 encoding one SIV insertion cassette (such as env and rev genes) was constructed as described previously in sections 2.2.4.2 to 2.2.4.4, and then large-scale expanded (section 2.2.4.8) and titrated (section 2.2.3.6). This single rFP9 was used as a backbone recombinant virus to add more SIVmac239 DNA sequences (such as tat and gag-pro genes) into a different insertion site to make double rFP9 (Figure 2.3).

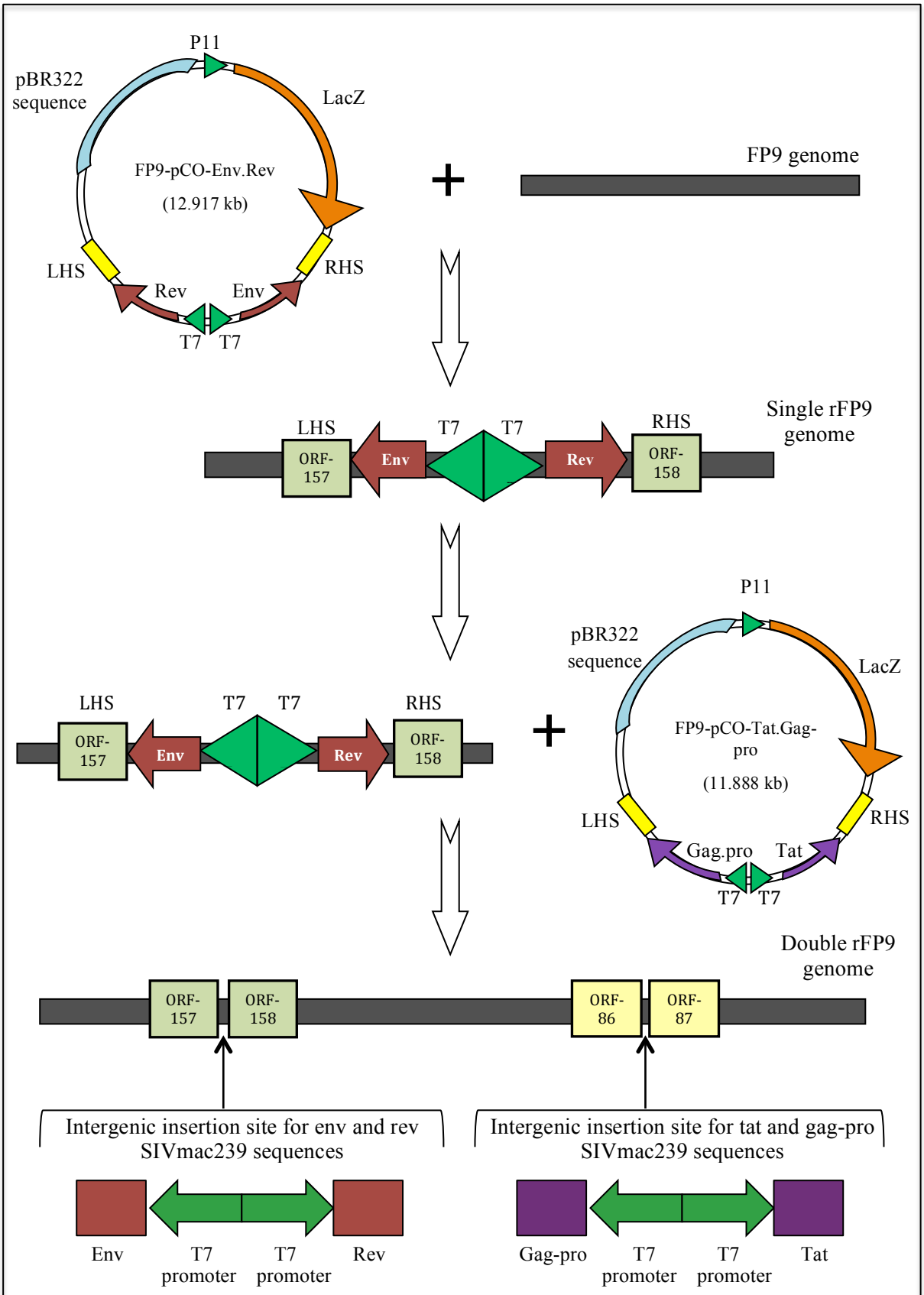

Figure 2.3: Schematic representation of construction of a double rFP9 encoding two insertion cassettes (env/rev and tat/gag-pro SIV sequences).

2.2.4.8 Large-scale expansion of pure rFP9

Purified and confirmed recombinant viruses were large-scale expanded through two passages. Firstly, two 100 mm tissue culture petri dishes containing near confluent (80–90%) pCEF monolayers were used for the 1st passage of each pure recombinant virus. In brief, the culture medium was aspirated from the dishes, and the cell monolayers were washed twice with 6 ml of pre-warmed 1X PBS. A volume of 150 µl of recombinant virus, which was expanded in one well of a 6-well plate, was diluted in 4 ml of serum-free DMEM to infect the cell monolayers of each 100 mm petri dish. The two dishes were incubated at 37˚C in 5% CO_2 incubator for 3 h with gentle mixing every 15 min. Thereafter, a volume of 8 ml of 10% growth DMEM was added to each dish, mixed gently and incubated at 37˚C in 5% CO_2 incubator for 3–5 days, until a maximal yield of CPE was achieved. Infected cell monolayers of each dish were then harvested using a 25 cm cell scraper, and pelleted by centrifugation at 1500 rpm for 10 min. The supernatant was discarded and the cell pellet was resuspended in 0.6 ml of 10 mM Tris-EDTA buffer (pH 7.5), and transferred into a sterile cryovial tube. The total cell-virus suspension from the two dishes (1.2 ml) was subjected to three freeze-thaw cycles, and then sonicated for 10 sec at 0.5 pulses using sonicator 3000-Ultrasonic liquid processor. The final virus suspension was either processed for 2nd passage or stored at -80˚C for further use.

Secondly, three T175 cm^2 tissue culture flasks containing near confluent (80–90%) pCEF monolayers were used for the 2nd passage of pure recombinant viruses. Briefly, the cell monolayers of the three flasks were washed twice with 12 ml of pre-warmed 1X PBS. A volume of 400 µl of the expanded virus stock from the previous passage (1st passage) was diluted in 10 ml of serum-free DMEM to infect the cell monolayers of each flask. The three flasks were incubated at 37˚C in 5% CO_2 incubator for 4–5 h with

gentle agitation every 15 min. Following incubation, a volume of 20 ml of 10% growth DMEM was added to each flask, mixed gently and incubated at 37˚C in 5% CO_2 incubator for 2–3 days, until a maximal yield of CPE was obtained. Infected cell monolayers were harvested as describe previously. The cell pellet of each flask was resuspended in 1 ml of 10 mM Tris-EDTA buffer (pH 7.5). The total cell-virus suspension from the three flasks (3 ml) was subjected to three freeze-thaw cycles, and then either titrated or stored at -80˚C for further use.

2.2.4.9 Genetic stability testing

Two rFP9s (rFP9.env.rev and rFP9.T7pol) had passaged more than 11 times in pCEFs, in order to prove the genetic stability of the insertion cassette within the recombinant genome. Around 10 passages were performed in 6-well cell culture plates and 100 mm dishes, and about 2 passages were performed in 3 T175 cm^2 tissue culture flasks. Thereafter, the recombinant viruses were large-scale expanded, titrated, and then confirmed of target sequence retention by PCR and protein expression assays.

2.2.5 SIV Protein expression and characterization

2.2.5.1 Expression of SIVmac239 proteins in different cell lines employing T7 RNA polymerase expression system

Cell monolayers (pCEFs/MRC-5/Vero) seeded in 6-well tissue culture plates at a density of 5 X 10^5 cells/well were co-infected simultaneously with two rFP9s at an MOI of 2 pfu/cell for each recombinant virus. One of these recombinants encoded the target SIV sequences flanked by T7 promoters, and the other one encoded the T7 RNA polymerase gene (GenBank accession no. M38308) flanked by poxvirus promoters. Briefly, the cell monolayers of each well of the 6-well plate were co-infected at an MOI of 4 pfu/cell (virus suspension was diluted in 1 ml of serum-free DMEM), and

incubated at 37ºC in 5% CO_2 incubator for 3 h with gentle mixing every 15–20 min. The virus inoculum was then carefully aspirated from each well and replaced with 2 ml of fresh 10% growth DMEM, and incubated at 37ºC in 5% CO_2 incubator. Infected cells were harvested using a 25 cm cell scraper at various time points (24, 48, 72, and 96 hours post-infection [hpi]) to analyse the expression levels of the target protein. In this assay, two negative controls were also included: uninfected and single-infected (with one recombinant virus that encoded either SIV sequences or T7 polymerase gene) cells. These controls were treated in the same way as the co-infected cells.

2.2.5.2 Protein extraction

After 24, 48, 72, and 96 hpi, uninfected and infected cell monolayers were washed twice with cold 1X PBS. The target cells were then harvested using a 25 cm cell scraper, and pelleted by centrifugation at 450 g for 10 min at 4ºC. The supernatant was discarded, and the cell pellet of each well was resuspended in 200 µl of prechilled radioimmunoprecipitation assay (RIPA) buffer (0.5% sodium deoxycholate, 0.1% SDS, 50 mM Tris, 150 mM NaCl, 1.0% IGEPAL® CA-630, pH 8.0) containing 15 µl of protease inhibitor cocktail (serine, cysteine, acid proteases, and aminopeptidases). The resuspended cells were incubated on ice for 5 min, and then centrifuged at 8000 g for 10 min at 4˚C. The resultant lysate (200 µl) containing the soluble protein was carefully transferred into a fresh 1.5 ml eppendorf tube, and then either used immediately for protein expression assay or stored at -80ºC for further use.

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

SDS-PAGE was used to analyse and determine the size of the expressed proteins, which were resolved by electrophoresis through a polyacrylamide gel. Briefly, aliquots of 20 µl of extracted protein samples (controls and test samples) were mixed with 10 µl of 2X

sample laemmli buffer (0.004% bromphenol blue, 10% 2-mercaptoethanol, 4% SDS, 0.125 M Tris HCl and 20% glycerol, pH 6.8), and denatured at 95˚C in a heat-block for 10 min. A volume of 20 µl of each heated sample was then loaded in a NuPAGE 4–12% Bis-Tris gel (1.0mm X 10 well). In addition, a volume of 15 µl of precision plus protein™ dual colour standard was used as a protein marker to estimate the size of the separated proteins. The gel cassette was submerged in 1X Nupage® MES SDS running buffer using XCell SureLock™ Mini-Cell electrophoresis tank, and electrophoresed at 100 V for 90 min.

2.2.5.4 Western blot (WB)

Following SDS-PAGE separations, protein samples were transferred from the gel to a polyvinylidene difluoride (PVDF) membrane using semi-dry transfer. Briefly, an appropriate size of PVDF membrane was soaked in absolute methanol for 2–3 min. Thereafter, the PVDF membrane together with some filter papers were soaked in ice cold 1X Nupage® transfer buffer for another 2–3 min. A sandwich was then made in a particular order (4 filter papers/PVDF membrane/gel/4 filter papers), and placed in Novex semi-dry blotter between positive and negative electrodes. A blotting roller was used to remove any air bubbles between the gel and the membrane. The transblotting was performed at 30 V, 200 mA for 2 h. The transblotted membrane was then removed from the transfer apparatus, and placed in WB blocking buffer (5% BSA) for 90 min with shaking at 100 rpm using an orbital shaker. After blocking nonspecific sites, the membrane was immediately incubated with primary monoclonal antibodies (1:1000–1:3000 diluted in 10 ml of 5% BSA buffer) for either 2 h at room temperature with shaking or overnight at 4˚C without shaking. Next, the membrane was washed 3 times in WB washing buffer with shaking at 100 rpm (fresh buffer was used for each 5 min wash step). The membrane was then incubated with Anti-mouse IgG, horseradish

peroxidase (HRP)-linked secondary antibody (1:2000 diluted in 10 ml of 5% BSA buffer) for 1 h at room temperature with shaking at 100 rpm, followed by 3 washes as describe previously. After the last washing step, the separated proteins were detected using the enhanced chemiluminescence detection system (Amersham^{TM} ECL Western blotting detection reagents) following the manufacturer's instructions. In brief, a mixture of 1 ml of ECL detection reagent No.1, 1 ml of ECL detection reagent No.2, and 2.5 µl of 30–31% hydrogen peroxide solution was used to treat each transplotted membrane for 1–2 min at room temperature. One of the detection reagents containing luminol, and the other is an activating compound. The detection mixture (1:1) solution should cover the whole surface area of the membrane. ChemiDoc™ XRS molecular imager system was then used to visualise and analyse the separated protein bands. Only a few seconds of exposure were required for protein visualisation.

2.2.5.5 Immunocytochemical (ICC) staining assay

At 48 hpi, the culture medium was aspirated from the 6-well tissue culture plate, and cell monolayers (pCEFs/MRC-5) were washed twice with ice cold 1X PBS. Uninfected, single-infected, and co-infected cells were then fixed by incubation in ice cold absolute methanol (2 ml for each well) for 5 min at room temperature. Thereafter, the fixative was aspirated, and the cells were immediately washed 3 times with ice cold 1X PBS for 5 min each wash (3 X 5 min). Cells were then permeablised with 2 ml of 0.1% Triton X-100 in PBS for 10 min at room temperature, followed by 3 washes with ice cold 1X PBS (3 X 5 min). A volume of 2 ml of 1% BSA was used to block the cell monolayers of each well for 1 h at room temperature. After blocking step, the cells were incubated with primary monoclonal antibodies (1:2000 diluted in 12 ml of 1% BSA buffer) for either 90 min at room temperature or overnight at 4˚C, followed by 3 washes with ice cold 1X PBS (3 X 5 min). The cells were then incubated with Anti-mouse IgG, HRP-

linked secondary antibody (1:2000 diluted in 12 ml of 1% BSA buffer) for 90 min at room temperature, followed by 3 washes as describe previously. Detection and staining of target proteins was performed using Sigma fast 3,3'-Diaminobenzidine (DAB) peroxidase tablets following the manufacturer's instructions. In brief, one DAB and one urea hydrogen peroxide tablets were completely dissolved in 5 ml of DNase/RNase free distal water. A volume of 1 ml of freshly prepared DAB mixture was used to cover each well in a 6-well plate. The plate was then incubated at room temperature in the dark for 10–15 min or until dark brown colours developed. Development of colours was monitored under an inverted microscope (Optika-XDS-3FL), and the images were acquired and analysed using Optika Vision® software. The DAB substrate reaction was stopped by washing the stained cell monolayers twice with 1X PBS. Harris haematoxylin and eosin (H&E) stain, which is a stronger form of haematoxylin, was then used as a counterstain to stain the nuclear materials with a deep purple-blue colour. A volume of 1 ml of H&E stain was applied into each well for 20–30 sec, followed by 3 washes with 1X PBS to remove excess stain.

2.2.5.6 Enzyme-linked immunosorbent assay (ELISA)

A sandwich ELISA system was used to measure the amount of protein expressed by recombinant viruses under the control of a T7 RNA polymerase gene. In brief, 96-well flat bottom microplates were coated with a suitable capture monoclonal antibody at concentration of 3 µg/ml (1:350) in carbonate-bicarbonate buffer, covered with an adhesive plate sealer, and incubated overnight at 4˚C. The plates were then washed twice with 200 µl of 1X PBS, and blocked with 200 µl of 3% BSA, per well. Blocking step was last for 2 h at room temperature, followed by 2 washes with 200 µl of 1X PBS/well.

Protein samples and standards (recombinant proteins) were diluted in 1X PBS, and a volume of 100 µl of each diluted sample was plated in triplicate wells. Dilution of 1:50 was used for the protein samples, while seven diluted solutions (1:1000, 1:100, and then 1:2 dilutions) were prepared for the standard samples. A volume of 100 µl of 1X PBS was included as a negative control, and plated again in triplicate wells. After adding the samples, the plates were sealed and incubated for 90 min at 37˚C. Thereafter, all the samples were removed, and the plates were washed twice with 200 µl of 1X PBS/well. A volume of 100 µl of a suitable detection polyclonal antibody (1:300 diluted in 12 ml of 1X PBS) was then added to each well and incubated for 2 h at room temperature, followed by 4 washes with 200 µl of 1X PBS/well. Next, a volume of 100 µl of a suitable conjugated secondary antibody (1:2000 diluted in 12 ml of 3% BSA) was added to each well and incubated for 2 h at room temperature, followed by 4 washes with PBS. Following the final wash step, a volume of 100 µl of 3,3',5,5' Tetramethylbenzidine (TMB) liquid substrate was added to each well, and the plate was incubated at room temperature in the dark for colour development. Once a strong colour developed in the standard protein wells, the reaction was stopped by adding 100 µl/well of stop reagent (2 M H_2SO_4) for TMB substrate. The optical densities (OD) of each well were then measured at 450 nm using an ELISA plate reader (ELX800). The OD values of each test and standard sample were calculated by subtracting the OD values of the negative control samples.

In this assay, different standard proteins of known concentration, supplied by NIBSC, were used as positive controls to quantify the expressed protein levels. Serial dilutions of each known standard protein were prepared to incorporate a standard curve into the ELISA assay. A standard curve was created for each assay by plotting the standard protein concentration against the mean of the absorbance values of each dilution, in

order to determine the protein concentration of test samples.

2.2.5.7 Demonstration of green-fluorescent protein (GFP) gene expression and immunofluorescence imaging

Expression of GFP under the control of T7 RNA polymerase system was demonstrated by fluorescent microscopy. In brief, rFP9 encoding the T7 RNA polymerase gene was first constructed (as previously described), and then was used as a backbone recombinant virus to add the defective SIVmac239 (DSIV) genome into a different insertion site (Figure 2.4). A GFP DNA sequence was already incorporated within the middle of the DSIV genome, in order to demonstrate and prove the principle of the T7 expression system within a single recombinant vector. The double rFP9, which encodes both the DSIV genome and the T7 RNA polymerase, was used to infect pCEFs monolayers in 6-well tissue culture plates and 100 mm petri dishes with 80–90% confluent cells. The monolayers were infected with serial 10-fold dilutions of the double rFP9, and incubated at 37˚C in 5% CO_2 incubator for 3 h with gentle mixing every 15–20 min. The virus inoculum was then replaced with agarose overlay, and the plate was incubated for 5–6 days at 37ºC in 5% CO_2 incubator until clear plaques were visible. The resultant GFP-positive plaques were identified and visualised under an inverted fluorescent microscope (Optika-XDS-3FL) using a green fluorescent filter. The images were acquired and analysed using Optika Vision® software.

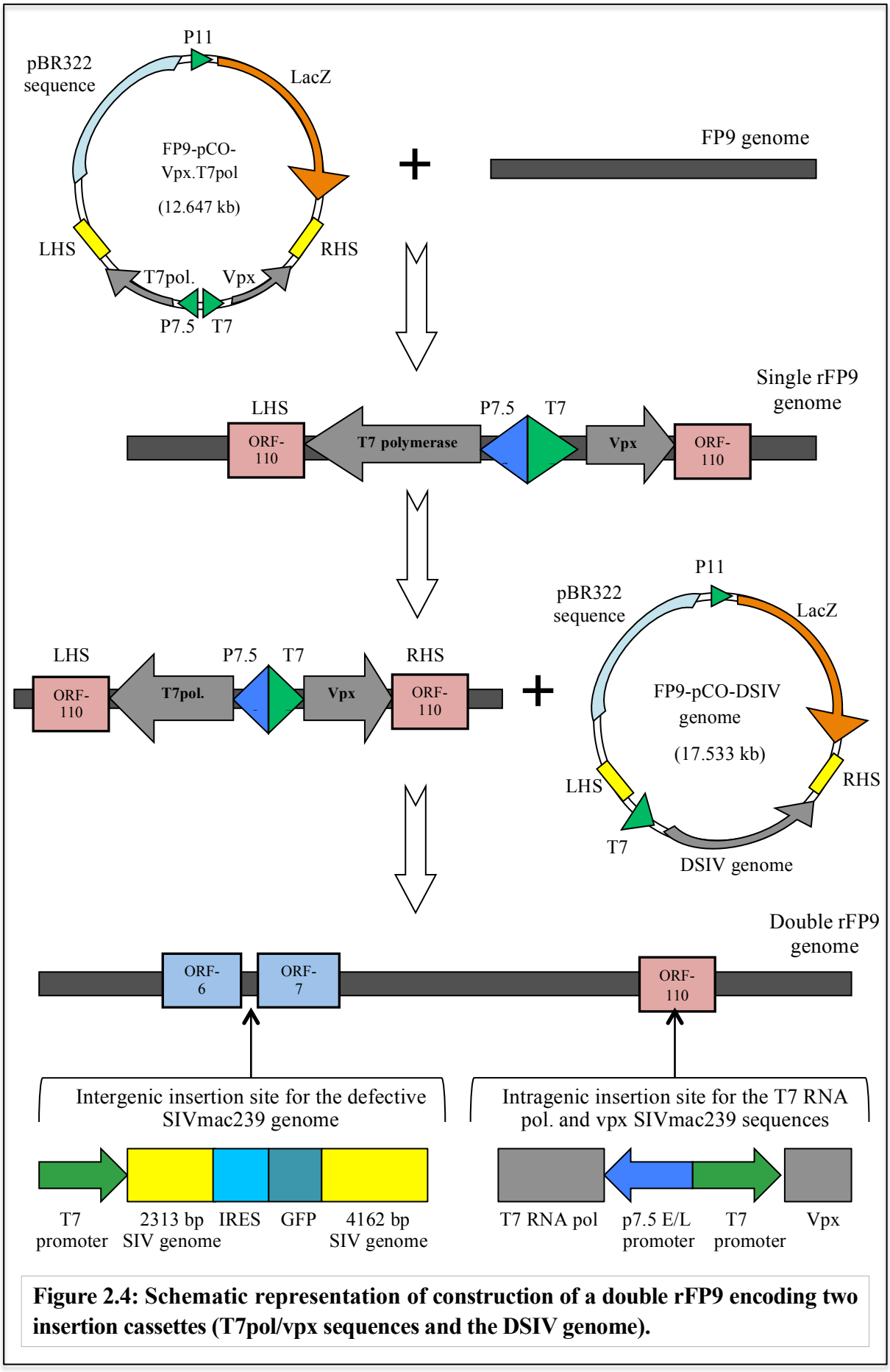

Figure 2.4: Schematic representation of construction of a double rFP9 encoding two insertion cassettes (T7pol/vpx sequences and the DSIV genome).

GFP positive plaques were marked on the bottom of the plate, and then a second X-gal overlay was carefully applied onto the primary overlay and incubated for 24 h at 37ºC in 5% CO_2 incubator. The GFP positive plaques (blue and colourless) were picked using a sterile fine point Pasteur pipette, and dispersed in 1 ml of serum-free DMEM. These positive plaques were subjected to three freeze-thaw cycles, and then underwent several rounds of plaque purification. As a negative control in this experiment, rFP9 encoding either the DSIV genome or T7 polymerase gene was used in order to demonstrate that the protein of interest (GFP) was not expressed.

2.2.5.8 Statistical analysis

Statistical analyses were performed using GraphPad prism software (version 6.0 for Mac). These analyses were conducted to determine whether there are significant differences in protein expression levels between the groups. To compare the means of two groups at four different time points, a two-way analysis of variance (ANOVA) followed by a Bonferroni post hoc test was applied. However, a one-way ANOVA followed by the same post hoc multiple comparisons test (a Bonferroni correction) was used to compare the averages of four different groups at each time point. The statistical significance of differences and associations between the groups were considered at p-values < 0.05.

3 Results

3.1 Molecular biology and DNA cloning techniques

3.1.1 Sequence maps for MVA transfer plasmid DNA carrying various SIVmac239 sequences

Five MVA transfer plasmids encoding various SIVmac239 sequences, MVA HS, and reporter expression cassettes were constructed by Dr. Thomas Blanchard and Dr. Gowda CPC, The University of Manchester. These MVA transfer plasmids were used for cloning the newly synthesised FP9 HS and generating five different FP9 transfer plasmids carrying SIVmac239 sequences. All MVA HS were flanked on both sides by unique RE sites that can be used to replace these sequences with FP9 HS (Table 3.1).

MVA transfer plasmid	Plasmid size	RE for right HS	RE for left HS	Encoding SIVmac239 sequences
pCO-Vpx.T7pol	12.902 kb	AsiSI and NotI	Acc651 and SpeI	Vpx gene and T7 polymerase
pCO-DSIV genome	17.735 kb	NotI and XhoI	AsiSI and Acc651	Defective SIV genome
pCO-Tat.Gag-pro	12.104 kb	NotI and SpeI	AsiSI and BsiWI	Tat and gag-pro genes
pCO-Env.Rev	13.114 kb	SacII and NotI	Acc651 and Bgl II	Env and rev genes
pCO-RT.RNase.Int.Vpr.Vif	13.476 kb	PacI and NotI	AsiSI and Acc651	RT.RNase, int, vpr and vif genes

Table 3.1: Shows all five MVA transfer plasmids with their encoding SIVmac239 sequences.

The following electronic versions of schematic maps for MVA transfer plasmid (Figures 3.1 to 3.5) were generated using Lasergene™ Core Suite Software, SeqBuilder programme. These maps provide all required information (such as the unique RE sites and the expected banding patterns of MVA HS) that can be used for RE digestion analysis.

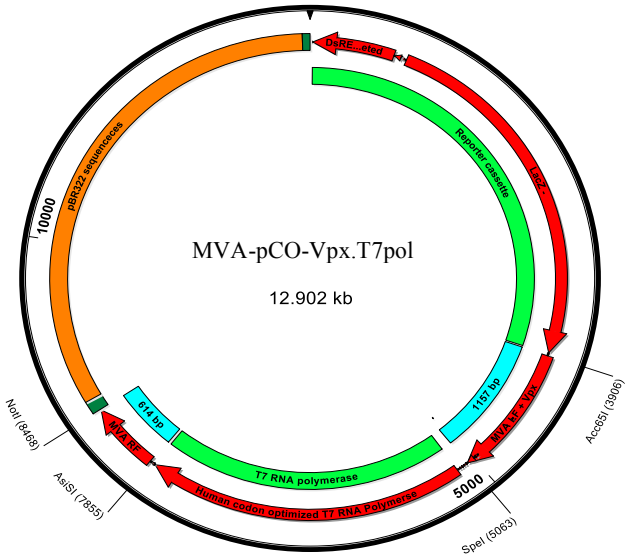

Figure 3.1: Schematic map of MVA-pCO-Vpx.T7pol plasmid (12.902 kb).

MVA right HS (614 bp) is flanked on both sides with two unique RE sites (AsiSI and NotI). MVA left HS (1157 bp) is also flanked on both sides with two unique RE sites (Acc651 and SpeI).

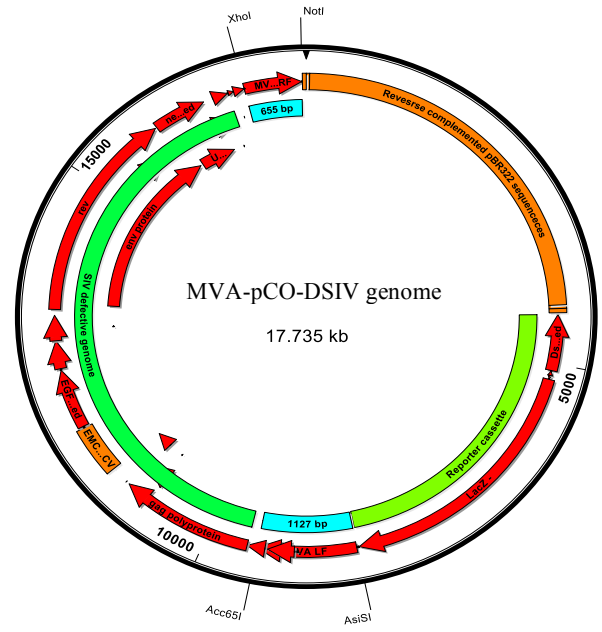

Figure 3.2: Schematic map of MVA-pCO-DSIV genome plasmid (17.735 kb).

MVA right HS (655 bp) is flanked on both sides with two unique RE sites (NotI and XhoI). MVA left HS (1127 bp) is also flanked on both sides with two unique RE sites (AsiSI and Acc651).

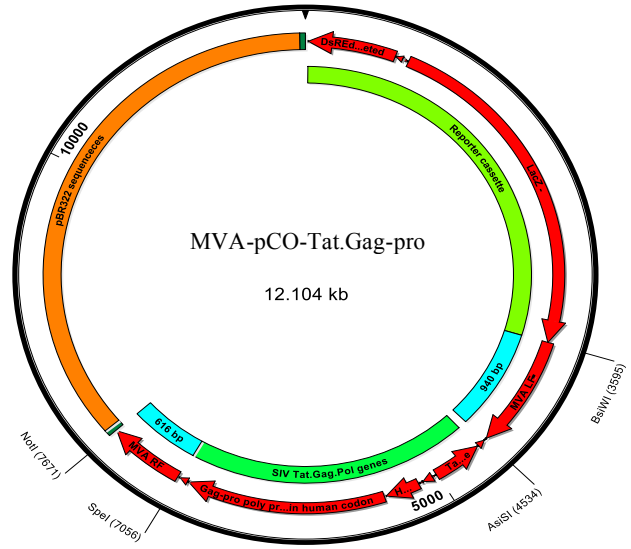

Figure 3.3: Schematic map of MVA-pCO-Tat.Gag-pro plasmid (12.104 kb).

MVA right HS (616 bp) is flanked on both sides with two unique RE sites (NotI and SpeI). MVA left HS (940 bp) is also flanked on both sides with two unique RE sites (AsiSI and BsiWI).

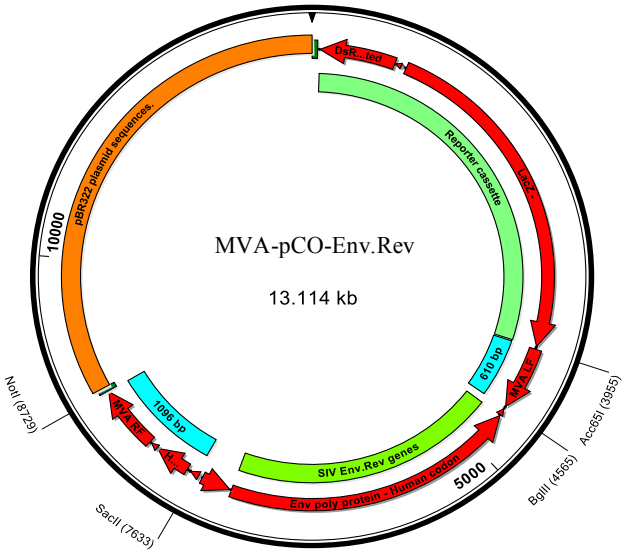

Figure 3.4: Schematic map of MVA-pCO-Env.Rev plasmid (13.114 kb).

MVA right HS (1096 bp) is flanked on both sides with two unique RE sites (SacII and NotI). MVA left HS (610 bp) is also flanked on both sides with two unique RE sites (Acc651 and Bgl II).

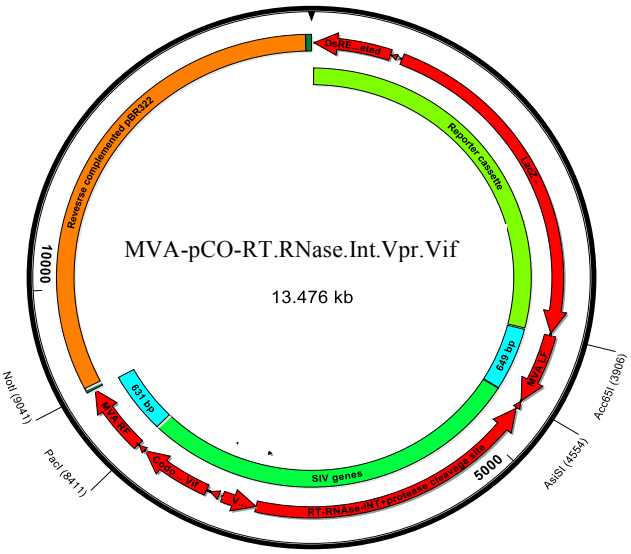

Figure 3.5: Schematic map of MVA-pCO-RT.RNase.Int.Vpr.Vif plasmid (13.476 kb).

MVA right HS (631 bp) is flanked on both sides with two unique RE sites (PacI and NotI). MVA left HS (649 bp) is also flanked on both sides with two unique RE sites (AsiSI and Acc651).

3.1.2 FP9 insertion sites

In order to construct rFP9 encoding SIVmac239 sequences, five potential insertion sites within the FP9 genome were chosen. Homologous flanking regions to these insertion sites were designed. These FP9 HS were flanked on both sides with the same unique RE sites, which were incorporated in the MVA transfer plasmid, in order to allow cloning of the FP9 HS into the previously constructed MVA transfer plasmid (Table 3.2). The following is a list of the five insertion sites within the FP9 genome:

- **F11L orthologue (ORF 110):**

F11L orthologue was used as an intragenic insertion site (nt 127003/4) for vpx sequence and T7 RNA polymerase cassette.

- **Thymidine kinase (TK) gene (ORF 86)**:

Non-essential region between the TK gene (ORF 86) and the downstream gene (ORF 87) was used as an intergenic insertion site (nt 88924/5) for tat and gag-pro SIVmac239 sequences.

- **Photolyase gene (ORF 158):**

Non-essential region between the photolyase gene (ORF 158) and the upstream gene (ORF 157) was used as an intergenic insertion site (nt 179664/5) for env and rev SIVmac239 sequences.

- **Intracellular mature virus (IMV) membrane protein (ORF 179.1)**:

IMV orthologue, which is located between ORFs 179 and 180, was used as an intragenic insertion site (nt 201566/7) for RT, RNase, int, vpr and vif SIVmac239 sequences.

- **Intergenic region between ORFs 6 and 7:**

Non-essential region between ORFs 6 and 7 was used as an intergenic insertion site (nt 7836/7) for the defective SIVmac239 (DSIV) genome.

FP9 insertion sites	FP9 right HS		FP9 left HS	
	Specific RE	Fragment size	Specific RE	Fragment size
F11L orthologue (ORF 110)	AsiSI and NotI	515 bp	KpnI and SpeI	543 bp
Between ORFs 6 and 7	NotI and XhoI	547 bp	AsiSI and KpnI	1037 bp
Between ORFs 86 and 87	NotI and SpeI	507 bp	AsiSI and BsiWI	831 bp
Between ORFs 157 and 158	SacII and NotI	1003 bp	Acc651 and Bgl II	506 bp
IMV (ORF 179.1)	PacI and NotI	521 bp	AsiSI and KpnI	543 bp

Table 3.2: Shows all five FP9 insertion sites with their homologous flanking regions. This table also shows the size of each FP9 HS with its specific RE sites.

3.1.3 Sequence maps for the newly synthesised FP9 HS plasmids

The following electronic versions of schematic maps for FP9 HS plasmids (Figures 3.6 to 3.10) were generated using the Lasergene™ Software. These maps provide all required information (such as the unique RE sites and the expected banding patterns of FP9 HS) that can be used for RE digestion analysis.

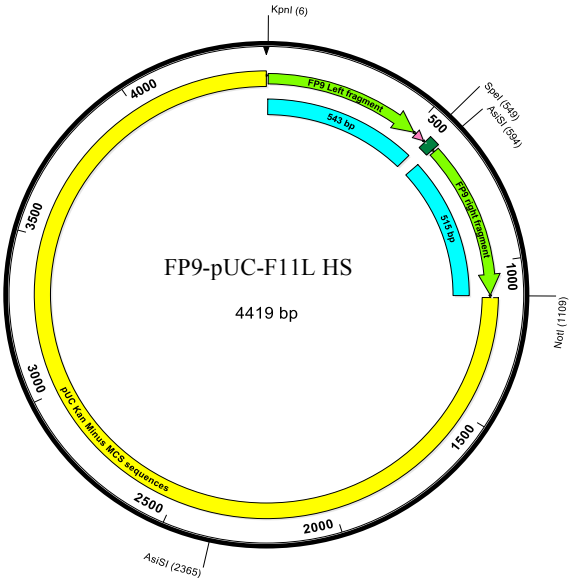

Figure 3.6: Schematic map of FP9-pUC-F11L HS plasmid (4419 bp).

FP9 right HS (515 bp) is flanked on both sides with two RE sites (AsiSI and NotI). AsiSI, which is not a unique RE, has two RE sites. However, FP9 left HS (543 bp) is flanked on both sides with two unique RE sites (KpnI and SpeI).

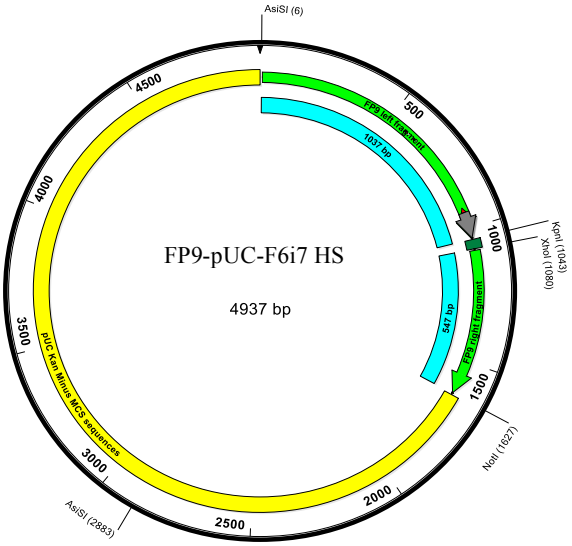

Figure 3.7: Schematic map of FP9-pUC-F6i7 HS plasmid (4937 bp).

FP9 right HS (547 bp) is flanked on both sides with two unique RE sites (NotI and XhoI). FP9 left HS (1037 bp) is flanked on both sides with two RE sites (AsiSI and KpnI). AsiSI, which is not a unique RE, has two RE sites.

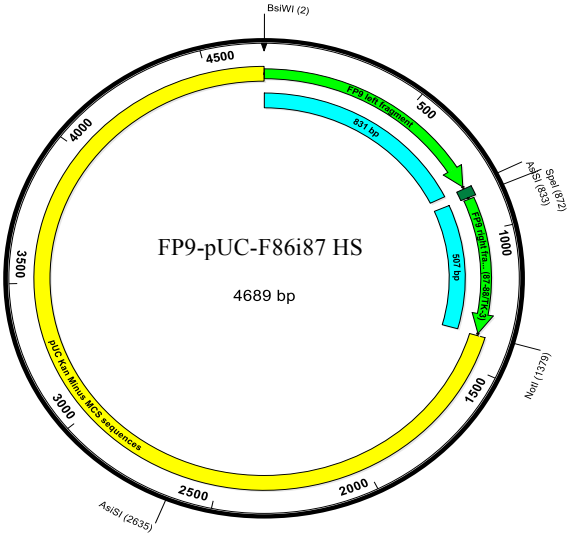

Figure 3.8: Schematic map of FP9-pUC-F86i87 HS plasmid (4689 bp).

FP9 right HS (507 bp) is flanked on both sides with two unique RE sites (NotI and SpeI). FP9 left HS (831 bp) is flanked on both sides with two RE sites (AsiSI and BsiWI). AsiSI, which is not a unique RE, has two RE sites.

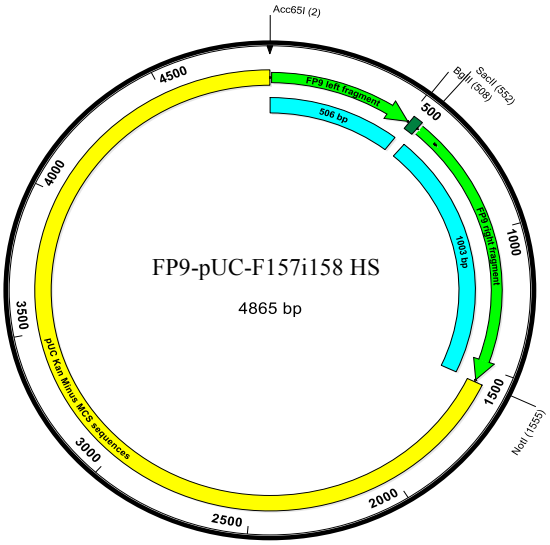

Figure 3.9: Schematic map of FP9-pUC-F157i158 HS plasmid (4865 bp).

FP9 right HS (1003 bp) is flanked on both sides with two unique RE sites (SacII and NotI). FP9 left HS (506 bp) is also flanked on both sides with two unique RE sites (Acc651 and Bgl II).

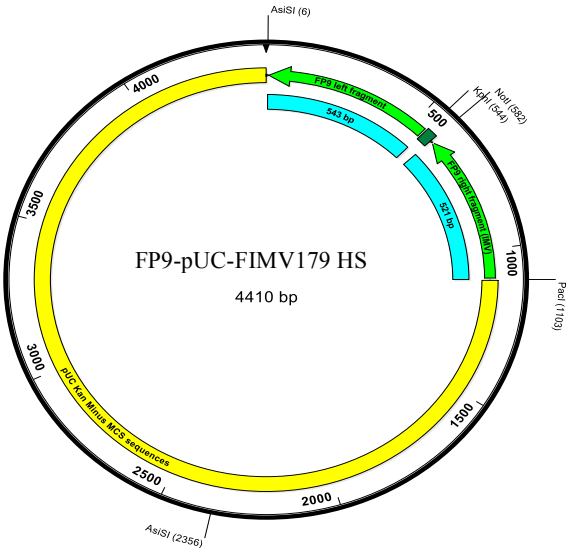

Figure 3.10: Schematic map of FP9-pUC-FIMV179 plasmid (4410 bp).

FP9 right HS (521 bp) is flanked on both sides with two unique RE sites (PacI and NotI). FP9 left HS (543 bp) is flanked on both sides with two RE sites (AsiSI and KpnI). AsiSI, which is not a unique RE, has two RE sites.

3.1.4 RE digestion analysis of MVA transfer plasmids

MVA transfer plasmids were redesigned as FP9 vectors encoding SIVmac239 sequences. In order for construction to occur, MVA transfer plasmids were double digested using unique RE sites to replace the MVA HS with the FP9 HS. However, in some of these MVA plasmids, MVA HS were not flanked with unique RE sites that can be used to remove the HS. Therefore, it was necessary to double digest the large fragment from the plasmid containing the target MVA HS. RE digestions were

performed to cleave out these HS. All MVA HS (10 DNA fragments) were successfully double digested and removed from the original MVA plasmid DNA. The following gel figures (Figures 3.11 and 3.12) represent the expected banding patterns for all the five MVA transfer plasmids. All observed banding patterns gave the expected molecular sizes based on the generated sequence maps.

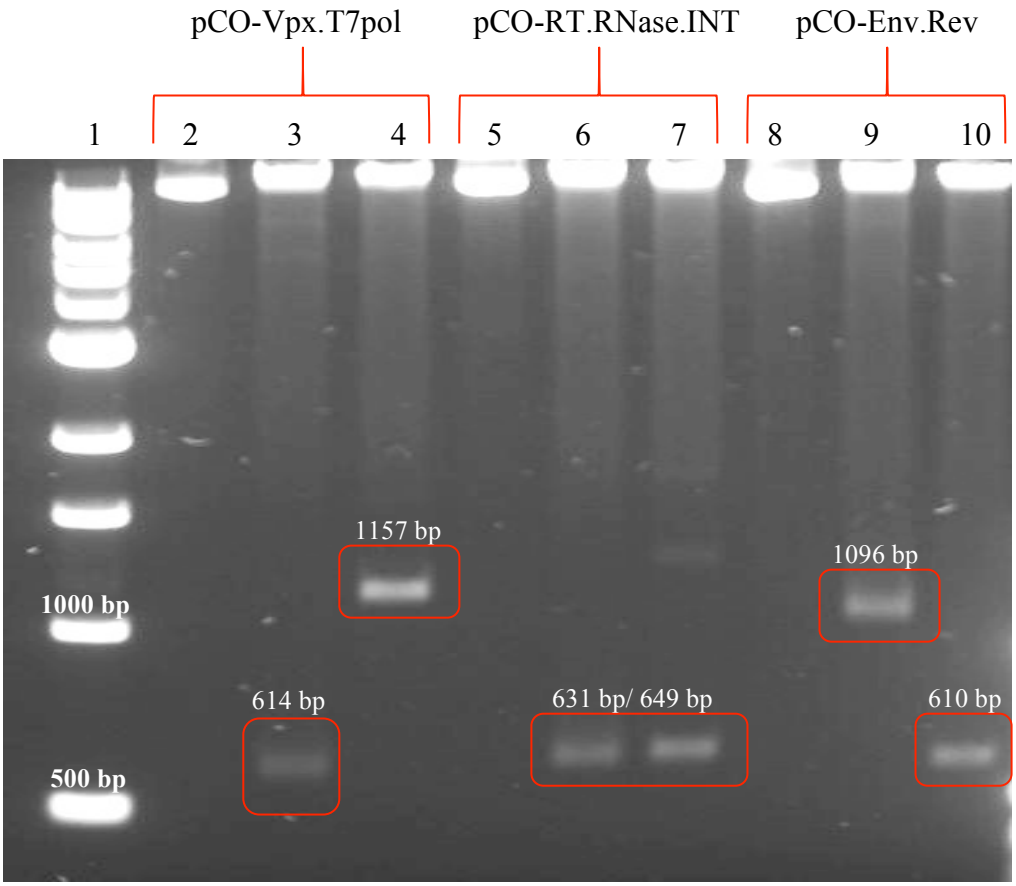

Figure 3.11: Shows the restriction banding profile of three MVA transfer plasmids (pCO-Vpx.T7pol, pCO-RTRNase.INT.Vpr.Vif, and pCO-Env.Rev).

Lane 1 represents the banding profile of a 1 kb DNA ladder, lanes 2, 5, and 8 represent different forms of the uncut plasmid DNA, lanes 3, 6, and 9 represent the expected two bands corresponding to the MVA right HS (RHS) and the linearised form of the plasmid, lanes 4, 7, and 10 represent the expected two bands corresponding to the MVA left HS (LHS) and the linearised form of the plasmid.

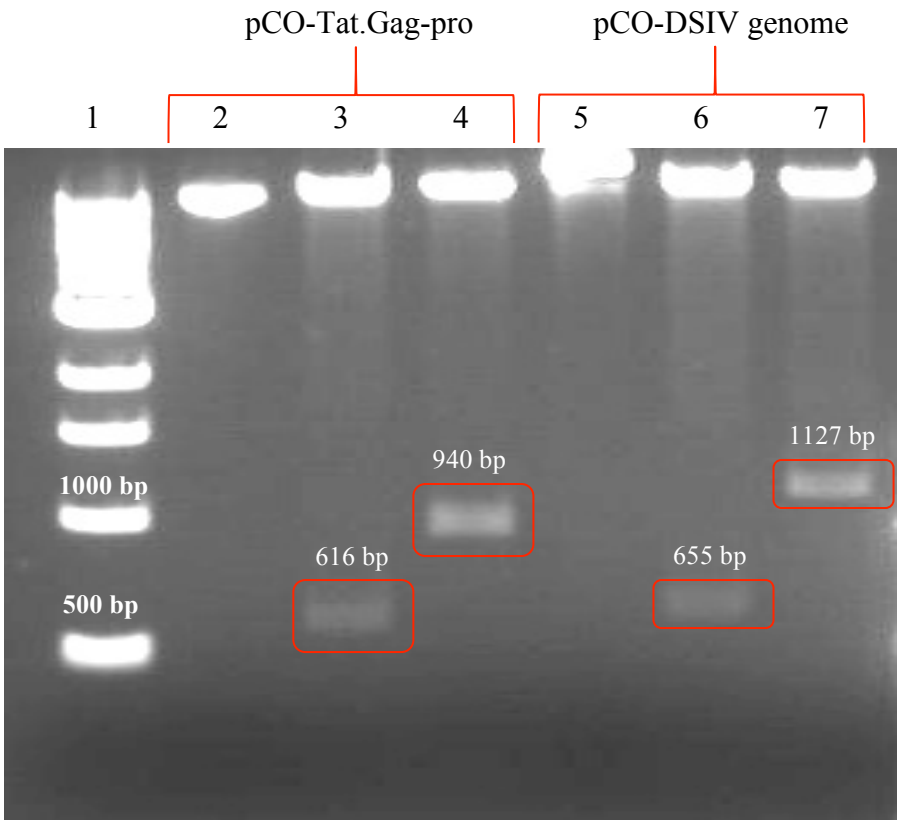

Figure 3.12: Shows the restriction banding profile of two MVA transfer plasmids (pCO-Tat.Gag-pro and pCO-DSIV genome).

Lane 1 represents the banding profile of a 1 kb DNA ladder, lanes 2 and 5 represent different forms of the uncut plasmid (original plasmid DNA), lanes 3 and 6 represent the expected two bands corresponding to the MVA RHS and the linearised form of the plasmid, lanes 4 and 7 represent the expected two bands corresponding to the MVA LHS and the linearised form of the plasmid.

3.1.5 RE digestion analysis of the synthesised FP9 HS

The synthesised FP9 HS were analysed using specific RE sites, in order to confirm that BlueHeron^{TM} Company has synthesised the correct DNA sequences based on the submitted sequence maps. For each plasmid construct, RE digestion analyses of four different transformed colonies plus their original plasmid DNA were carried out, followed by gel electrophoresis. Banding patterns from these four digested colonies were compared with the original (purified) plasmid DNA supplied by the BlueHeron^{TM} Company (Figures 3.13 to 3.17). In addition, sequence trace data for all five FP9 plasmids were aligned to the submitted sequences using SeqMan and MegAlign alignment programmes (Lasergene^{TM} Software) (Data not shown). All FP9 plasmids were successfully analysed (through RE digestion analysis and sequence alignment) and confirmed. The following gel figures (Figures 3.13 to 3.17) represent the expected banding patterns for all the five FP9 HS plasmids. All the observed banding patterns gave the expected molecular sizes based on the generated sequence maps.

All the following electrophoretic gels have a standard layout. Inner and outer lanes are molecular weight markers. Lanes 2, 3, and 4 are original plasmid DNA supplied by Blue Heron. Lane 2 is uncut DNA, lanes 3 and 4 are double digests to isolate the right homologous sequence (RHS) and left homologous sequence (LHS) recombination flanks respectively. Lanes 5–7 follows the same pattern with transformant colony 1, then lanes 8–9; 10–11; 12–13 are three additional transformant colonies showing double digests for RHS and LHS but not uncut plasmid DNA.

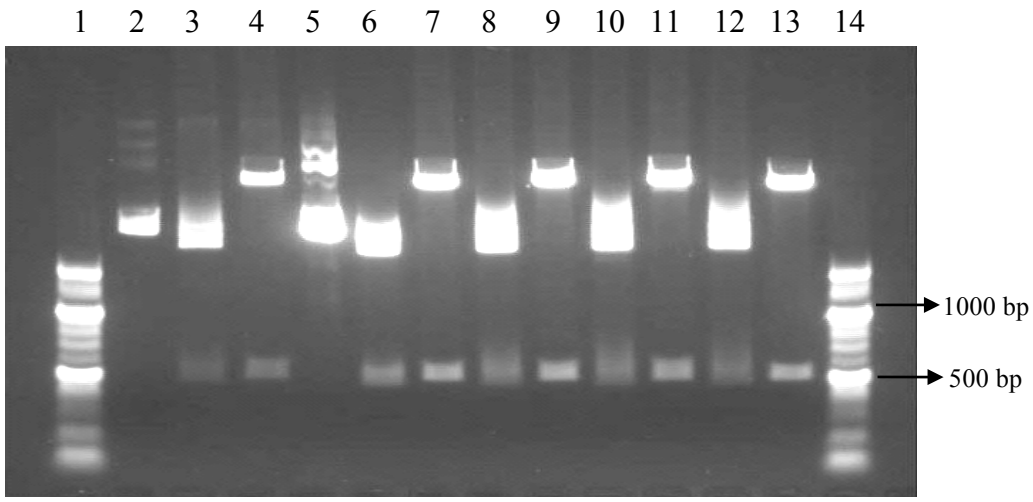

Figure 3.13: Shows the restriction banding profile of FP9-pUC-F11L HS plasmid. Lanes 1 and 14 represent the banding profile of a 100 bp DNA ladder, lanes 2 and 5 represent different forms of the uncut plasmid (original plasmid DNA and 1st colony, respectively), lanes 3, 6, 8, 10, and 12 represent the expected two bands corresponding to the RHS (515 bp) and the linearised form of the plasmid, and finally lanes 4, 7, 9, 11, and 13 represent the expected two bands corresponding to the LHS (543 bp) and the linearised form of the plasmid.

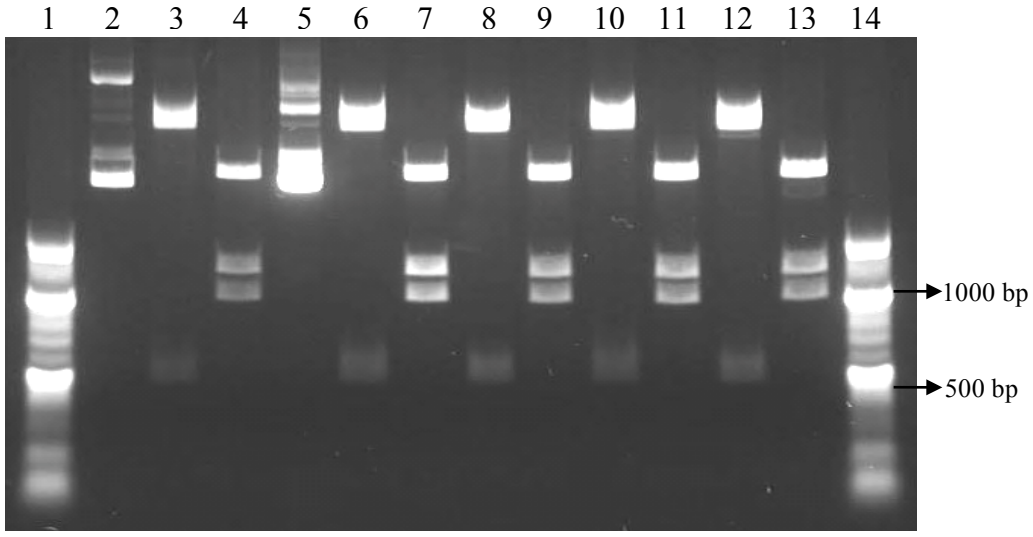

Figure 3.14: Shows the restriction banding profile of FP9-pUC-F6i7 HS plasmid. Lanes 1 and 14 represent the banding profile of a 100 bp DNA ladder, lanes 2 and 5 represent different forms of the uncut plasmid (original plasmid DNA and 1st colony, respectively), lanes 3, 6, 8, 10, and 12 represent the expected two bands corresponding to the RHS (547 bp) and the linearised form of the plasmid, and finally lanes 4, 7, 9, 11, and 13 represent the expected three bands corresponding to the LHS (1037 bp) and two others expected from the plasmid.

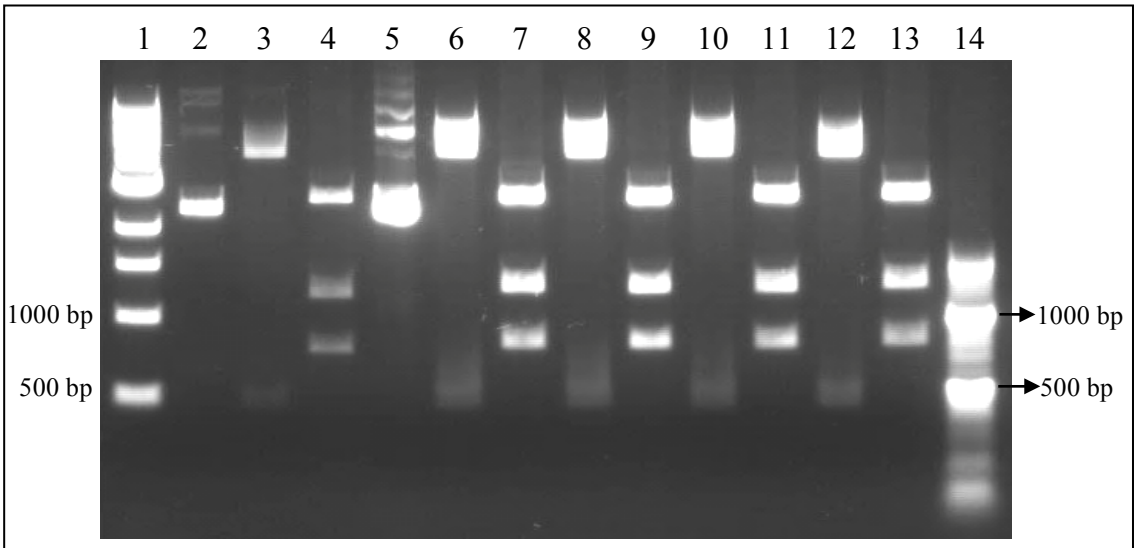

Figure 3.15: Shows the restriction banding profile of FP9-pUC-F86i87 HS plasmid. Lane 1 represents the banding profile of a 1 kb DNA ladder, lanes 2 and 5 represent different forms of the uncut plasmid (original plasmid DNA and 1st colony, respectively), lanes 3, 6, 8, 10, and 12 represent the expected two bands corresponding to the RHS (507 bp) and the linearised form of the plasmid, lanes 4, 7, 9, 11, and 13 represent the expected three bands corresponding to the LHS (831 bp) and two others expected from the plasmid, and finally lane 14 represents the banding profile of a 100 bp DNA ladder.

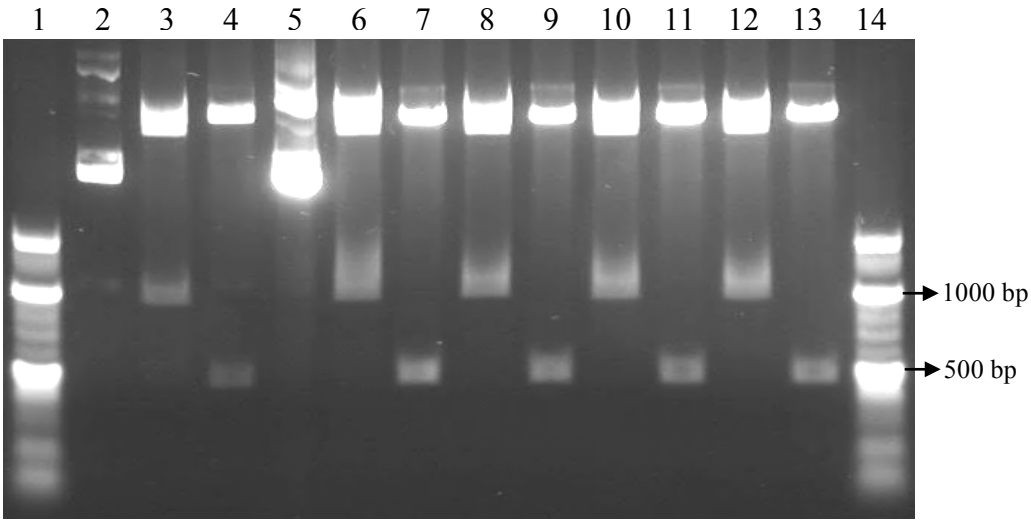

Figure 3.16: Shows the restriction banding profile of FP9-pUC-F157i158 HS plasmid. Lanes 1 and 14 represent the banding profile of a 100 bp DNA ladder, lanes 2 and 5 represent different forms of the uncut plasmid (original plasmid DNA and 1st colony, respectively), lanes 3, 6, 8, 10, and 12 represent the expected two bands corresponding to the RHS (1003 bp) and the linearised form of the plasmid, and finally lanes 4, 7, 9, 11, and 13 represent the expected two bands corresponding to the LHS (506 bp) and the linearised form of the plasmid.

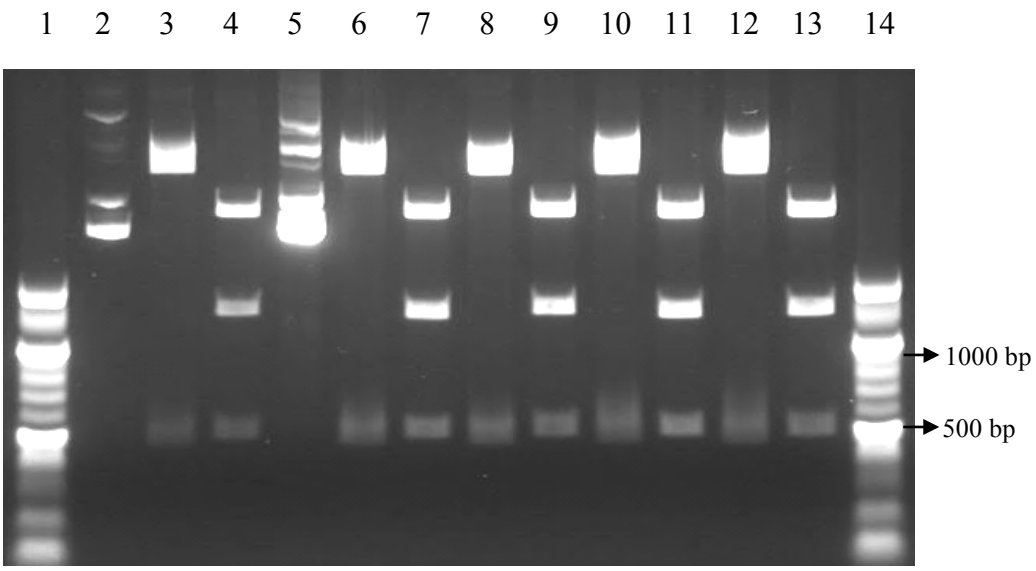

Figure 3.17: Shows the restriction banding profile of FP9-pUC-FIMV179 plasmid. Lanes 1 and 14 represent the banding profile of a 100 bp DNA ladder, lanes 2 and 5 represent different forms of the uncut plasmid (original plasmid DNA and 1st colony, respectively), lanes 3, 6, 8, 10, and 12 represent the expected two bands corresponding to the RHS (521 bp) and the linearised form of the plasmid, and finally lanes 4, 7, 9, 11, and 13 represent the expected three bands corresponding to the LHS (543 bp) and two others expected from the plasmid.

3.1.6 Generation of five FP9 transfer plasmids encoding various SIVmac239 sequences

All ten FP9 HS were cloned into five different MVA transfer plasmids (two FP9 HSs for each MVA plasmid). MVA HS were cleaved out using double digestion reaction, and FP9 HS were then ligated into each target plasmid DNA through two-step gene cloning. Recombinant plasmids DNA were then transformed into SCS1 supercompetent *E.coli* cells. Different single transformed colonies (Figure 3.18) were picked and their recombinant DNA was extracted, digested, and successfully analysed using RE digestion analysis.

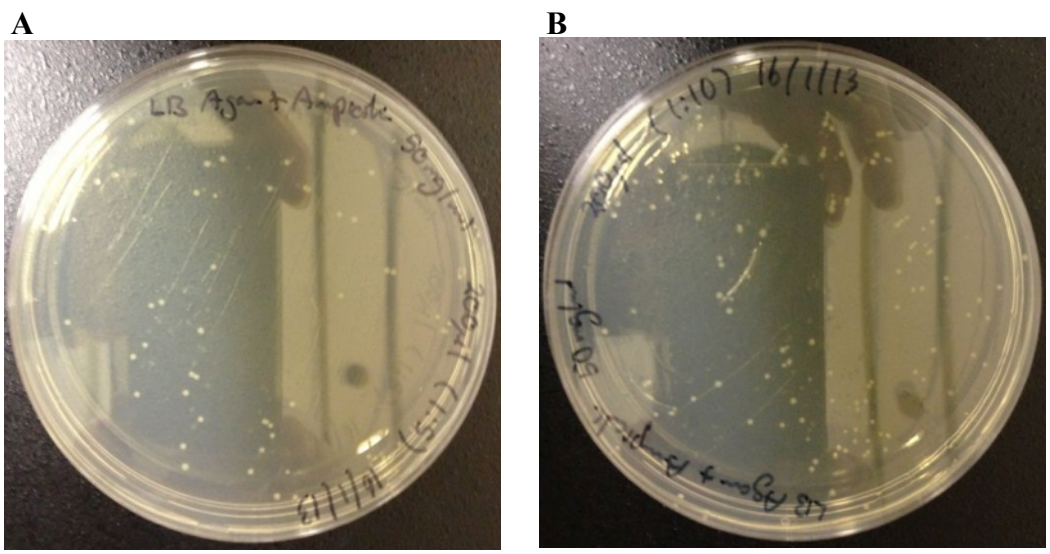

Figure 3.18: Shows selective LB nutrient agar plates with many transformed colonies after successful transformation of SCS1 supercompetent cells with the target recombinant plasmid DNA.

A) shows the results of using 1:5 molar ratio of vector to insert in ligation step, while B) shows that ligation efficiency was improved by increasing the ratio to 1:10.

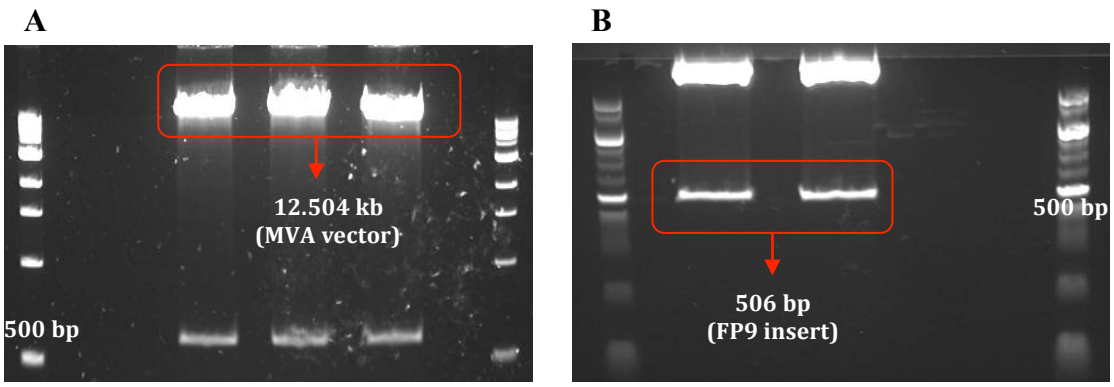

Figure 3.19: Shows an example of complete RE digestion of both MVA vector (A) and FP9 insert (B) prior to the ligation step.

These digested bands were then excised from the gel, purified, ligated and transformed.

The following electrophoretic gel figures show the positive transformed colonies that gave digested banding patterns with the expected molecular size. These banding patterns were compared with the banding profile of the original FP9 HS plasmid (+ve FP9 control) containing the FP9 HS to confirm the presence of the intended FP9 insert. The figures also show that only the original MVA transfer plasmid (+ve MVA control) produced digested bands corresponding to the previous MVA HS that have been replaced. These results indicate that all FP9 HS were successfully cloned into the previously constructed MVA transfer plasmids.

In terms of control plates, few single colonies were observed on the negative control plate containing the linear plasmid alone. However, the control plates that contain only competent cells, gave the expected results (many single colonies were observed on the positive control plate, while no colonies were seen on the negative control plate). These results prove the viability of the competent cells and the validity of the selection method that were used in this experiment.

3.1.6.1 Construction of FP9 transfer plasmid encoding env and rev SIVmac239 genes

FP9 transfer plasmid carrying env and rev sequences was constructed by replacing both MVA HS (right and left) of the MVA-pCO-Env.Rev transfer plasmid with FP9 HS from the FP9-pUC-F157i158 plasmid (Figures 3.20 and 3.21).

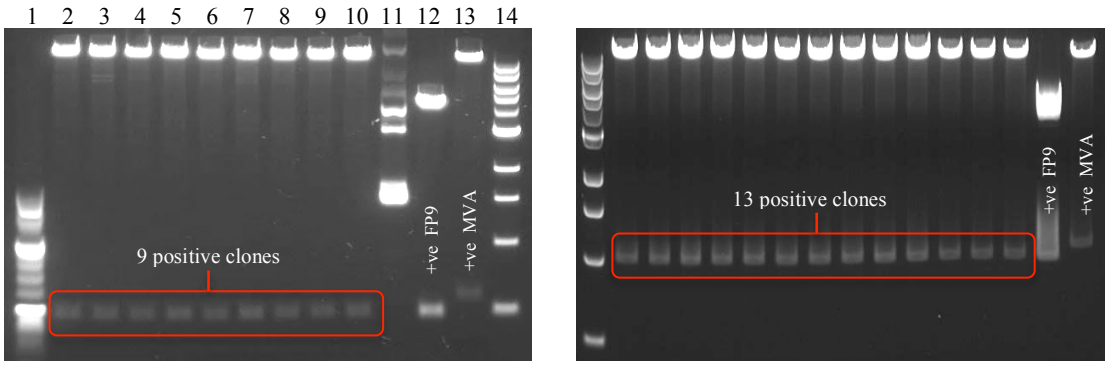

Figure 3.20: Gel electrophoresis to analyse the positive recombinant FP9-pCO-Env.Rev plasmids DNA of different transformed colonies after double digestion the LF with Acc651 and Bgl II RE, and the RF with NotI and SacII RE.

Lane 1 100 bp DNA ladder, lanes 2–10 plasmids DNA from nine different transformed colonies (all pBR322-based), lane 12 pUC plasmid containing newly synthesised FP9 flanking sequences, lane 13 pBR322 plasmid containing MVA flanking region being replaced, and lane 14 1 kb DNA ladder.

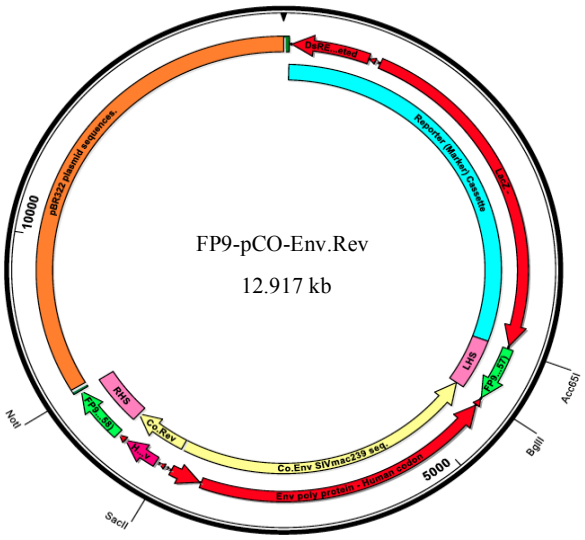

Figure 3.21: Schematic map of FP9-pCO-Env.Rev transfer plasmid DNA containing env and rev SIVmac239 sequences.

3.1.6.2 Construction of FP9 transfer plasmid encoding T7 RNA polymerase and vpx SIVmac239 genes

FP9 transfer plasmid containing T7 polymerase and vpx sequences was constructed by replacing both MVA HS of the MVA-pCO-Vpx.T7pol transfer plasmid with FP9 HS from the FP9-pUC-F11L plasmid (Figures 3.22 and 3.23).

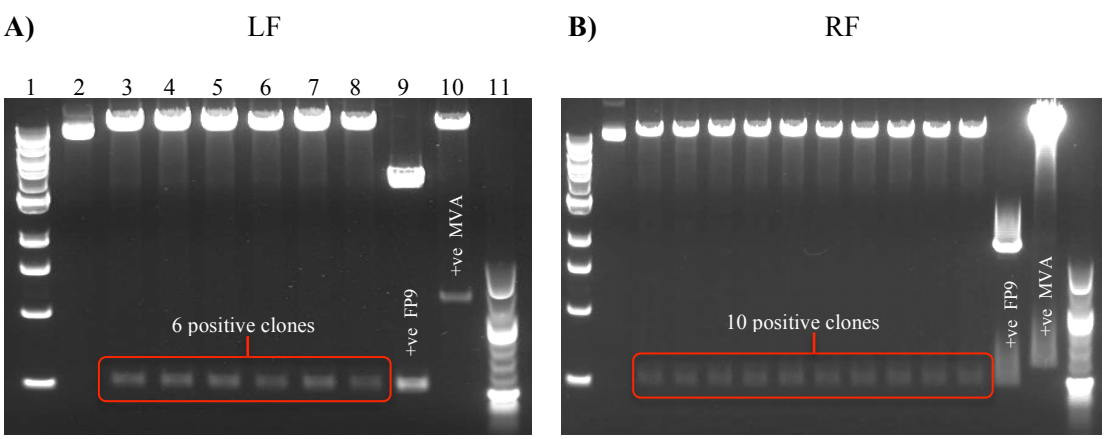

Figure 3.22: Gel electrophoresis to analyse the positive recombinant FP9-pCO-Vpx.T7pol plasmids DNA of different transformed colonies after double digestion the LF with SpeI and KpnI RE, and the RF with NotI and AsiSI RE.

Lane 1 1 kb DNA ladder, lane 2 uncut plasmid DNA from the first transformed colony, lanes 3–8 plasmids DNA from six different transformed colonies (all pBR322-based), lane 9 pUC plasmid containing newly synthesised FP9 flanking sequences, lane 10 pBR322 plasmid containing MVA flanking region being replaced, and lane 11 100 bp DNA ladder.

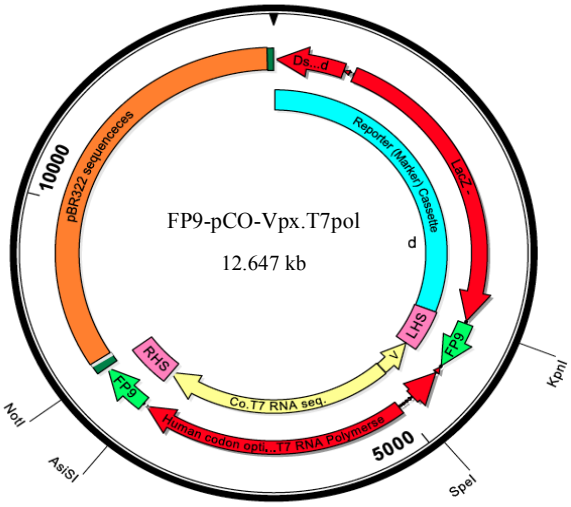

Figure 3.23: Schematic map of FP9-pCO-Vpx.T7pol transfer plasmid DNA containing T7 polymerase and Vpx SIVmac239 sequences.

3.1.6.3 Construction of FP9 transfer plasmid encoding tat and gag-pro SIVmac239 genes

FP9 transfer plasmid encoding tat and gag-pro sequences was constructed by replacing both MVA HS of the MVA-pCO-Tat.Gag-pro transfer plasmid with FP9 HS from the FP9-pUC-F86i87 plasmid (Figures 3.24 and 3.25).

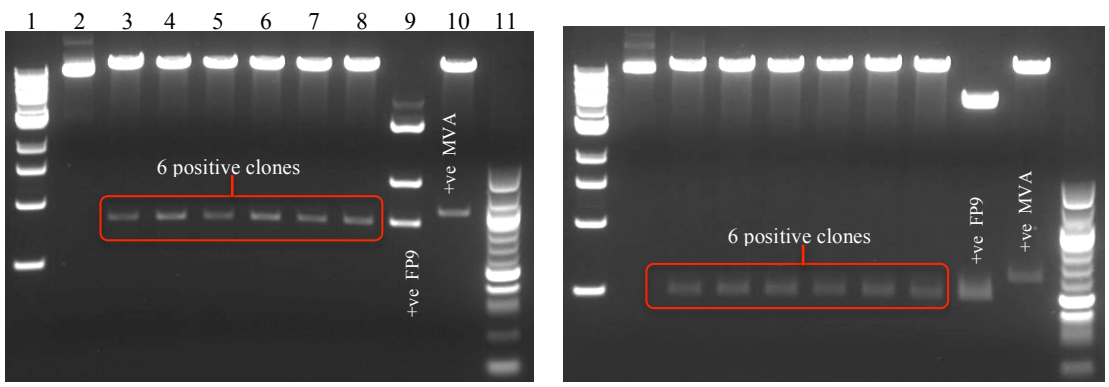

Figure 3.24: Gel electrophoresis to analyse the positive recombinant FP9-pCO-Tat.Gag-pro plasmids DNA of different transformed colonies after double digestion the LF with AsiSI and BsiWI RE, and the RF with NotI and SpeI RE.

Lane 1 1 kb DNA ladder, lane 2 uncut plasmid DNA from the first transformed colony, lanes 3–8 plasmids DNA from six different transformed colonies (all pBR322-based), lane 9 pUC plasmid containing newly synthesised FP9 flanking sequences, lane 10 pBR322 plasmid containing MVA flanking region being replaced, and lane 11 100 bp DNA ladder.

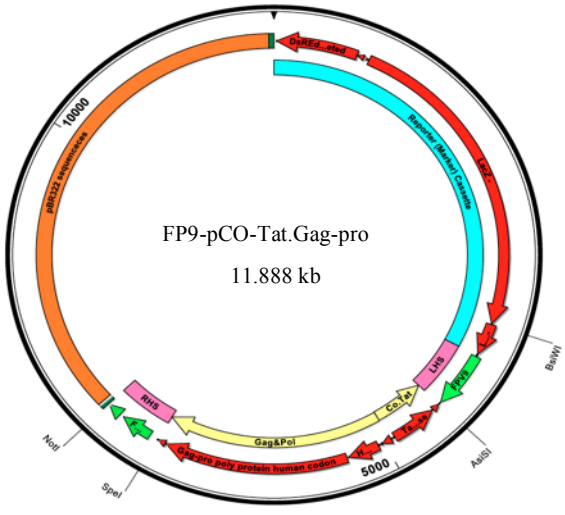

Figure 3.25: Schematic map of FP9-pCO-Tat.Gag-pro transfer plasmid DNA containing tat and gag-pro SIVmac239 sequences.

3.1.6.4 Construction of FP9 transfer plasmid encoding RT, RNase, int, vpr, and vif SIVmac239 genes

FP9 transfer plasmid carrying RT, RNase, int, vpr and vif sequences was constructed by replacing both MVA HS of the MVA-pCO-RT.RNase.Int.Vpr.Vif transfer plasmid with FP9 HS from the FP9-pUC-FIMV179 plasmid (Figures 3.26 and 3.27).

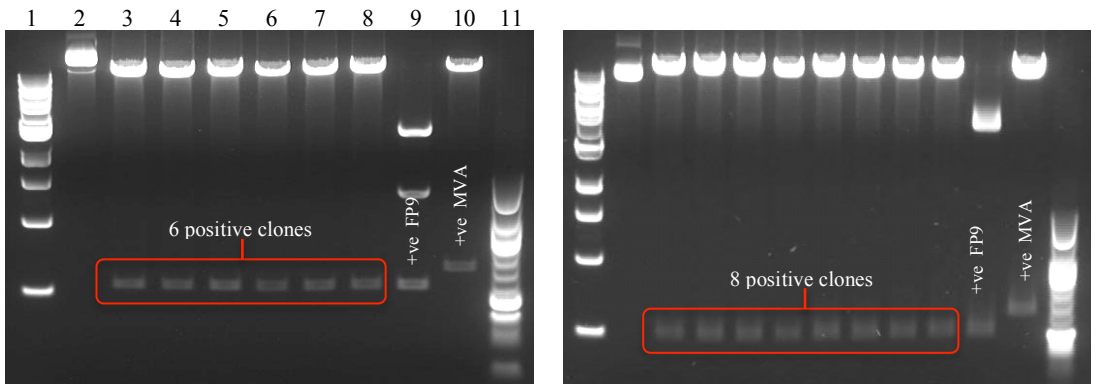

Figure 3.26: Gel electrophoresis to analyse the positive recombinant FP9-pCO-RT.RNase.INT.Vpr.Vif plasmids DNA of different transformed colonies after double digestion the LF with AsiSI and KpnI, and the RF with NotI and PacI RE.

Lane 1 1 kb DNA ladder, lane 2 uncut plasmid DNA from the first transformed colony, lanes 3–8 plasmids DNA from six different transformed colonies (all pBR322-based), lane 9 pUC plasmid containing newly synthesised FP9 flanking sequences, lane 10 pBR322 plasmid containing MVA flanking region being replaced, and lane 11 100 bp DNA ladder.

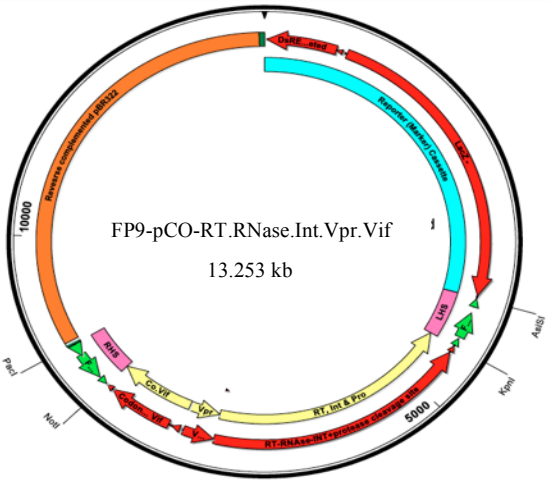

Figure 3.27: Schematic map of FP9-pCO-RT.RNase.Int.Vpr.Vif transfer plasmid containing RT, int, vpr, and vif SIVmac239 sequences.

3.1.6.5 Construction of FP9 transfer plasmid encoding the defective SIVmac239 (DSIV) genome

FP9 transfer plasmid encoding the DSIV genome was constructed by replacing both MVA HS of the MVA-pCO-DSIV transfer plasmid with FP9 HS from the FP9-pUC-F6i7 plasmid (Figures 3.28 and 3.29).

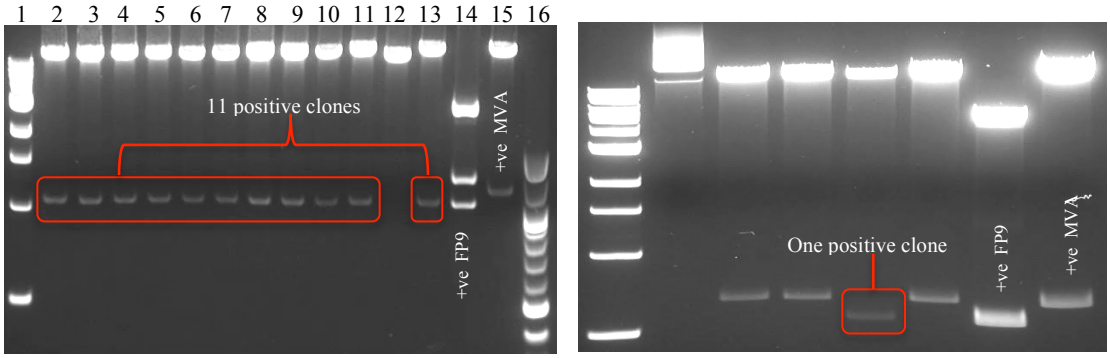

Figure 3.28: Gel electrophoresis to analyse the positive recombinant FP9-pCO-DSIV plasmids DNA of different transformed colonies after double digestion the LF with AsiSI and KpnI RE, and the RF with NotI and XhoI RE.

Lane 1 1 kb DNA ladder, lanes 2–13 plasmids DNA from twelve different transformed colonies (all pBR322-based), lane 14 pUC plasmid containing newly synthesised FP9 flanking sequences, lane 15 pBR322 plasmid containing MVA flanking region being replaced, and lane 16 100 bp DNA ladder.

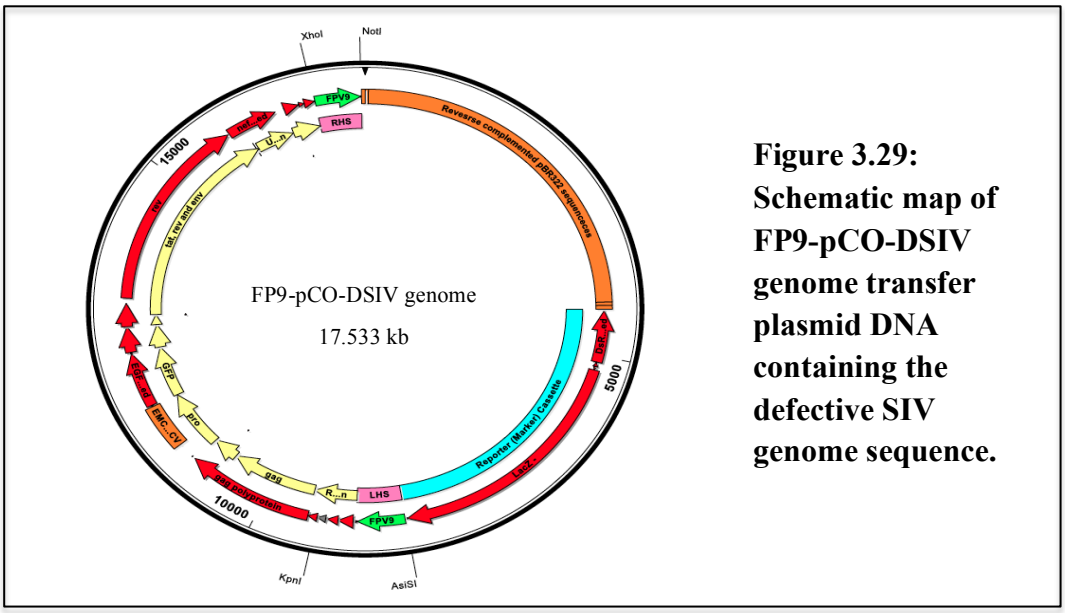

Figure 3.29: Schematic map of FP9-pCO-DSIV genome transfer plasmid DNA containing the defective SIV genome sequence.

3.2 Construction of rFP9

3.2.1 Cell processing of pCEFs

25–30 SPF eggs were incubated for 10 days and then processed to produce pCEFs. These primary cell lines were checked for viability and sterility. Following 2 days of incubation at 37˚C in 5% CO_2 incubator, they were grown to almost 95% confluence and achieved a good growth rate. In addition, there was no bacterial or fungal growth (contamination) in the tissue culture flask that was incubated for 8 days. This suggests that the entire batch of the pCEFs is viable and suitable for use.

3.2.2 Virus titration

Plaque assay was used to titrate nonrecombinant and recombinant viruses. These viruses were able to produce clear and large plaques on pCEF monolayers within 5–6 days after virus infection. Many plaques (uncountable) were observed in wells infected with lower virus dilutions (10^{-3} and 10^{-4}). However, isolated and countable plaques were observed in higher virus dilutions wells (from 10^{-6} to 10^{-8}). The negative control wells showed no plaques indicating that there was no contamination with virus infection. The virus titre was calculated in plaque-forming units (pfu)/ml (Table 3.3).

Virus name	Encoding sequences	NO. of passages	Virus titre
Nonrecombinant FP9	------	4	6×10^7 pfu/ml
rFP9.Env.Rev	Env and rev SIVmac239 sequences	13	4×10^8 pfu/ml
rFP9.Vpx.T7pol	T7 polymerase and vpx SIVmac239 sequences	11	2×10^8 pfu/ml

Table 3.3: Shows the results of the titration assay for nonrecombinant and two rFP9s.

3.2.3 HR in pCEFs

HR between FP9 HS in the transfer plasmid DNA flanking the target genes and the HS of the FP9 genome was carried out in pCEFs to construct rFP9. After 48–72 h of infection/transfection, clear CPE and numerous changes were observed in the infected pCEFs (Figure 3.30). However, normal cell morphology (fibroblasts) was seen in the cell control well (Figure 3.31). The transfection efficiency and toxicity were also assessed, and it was found that the SuperFect reagent was highly efficient and had no CPE against the primary cell line used (Figures 3.32 and 3.33).

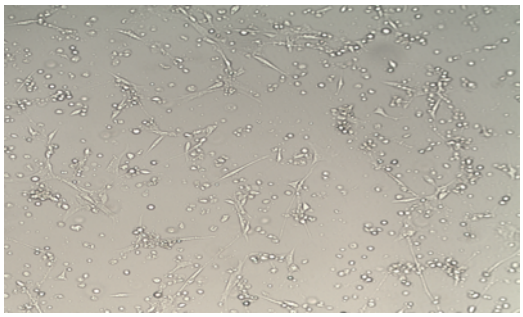

Figure 3.30: Microscopic image (at 10X) shows a clear CPE in pCEFs infected with rFP9.

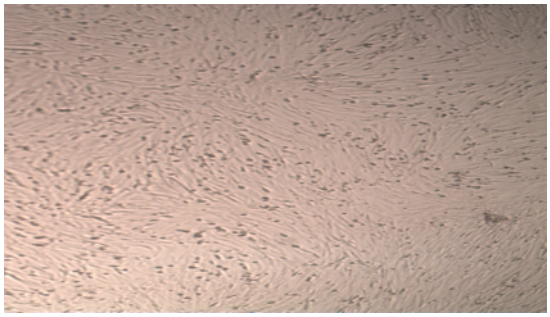

Figure 3.31: Microscopic image (at 4X) of normal cell morphology of pCEFs in the cell control well.

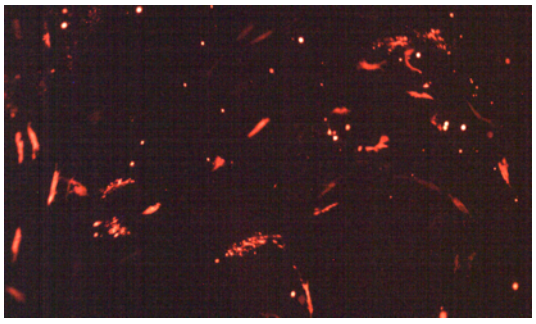

Figure 3.32: Microscopic image (at 10X) of RFP expression in pCEFs indicating high transfection efficiency.

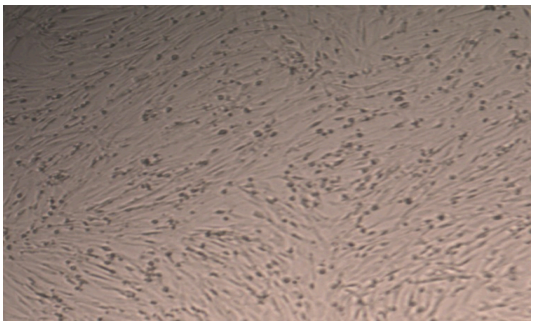

Figure 3.33: Microscopic image (at 4X) shows the normal cell appearance of pCEFs after transfection reagent addition.

3.2.4 Screening of rFP9 plaques under X-gal

rFP9 viruses were identified using plaque assay on the basis of β-gal expression under X-gal. These recombinant viruses produce β-galactosidase enzyme that cleaves the X-gal substrate to form a blue pigment. Hence, successful HR was confirmed by blue plaque formation that can be clearly visualised, particularly in the fourth or fifth round of plaque purification (PP4/PP5) (Figure 3.34). However, during the first round of purification, it was difficult to identify and pick the blue plaques due to their small size and faint blue colour. It was estimated that 0.8% of rFP9 plaques from the infection/transfection mixture expressed β-galactosidase. Around 50% of these recombinant viruses could be recovered, and all were PCR positive for target sequences. Single and well-isolated blue plaques were picked only from wells infected with the highest virus dilutions (10^{-5} and 10^{-6}), in order to reduce the number of plaque purification assays needed to isolate pure recombinant virus (Figure 3.35). Figures 3.36 and 3.37 show the microscopic appearance of rFP9 plaques before and after X-gal substrate addition (Figures 3.36 and 3.37).

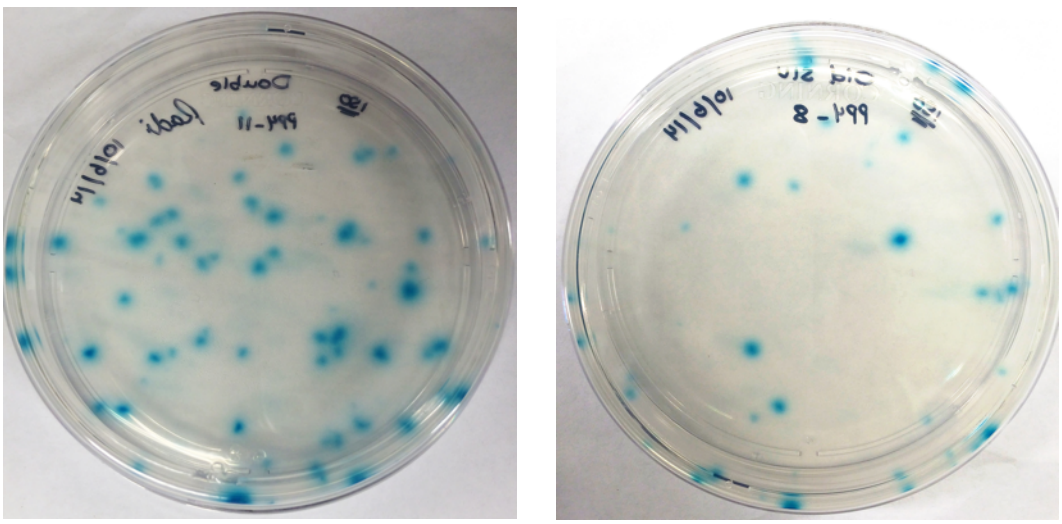

Figure 3.34: Appearance of lacZ positive rFP9 plaques under a second agarose overlay containing X-gal.

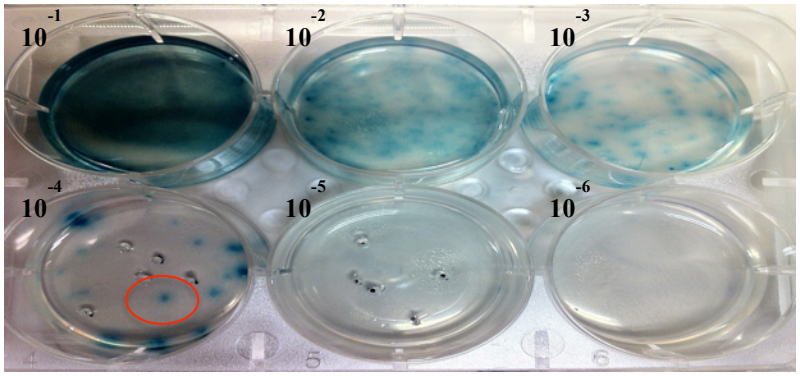

Figure 3.35: Shows blue plaque formation on screening positive rFP9.

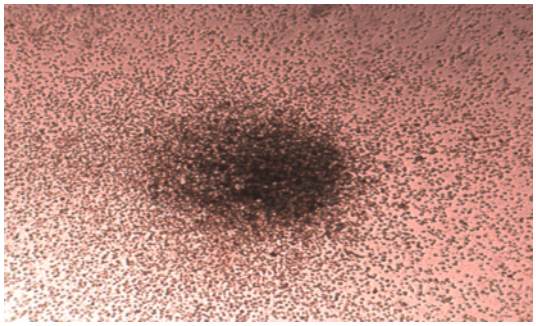

Figure 3.36: Microscopic image of a colourless plaque formation in pCEFs prior to the second X-gal overlay.

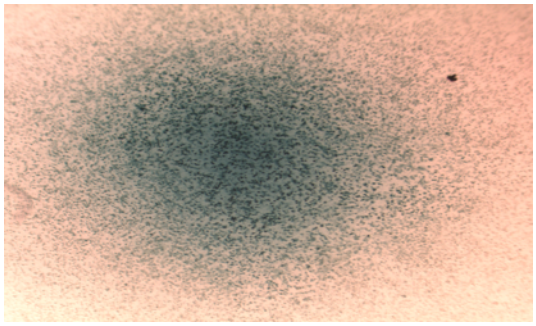

Figure 3.37: Microscopic image of a blue plaque formation in pCEFs under the X-gal overlay.

3.2.5 PCR confirmation of insertion cassette within recombinant blue plaques

Conventional PCR assay was carried out to confirm the presence of the target sequences (insertion cassette) within the rFP9 genome. Following the first and fifth rounds of blue plaque purification, several plaque samples were expanded and PCR screened to ensure the absence of the nonrecombinant virus and to avoid needless plaque passages. Successful integration of SIVmac239 sequences was demonstrated by amplifying the target regions within the recombinant genome using specific sets of primers. PCR was used to confirm the preliminary construction of rFP9 carrying the gene of interest, and to discriminate the recombinant genome form the nonrecombinant virus genome.

Appropriate negative and positive control samples were included to ensure the reliability and the validity of the experiment. All three-control samples gave the

expected results. A strong banding pattern with the expected molecular size of the amplified products appeared in the positive control well (FP9 transfer plasmid DNA). However, no bands (amplification signals) were seen on the two negative control wells (total FP9 and pCEFs DNA). Sizes of PCR products for target sequences were compared with the plasmid positive control. A 100 bp DNA ladder was also included in the same gel as a size reference for the PCR products.

3.2.5.1 rFP9 encoding env and rev SIVmac239 sequences

Env and rev SIVmac239 sequences were inserted into the non-essential region (intergenic site) between ORFs 158 and 157 within the FP9 genome. The following figures show the PCR-positive recombinant plaques for both env (515 bp) and rev (458 bp) sequences in the fifth round of blue plaque purification (Figures 3.38 and 3.39). These PCR results demonstrate the successful integration of the SIV env and rev sequences into the FP9 genome.

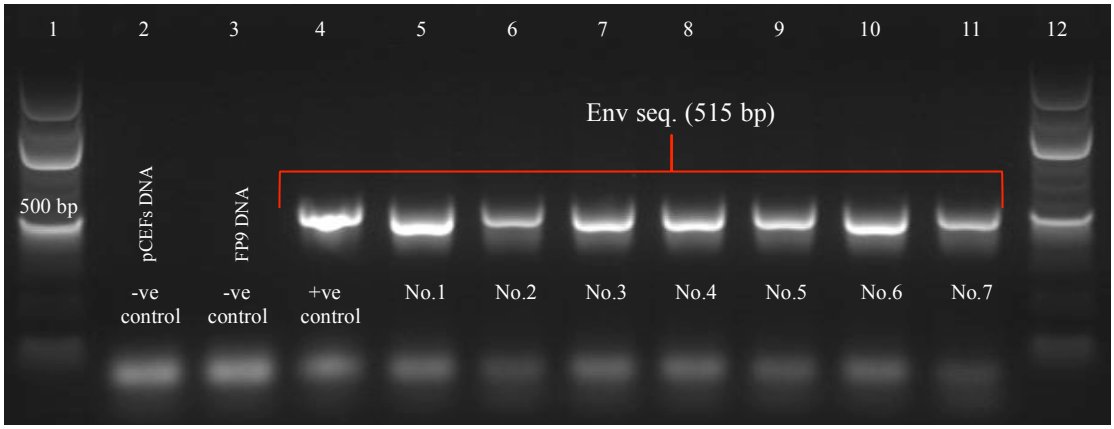

Figure 3.38: Detection of SIVmac239 env sequence within seven recombinant plaque samples after the 5th round of blue plaque purification of the rFP9 encoding env and rev sequences.

Lanes 1 and 12 100 bp ladder, lane 2 pCEFs total DNA (-ve control), lane 3 FP9 total DNA (-ve control), lane 4 FP9.Env.Rev transfer plasmid DNA (+ve control for env sequence), and lanes 5–11 positive blue plaque samples for SIV env sequence.

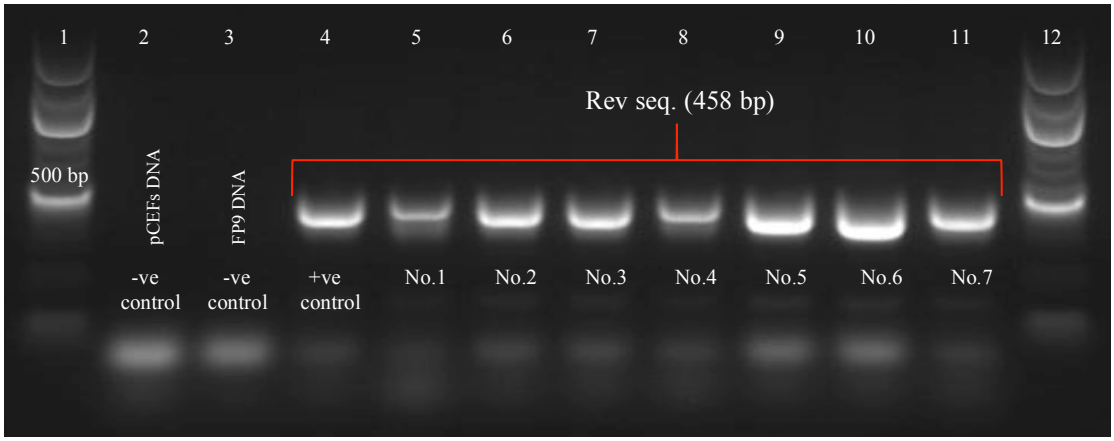

Figure 3.39: Detection of SIVmac239 rev sequence within seven recombinant plaque samples after the 5th round of blue plaque purification of the single rFP9 encoding env and rev sequences.

Lanes 1 and 12 100 bp ladder, lane 2 pCEFs total DNA (-ve control), lane 3 FP9 total DNA (-ve control), lane 4 FP9.Env.Rev transfer plasmid DNA (+ve control for rev sequence), and lanes 5–11 positive blue plaque samples for SIV rev sequence.

3.2.5.2 Double rFP9 encoding env/rev and tat/gag-pro SIVmac239 sequences

rFP9 containing env and rev SIVmac239 sequences was used as a backbone recombinant virus to add tat and gag-pro SIV sequences into the non-essential region (intergenic site) between the TK gene (ORF 86) and ORF 87 to make double rFP9. The following figures show the PCR-positive recombinant plaques for both insertion cassettes (Env/rev and tat [477 bp]/gag [577 bp]) after the fifth round of blue plaque purification (Figures 3.40, 3.41, 3.42, and 3.43). These PCR results illustrate the successful preliminary construction of double rFP9 encoding env/rev and tat/gag-pro SIV sequences.

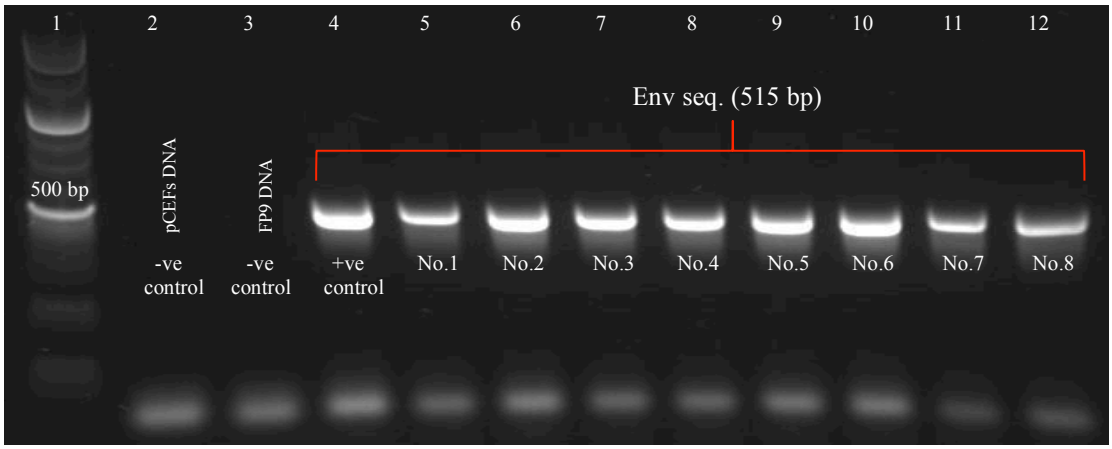

Figure 3.40: Detection of SIVmac239 env sequence within eight recombinant plaque samples after the 5th round of blue plaque purification of the double rFP9 encoding env/rev and tat/gag-pro sequences.

Lane 1 100 bp ladder, lane 2 pCEFs total DNA (-ve control), lane 3 FP9 total DNA (-ve control), lane 4 FP9.Env.Rev transfer plasmid DNA (+ve control for env sequence), and lanes 5–12 positive blue plaque samples for SIV env sequence.

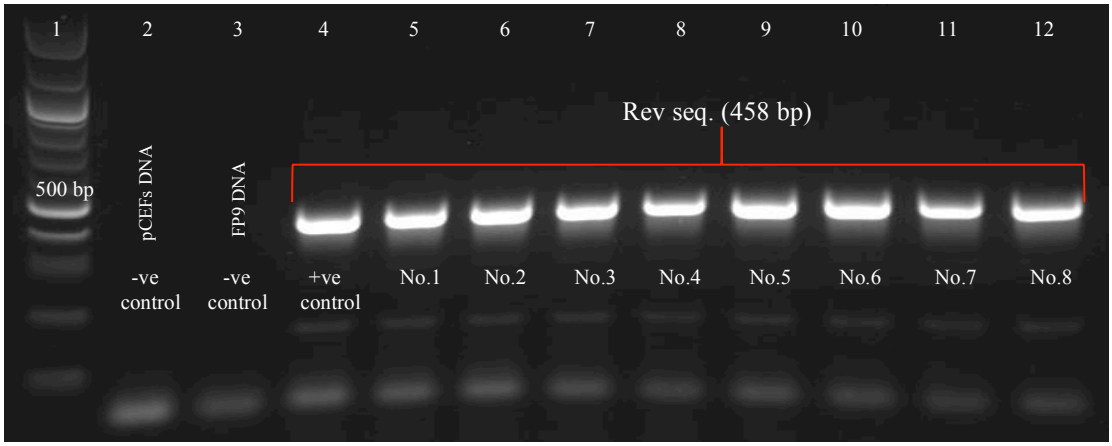

Figure 3.41: Detection of SIVmac239 rev sequence within eight recombinant plaque samples after the 5th round of blue plaque purification of the double rFP9 encoding env/rev and tat/gag-pro sequences.

Lane 1 100 bp ladder, lane 2 pCEFs total DNA (-ve control), lane 3 FP9 total DNA (-ve control), lane 4 FP9.Env.Rev transfer plasmid DNA (+ve control for rev sequence), and lanes 5–12 positive blue plaque samples for SIV rev sequence.

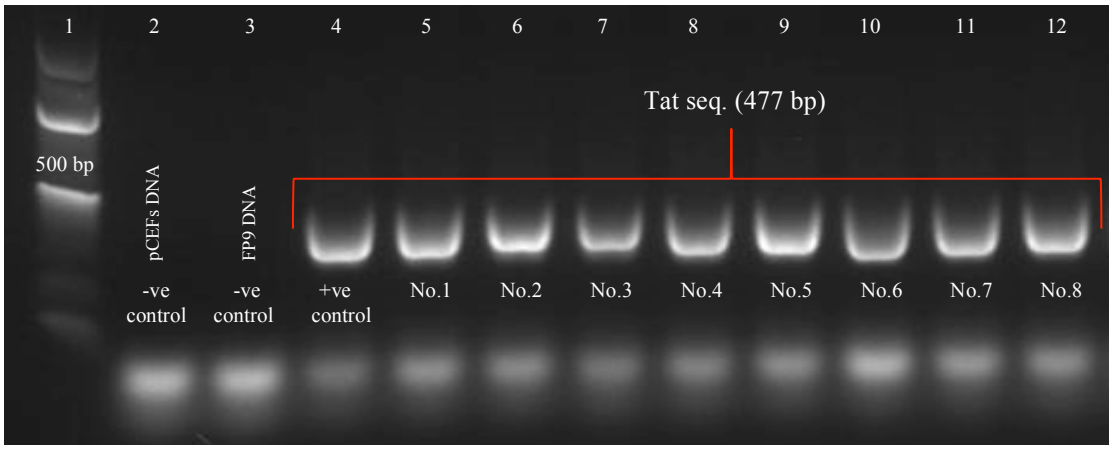

Figure 3.42: Detection of SIVmac239 tat sequence within eight recombinant plaque samples after the 5th round of blue plaque purification of the double rFP9 encoding env/rev and tat/gag-pro sequences.

Lane 1 100 bp ladder, lane 2 pCEFs total DNA (-ve control), lane 3 FP9 total DNA (-ve control), lane 4 FP9.Tat.Gag-pro transfer plasmid DNA (+ve control for tat sequence), and lanes 5–12 positive blue plaque samples for SIV tat sequence.

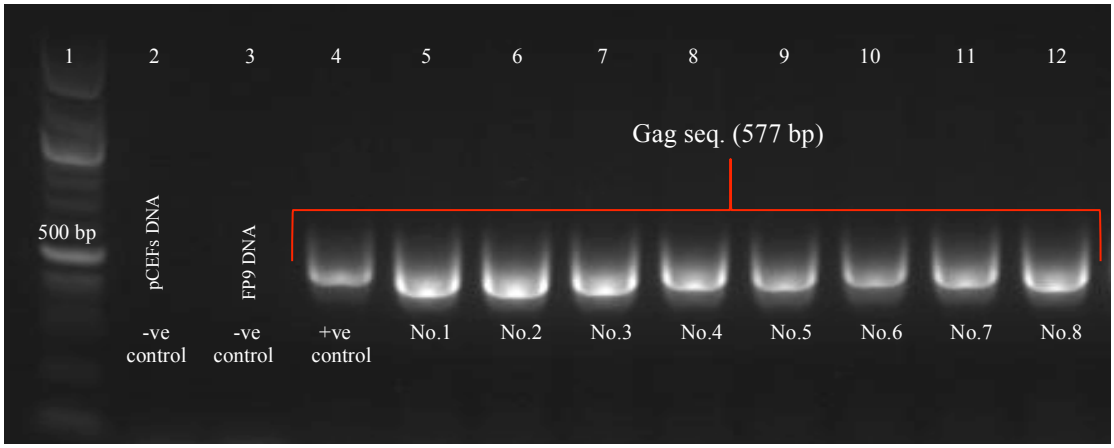

Figure 3.43: Detection of SIVmac239 gag sequence within eight recombinant plaque samples after the 5th round of blue plaque purification of the double rFP9 encoding env/rev and tat/gag-pro sequences.

Lane 1 100 bp ladder, lane 2 pCEFs total DNA (-ve control), lane 3 FP9 total DNA (-ve control), lane 4 FP9.Tat.Gag-pro transfer plasmid DNA (+ve control for gag sequence), and lanes 5–12 positive blue plaque samples for SIV gag sequence.

3.2.5.3 rFP9 encoding T7 RNA polymerase and vpx SIVmac239 sequences

T7 polymerase and vpx SIV sequences were inserted into the F11L orthologue (OFR 110) within the FP9 genome. The following figure shows the PCR-positive recombinant plaque samples for both T7 polymerase (404 bp) and vpx (439 bp) SIV sequences after the fifth round of blue plaque purification (Figure 3.44). This PCR result demonstrates the successful integration of the T7 and vpx sequences into the FP9 genome.

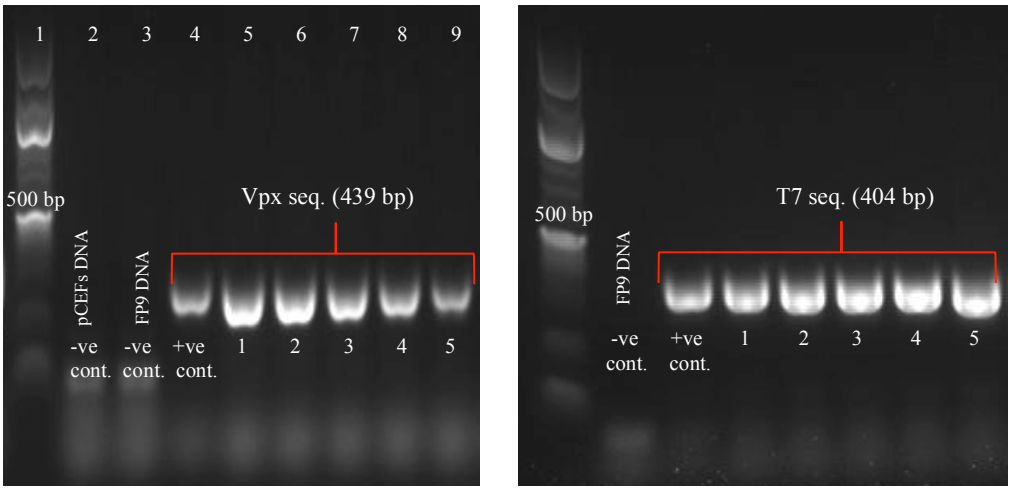

Figure 3.44: Detection of vpx and T7 polymerase sequences within five recombinant plaque samples after the 5th round of blue plaque purification of the single rFP9 encoding T7 and vpx sequences.

Lane 1 100 bp ladder, lane 2 pCEFs total DNA (-ve control), lane 3 FP9 total DNA (-ve control), lane 4 FP9.Vpx.T7pol transfer plasmid DNA (+ve control), and lanes 5–9 positive blue plaque samples for vpx sequence.

3.2.5.4 Double rFP9 encoding vpx/T7pol and the DSIV genome

rFP9 containing T7 polymerase and vpx SIVmac239 sequences was used as a backbone recombinant virus to add the DSIV genome into a different insertion site to make double rFP9. The following figures show the PCR-positive recombinant plaque samples for both insertion cassettes (vpx/T7pol and the DSIV genome) after the fifth round of blue plaque purification (Figure 3.45 and 3.46). These PCR results illustrate the

successful preliminary construction of double rFP9 encoding both vpx/T7pol sequences and the DSIV genome.

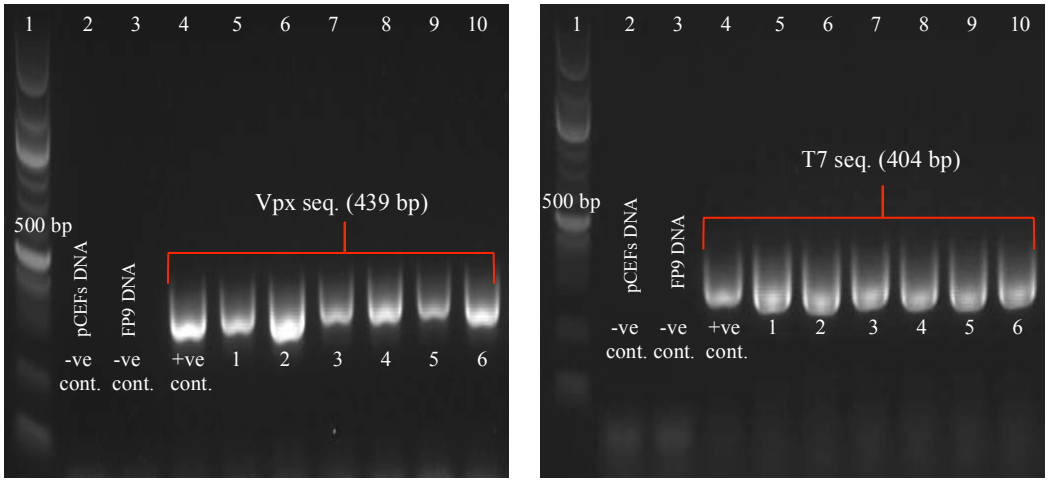

Figure 3.45: Detection of vpx and T7 polymerase sequences within six recombinant plaque samples after the 5th round of blue plaque purification of the double rFP9 encoding both vpx/T7pol and the DSIV genome.

Lane 1 100 bp ladder, lane 2 pCEFs total DNA (-ve control), lane 3 FP9 total DNA (-ve control), lane 4 FP9.Vpx.T7pol transfer plasmid DNA (+ve control), and lanes 5–10 positive blue plaque samples for both vpx and T7 sequences.

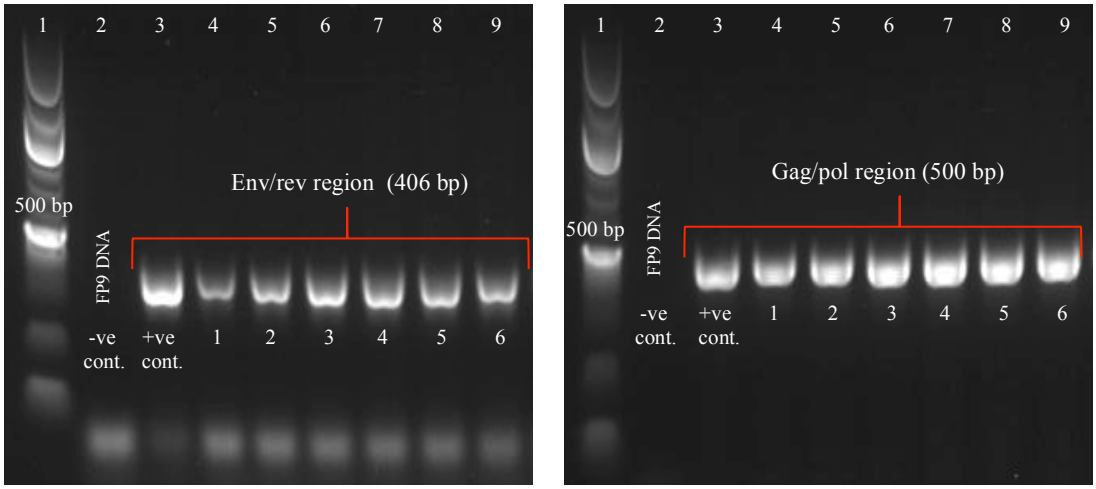

Figure 3.46: Detection of two SIV genomic regions (env/rev and gag/pol) within six recombinant plaque samples after the 5th round of blue plaque purification of the double rFP9 encoding both vpx/T7pol and the DSIV genome.

Lane 1 100 bp ladder, lane 2 FP9 total DNA (-ve control), lane 3 FP9.DSIV genome transfer plasmid DNA (+ve control), and lanes 4–9 positive blue plaque samples for both env/rev and gag/pol SIV genomic regions.

3.2.5.5 rFP9 encoding RT, RNase, int, vpr, and vif SIVmac239 sequences

RT, RNase, int, vpr and vif SIV sequences were inserted into the IMV orthologue (ORF 179.1) within the FP9 genome. The following figures show the PCR-positive recombinant plaque samples for RT (568 bp) and vif (452 bp) SIV sequences after the third round of blue plaque purification (Figures 3.47 and 3.48). These PCR results demonstrate the successful integration of both RT and vif sequences into the FP9 genome.

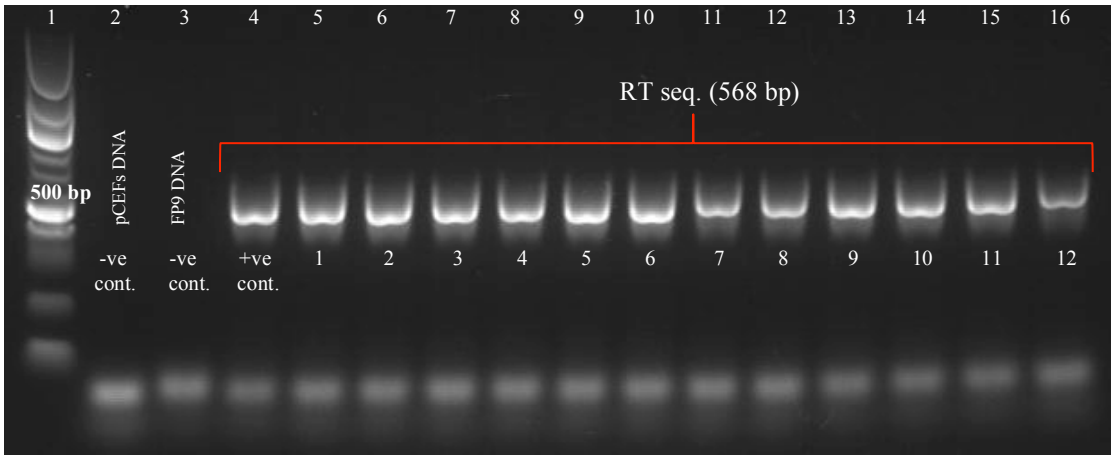

Figure 3.47: Detection of SIVmac239 RT sequence within twelve recombinant plaque samples after the 3rd round of blue plaque purification of the single rFP9.

Lane 1 100 bp ladder, lane 2 pCEFs total DNA (-ve control), lane 3 FP9 total DNA (-ve control), lane 4 FP9.RT.RNase.int.vpr.vif transfer plasmid DNA (+ve control for RT sequence), and lanes 5–16 positive blue plaque samples for SIV RT sequence.

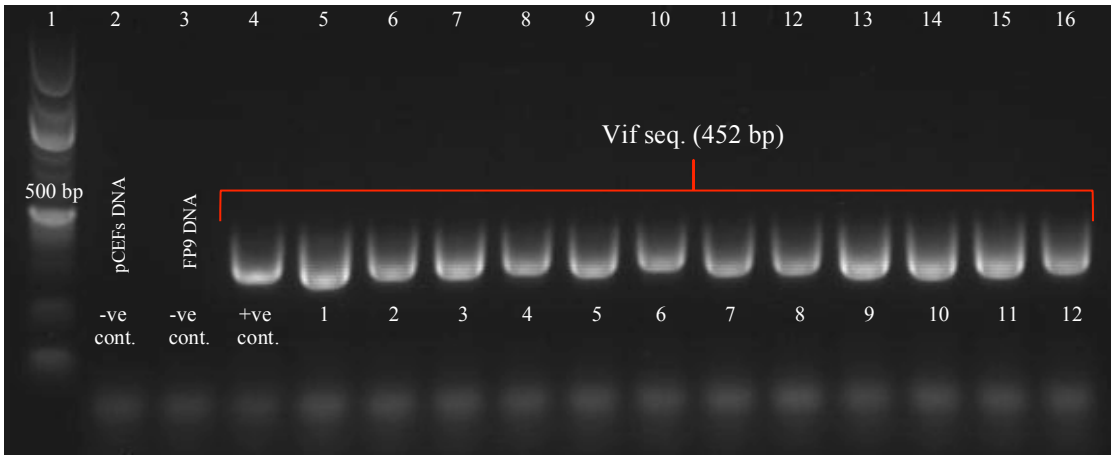

Figure 3.48: Detection of SIVmac239 vif sequence within twelve recombinant plaque samples after the 3rd round of blue plaque purification of the single rFP9.

Lane 1 100 bp ladder, lane 2 pCEFs total DNA (-ve control), lane 3 FP9 total DNA (-ve control), lane 4 FP9.RT.RNase.int.vpr.vif transfer plasmid DNA (+ve control for vif sequence), and lanes 5–16 positive blue plaque samples for SIV vif sequence.

3.2.6 PCR confirmation of pure markerless recombinant plaques

As the marker gene (LacZ) was designed to be efficiently eliminated from the recombinant genome, PCR-positive blue plaque samples underwent several rounds of subsequent colourless plaque purification to eliminate the marker gene and to ensure the purity of the recombinant virus. Four to five consecutive rounds of colourless plaque purification were performed on pCEFs. At this stage of purification, pure recombinants and nonrecombinant viruses had to be distinguished by PCR assay.

PCR results of markerless recombinant plaques illustrate the successful integration of insertion cassette into the FP9 genome, and by repetitive screening of 12–14 colourless plaques proves the genomic stability of both the inserted DNA sequences and the FP9 insertion sites. The stability of these pure rFP9s was maintained after a total of ten passages in pCEFs. In addition, large-scale expansion of the pure recombinant virus was

performed in three T-175 flasks to further assess the stability of the inserted materials, and to prepare large virus stocks for further manipulation using different insertion sites.

3.2.6.1 Pure (markerless) single rFP9 encoding env and rev SIVmac239 sequences

Figures 3.49, 3.50, 3.51, and 3.52 show PCR results of fourteen colourless plaque samples following the fourth round of colourless plaque purification, with positive amplification results for both env and rev SIV sequences, and negative results for the marker gene (LacZ) and the plasmid (pBR322) sequences. These PCR results confirm both the absence of the selectable marker gene, and also the stability of the SIV insertion cassettes within the single rFP9 construct.

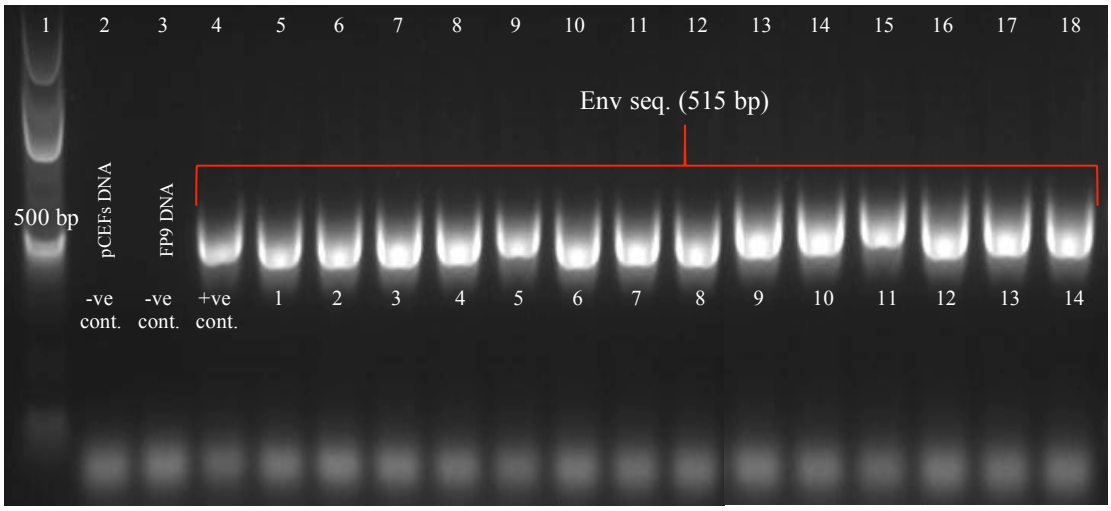

Figure 3.49: Detection of SIVmac239 env sequence within fourteen recombinant plaque samples after the 4th round of colourless plaque purification of the single rFP9 encoding env and rev sequences.

Lane 1 100 bp ladder, lane 2 pCEFs total DNA (-ve control), lane 3 FP9 total DNA (-ve control), lane 4 FP9.Env.Rev transfer plasmid DNA (+ve control for env sequence), and lanes 5–18 positive colourless plaque samples for SIV env sequence.

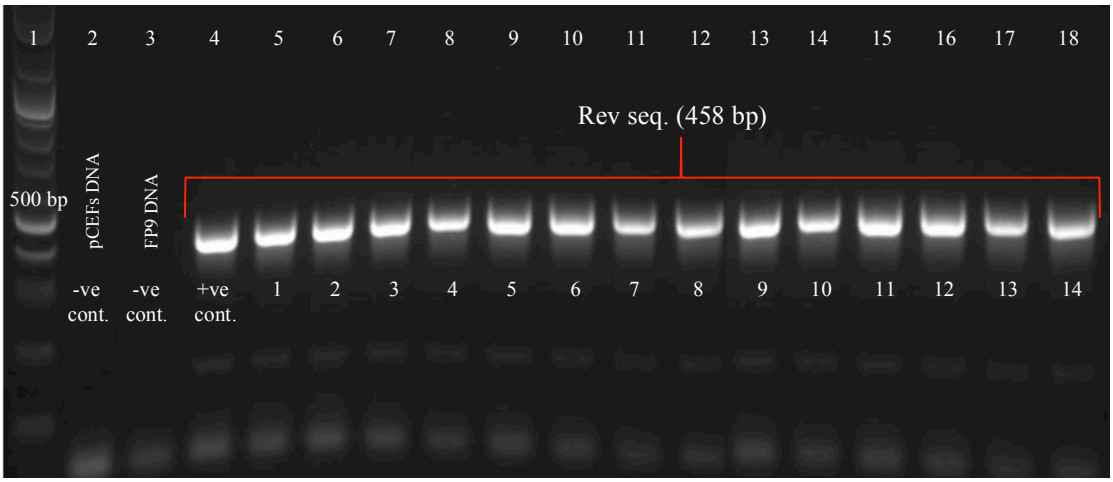

Figure 3.50: Detection of SIVmac239 rev sequence within fourteen recombinant plaque samples after the 4th round of colourless plaque purification of the single rFP9 encoding env and rev sequences.

Lane 1 100 bp ladder, lane 2 pCEFs total DNA (-ve control), lane 3 FP9 total DNA (-ve control), lane 4 FP9.Env.Rev transfer plasmid DNA (+ve control for rev sequence), and lanes 5–18 positive colourless plaque samples for SIV rev sequence.

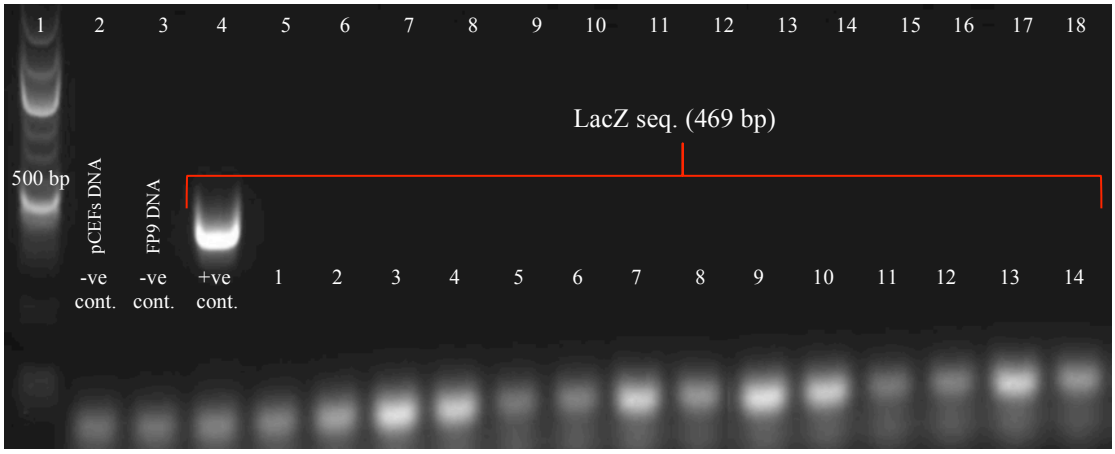

Figure 3.51: Absence of LacZ (marker gene) PCR products in all fourteen recombinant plaque samples after the 4th round of colourless plaque purification of the single rFP9 encoding env and rev sequences.

Lane 1 100 bp ladder, lane 2 pCEFs total DNA (-ve control), lane 3 FP9 total DNA (-ve control), lane 4 FP9.Env.Rev transfer plasmid DNA (+ve control), and lanes 5–18 negative colourless plaque samples for LacZ sequence.

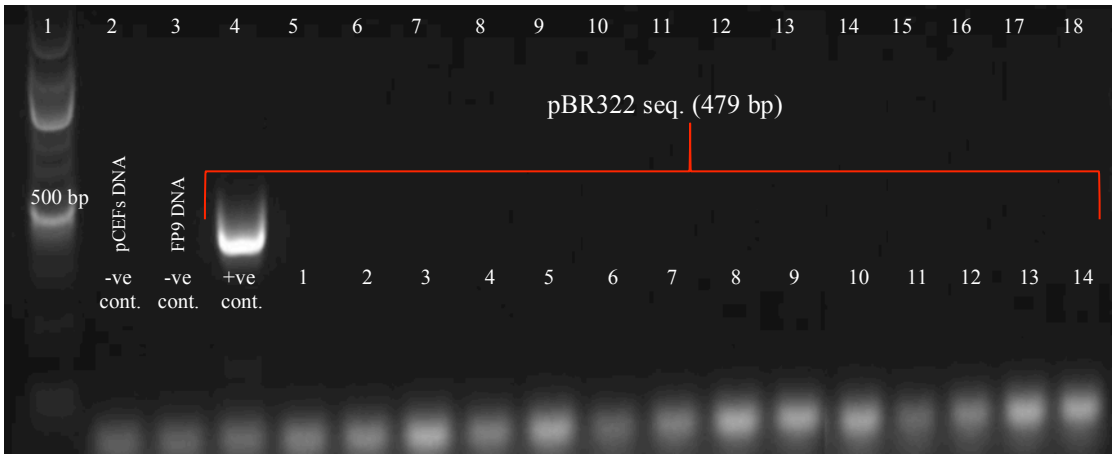

Figure 3.52: Absence of pBR322 (plasmid) PCR products in all fourteen recombinant plaque samples after the 4th round of colourless plaque purification of the single rFP9 encoding env and rev sequences.

Lane 1 100 bp ladder, lane 2 pCEFs total DNA (-ve control), lane 3 FP9 total DNA (-ve control), lane 4 FP9.Env.Rev transfer plasmid DNA (+ve control), and lanes 5–18 negative colourless plaque samples for plasmid sequence.

3.2.6.2 Pure (markerless) single rFP9 encoding T7 polymerase and vpx SIVmac239 sequences

Figures 3.53, 3.54, and 3.55 show PCR results of fourteen recombinant plaque samples following the fourth round of colourless plaque purification, with positive amplification results for both T7 and vpx SIVmac239 sequences, and negative results for the marker gene (LacZ). These PCR results confirm both the absence of the selectable marker gene, and also the stability of the insertion cassette within the single rFP9 construct.

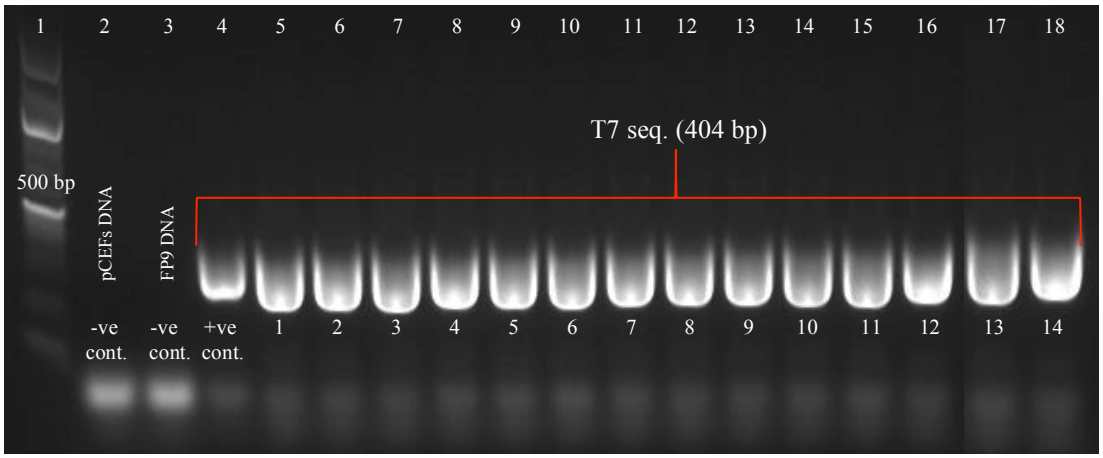

Figure 3.53: Detection of T7 polymerase sequence within fourteen recombinant plaque samples after the 4th round of colourless plaque purification of the single rFP9 encoding T7 and vpx sequences.

Lane 1 100 bp ladder, lane 2 pCEFs total DNA (-ve control), lane 3 FP9 total DNA (-ve control), lane 4 FP9.Vpx.T7pol transfer plasmid DNA (+ve control for T7 sequence), and lanes 5–18 positive colourless plaque samples for T7 sequence.

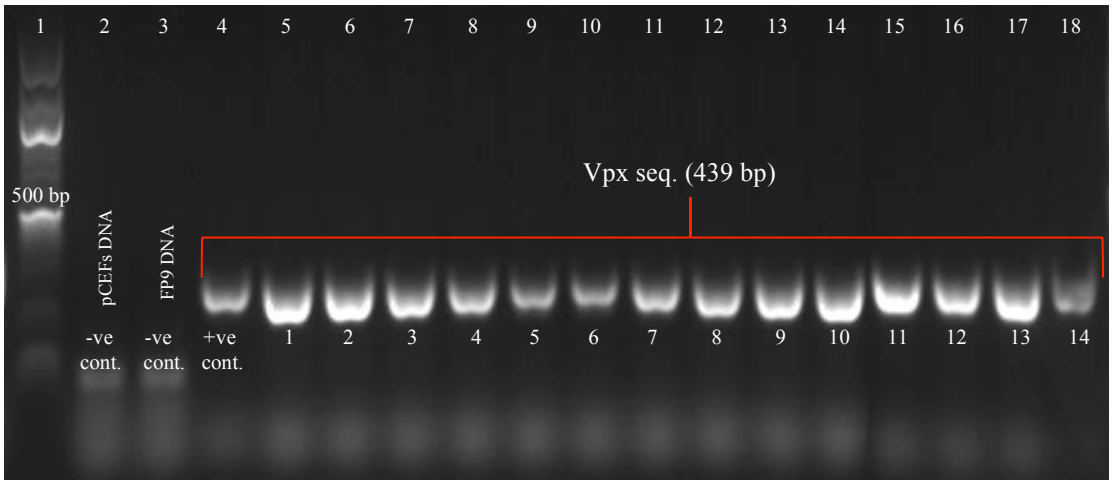

Figure 3.54: Detection of SIVmac239 vpx sequence within fourteen recombinant plaque samples after the 4th round of colourless plaque purification of the single rFP9 encoding T7 and vpx sequences.

Lane 1 100 bp ladder, lane 2 pCEFs total DNA (-ve control), lane 3 FP9 total DNA (-ve control), lane 4 FP9.Vpx.T7pol transfer plasmid DNA (+ve control for vpx sequence), and lanes 5–18 positive colourless plaque samples for SIV vpx sequence.

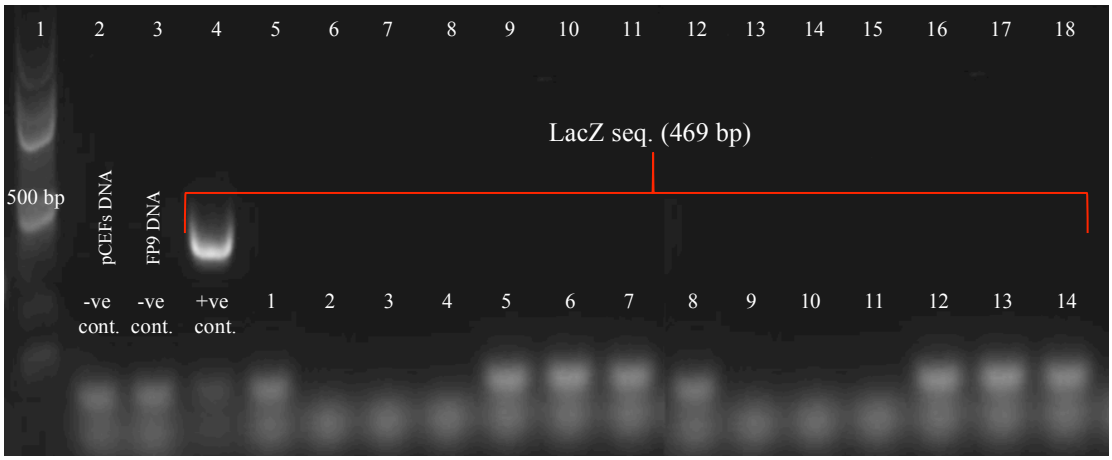

Figure 3.55: Absence of LacZ (marker gene) PCR products in all fourteen recombinant plaque samples after the 4th round of colourless plaque purification of the single rFP9 encoding T7 and vpx sequences.

Lane 1 100 bp ladder, lane 2 pCEFs total DNA (-ve control), lane 3 FP9 total DNA (-ve control), lane 4 FP9.Vpx.T7pol transfer plasmid DNA (+ve control), and lanes 5–18 negative colourless plaque samples for LacZ sequence.

3.2.6.3 Pure (markerless) double rFP9 encoding env/rev and tat/gag-pro SIVmac239 sequences

Figures 3.56, 3.57, and 3.58 show PCR results of only four recombinant plaque samples following the second round of colourless plaque purification, with positive amplification results for both insertion cassettes (env/rev and tat/gag-pro), and negative results for the marker gene (LacZ). These PCR results demonstrate the absence of the selectable marker gene and the purity of the double rFP9 construct. However, ideally this double recombinant needs further plaque purification in order to confirm the stability of the insertion target sequences.

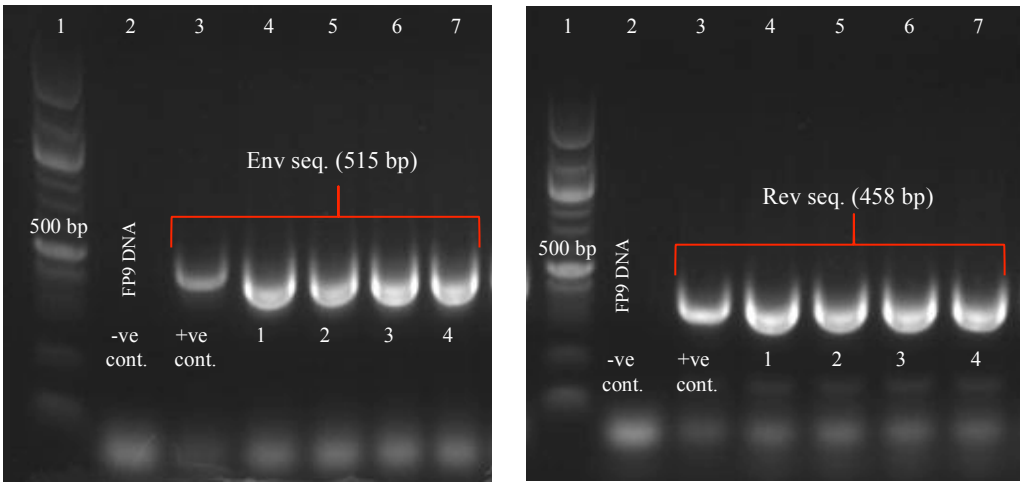

Figure 3.56: Detection of SIVmac239 env and rev sequences within four recombinant plaque samples after the 2nd round of colourless plaque purification of the double rFP9 encoding both env/rev and tat/gag-pro sequences.

Lane 1 100 bp ladder, lane 2 FP9 total DNA (-ve control), lane 3 FP9.Env.Rev transfer plasmid DNA (+ve control), and lanes 4–7 positive colourless plaque samples for SIV env and rev sequences.

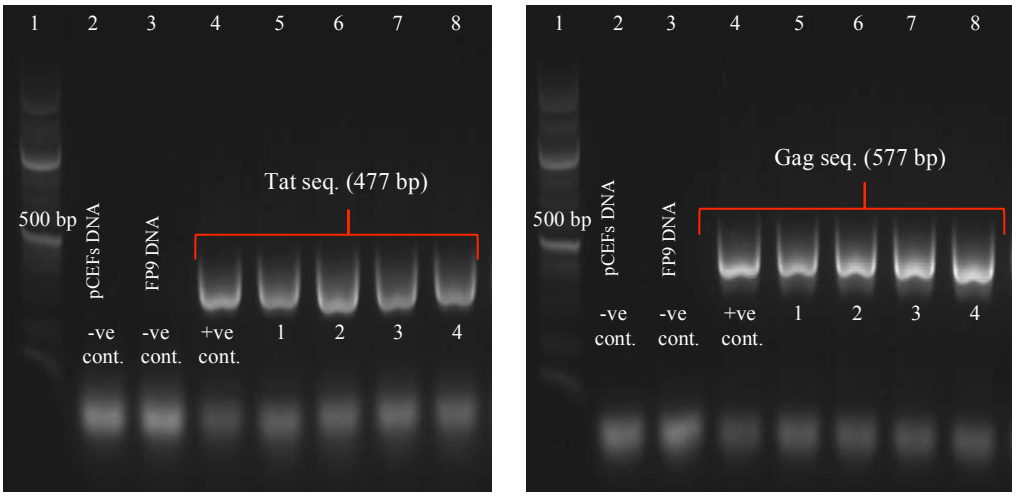

Figure 3.57: Detection of SIVmac239 tat and gag sequences within four recombinant plaque samples after the 2nd round of colourless plaque purification of the double rFP9 encoding both env/rev and tat/gag-pro sequences.

Lane 1 100 bp ladder, lane 2 pCEFs total DNA (-ve control), lane 3 FP9 total DNA (-ve control), lane 4 FP9.Tat.Gag-pro transfer plasmid DNA (+ve control), and lanes 5–8 positive colourless plaque samples for SIV tat and gag sequences.

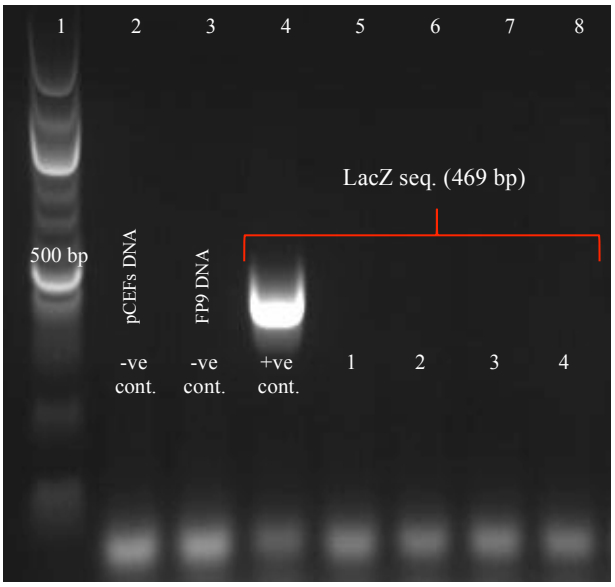

Figure 3.58: Absence of LacZ (marker gene) PCR products in all four recombinant plaque samples after the 2nd round of colourless plaque purification of the double rFP9 encoding both env/rev and tat/gag-pro sequences.

Lane 1 100 bp ladder, lane 2 pCEFs total DNA (-ve control), lane 3 FP9 total DNA (-ve control), lane 4 FP9 transfer plasmid DNA (+ve control), and lanes 5–8 negative colourless plaque samples for LacZ sequence.

3.2.6.4 Pure (markerless) double rFP9 encoding T7pol/vpx and DSIV genome

Figures 3.59, 3.60, 3.61, 3.62, and 3.63 show PCR results of twelve recombinant plaque samples following the second round of colourless plaque purification, with positive amplification results for both insertion cassettes (vpx/T7pol and DSIV genome), and negative results for the marker gene (LacZ). These PCR results confirm both the absence of the selectable marker gene, and also the stability of the insertion cassettes within the double rFP9 construct.

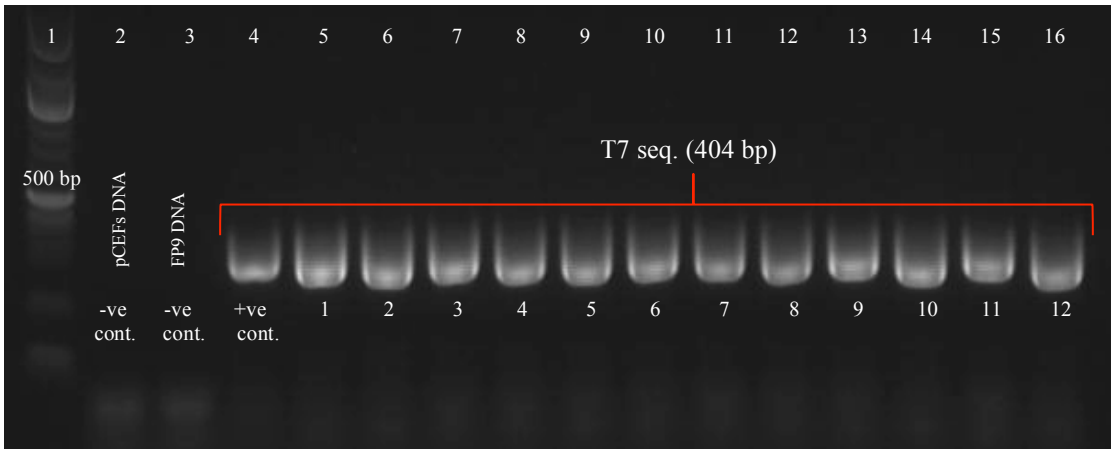

Figure 3.59: Detection of T7 polymerase sequence within twelve recombinant plaque samples after the 2nd round of colourless plaque purification of the double rFP9 encoding both T7/vpx sequences and the DSIV genome.

Lane 1 100 bp ladder, lane 2 pCEFs total DNA (-ve control), lane 3 FP9 total DNA (-ve control), lane 4 FP9.Vpx.T7pol transfer plasmid DNA (+ve control for T7 sequence), and lanes 5–16 positive colourless plaque samples for T7 sequence.

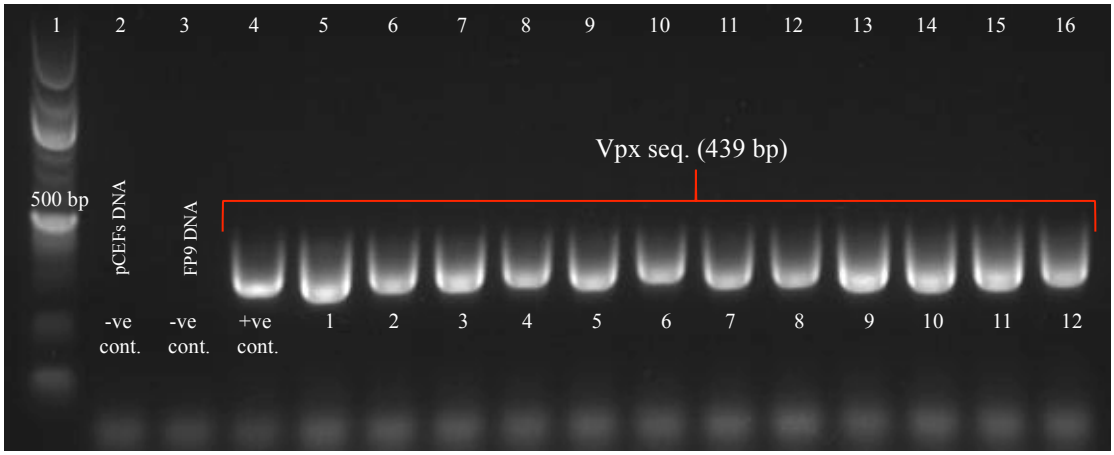

Figure 3.60: Detection of SIVmac239 vpx sequence within twelve recombinant plaque samples after the 2nd round of colourless plaque purification of the double rFP9 encoding both T7/vpx sequences and the DSIV genome.

Lane 1 100 bp ladder, lane 2 pCEFs total DNA (-ve control), lane 3 FP9 total DNA (-ve control), lane 4 FP9.Vpx.T7pol transfer plasmid DNA (+ve control for vpx sequence), and lanes 5–16 positive colourless plaque samples for SIV vpx sequence.

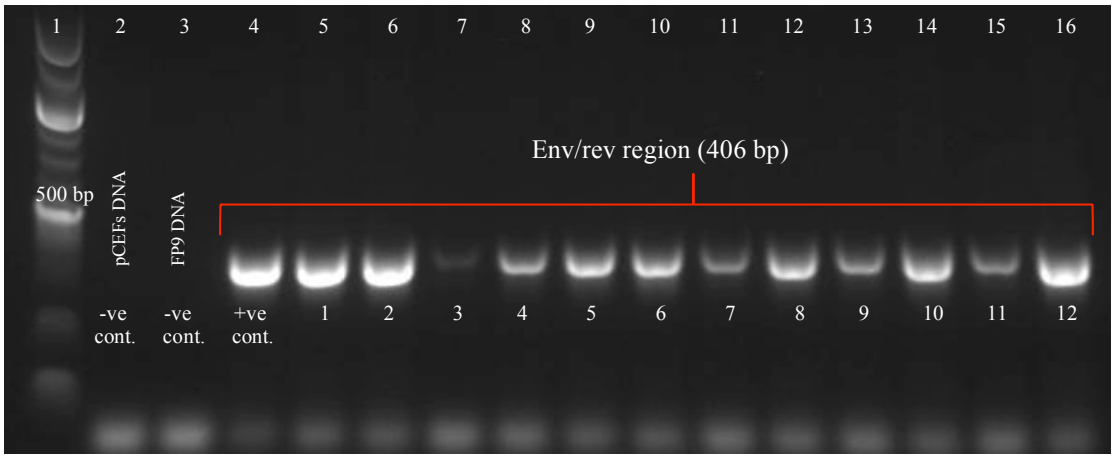

Figure 3.61: Detection of SIV env/rev genomic region within twelve recombinant plaque samples after the 2nd round of colourless plaque purification of the double rFP9 encoding both T7/vpx sequences and the DSIV genome.

Lane 1 100 bp ladder, lane 2 pCEFs total DNA (-ve control), lane 3 FP9 total DNA (-ve control), lane 4 FP9.DSIV genome transfer plasmid DNA (+ve control), and lanes 5–16 positive colourless plaque samples for SIV env/rev genomic region.

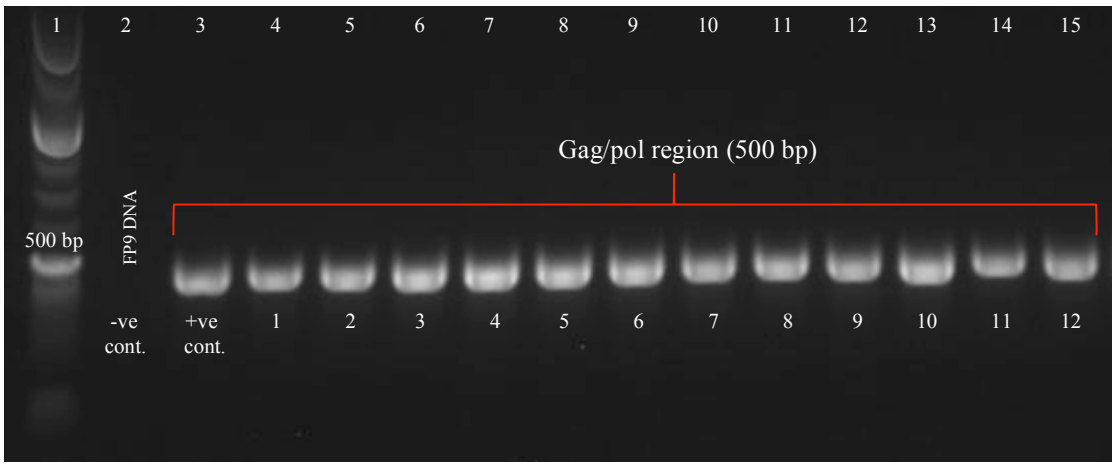

Figure 3.62: Detection of SIV gag/pol genomic region within twelve recombinant plaque samples after the 2nd round of colourless plaque purification of the double rFP9 encoding both T7/vpx sequences and the DSIV genome.

Lane 1 100 bp ladder, lane 2 FP9 total DNA (-ve control), lane 3 FP9.DSIV genome transfer plasmid DNA (+ve control), and lanes 4–15 positive colourless plaque samples for SIV gag/pol genomic region.

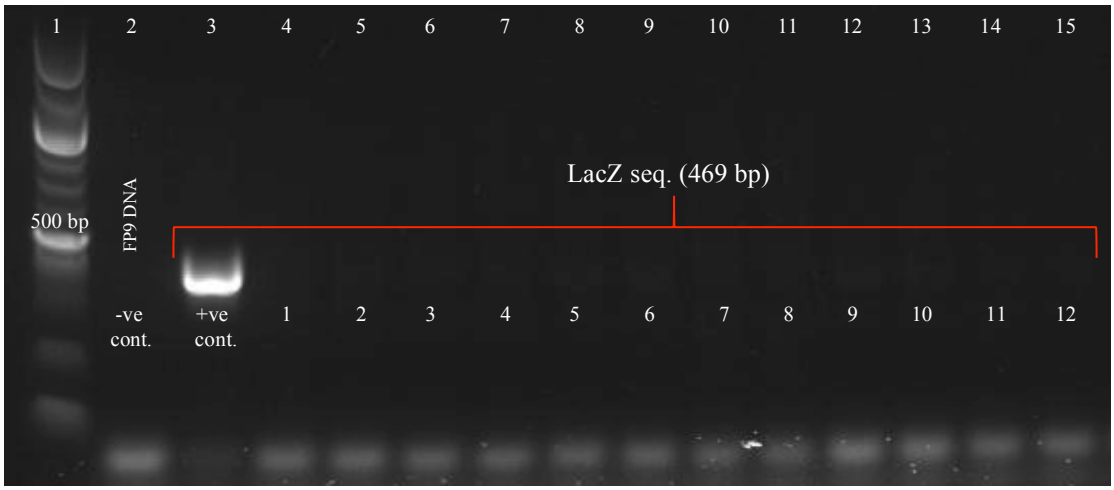

Figure 3.63: Absence of LacZ (marker gene) PCR products in all twelve recombinant plaque samples after the 2nd round of colourless plaque purification of the double rFP9 encoding both T7/vpx sequences and the DSIV genome.

Lane 1 100 bp ladder, lane 2 FP9 total DNA (-ve control), lane 3 FP9 transfer plasmid DNA (+ve control), and lanes 4–15 negative colourless plaque samples for LacZ sequence.

3.3 SIV protein expression and characterization in avian and mammalian cells

3.3.1 SDS-PAGE and Western blot (WB)

SDS-PAGE and WB were used to analyse and determine the correct size of the encoded SIVmac239 proteins that were expressed in rFP9-infected cells under the control of a T7 RNA polymerase expression system. Vero/pCEFs cells were co-infected simultaneously with two rFP9s at an MOI of 2 pfu/cell for each recombinant virus. One of these recombinants containing human codon-optimised SIVmac239 target sequences, and the other one encoding human codon-optimised T7 RNA polymerase expression cassette. At different harvesting time points, cell lysates were prepared and the target proteins were analysed and characterised by SDS-PAGE and WB using specific monoclonal antibodies to individual products.

Two appropriate negative controls (nonrecombinant FP9-infected pCEFs and uninfected pCEFs) were treated in the same way as the experimental protein samples, and incorporated in this assay to ensure the reliability and the validity of the experiment. Both negative controls gave the expected results with no separated protein bands corresponding to the desired protein size. However, irrelevant background proteins appeared on the negative controls suggesting cross-reactivity of the monoclonal antibodies with some components of the cell lines used in the assay. Sizes of the separated proteins were compared with the molecular weight protein marker as a size reference.

3.3.1.1 Expression of SIVmac239 env protein in pCEFs and Vero cells

The following WB figures of SIVmac239 env protein demonstrate that rFP9 efficiently expressed the desired SIV env protein in both avian (pCEFs) and mammalian (Vero) cell lines. The specific monoclonal antibody to SIV env gp160/32 (ARP3044-KK41) was used to detect and analyse the SIV env protein in the rFP9-infected cells.

Figure 3.64 demonstrates the expression of the SIV env protein in pCEFs. It shows that rFP9 was able to express the three different immunospecific bands of the env protein in pCEFs with molecular masses of approximately 160, 120, and 32 kDa (Figure 3.64). On the other hand, figure 3.65 demonstrates the ability of rFP9 to express proteins of six distinct molecular weights of the SIV env protein in mammalian cell line (Vero cells). As shown in the figure 3.65, four major immunospecific bands migrating on WB between 100 and 250 kDa (gp120, gp130, gp140, and gp160), one band between 50 and 75 kDa (gp70), and the last band between 25 and 37 kDa (gp32) (Figure 3.65). The unexpected protein bands of intermediate molecular weight (140, 130, and 70 kDa) probably represent proteolytically cleaved products of the main precursor gp160 in Vero cells.

Env protein expression in pCEFs was evaluated at 24, 48, 72, and 96 h time points, while the expression in Vero cells was only assessed at 48 and 72 hpi. As can be seen from the figures, the maximal protein expression levels in the pCEFs were obtained at 72 h, however the peak of env production in the Vero cells was observed at 48 h time point (Figures 3.64 and 3.65).

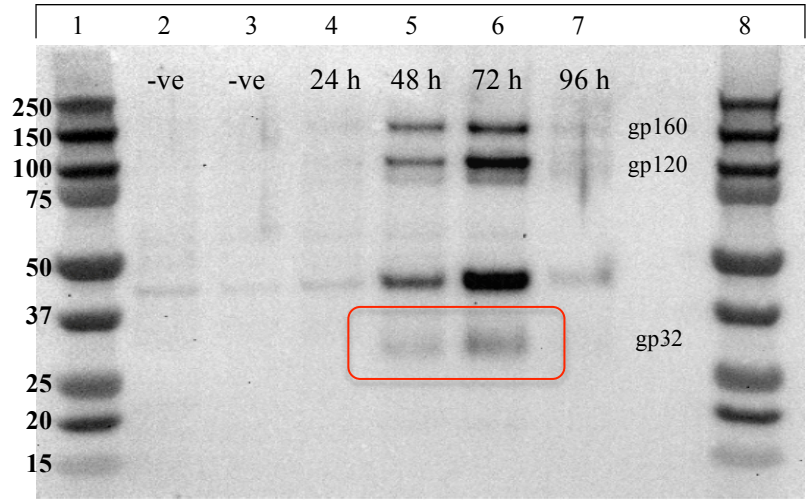

Figure 3.64: Demonstration of SIVmac239 env protein expression in pCEFs co-infected simultaneously with two rFP9s.

Lanes 1 and 8 molecular weight protein marker, lane 2 cell lysate of uninfected pCEFs (represented -ve control), lane 3 cell lysate of infected pCEFs with nonrecombinant FP9 at an MOI of 2 pfu/cell (represented another -ve control), lanes 4, 5, 6, and 7 cell lysates of co-infected pCEFs with two rFP9s and were harvested at 24, 48, 72, and 96 hpi respectively.

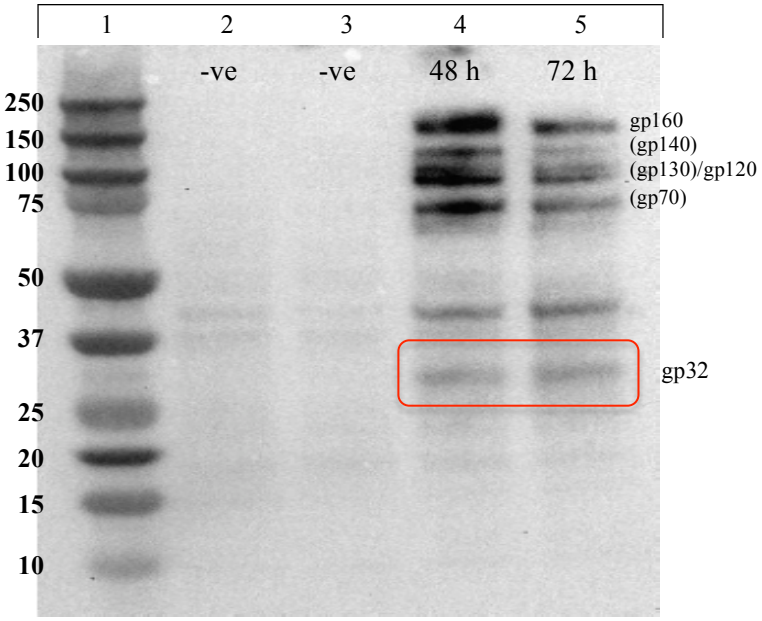

Figure 3.65: Demonstration of SIVmac239 env protein expression in Vero cells co-infected simultaneously with two rFP9s.

Lane 1 molecular weight protein marker, lane 2 cell lysate of uninfected Vero (represented -ve control), lane 3 cell lysate of infected Vero with nonrecombinant FP9 at an MOI of 2 pfu/cell (represented another -ve control), lanes 4 and 5 cell lysates of co-infected Vero with two rFP9s and were harvested at 48 and 72 hpi respectively.

3.3.1.2 Expression of SIVmac239 rev protein in pCEFs and Vero cells

SIVmac239 rev protein was also analysed and evaluated in pCEFs and Vero cell lines using the specific monoclonal antibodies to SIV rev EVA3072.4 and EVA3072.2, respectively. The following WB analysis figure demonstrates that rFP9 successfully expressed the rev protein in pCEFs and Vero cells with the expected molecular size (18 kDa) (Figure 3.66). As can be seen from the result, the rev protein was expressed at two time points (48 and 72 hpi) in both cell types (Figure 3.66). Please note that different monoclonal antibodies were used for pCEFs and Vero cells due to a shortage of reagents.

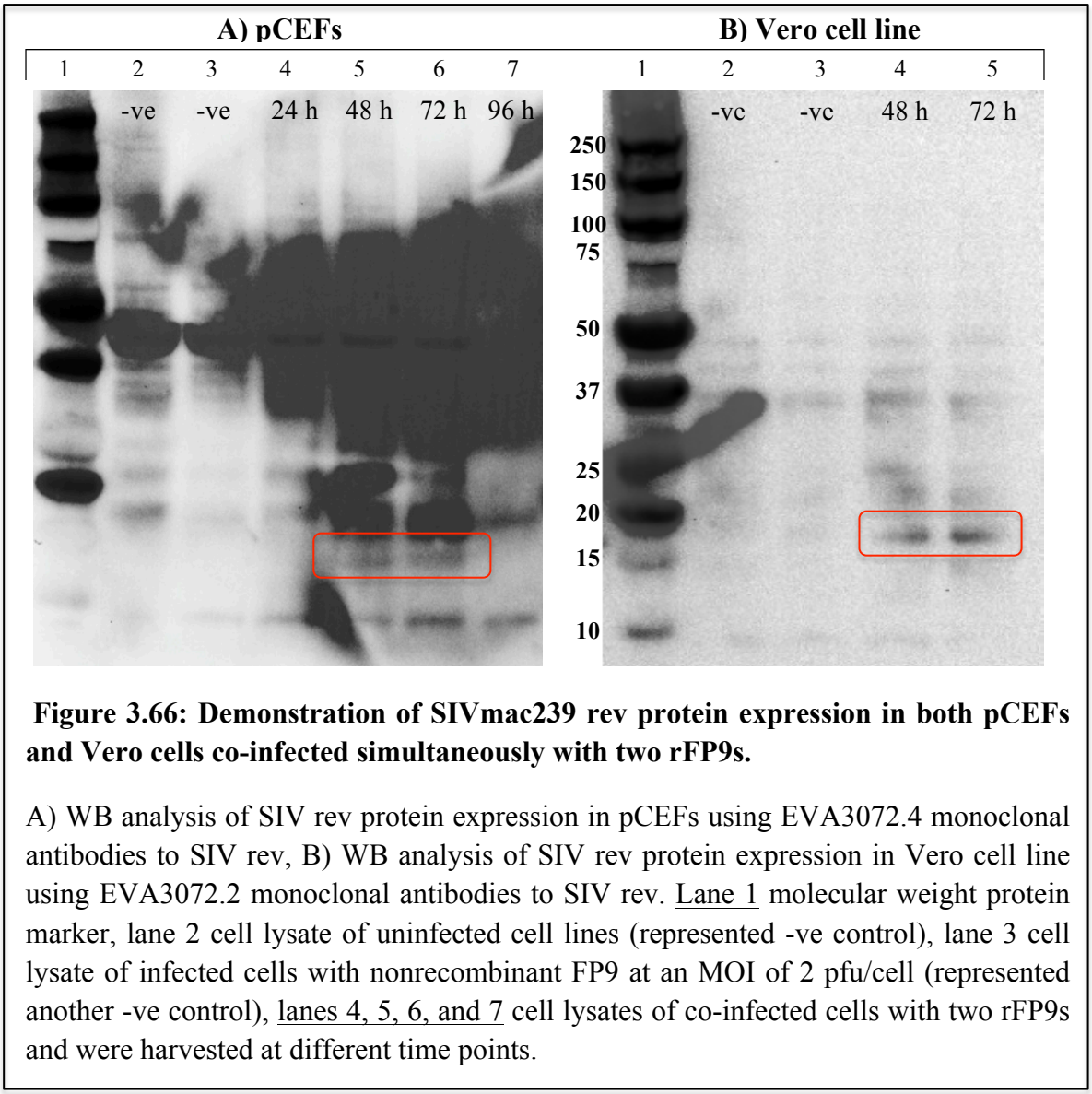

Figure 3.66: Demonstration of SIVmac239 rev protein expression in both pCEFs and Vero cells co-infected simultaneously with two rFP9s.

A) WB analysis of SIV rev protein expression in pCEFs using EVA3072.4 monoclonal antibodies to SIV rev, B) WB analysis of SIV rev protein expression in Vero cell line using EVA3072.2 monoclonal antibodies to SIV rev. Lane 1 molecular weight protein marker, lane 2 cell lysate of uninfected cell lines (represented -ve control), lane 3 cell lysate of infected cells with nonrecombinant FP9 at an MOI of 2 pfu/cell (represented another -ve control), lanes 4, 5, 6, and 7 cell lysates of co-infected cells with two rFP9s and were harvested at different time points.

3.3.1.3 Expression of SIVmac239 gag protein in pCEFs and Vero cells

Double rFP9 encoding two SIV expression cassettes (env/rev and tat/gag-pro) was able to express the SIV gag protein in both pCEFs and Vero cell lines. The target gag protein was detected using the specific monoclonal antibody to SIV gag p27 (ARP396/397-SIV 27e/27f). Figure 3.67 demonstrates that the double rFP9 expressed only the gag precursor protein with the expected molecular mass of approximately 55 kDa. At both time points (48 and 72 h), gag protein expression levels were found to be slightly higher in Vero compared to pCEFs cells (Figure 3.67).

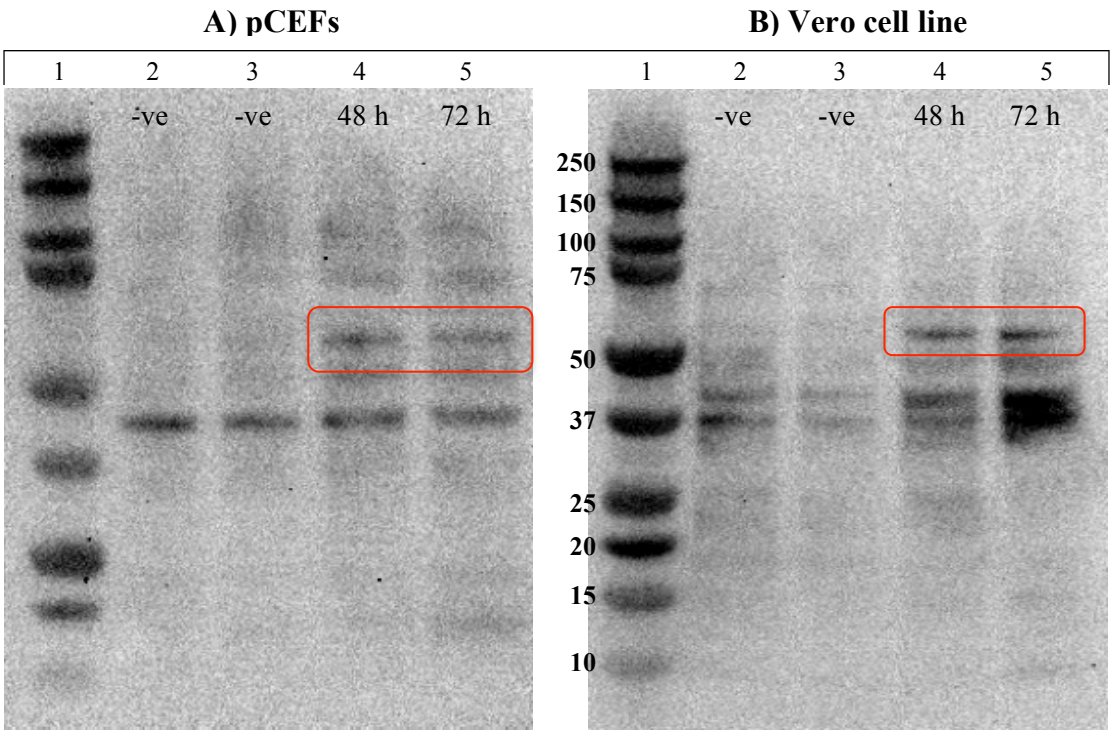

Figure 3.67: Demonstration of SIVmac239 gag protein expression in both pCEFs and Vero cells co-infected simultaneously with two rFP9s.

WB analysis of SIV gag protein expression in A) pCEFs and B) Vero cells using ARP396/397 monoclonal antibodies to SIV gag p27. Lane 1 molecular weight protein marker, lane 2 cell lysate of uninfected cell lines (represented -ve control), lane 3 cell lysate of infected cells with nonrecombinant FP9 at an MOI of 2 pfu/cell (represented another -ve control), lanes 4 and 5 cell lysates of co-infected cells with two rFP9s and were harvested at 48 and 72 hpi respectively.

3.3.2 Immunocytochemical (ICC) staining assay

ICC assay was used to confirm the SIVmac239 protein expression and to visualise the localisation of the target proteins within infected cells. MRC-5/pCEFs were co-infected simultaneously with two rFP9s at an MOI of 2 pfu/cell for each recombinant virus (one containing the target SIVmac239 sequences, and the other one encoding the T7 RNA polymerase expression cassette). At 48 hpi, infected cells were fixed and the target SIV proteins were detected using specific monoclonal antibodies to individual products. The antigen-antibody complexes were then stained using 3,3'-Diaminobenzidine (DAB) peroxidase substrate.

Four appropriate negative controls were included in this assay to ensure the reliability and the validity of the experiment: uninfected cell control, cells infected with nonrecombinant FP9, cells infected with rFP9 encoding only the target SIVmac239 sequences, and cells infected with rFP9 encoding only the T7 RNA polymerase gene. As shown in figures 3.68 and 3.69, all four negative controls gave the same expected results with no expression of the target SIV proteins in both pCEFs and MRC-5 cells (Figures 3.68 and 3.69).

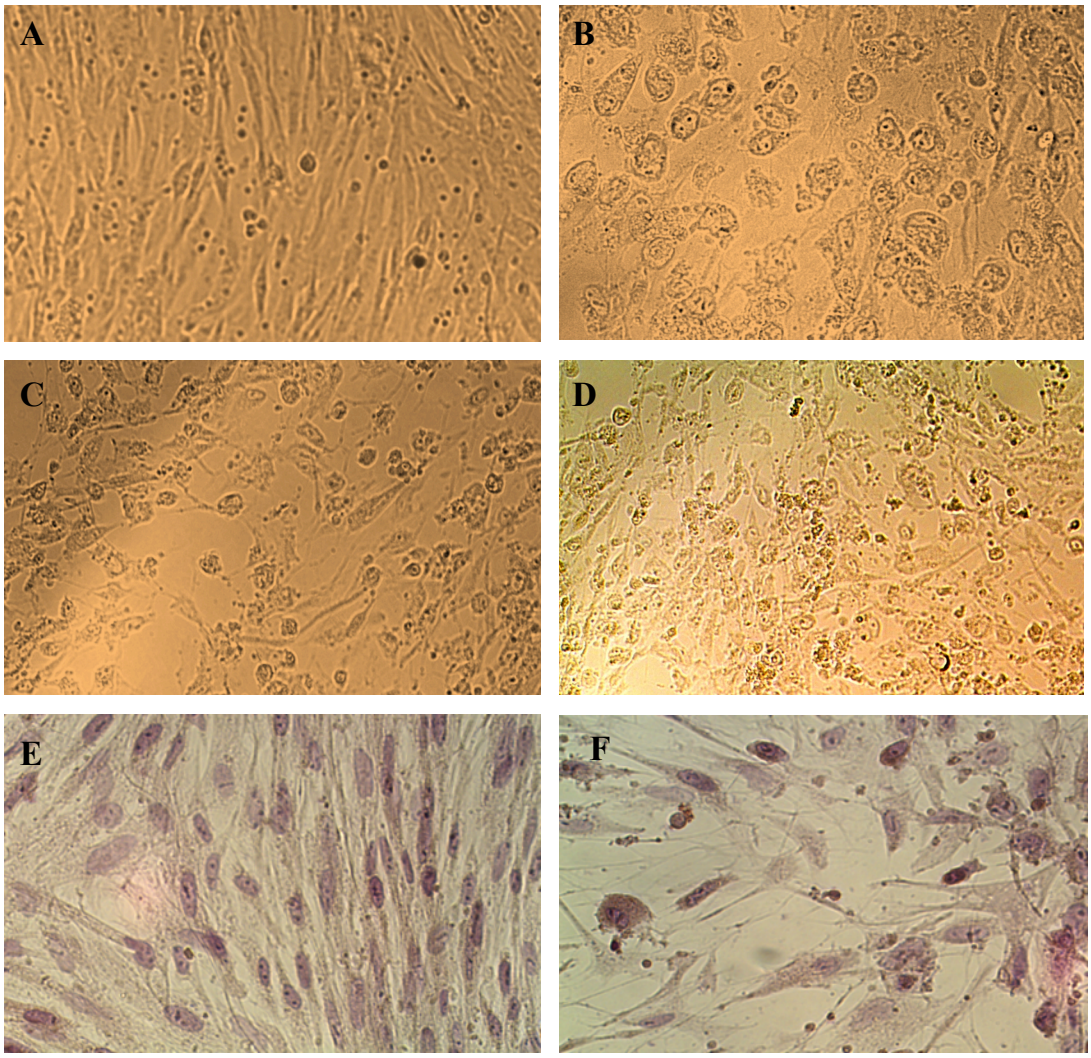

Figure 3.68: Shows the morphological and cytopathic appearance of pCEFs of different negative controls included in the ICC assay.

At 48 hpi, all negative controls show no expression of the target SIV proteins in pCEFs. A) Normal uninfected pCEFs, B) pCEFs infected with nonrecombinant FP9, C) pCEFs infected with rFP9 encoding only the target SIVmac239 sequences, D) pCEFs infected with rFP9 encoding only the T7 RNA polymerase gene, E) H&E stained figure of normal uninfected pCEFs, F) H&E stained figure of pCEFs infected with rFP9 containing the desired SIVmac239 sequences.

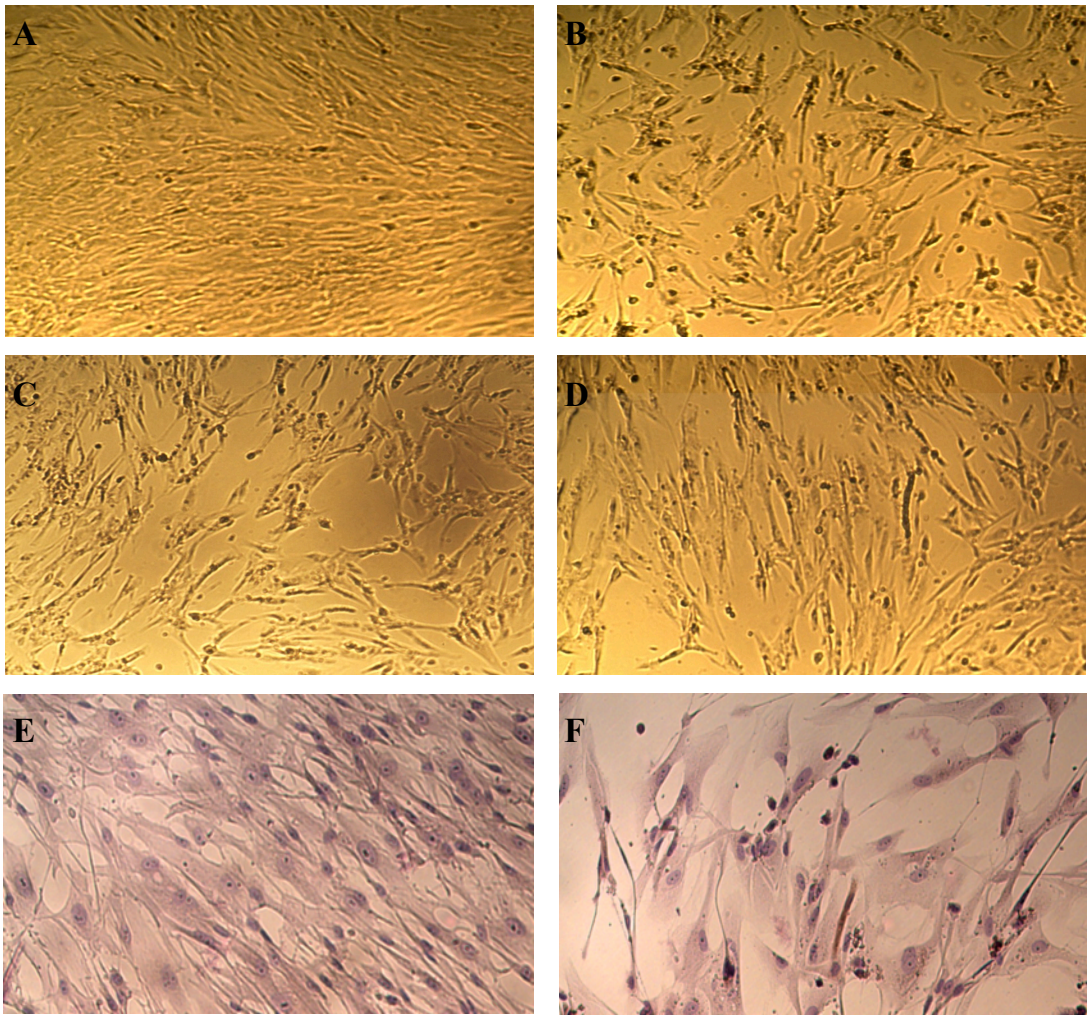

Figure 3.69: Shows the morphological and cytopathic appearance of MRC-5 of different negative controls included in the ICC assay.

At 48 hpi, all negative controls show no expression of the target SIV proteins in MRC-5. A) Normal uninfected MRC-5, B) MRC-5 infected with nonrecombinant FP9, C) MRC-5 infected with rFP9 encoding only the target SIVmac239 sequences, D) MRC-5 infected with rFP9 encoding only the T7 RNA polymerase gene, E) H&E stained figure of normal uninfected MRC-5, F) H&E stained figure of MRC-5 infected with rFP9 containing the desired SIVmac239 sequences.

3.3.2.1 ICC for SIVmac239 env protein detection in pCEFs and MRC-5 cells

ICC assay was used to localise env-expressing cells in pCEFs and MRC-5 cell lines using the specific monoclonal antibody to SIV env gp120 (ARP3045-KK9). Two rFP9s (one encoding the SIVmac239 env sequence and the other one containing the T7

polymerase gene) were used to co-infect the target cells. Figures 3.70 and 3.71 demonstrate the detection of the SIV env protein at 48 hpi in pCEFs and MRC-5 cells, respectively. These ICC results proved the efficient transport of the expressed SIV env protein into infected cells. As shown in the following figures 3.70 and 3.71, positive SIV env-expressing cells appear as light to dark brown colour cells after DAB staining. It was found that the number of env-expressing cells in pCEFs was higher than MRC-5. In addition, the target env protein was expressed qualitatively at high levels in avian compared to mammalian cells (Figures 3.70 and 3.71).

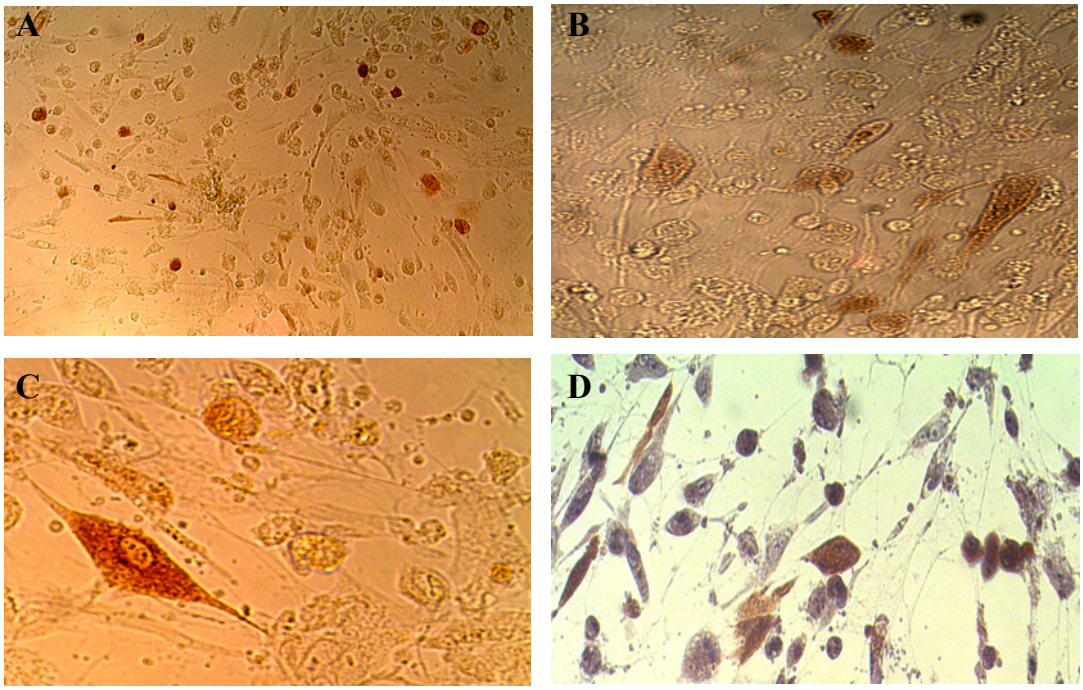

Figure 3.70: Detection of SIVmac239 env protein in pCEFs using ICC assay.

The SIV env protein was detected at 48 h after the co-infection. Simultaneous infection of pCEFs with rFP9.env and rFP9.T7pol (each at an MOI of 2 pfu/cell) resulted in expression of the target env protein. Figures A, B, C, and D are representative examples of positive SIV env-expressing cells at different magnification. A) 10X, B) 20X, C) 40X, and D) H&E stained figure of co-infected pCEFs at 40X.

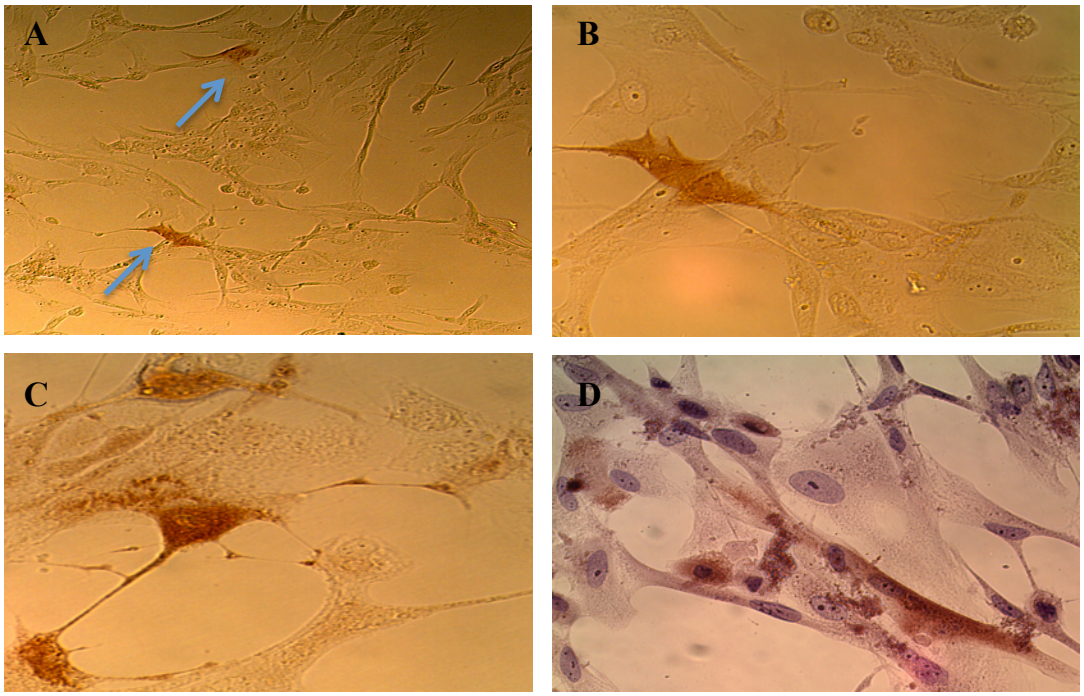

Figure 3.71: Detection of SIVmac239 env protein in MRC-5 using ICC assay.

The SIV env protein was detected at 48 h after the co-infection. Simultaneous infection of MRC-5 with rFP9.env and rFP9.T7pol (each at an MOI of 2 pfu/cell) resulted in expression of the target env protein. Figures A, B, C, and D are representative examples of positive SIV env-expressing cells at different magnification. A) 20X, B and C) 40X, and D) H&E stained figure of co-infected MRC-5 at 40X.

3.3.2.2 ICC for SIVmac239 rev protein detection in pCEFs and MRC-5 cells

SIVmac239 rev protein was detected and localised within the co-infected pCEFs and MRC-5 using the specific monoclonal antibody to SIV rev (EVA3072.4). The cells were co-infected with two rFP9s (one encoding the SIVmac239 rev sequence and the other one containing the T7 polymerase gene) each at an MOI of 2 pfu/cell. Figures 3.72 and 3.73 show some examples of positive SIV rev-expressing cells (light to dark brown colour cells) in pCEFs and MRC-5, respectively. In both cell types, small to moderate numbers of rev-positive cells were identified and the protein expression levels were qualitatively similar at 48 hpi (Figures 3.72 and 3.73).

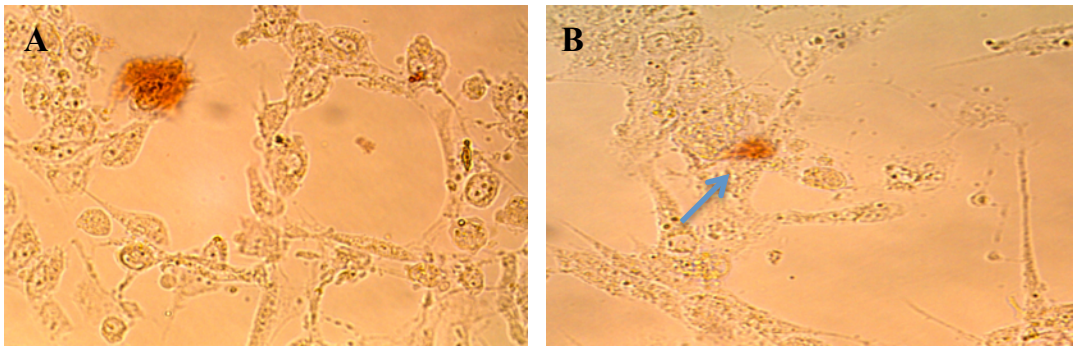

Figure 3.72: Detection of SIVmac239 rev protein in pCEFs using ICC assay.

The SIV rev protein was detected at 48 h after the co-infection. Simultaneous infection of pCEFs with rFP9.rev and rFP9.T7pol (each at an MOI of 2 pfu/cell) resulted in expression of the target rev protein. Figures A and B are representative examples of positive SIV rev-expressing cells at 40X magnification.

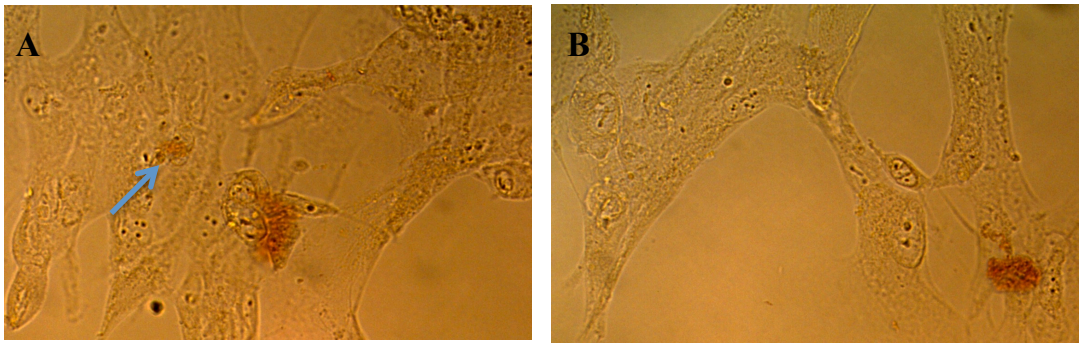

Figure 3.73: Detection of SIVmac239 rev protein in MRC-5 using ICC assay.

The SIV rev protein was detected at 48 h after the co-infection. Simultaneous infection of MRC-5 with rFP9.rev and rFP9.T7pol (each at an MOI of 2 pfu/cell) resulted in expression of the target rev protein. Figures A and B are representative examples of positive SIV rev-expressing cells at 40X magnification.

3.3.2.3 ICC for SIVmac239 tat and gag proteins detection in pCEFs and MRC-5 cells

The specific monoclonal antibodies to SIV tat (ARP3248-MH6) and SIV gag p27 (ARP396) were used to detect and localise the SIVmac239 tat and gag proteins within the infected cells, respectively. MRC-5/pCEFs were co-infected simultaneously with two rFP9s (one recombinant encoding the SIVmac239 tat and gag-pro sequences and

the other one containing the T7 polymerase gene) each at an MOI of 2 pfu/cell. As shown in the following ICC figures, the SIV tat and gag proteins were successfully detected in both avian and mammalian cells. However, only a few positive SIV tat and gag-expressing cells (light to dark brown colour cells) were identified in pCEFs and MRC-5 cells. The protein expression levels were also weak in both cell types at 48 hpi (Figures 3.74 and 3.75).

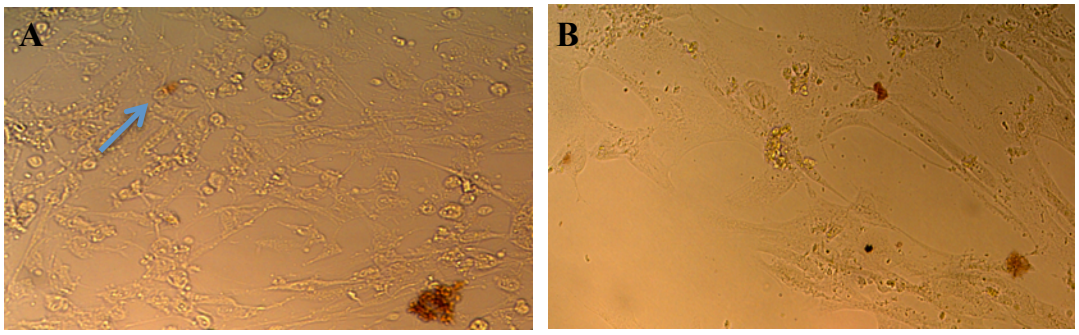

Figure 3.74: Detection of SIVmac239 tat protein in pCEFs and MRC-5 using ICC assay.

The SIV tat protein was detected at 48 h after the co-infection. Simultaneous infection of the cells with rFP9.tat.gag-pro and rFP9.T7pol (each at an MOI of 2 pfu/cell) resulted in expression of the target tat protein. These figures show positive SIV tat-expressing cells in A) pCEFs at 40X and B) MRC-5 at 20X magnification.

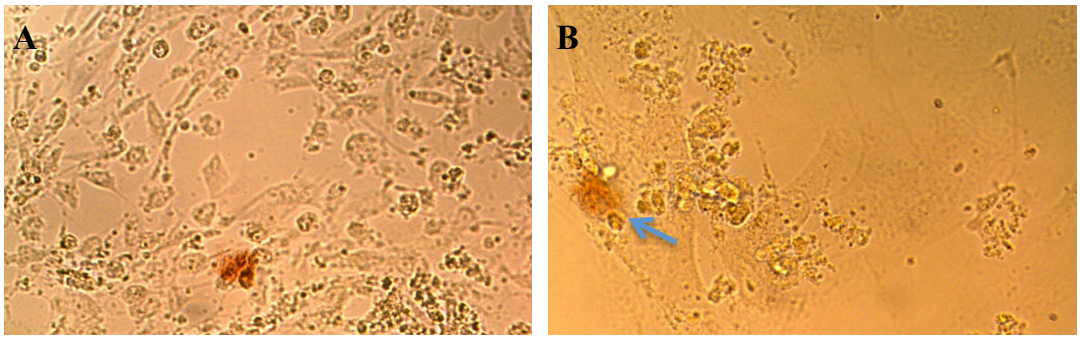

Figure 3.75: Detection of SIVmac239 gag protein in pCEFs and MRC-5 using ICC assay.

The SIV gag protein was detected at 48 h after the co-infection. Simultaneous infection of the cells with rFP9.tat.gag-pro and rFP9.T7pol (each at an MOI of 2 pfu/cell) resulted in expression of the target gag protein. These figures show positive SIV gag-expressing cells in A) pCEFs and B) MRC-5 at 40X magnification.

3.3.3 Green-fluorescent protein (GFP) expression for identification of double rFP9 encoding the DSIV genome and the T7 RNA polymerase gene

GFP, which was incorporated within the middle of the DSIV genome, was used as a model protein to study the expression efficiency of the T7 polymerase system within a single recombinant virus. Toward this goal, the DSIV genome and the T7 polymerase gene were integrated with their specific promoters into two separate sites within the FP9 genome to construct a single rFP9 vector. It was decided to use a strong poxvirus (VACV p7.5 early/late) promoter to direct the transcription of the T7 RNA polymerase gene, while the target DSIV genome was driven by the bacteriophage T7 promoter.

Monolayers of pCEFs were infected with the constructed rFP9 encoding two insertion cassettes (DSIV genome and T7 polymerase gene). At 5–6 days after infection, GFP-positive plaques were identified and visualised under an inverted fluorescent microscope (Optika-XDS-3FL). Figure 3.76 shows various examples of positive GFP-expressing cells infected with rFP9. These fluorescence images were taken at different rounds of blue and colourless plaque purification. It is clear that the T7 RNA polymerase was able to drive the GFP gene expression in rFP9 (Figure 3.76). These data provide a proof of principle that the T7 expression system is functional and able to direct selective transcription of target genes within a single recombinant virus (rFP9).

On the other hand, as demonstrated in figure 3.77, no green fluorescent signal was observed in all three negative controls used in the assay: uninfected pCEFs, pCEFs infected with rFP9 encoding only the DSIV genome, and pCEFs infected with rFP9 containing only the T7 polymerase gene (Figure 3.77).

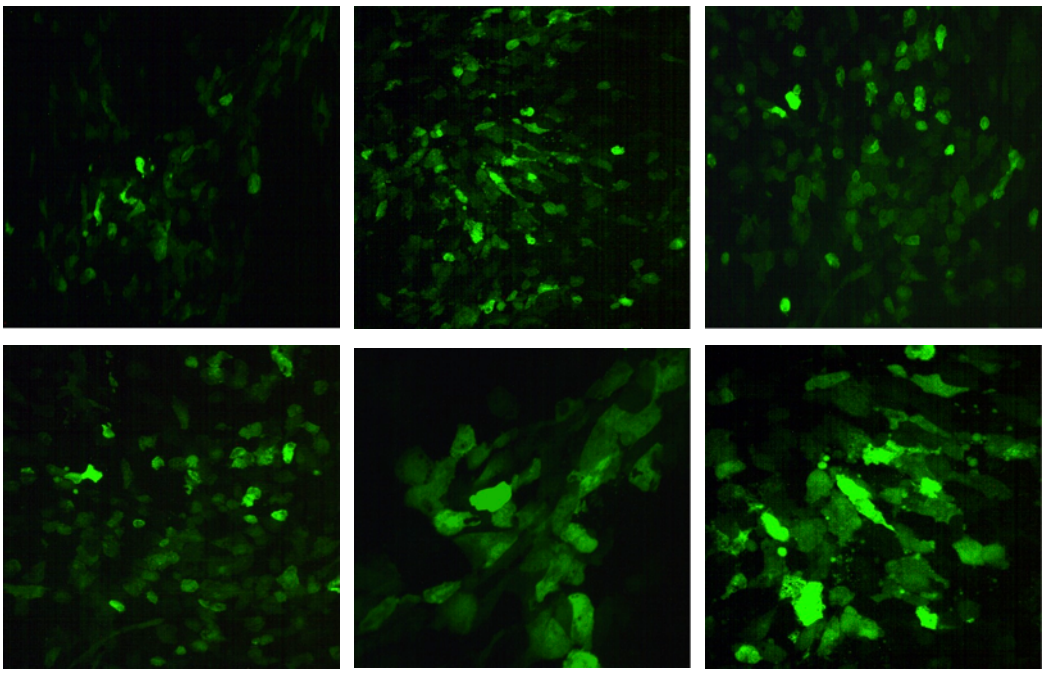

Figure 3.76: Demonstrates the expression of GFP in pCEFs infected with single rFP9 vector.

The GFP was part of the DSIV genome that was integrated together with the T7 polymerase gene into the FP9 genome. pCEFs were infected with the constructed rFP9 and the GFP-positive plaques were identified at 5–6 days post-infection. These GFP expression results proved the functionality of the T7 RNA polymerase system within a single rFP9 vector. Multiple fluorescence images were taken at different passages of the pure rFP9.

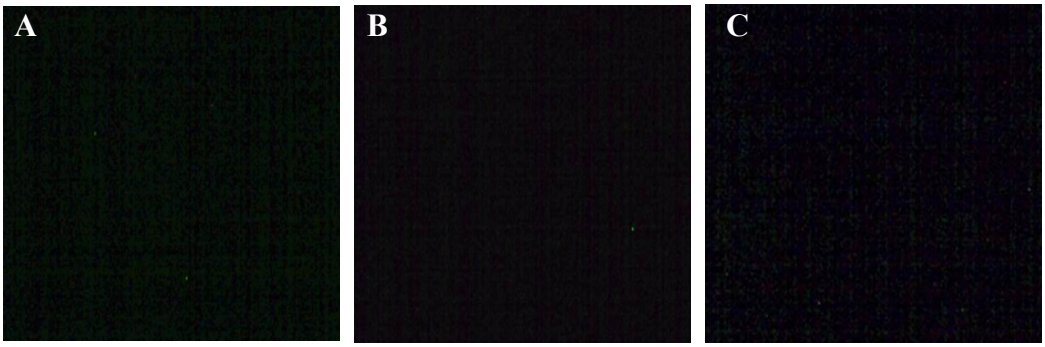

Figure 3.77: Shows three different GFP-negative controls included in the assay.

No green fluorescent signal was detected in all three negative controls. A) Uninfected pCEFs, B) pCEFs infected with rFP9 encoding only the DSIV genome, C) pCEFs infected with rFP9 encoding only the T7 polymerase gene.

3.3.4 Enzyme-linked immunosorbent assay (ELISA)

For the quantitative determination of SIVmac239 protein concentrations in avian and mammalian cells, pCEFs/MRC-5/Vero were co-infected simultaneously with two rFP9s (one encoding SIVmac239 target sequences and the other one containing the T7 polymerase gene) each at an MOI of 2 pfu/cell. The expressed SIV proteins were quantified at multiple time points using a sandwich ELISA.

ELISA data are presented as mean values ± standard deviation (SD) of protein expression levels of three replicate samples at each time point. The following ELISA results prove that the rFP9 candidate used in this project was able to express the SIVmac239 env, rev, gag, and tat proteins in both avian and mammalian cells. These data were found to correlate with the results obtained from the ICC and WB assays. Moreover, the expression of SIV proteins in avian cells was generally stronger and achieved higher levels than that observed in mammalian cells, with the exception of some SIV proteins at particular time points.

In order to determine whether there is a statistical significant difference in the level of protein expression between avian (pCEFs) and mammalian (Vero/MRC-5) cells, a two-way ANOVA followed by a Bonferroni post hoc test was used to compare the means of protein levels between the two cell lines at each time point. In all comparison studies, p-value below 0.05 ($p < 0.05$) was considered as a statistically significant difference.

3.3.4.1 ELISA for evaluating the expression level of SIVmac239 env and rev proteins in pCEFs and MRC-5 cells

rFP9 encoding SIV env/rev sequences and rFP9 containing T7 polymerase gene were used to co-infect the cells and express the target env and rev proteins. The ELISA results demonstrate that the expression levels of SIV env protein in pCEFs were significantly higher than MRC-5 at 24, 48, and 72 hpi with the p-value of <0.0001, <0.0001, and 0.005 respectively (Table 3.4). As shown in table 3.4, the env protein levels expressed in pCEFs were more than double the amount (>100%) that expressed in MRC-5 at 48 h (Table 3.4). In pCEFs, env protein expression level peaked at 48 hpi and then decreased sharply between 48 and 72 h followed by a slight increase at 96 hpi. In MRC-5, however, there was a gradual decrease in the env expression levels between 24 and 72 hpi, followed by a peak at 96 h time point. Interestingly, at 96 hpi, the expression level of env protein in MRC-5 became higher than that seen in pCEFs (Figure 3.78).

	SIVmac239 env protein		
Time points	pCEFs	MRC-5	P-values
24 h	43.7 ± 0.22	22.9 ± 0.37	<0.0001
48 h	47.9 ± 5.31	13.3 ± 2.97	<0.0001
72 h	23.4 ± 1.81	12.1 ± 2.55	0.005
96 h	27.7 ± 6.68	30.8 ± 2.87	0.99–NS

Table 3.4: Expression levels of SIVmac239 env protein (µg/ml) in pCEFs and MRC-5 cells at different time points after infection. Data are presented as mean ± SD (n=3). (Ns= no significant).

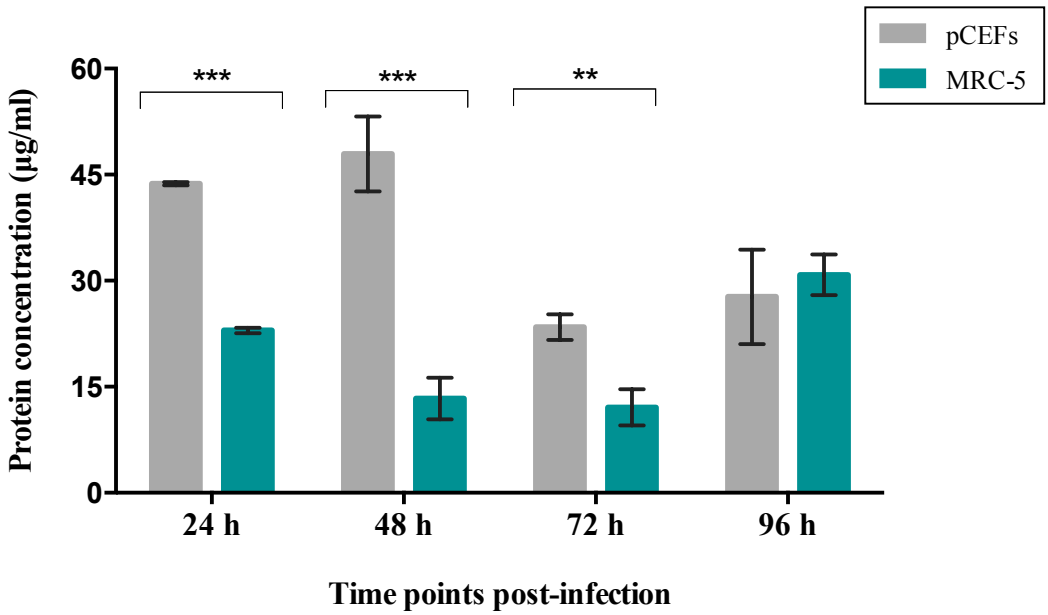

Figure 3.78: Shows the significant differences in the SIV env protein expression levels (µg/ml) between pCEFs and MRC-5 cells at multiple time points post-infection.

The expression levels were determined by ELISA and these data represent the mean ± SD (n=3). Significant differences were revealed in the SIV env protein expression levels between the two cell types at 24, 48, and 72 hpi (**P<0.01, ***P<0.001).

For SIVmac239 rev protein, the following figure shows that the expression amounts in MRC-5 were lower than those produced in pCEFs cells with the exception of the 72 h time point (Figure 3.79). Statistically significant differences were noted in the expression levels of the rev protein between the two cell types at 48 and 96 hpi with the p-value of <0.0001 and 0.024 respectively (Table 3.5). In pCEFs, the optimal expression levels of the rev protein were obtained at 48 hpi, whereas the peak production in MRC-5 was observed at 24 h time point. Between 48 and 72 hpi, the expression levels decreased sharply in pCEFs followed by a significant increase at 96 h. By contrast, similar amounts of the rev protein were expressed in MRC-5 among all different time points (Figure 3.79).

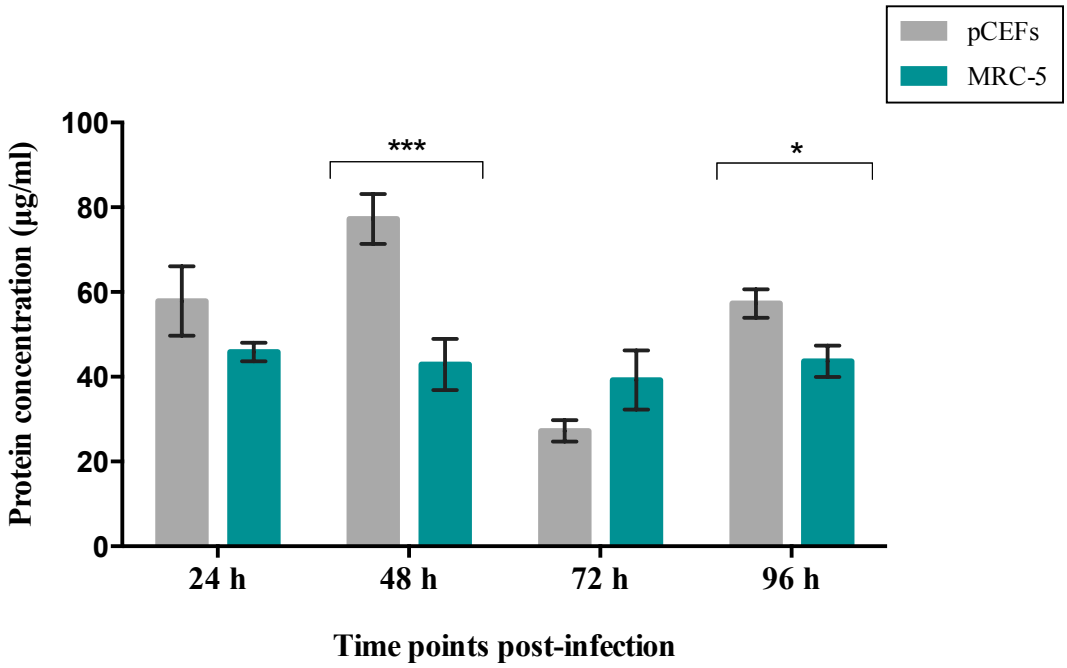

Figure 3.79: Shows the significant differences in the SIV rev protein expression levels (µg/ml) between pCEFs and MRC-5 cells at multiple time points post-infection.

The expression levels were determined by ELISA and these data represent the mean ± SD (n=3). Significant differences were revealed in the SIV rev protein expression levels between the two cell types at 48 and 96 hpi (*P<0.05, ***P<0.001).

	SIVmac239 rev protein		
Time points	pCEFs	MRC-5	P-values
24 h	57.9 ± 8.19	45.8 ± 2.19	0.053–NS
48 h	77.2 ± 5.90	42.9 ± 6.03	<0.0001
72 h	27.2 ± 2.54	39.2 ± 6.97	0.053–NS
96 h	57.3 ± 3.37	43.7 ± 3.69	0.024

Table 3.5: Expression levels of SIVmac239 rev protein (µg/ml) in pCEFs and MRC-5 cells at different time points after infection. Data are presented as mean ± SD (n=3).

3.3.4.2 ELISA for evaluating the expression level of SIVmac239 gag and tat proteins in pCEFs and Vero cells

Double rFP9 encoding env.rev/tat.gag-pro SIVmac239 sequences and rFP9 containing the T7 polymerase gene were used to co-infect the cells and express the gag and tat proteins. Expression levels of the target proteins were evaluated only at 48 and 72 hpi in pCEFs and Vero cells (Table 3.6). In both cell types, stronger levels of transgene expression were observed at 48 h compared to the 72 hpi. It was also found that the double rFP9 was able to express slightly higher amounts of the gag and tat proteins in pCEFs than Vero cells. Strikingly, at 72 h, the expression level of the tat protein in Vero cell line increased and became higher than that produced in pCEFs (Figure 3.80). Nevertheless, the results show that there were no significant differences in the expression levels of the SIV gag and tat proteins between avian and mammalian cells at both time points (Table 3.6).

	SIVmac239 gag protein			SIVmac239 tat protein		
Time points	pCEFs	Vero	P-values	pCEFs	Vero	P-values
48 h	13.5 ± 2.53	13.1 ± 2.71	0.99–NS	35.5 ± 0.82	31.4 ± 3.27	0.26–NS
72 h	9.67 ± 2.29	7.35 ± 2.24	0.56–NS	16.7 ± 4.44	23.9 ± 2.18	0.06–NS

Table 3.6: Expression levels of SIVmac239 gag and tat proteins (µg/ml) in pCEFs and Vero cells at two different time points after infection. Data are presented as mean ± SD (n=3).

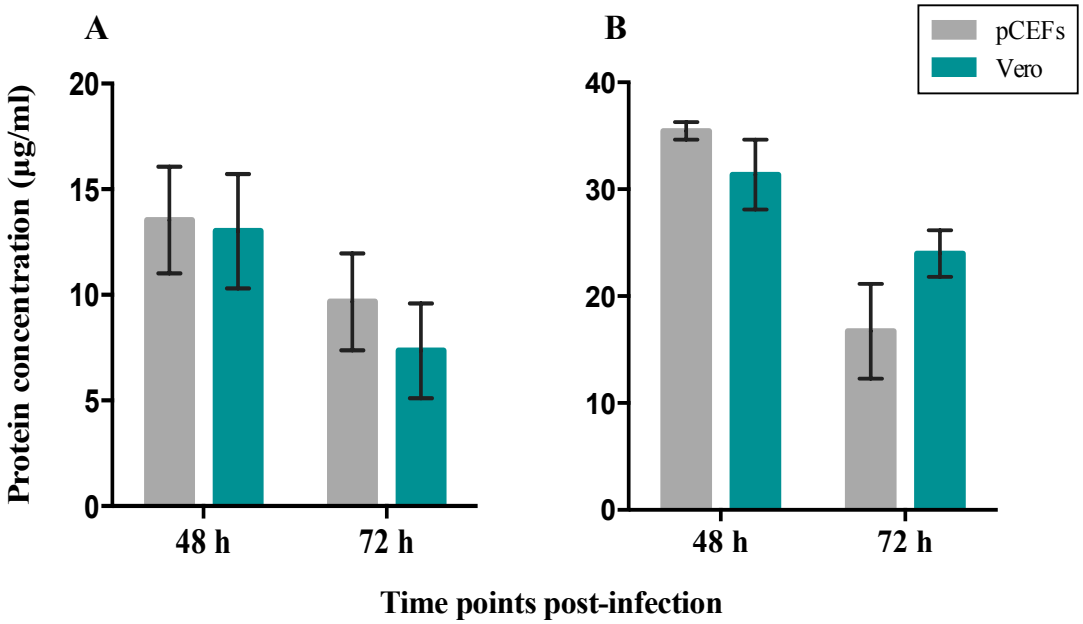

Figure 3.80: Shows the expression levels of SIVmac239 A) gag and B) tat proteins (µg/ml) in pCEFs and Vero cells at two different time points after infection.

The expression levels were determined by ELISA and these data represent the mean ± SD (n=3). There were no significant differences in the SIV gag and tat expression levels between the two cell types.

3.4 Comparison of gene expression profiles between rFP9 and rMVA encoding env/rev SIVmac239 sequences under the control of T7 RNA polymerase expression system

Gene expression profiles of rFP9 and rMVA encoding env and rev components of SIVmac239 were studied *in vitro*. In addition, combinations between these recombinants were assessed for effect on expression of target SIV proteins in avian and mammalian cells.

Four different groups of recombinant viruses expressing identical gene expression cassettes were included in this comparative study: group 1 consists of rFP9.env.rev and

rFP9.T7pol (two rFP9s), group 2 contains rMVA.env.rev and rMVA.T7pol (two rMVAs), group 3 includes rFP9.env.rev and rMVA.T7pol (rFP9/rMVA), and group 4 incorporates rMVA.env.rev and rFP9.T7pol (rMVA/rFP9). rFP9.T7pol and rMVA.T7pol employed the same codon-optimised T7 polymerase gene. The only difference between the two recombinants is the promoter used to drive the expression of the T7 polymerase gene. In rFP9.T7.pol, the expression of the T7 was driven by an early/late p7.5 VACV promoter, while in rMVA.T7pol by an I3L promoter. All recombinant viruses used in this study were propagated and titrated on pCEFs and their encoded target genes were human codon-optimised for high-level protein expression in mammalian cells.

Qualitative and quantitative comparative analyses of transgene expression levels between different virus combinations were performed using two different expression assays: ICC and ELISA. To evaluate whether the expression levels of the target proteins by two rFP9s, two rMVAs, rFP9/rMVA, and rMVA/rFP9 were equal in avian and mammalian cell lines, pCEFs and MRC-5 cells were co-infected simultaneously with these various groups of recombinants. The target cells were also singly infected with a recombinant virus encoding either the target SIVmac239 sequences or the T7 polymerase gene as negative controls.

3.4.1 Qualitative ICC staining assay

The ICC assay was used for qualitative determination of the target SIVmac239 env and rev proteins that were expressed by different groups of recombinants. The specific monoclonal antibodies to SIV env gp120 (ARP3045-KK9) and SIV rev (EVA3072.4) were used to detect and localise the SIV env and rev proteins within the infected cells, respectively. Env and rev-expressing cells (light to dark brown colour cells) were

successfully detected in avian and mammalian cells co-infected simultaneously with recombinant viruses. On the other hand, all negative controls show no expression of the target SIV proteins in pCEFs and MRC-5 cells (Figure 3.81).

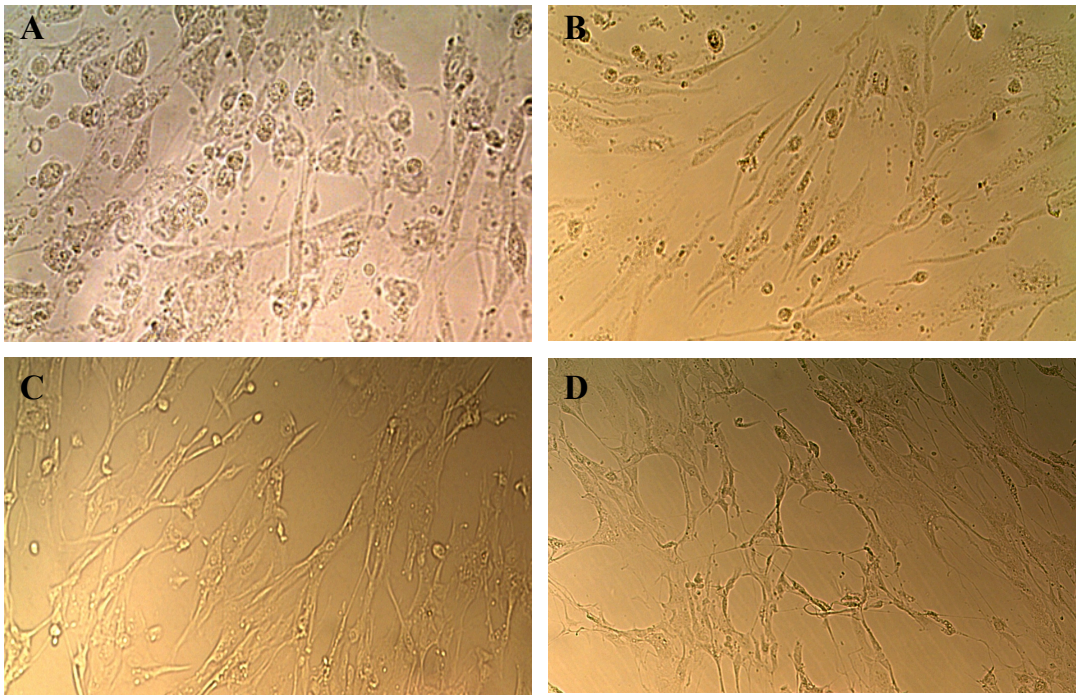

Figure 3.81: Shows the morphological and cytopathic appearance of pCEFs (top two figures) and MRC-5 (bottom two figures) of different negative controls included in the ICC assay.

At 48 hpi, all negative controls show no expression of the target SIV env and rev proteins. A) pCEFs infected with rFP9.env.rev, B) pCEFs infected with rMVA.env.rev, C) MRC-5 infected with rFP9.env.rev, D) MRC-5 infected with rMVA.env.rev.

3.4.1.1 ICC for qualitative analysis of SIVmac239 env protein expressed by different virus combinations in pCEFs and MRC-5 cells

rFP9 and rMVA were compared for their ability to express the SIV env protein in avian and mammalian cells. As shown in the following figures, both recombinants successfully expressed the target env protein in pCEFs and MRC-5 cells at 48 hpi. However, the qualitative analysis of the ICC figures demonstrates that the expression of

the target env protein by rMVA was more distinct in comparison to rFP9. In both cell types, co-infection with two rMVAs (group 2) expressed higher levels of the env protein than that produced by two rFP9s (group 1) (Figures 3.82 and 3.83). Generally, rMVA was able to express the env protein in MRC-5 to a visually noticeable extent in comparison to rFP9 as shown by the qualitative analysis of the two recombinants (Figure 3.83).

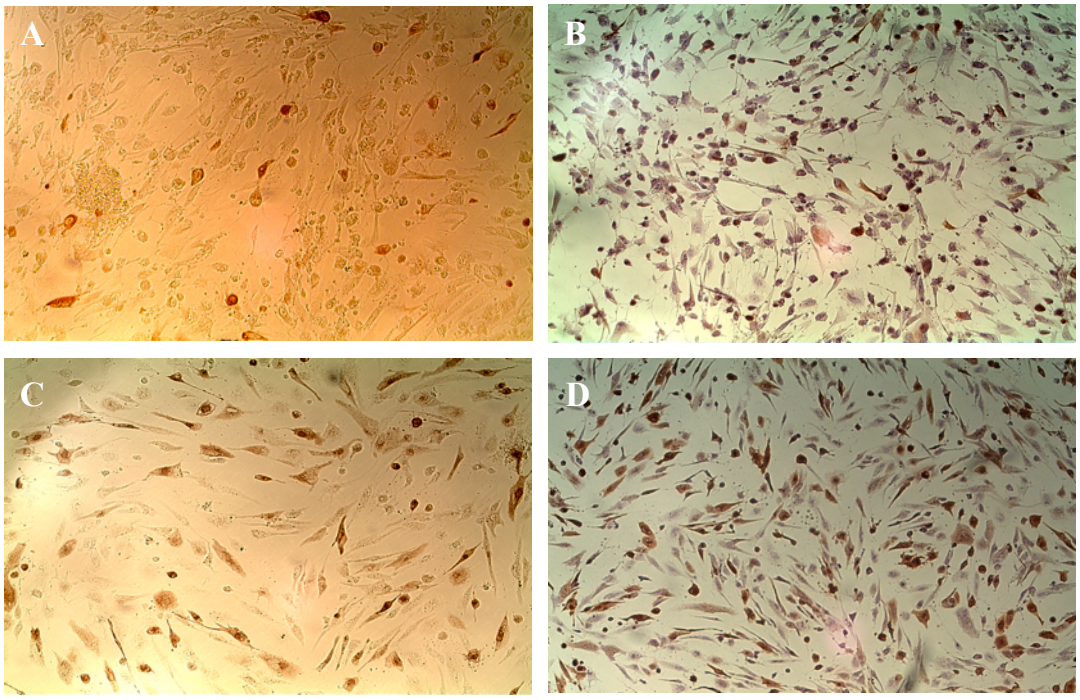

Figure 3.82: Detection of SIVmac239 env protein expressed by rFP9 (top two figures) and rMVA (bottom two figures) in pCEFs using ICC assay.

The SIV env protein was detected at 48 h after the co-infection. Simultaneous infection of pCEFs with either two rFP9s or two rMVAs (each at an MOI of 2 pfu/cell) resulted in expression of the target env protein. A and B show positive SIV env-expressing cells as a result of co-infection of pCEFs with rFP9.env and rFP9.T7pol, C and D show positive SIV env-expressing cells as a result of co-infection of pCEFs with rMVA.env and rMVA.T7pol. B and D show H&E stained figures of co-infected pCEFs.

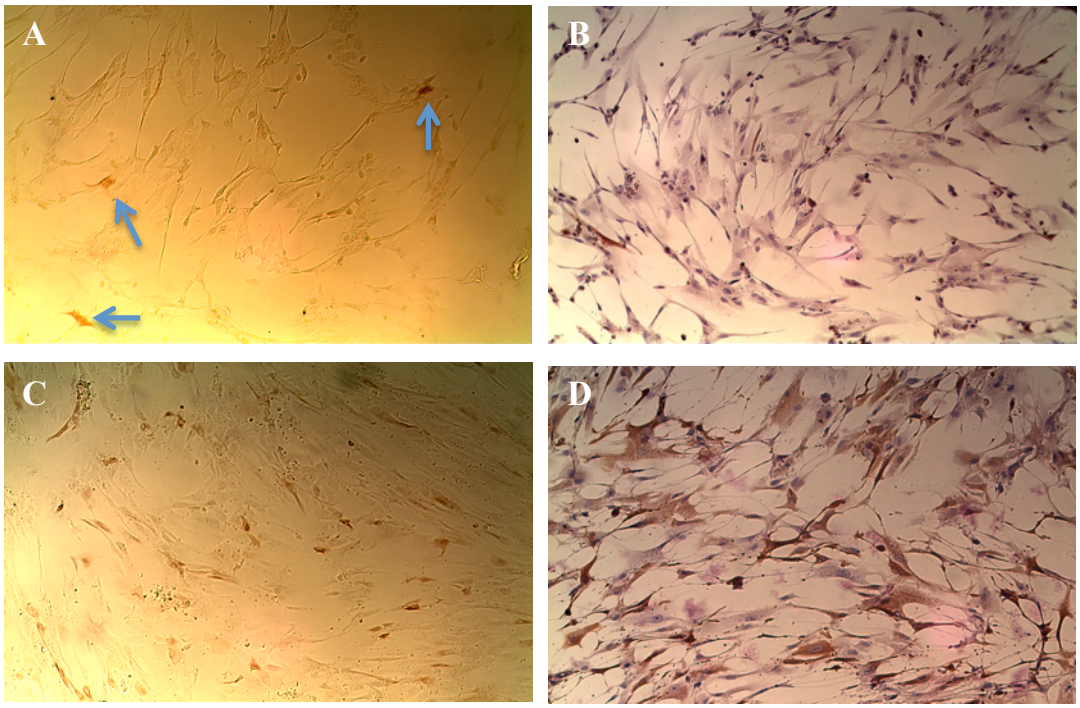

Figure 3.83: Detection of SIVmac239 env protein expressed by rFP9 (top two figures) and rMVA (bottom two figures) in MRC-5 using ICC assay.

The SIV env protein was detected at 48 h after the co-infection. Simultaneous infection of MRC-5 with either two rFP9s or two rMVAs (each at an MOI of 2 pfu/cell) resulted in expression of the target env protein. A and B show small number of positive SIV env-expressing cells as a result of co-infection of MRC-5 with rFP9.env and rFP9.T7pol, C and D show large number of positive SIV env-expressing cells as a result of co-infection of MRC-5 with rMVA.env and rMVA.T7pol. B and D show H&E stained figures of co-infected MRC-5.

Combination between rFP9 and rMVA was also used to enhance the expression of the target SIV proteins in both avian and mammalian cells. Figures 3.84 and 3.85 demonstrate the successful expression of the env protein in pCEFs and MRC-5 cells cross-infected with both recombinants. As can be seen from the results, cross-infection with rFP9 and rMVA (either rFP9.env and rMVA.T7pol or rMVA.env and rFP9.T7pol) reduced the expression efficiency in pCEFs (Figure 3.84). In contrast, high levels of the env protein were expressed in MRC-5 cross-infected with rFP9.env and rMVA.T7pol (group 3) (Figure 3.85). In MRC-5, the difference in expression of the env protein was more obvious by using rMVA.T7pol in comparison to rFP9.T7pol. Basically, no

noticeable improvement was achieved in the env expression level in MRC-5 cells that were cross-infected with rMVA.env and rFP9.T7pol (group 4) (Figure 3.85).

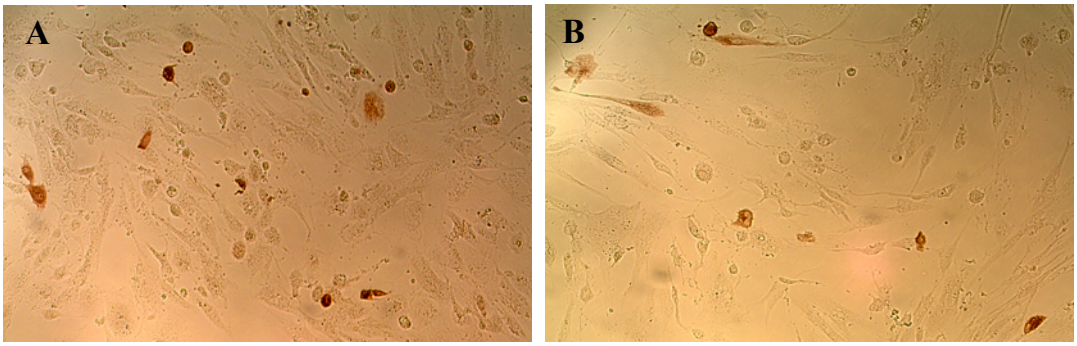

Figure 3.84: Demonstrates the detection of SIVmac239 env protein in pCEFs cross-infected with both rFP9 and rMVA.

The SIV env protein was detected at 48 h after the cross-infection. A) pCEFs cross-infected with rFP9.env and rMVA.T7pol, B) pCEFs cross-infected with rMVA.env and rFP9.T7pol.

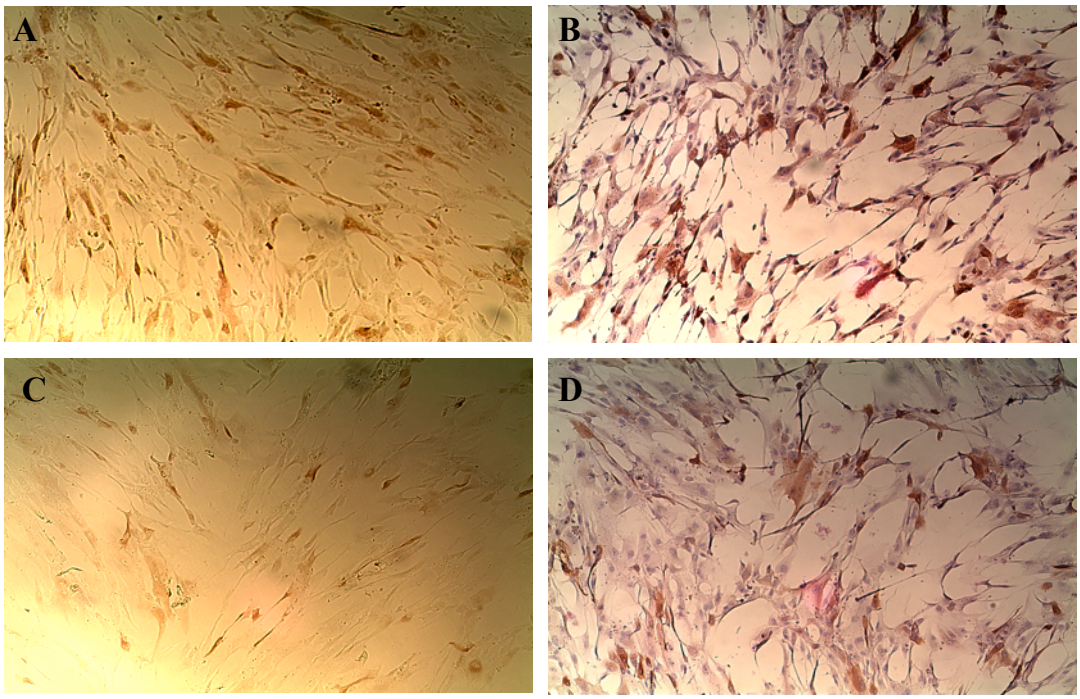

Figure 3.85: Demonstrates the detection of SIVmac239 env protein in MRC-5 cross-infected with both rFP9 and rMVA.

The SIV env protein was detected at 48 h after the cross-infection. A and B) MRC-5 cross-infected with rFP9.env and rMVA.T7pol, C and D) MRC-5 cross-infected with rMVA.env and rFP9.T7pol. B and D) show H&E stained figures of cross-infected MRC-5.

3.4.1.2 ICC for qualitative analysis of SIVmac239 rev protein expressed by different virus combinations in pCEFs and MRC-5 cells

All virus combinations were able to express the target SIV rev protein in pCEFs and MRC-5 cells at 48 hpi. However, it was difficult to compare the rev expression levels between the different groups of recombinants due to the fact that small numbers of rev-positive cells were identified in both cell types. As shown in figure 3.86, the rev protein expressed by two rFP9s (group1) was generally indistinguishable from that produced by two rMVAs (group 2) in terms of the number of positive rev-expressing cells (Figure 3.86).

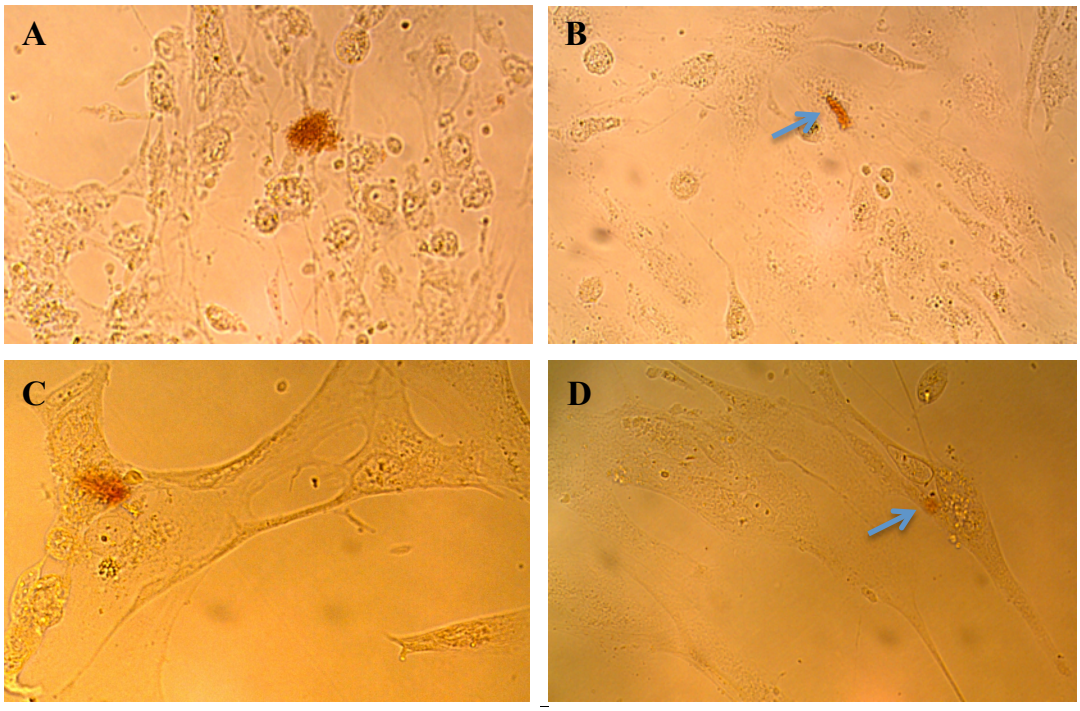

Figure 3.86: Detection of SIVmac239 rev protein expressed by rFP9 and rMVA in pCEFs (top two figures) and MRC-5 (bottom two figures) using ICC assay.

The SIV rev protein was detected at 48 h after the co-infection. Simultaneous infection of pCEFs and MRC-5 with either two rFP9s or two rMVAs (each at an MOI of 2 pfu/cell) resulted in expression of the target rev protein. A and B show positive SIV rev-expressing cells in pCEFs as a result of co-infection with rFP9.rev and rFP9.T7pol (A), rMVA.rev and rMVA.T7pol (B). C and D show positive SIV rev-expressing cells in MRC-5 as a result of co-infection with rFP9.rev and rFP9.T7pol (C), rMVA.rev and rMVA.T7pol (D).

In terms of cross-infection with the two recombinants (rFP9 and rMVA), no improvement was achieved in the rev expression levels in both pCEFs and MRC-5 cells that were cross-infected with either rFP9.rev and rMVA.T7pol (group 3) or rMVA.rev and rFP9.T7pol (group 4) (Figure 3.87).

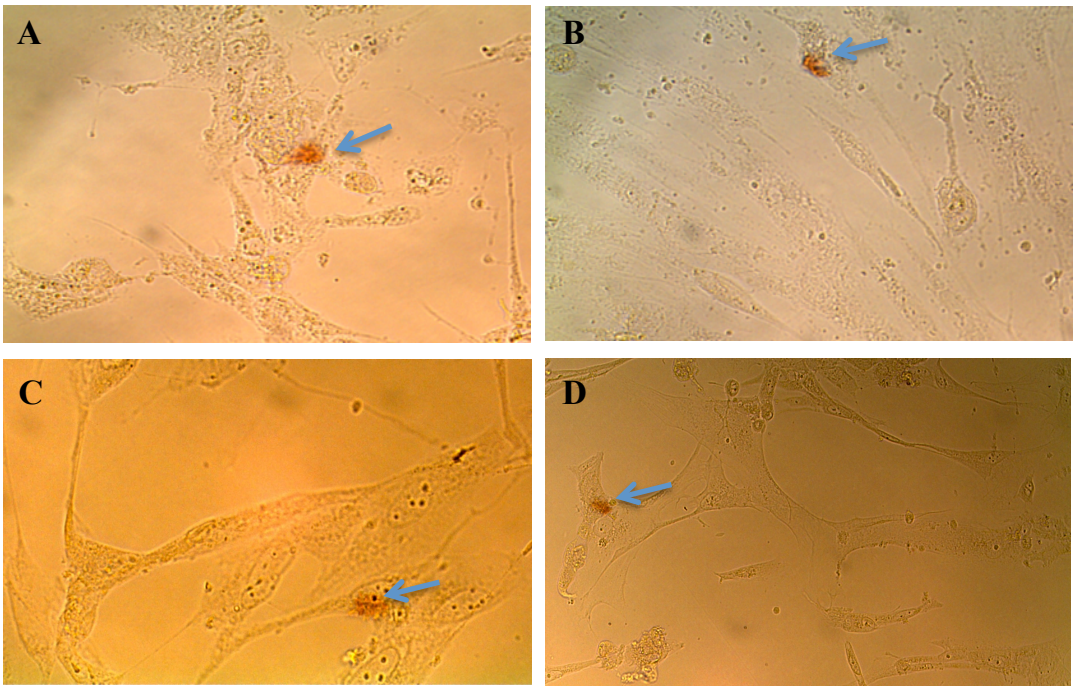

Figure 3.87: Demonstrates the detection of SIVmac239 rev protein in pCEFs (top two figures) and MRC-5 (bottom two figures) cross-infected with both rFP9 and rMVA.

The SIV rev protein was detected at 48 h after the cross-infection. A and B show positive SIV rev-expressing cells in pCEFs as a result of cross-infection with rFP9.rev and rMVA.T7pol (A), rMVA.rev and rFP9.T7pol (B). C and D show positive SIV rev-expressing cells in MRC-5 as a result of cross-infection with rFP9.rev and rMVA.T7pol (C), rMVA.rev and rFP9.T7pol (D).

Among the two SIVmac239 env and rev proteins studied, it was demonstrated that vector combination between rFP9 and rMVA (rFP9.env and rMVA.T7pol) can be used to enhance the expression levels of the SIV env protein in mammalian cells (MRC-5) only.

3.4.2 Quantitative ELISA

The same four groups of recombinant viruses, which were used in the previous ICC assay, were involved in this quantitative comparative study; group 1 (rFP9.env.rev and rFP9.T7pol), group 2 (rMVA.env.rev and rMVA.T7pol), group 3 (rFP9.env.rev and rMVA.T7pol), and group 4 (rMVA.env.rev and rFP9.T7pol). To compare the gene expression efficiency of these recombinant viruses in avian and mammalian cell lines, pCEFs and MRC-5 cells were co-infected simultaneously and the cell lysates were then prepared. The expression levels of the SIV env and rev proteins were quantified in both cell types at four different time points using a sandwich ELISA.

ELISA data are presented as mean values ± SD of protein expression levels of three replicate samples at each time point. The following ELISA results compare the production levels of the two SIV env and rev proteins expressed by four different groups of recombinant viruses in both avian and mammalian cells. In order to determine whether there is a statistical significant difference in the protein expression levels (at each time point) between the different virus combinations, a one-way ANOVA followed by a Bonferroni post hoc test was applied. In all comparison studies, p-value below 0.05 ($p < 0.05$) was considered as a statistically significant difference.

3.4.2.1 ELISA for quantitative analysis of SIVmac239 env protein expressed by different virus combinations in pCEFs and MRC-5 cells

The amounts of SIVmac239 env protein expressed by four groups of recombinants were quantified in two cell types at multiple time points using specific monoclonal antibodies to individual products. Table 3.7 represents the mean values ± SD (n=3) of the env protein expression levels in pCEFs cells that were co-infected simultaneously with different recombinant viruses employing identical gene expression cassettes (Table

3.7). As shown in figure 3.88, the optimal levels of the env protein were expressed by group 3 (rFP9.env and rMVA.T7pol) at 24 hpi. However, the gene expression levels of this group dropped sharply after the highest peak and became the lowest at 48, 72, and 96 hpi (Figure 3.88). At 72 h time point, it was found that using rFP9.T7pol with rMVA.env (group 4) produced significantly higher amount of the env protein than that expressed by rFP9.env and rMVA.T7pol (group 3) (Figure 3.89). In terms of co-infection, the amount of the env protein expressed by two rFP9s (group 1) was found to be higher than that produced either by two rMVAs (group 2) or rFP9.env and rMVA.T7pol (group 3) at 48, 72, and 96 hpi (Figure 3.88). Basically, a significant difference in the env expression level was observed between groups 1 and 3 at 48 and 72 hpi (Figure 3.89).

Time points	SIVmac239 env protein in pCEFs				
	Group 1	Group 2	Group 3	Group 4	P-values
	rFP9.env+ rFP9.T7pol	rMVA.env+ rMVA.T7pol	rFP9.env+ rMVA.T7pol	rMVA.env+ rFP9.T7pol	
24 h	43.7 ± 0.22	47.5 ± 6.51	53.9 ± 0.44	48.1 ± 6.91	0.15–NS
48 h	47.9 ± 5.31	34.8 ± 6.80	27.9 ± 1.95	43.5 ± 7.72	0.013
72 h	23.4 ± 1.81	18.2 ± 5.40	10.1 ± 0.54	21.9 ± 5.17	0.012
96 h	27.7 ± 6.68	20.4 ± 7.64	16.9 ± 5.85	32.2 ± 4.54	0.062–NS

Table 3.7: Shows the production levels of SIVmac239 env protein (µg/ml) expressed by four groups of recombinant viruses in pCEFs at different time points after infection. Data are presented as mean ± SD (n=3).

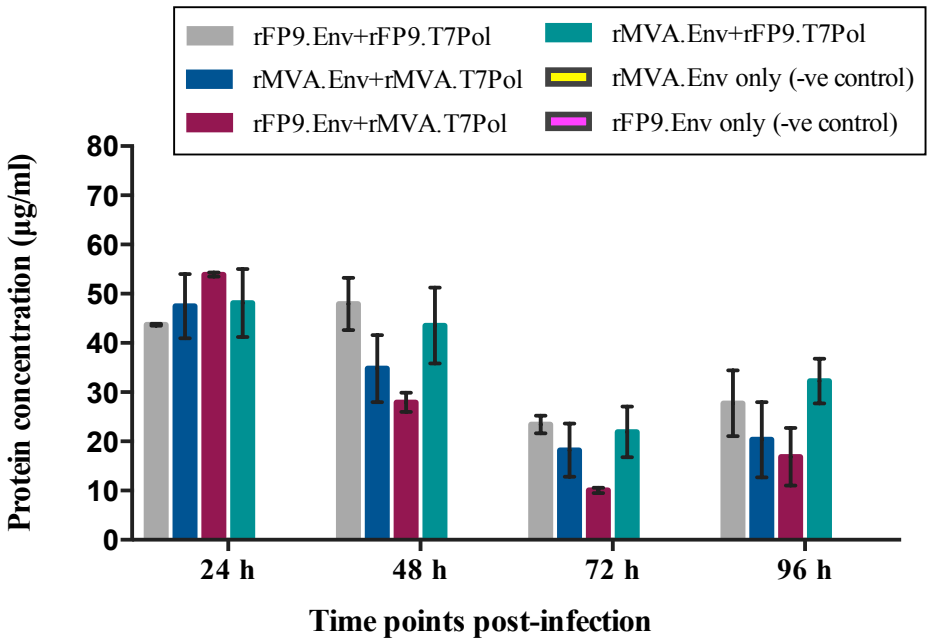

Figure 3.88: Compares the SIVmac239 env protein expression levels (µg/ml) in pCEFs between different recombinant viruses at multiple time points post-infection.

pCEFs cells were infected simultaneously with the recombinant viruses at a constant MOI (2 pfu/cell) for each recombinant. At different time points, pCEFs-infected cells were harvested and the target proteins were then extracted and quantified using a sandwich ELISA. These data are expressed as mean ± SD (n=3) of the env protein expression levels.

Generally, no remarkable differences were observed in the expression levels of the env protein in avian cells between all different groups of recombinant viruses at 24 and 96 hpi. However, a statistically significant difference was noted between groups 1 and 3 at 48 and 72 hpi with the p-value of 0.018 and 0.017 respectively. In addition, there were significant differences between groups 3 and 4 at 72 hpi with the p-value of 0.033 (Figure 3.89).

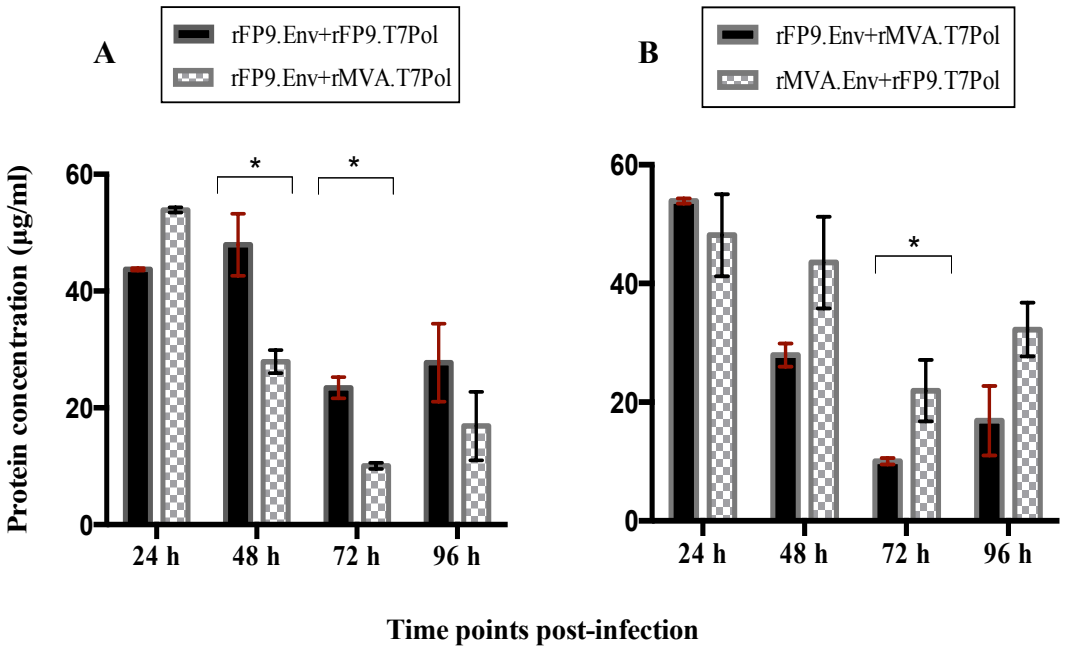

Figure 3.89: Shows the significant differences in the env protein expression levels in pCEFs between virus combinations.

These data are expressed as mean ± SD (n=3) of the env protein expression levels at each time point (*P<0.05). A) Groups 1 (rFP9.env+rFP9.T7pol) and 3 (rFP9.env+rMVA.T7pol), B) Groups 3 (rFP9.env+rMVA.T7pol) and 4 (rMVA.env+rFP9.T7pol). **The data presented here is summarised in figure 3.88**.

The amounts of the SIV env protein expressed by these different groups of recombinants were also assessed in mammalian (MRC-5) cells at multiple time points. Table 3.8 represents the mean values ± SD (n=3) of the env protein expression levels in MRC-5 cell line (Table 3.8). The optimal expression levels of the env protein in MRC-5 were expressed by group 4 (rMVA.env and rFP9.T7pol) at 96 hpi. However, at 24, 48, and 72 h time points, group 2 (rMVA.env and rMVA.T7pol) was the most effective and produced the highest amounts of the target env protein in MRC-5 cell line (Table 3.8).

Time points	SIVmac239 env protein in MRC-5				
	Group 1	Group 2	Group 3	Group 4	P-values
	rFP9.env+ rFP9.T7pol	rMVA.env+ rMVA.T7pol	rFP9.env+ rMVA.T7pol	rMVA.env+ rFP9.T7pol	
24 h	22.9 ± 0.37	27.3 ± 0.87	5.83 ± 1.57	21.7 ± 3.15	<0.0001
48 h	13.3 ± 2.97	29.8 ± 1.76	6.49 ± 0.37	18.2 ± 2.22	<0.0001
72 h	12.1 ± 2.55	30.9 ± 5.72	14.9 ± 0.74	27.7 ± 5.65	0.001
96 h	30.8 ± 2.87	38.3 ± 1.15	31.9 ± 4.63	40.3 ± 4.72	0.052–NS

Table 3.8: Shows the production levels of SIVmac239 env protein (µg/ml) expressed by four groups of recombinant viruses in MRC-5 at different time points after infection. Data are presented as mean ± SD (n=3).

Generally speaking, the env expression level by group 1 (rFP9.env and rFP9.T7pol) was characterised by a gradual decrease between 24 and 72 hpi, followed by a peak at 96 h. However, there was a gradual increase in the env expression levels by groups 2 (rMVA.env and rMVA.T7pol) and 3 (rFP9.env and rMVA.T7pol) over time (Figure 3.90).

As shown in figure 3.91, there were significant differences in the expression levels of the env protein in MRC-5 cells between: groups 1 and 2 at 48 and 72 hpi (p-value= <0.0001 and 0.004 respectively), groups 1 and 4 at 72 hpi (p-value= 0.012), groups 2 and 3 at 24, 48, and 72 hpi (p-value= <0.0001, <0.0001 and 0.01 respectively), groups 3 and 4 at 24, 48, and 72 hpi (p-value= <0.0001, 0.001 and 0.036 respectively) (Figure 3.91).

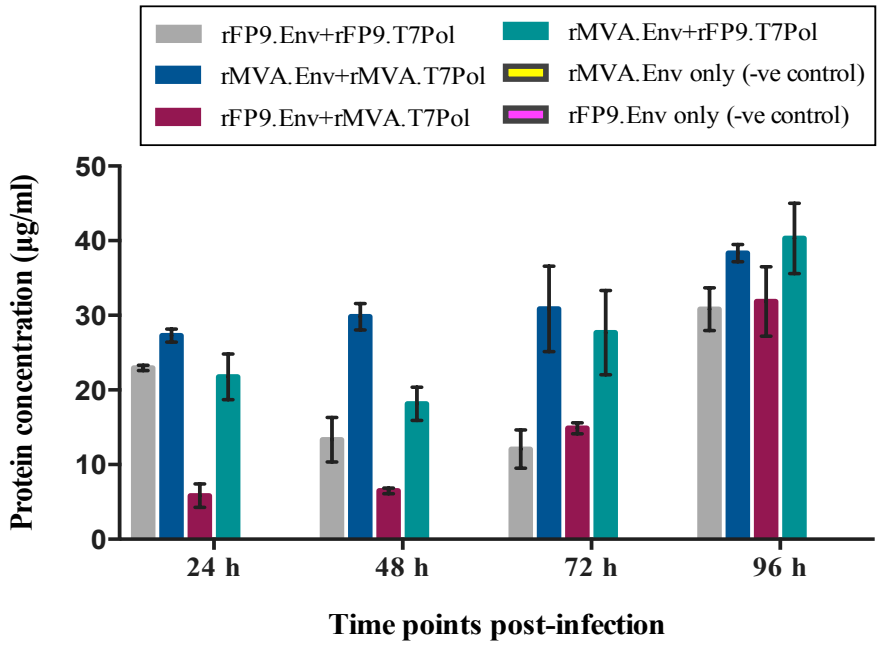

Figure 3.90: Compares the SIVmac239 env protein expression levels (µg/ml) in MRC-5 between different recombinant viruses at multiple time points post-infection.

MRC-5 cells were infected simultaneously with the recombinant viruses at a constant MOI (2 pfu/cell) for each recombinant. At different time points, MRC-5-infected cells were harvested and the target proteins were then extracted and quantified using a sandwich ELISA. These data are expressed as mean ± SD (n=3) of the env protein expression levels.

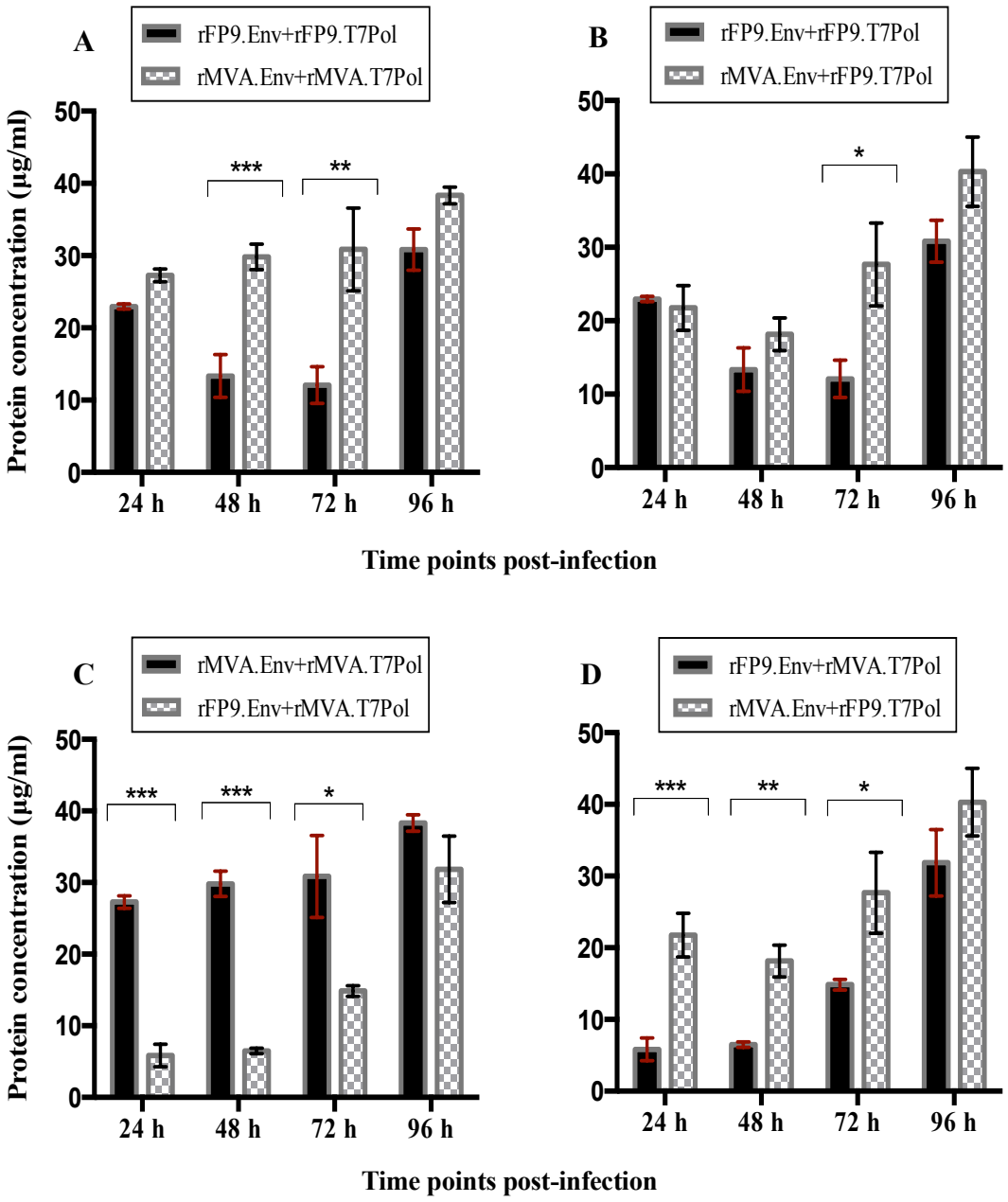

Figure 3.91: Shows the significant differences in the env protein expression levels in MRC-5 between virus combinations.

These data are expressed as mean ± SD (n=3) of the env protein expression levels at each time point (*P<0.05, **P<0.01, ***P<0.001). A) Groups 1 (rFP9.env+rFP9.T7pol) and 2 (rMVA.env+rMVA.T7pol), B) Groups 1 (rFP9.env+rFP9.T7pol) and 4 (rMVA.env+rFP9.T7pol), C) Groups 2 (rMVA.env+rMVA.T7pol) and 3 (rFP9.env+rMVA.T7pol), D) Groups 3 (rFP9.env+rMVA.T7pol) and 4 (rMVA.env+rFP9.T7pol). **The data presented here is summarised in figure 3.90**.

Figure 3.92 shows that co-infection of MRC-5 cells with the same two recombinant viruses (either two rFP9s or two rMVAs) expressed significantly higher levels of the target env protein than cross-infection with two different recombinants at 24 and 48 hpi. As can be seen from the figure 3.92, there were significant differences between: groups 1 and 3 at 24 and 48 hpi (p-value= <0.0001 and 0.021 respectively), and groups 2 and 4 at 24 and 48 hpi (p-value= 0.032 and 0.001 respectively). However, expression at 72 and 96 h was practically similar between co- and cross infection (Figure 3.92).

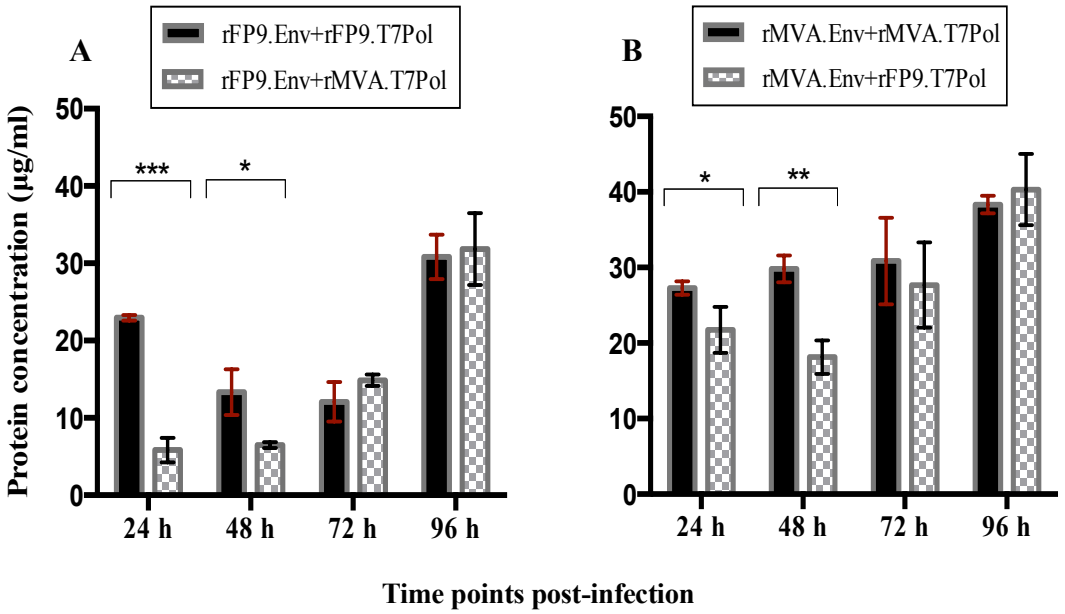

Figure 3.92: Shows the significant differences in the expression efficiency of the env protein between co- and cross-infection of MRC-5.

These data are expressed as mean ± SD (n=3) of the env protein expression levels at each time point (*P<0.05, **P<0.01, ***P<0.001). A) Groups 1 (rFP9.env+rFP9.T7pol) and 3 (rFP9.env+rMVA.T7pol), B) Groups 2 (rMVA.env+rMVA.T7pol) and 4 (rMVA.env+rFP9.T7pol). **The data presented here is summarised in figure 3.90**.

3.4.2.2 ELISA for quantitative analysis of SIVmac239 rev protein expressed by different virus combinations in pCEFs and MRC-5 cells

The production levels of SIVmac239 rev protein that was expressed by different groups of recombinant viruses were quantified in avian (pCEFs) and mammalian (MRC-5) cells at multiple time points. Table 3.9 represents the mean values ± SD (n=3) of the rev protein expression levels in pCEFs (Table 3.9). It was found that cross-infected pCEFs with rFP9.rev and rMVA.T7pol (group 3) expressed the optimal levels of the target rev protein at 96 hpi. Generally, the expression levels of the rev protein in pCEFs by groups 1 and 3 were higher than that expressed by groups 2 and 4 at 24, 48, and 96 hpi (Figure 3.93).

	SIVmac239 rev protein in pCEFs				
Time points	Group 1	Group 2	Group 3	Group 4	P-values
	rFP9.rev+ rFP9.T7pol	rMVA.rev+ rMVA.T7pol	rFP9.rev+ rMVA.T7pol	rMVA.rev+ rFP9.T7pol	
24 h	57.8 ± 8.19	31.1 ± 15.3	71.4 ± 16.6	34.9 ± 13.0	0.020
48 h	77.2 ± 5.90	25.5 ± 11.3	65.4 ± 1.41	25.5 ± 8.81	<0.0001
72 h	27.2 ± 2.53	46.1 ± 3.37	68.3 ± 12.1	50.4 ± 5.63	0.001
96 h	57.3 ± 3.37	25.7 ± 8.13	109 ± 18.6	35.6 ± 8.69	<0.0001

Table 3.9: Shows the production levels of SIVmac239 rev protein (µg/ml) expressed by four groups of recombinant viruses in pCEFs at different time points after infection. Data are presented as mean ± SD (n=3).

In terms of co-infection, group 1 (rFP9.rev and rFP9.T7pol) was more effective in expressing higher levels of the rev protein at 24, 48, and 96 hpi in comparison to group 2 (rMVA.rev and rMVA.T7pol). Only at 72 h, group 2 was able to produce higher amounts of the target rev protein than that expressed by group 1. Overall, there were fluctuations in the rev expression levels by groups 1, 2, and 4 over time between 24 and 96 hpi (Figure 3.93).

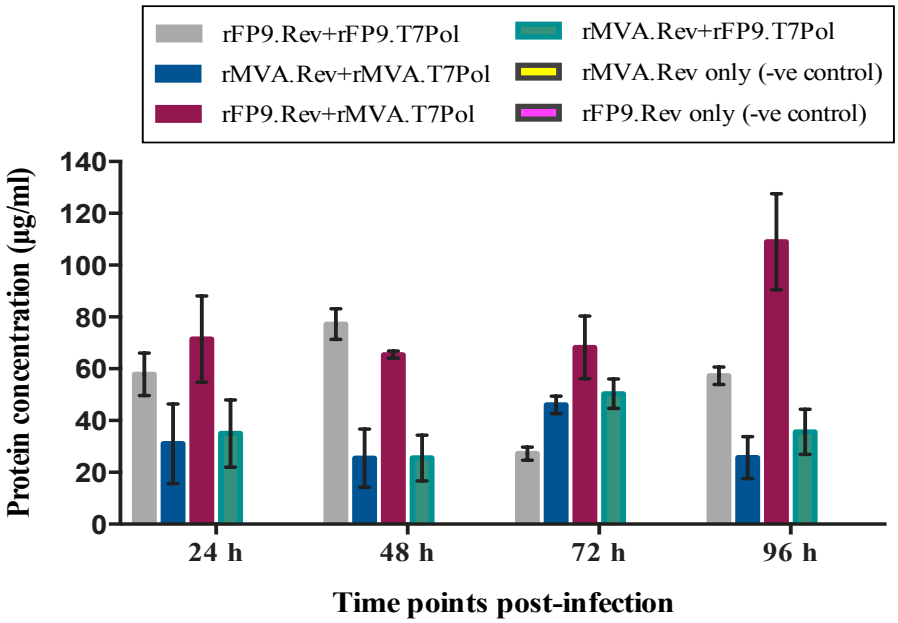

Figure 3.93: Compares the SIVmac239 rev protein expression levels (µg/ml) in pCEFs between different recombinant viruses at multiple time points post-infection.

pCEFs cells were infected simultaneously with the recombinant viruses at a constant MOI (2 pfu/cell) for each recombinant. At different time points, pCEFs-infected cells were harvested and the target proteins were then extracted and quantified using a sandwich ELISA. These data are expressed as mean ± SD (n=3) of the rev protein expression levels.

Among all different time points, statistically significant differences were noted in the rev expression levels in pCEFs between virus combinations (Table 3.9). As demonstrated in figures 3.94, there were significant differences between: groups 1 and 2 at 48 h (p-value= <0.0001), groups 1 and 4 at 48 and 72 h (p-value= <0.0001 and 0.022 respectively), groups 2 and 3 at 24, 48, 72, and 96 h (p-value= 0.041, 0.001, 0.027, and <0.0001 respectively), groups 3 and 4 at 48 and 96 h (p-value= 0.001 and <0.0001 respectively) (Figures 3.94).

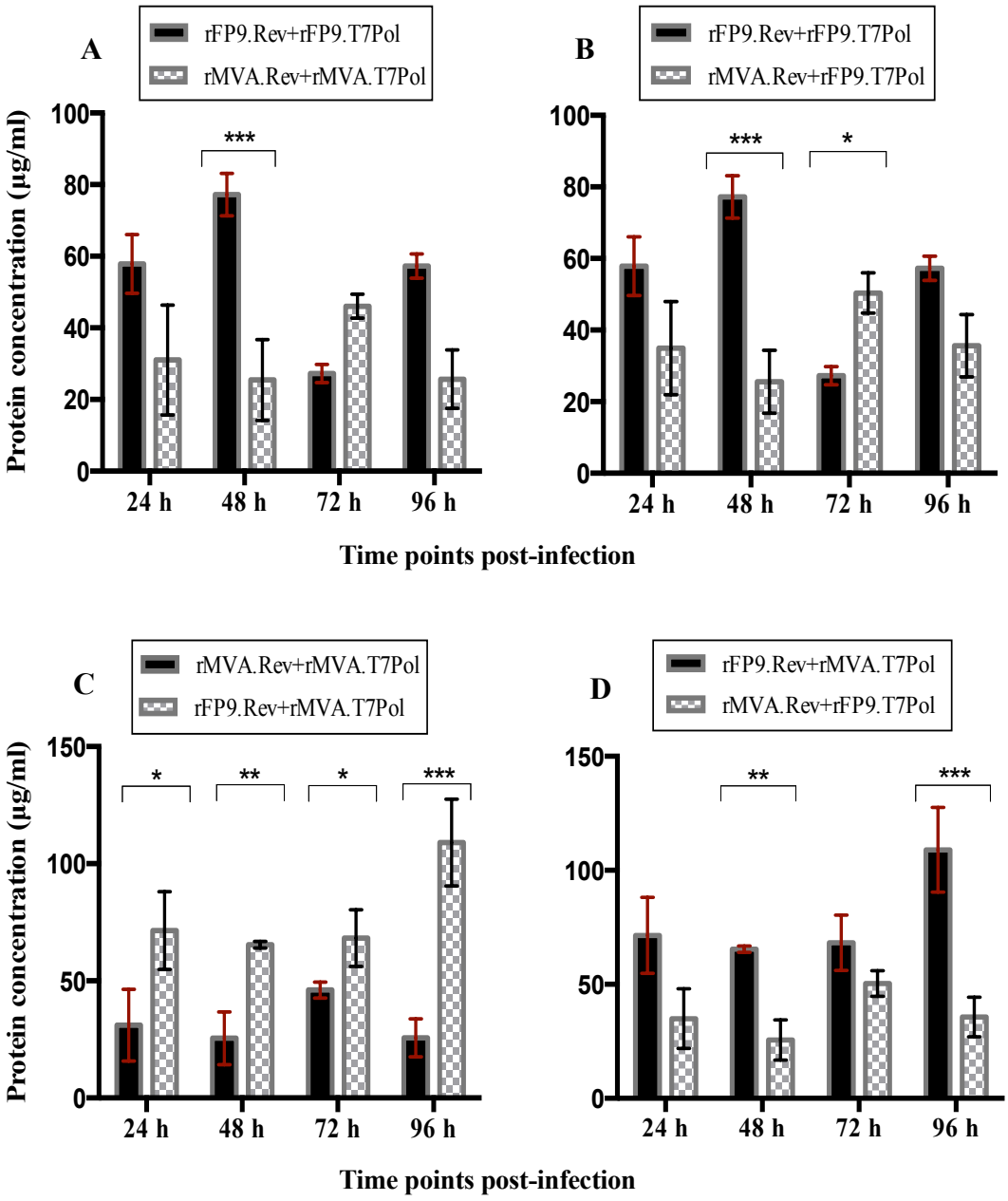

Figure 3.94: Shows the significant differences in the rev protein expression levels in pCEFs between virus combinations.

These data are expressed as mean ± SD (n=3) of the rev protein expression levels at each time point (*P<0.05, **P<0.01, ***P<0.001). A) Groups 1 (rFP9.rev+rFP9.T7pol) and 2 (rMVA.rev+rMVA.T7pol), B) Groups 1 (rFP9.rev+rFP9.T7pol) and 4 (rMVA.rev+rFP9.T7pol), C) Groups 2 (rMVA.rev+rMVA.T7pol) and 3 (rFP9.rev+rMVA.T7pol), D) Groups 3 (rFP9.rev+rMVA.T7pol) and 4 (rMVA.rev+rFP9.T7pol). **The data presented here is summarised in figure 3.93**.

In addition, it was found that cross-infection of pCEFs with two different recombinants (rFP9.rev and rMVA.T7pol) expressed significantly higher levels of the target rev protein than that produced by co-infection with the same two recombinant viruses (rFP9.rev and rFP9.T7pol) at 72 and 96 hpi with the p-value of 0.001 and 0.003 respectively (Figure 3.95).

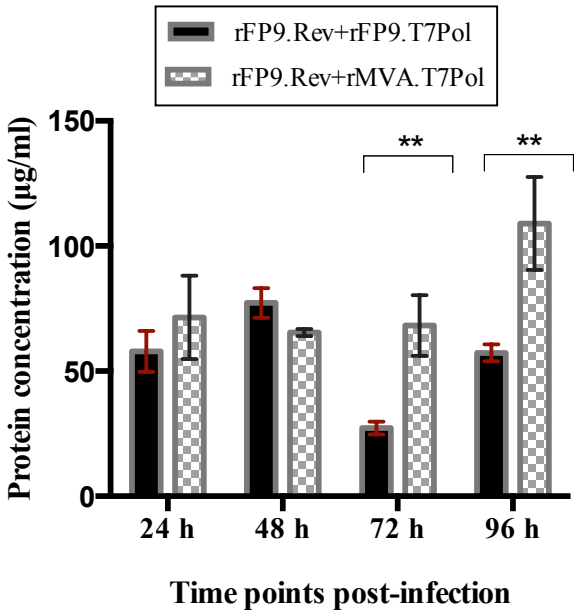

Figure 3.95: Shows the significant differences in the expression efficiency of the rev protein between co- and cross-infection of pCEFs.

These data are expressed as mean ± SD (n=3) of the rev protein expression levels at each time point (**P<0.01). Group 1 (rFP9.rev+rFP9.T7pol) expressed a significantly higher level of the rev protein than that produced by group 3 (rFP9.rev+rMVA.T7pol) at 72 and 96 h time points. **The data presented here is summarised in figure 3.93**.

A quantitative comparison of the rev protein expression by different recombinants was also performed in MRC-5 cells at various time points. Table 3.10 represents the mean values ± SD (n=3) of the rev protein expression levels in MRC-5 cell line (Table 3.10). Figure 3.96 shows that group 1 (rFP9.rev and rFP9.T7pol) was the most effective among all different groups of recombinant viruses in expressing the target rev protein in MRC-5 over time (Figure 3.96). However, there were no statistically significant differences in the expression levels of the rev protein between all virus combinations (Table 3.10).

Time points	SIVmac239 rev protein in MRC-5				
	Group 1	Group 2	Group 3	Group 4	P-values
	rFP9.rev+ rFP9.T7pol	rMVA.rev+ rMVA.T7pol	rFP9.rev+ rMVA.T7pol	rMVA.rev+ rFP9.T7pol	
24 h	45.9 ± 2.19	31.6 ± 12.3	28.5 ± 7.29	39.4 ± 10.2	0.148–NS
48 h	42.9 ± 6.03	33.1 ± 4.97	30.1 ± 7.56	25.1 ± 7.62	0.057–NS
72 h	39.2 ± 6.97	36.5 ± 4.72	33.4 ± 5.59	33.1 ± 3.43	0.491–NS
96 h	43.7 ± 3.69	26.4 ± 8.73	30.9 ± 7.86	40.3 ± 2.58	0.054–NS

Table 3.10: Shows the production levels of SIVmac239 rev protein (µg/ml) expressed by four groups of recombinant viruses in MRC-5 at different time points after infection. Data are presented as mean ± SD (n=3).

In terms of co-infection of MRC-5, the production levels of the rev protein by two rFP9s (group 1) were greater compared to that expressed by two rMVAs (group 2) among all different time points (Figure 3.96). As a cross-infection, no improvement was achieved in the rev protein expression levels by using rMVA.T7pol with rFP9.rev (group 3). However, using rFP9.T7pol with rMVA.rev (group 4) was effective and produced higher levels of the rev protein than that expressed by two rMVAs (group 2) at 24 and 96 hpi (Figure 3.96).

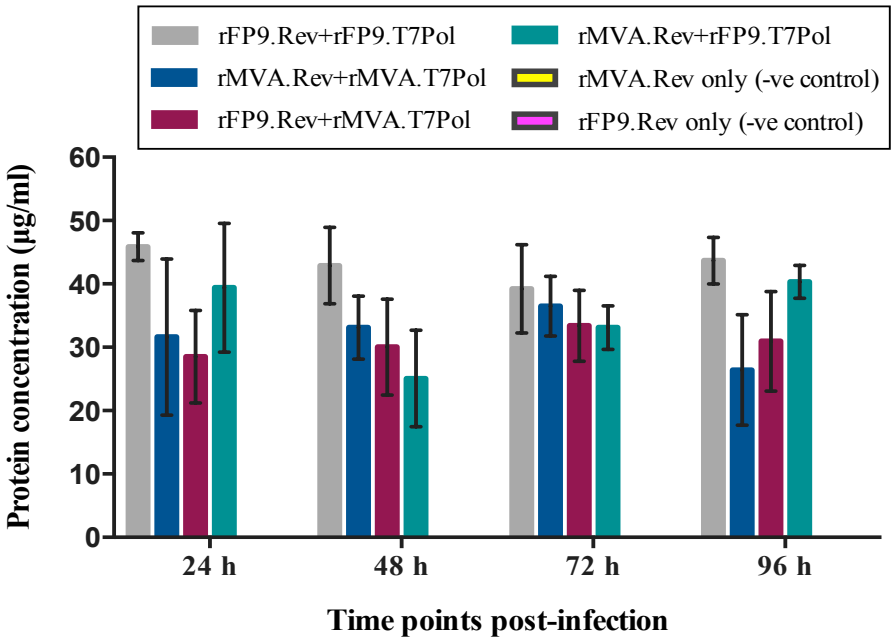

Figure 3.96: Compares the SIVmac239 rev protein expression levels (µg/ml) in MRC-5 between different recombinant viruses at multiple time points post-infection.

MRC-5 cells were infected simultaneously with the recombinant viruses at a constant MOI (2 pfu/cell) for each recombinant. At different time points, MRC-5-infected cells were harvested and the target proteins were then extracted and quantified using a sandwich ELISA. These data are expressed as mean ± SD (n=3) of the rev protein expression levels.

4 Discussion

FP9 is a highly attenuated poxvirus strain that has lost several gene families of the wild-type FPV during the extensive serial tissue culture passages (Mayr and Malicki, 1966). rFP9 vectors have an enhanced ability to induce stronger immune responses compared to the FPV. These recombinants have been shown to generate specific immune responses to target recombinant proteins in humans with no systemic side effects beyond minor local reactions at the immunisation site (Anderson et al., 2004; Webster et al., 2006). They are considered promising vaccine candidates due to their safety profile in humans and also their large genome size that can encode a significant amount of foreign DNA fragments which can be successfully expressed in mammalian hosts (Boyle et al., 2004; Webster et al., 2006).

This PhD project aims to construct complex rFP9 vectors encoding various genomic components of SIVmac239 (env, gag, pol, and accessory genes except nef). In addition, a single rFP9 vector containing a defective SIVmac239 genome under the control of phage T7 RNA polymerase gene was to be generated. These constructed complex rFP9 will later be used as a retroviral vaccine candidate for SIVmac239/macaque model of HIV infection. The idea in this study is original in the field of vaccine design. The novelty of this project lies in using the FP9 as a viral vector encoding SIVmac239 sequences in combination with the T7 RNA polymerase expression system to generate a replication-restricted retrovirus.

4.1 Molecular biology and DNA cloning techniques

In order to construct rFP9, different insertion sites within the FP9 genome were first designed and synthesised. Homologous flanking regions to these insertion sites were

designed to facilitate HR between FP9 transfer plasmids and the FP9 genome to integrate the target SIVmac239 sequences into the FP9 genome at various locations.

4.1.1 Design of FP9 insertion sites

Five potential insertion sites within the FP9 genome were chosen to construct five rFP9s carrying different SIVmac239 sequences. Due to the fact that FP9 does not have many insertion sites characterised, it was decided in this study to use three non-essential regions and only two intragenic insertion sites that have already been used in previous studies. Firstly, F11L orthologue (ORF 110), which is localised in the central region of the FP9 genome, was used in this project as an intragenic insertion site for vpx gene and T7 RNA polymerase cassette. In 2002, this orthologue (ORF 110) was used successfully as a suitable insertion locus to construct recombinant FPV encoding melanoma-associated antigen tyrosinase (Boulanger et al., 2002). As proved by Boulanger et al. (2002), disturbing the F11L gene does not affect the virus replication or the expression of encoded target genes.

Secondly, thymidine kinase (TK) gene (ORF 86), which is a highly conserved region among fowlpox viruses, is considered another non-essential gene that can be used for insertion of target DNA sequences (Boyle et al., 1987). However, Scheiflinger et al. (1997) reported that the TK gene can be considered as one of the essential genes for viral growth, particularly in the highly attenuated poxviruses such as fowlpox strain HP1.441. As a consequence, in order to avoid disturbing the TK gene, the non-essential region between the TK gene (ORF 86) and the downstream gene (ORF 87) was used in this project as an intergenic insertion site for tat and gag-pro SIVmac239 genes. This example describes the first use of this intergenic region within the FP9 genome.

Thirdly, the photolyase gene (ORF 158), which is expressed late in the viral replication cycle, can also be considered as one of the non-essential genes for viral growth in cell cultures (Srinivasan et al., 2001). However, this gene encodes an enzyme that has the ability to protect and repair the fowlpox genome from the UV light-induced DNA damage (Srinivasan et al., 2001; Swenson et al., 2008). Therefore, it was decided in this study to use the non-essential region between the photolyase gene (ORF 158) and the upstream gene (ORF 157) as an intergenic insertion site for env and rev SIVmac239 genes. This example describes the first use of this intergenic region within the FP9 genome.

Fourthly, intracellular mature virus (IMV) membrane protein (ORF 179.1), which may have a role in the viral virulence, is another non-essential gene (Laidlaw and Skinner, 2004). In 2004, Laidlaw and Skinner reported that the IMV protein is not required for viral replication in tissue culture. Hence, this gene was used in this project as an intragenic insertion site for RT, RNase, int, vpr, and vif SIVmac239 genes.

Finally, in the present study, the non-essential region between ORFs 6 and 7 was used as an intergenic insertion site for the defective SIVmac239 genome. The same insertion site was used successfully by Alvarez-Lajonchere et al. (2008) to construct rFP9 vector encoding HCV core-E1 polyprotein (Alvarez-Lajonchere et al., 2008).

All the designed DNA sequences (FP9 homologous flanking regions) were submitted to BlueHeron^{TM} Biotechnology for synthesis. They were subcloned into a variant of pUC, a high copy number plasmid vector lacking a multiple cloning site and encoding a kanamycin resistance gene. The absence of the multiple cloning site (MCS) allows the easy incorporation of unique restriction sites in synthetic DNA, thereby facilitating subsequent subcloning. This pUC plasmid encodes kanamycin resistance and lacks

ampicillin resistance gene; another factor that aids subcloning into the ampicillin-resistant MVA recombination plasmids.

4.1.2 RE digestion analysis of transfer plasmid DNA

As an initial step in generating FP9 transfer plasmid DNA, it was necessary to confirm the actual sequence of both the newly synthesised FP9 HS plasmids and the previously constructed MVA transfer plasmids using RE digestion analysis. In each digestion reaction, the total volume of RE, which are supplied in a 50% glycerol solution, did not exceed 10% of the total reaction volume. This was due to the fact that some enzymes can exhibit star activity that can alter or reduce their cleavage specificity (unwanted cleavage or product degradation) (Wei et al., 2008). This loss of fidelity commonly occurs when the optimal reaction conditions are changed, such as the use of a high percentage of glycerol (>5%), low ionic strength (<25 mM salt), or an incompatible buffer (Wei et al., 2008). To ensure good restriction digests, fresh sets of high-fidelity (HF) enzymes (NEB Inc., UK) were used to reduce star activity and increase accuracy.

All five MVA transfer plasmids and two out of five FP9 HS plasmids (FP9-pUC-F11L and FP9-pUC-F157i158) were double digested using unique enzymes that have only one cutting site within the whole plasmid. Therefore, the observed banding patterns of each plasmid of these seven constructs were two fragments (the smaller fragments corresponded to the target DNA sequences and the larger fragments corresponded to the linearized form of the plasmid). However, only three FP9 HS plasmids (FP9-pUC-F86i87, FP9-pUC-FIMV179 and FP9-pUC-F6i7) were double digested using non-unique enzymes that have two cutting sites. As a result, three digested fragments were observed in the restriction banding profile of these FP9 plasmids. Despite the fact that three fragments were seen upon the agarose gel, the targeted DNA sequences were

easily distinguished from the background due to their unique small sizes. Based on the generated sequence maps, all five MVA transfer vectors and the five FP9 HS plasmids were successfully analysed and confirmed for use in subsequent cloning experiments.

4.1.3 Generation of FP9 transfer plasmid DNA

To facilitate the integration of SIVmac239 sequences into the FP9 genome, five FP9 transfer plasmids employing a TCS strategy were constructed. The SIVmac239 genome was a huge insertion and therefore performed in isolation. In order to minimise the number of recombination procedures, general combinations of SIV sequences were inserted simultaneously. No more than two SIV sequences (genes) were inserted at one time in order to avoid direct duplication of identical T7 promoters and termination sequences that would have led to instability. It was decided to couple large and small SIV sequences generally because they were easy to verify on restriction digests and in order to avoid very large inserts.

The five FP9 transfer plasmids were generated using five previously constructed MVA transfer plasmids encoding various SIVmac239 sequences (provided by Dr. Thomas Blanchard and Dr. Gowda CPC). All ten newly synthesised FP9 HSs (flanking regions) were cloned into these five different MVA transfer plasmids (two FP9 HSs for each MVA plasmid) to construct FP9 transfer plasmid DNA carrying SIVmac239 sequences. In agreement with other studies, some of the encoded SIV sequences were human codon-optimised for eventual optimal protein expression in mammalian cells, and also to reduce the similarity between the sequences within the recombinant virus (Narum et al., 2001). This decreases the risk of generating a replication competent SIV particle, and hence increases the safety profile of the vaccine candidate.

In the present study, it was decided to double digest the target DNA sequences (both the MVA vector and the FP9 insert) using two different REs (one enzyme on the 5' end and the other on the 3' end) that are capable of producing sticky (cohesive) ends. The reason behind this is to prevent self-ligation (re-ligation) of the vector and to confirm the correct orientation of the inserted sequences. Using compatible REs (single digestion) can increase the chance for the vector to re-ligate to itself (Patel et al., 2012). In order to set up a double digest reaction, the two REs should have the same optimal reaction conditions, such as the reaction buffer, incubation time for digestion, activation temperature, and requirement for BSA (Patel et al., 2012).

For the cloning step, several attempts were made to ligate and transform FP9 HS into MVA transfer plasmid DNA, but they all failed, and no transformed colonies were detected after 24 h of transformation. The lack of transformants was likely due to the low concentration of both vector and insert plasmid DNA used in the ligation stage. However, the control plates, which contain the competent cells without the plasmid, gave the expected results. Many single colonies were seen on the positive control plate (LB agar medium without antibiotic), and no colonies were seen on the negative control plate (selective LB agar medium). The results of these control plates have proven the viability of the used competent cells and the selection process. On the other hand, few single colonies were observed on the negative control plate containing the linear plasmid alone. This result indicates that the MVA vector was not completely digested, and that there was some amount of background.

Cloning conditions recommended by the manufacturer were then optimised in order to increase the ligation and transformation efficiency. The vector to insert the molar ratio was increased from 1:3 to 1:5 and 1:10. The concentration of the linearized plasmid vector was also increased from 50 ng to 200 ng. Moreover, the length of time of heat

shock (at 42°C) was increased from 30 to 50 sec. Following these modifications, the efficiency of the ligation and transformation was increased, and many transformed colonies were seen on the selective LB agar medium.

4.2 Construction of rFP9

To date, there have not been any studies reporting the construction of rFP9 containing SIVmac239 sequences. This PhD project is the first to use FP9 as a viral vector to deliver various genomic components of SIVmac239 under the control of a T7 RNA polymerase expression system.

4.2.1 HR in pCEFs

The HR method was used to construct rFP9 encoding various SIVmac239 sequences. This recombination was carried out in pCEFs between the FP9 homologous flanking sequences within the transfer plasmid vector and the FP9 genome. There are two common possibilities of HR that may take place: single and double crossover recombination. Single recombination occurs between either the right or left FP9 HS and the FP9 genome. This type of recombination results in the integration of the entire circular plasmid vector into the FP9 genome and hence the generation of unstable recombinant blue plaques containing the marker gene (Parks et al., 1994). Duplication of the HS within the recombinant genome of the blue plaque leads to a high rate of subsequent intragenomic HR between the identical sequences, resulting in either the construction of a pure (markerless) recombinant virus or the restoration of the parent virus (Moss et al., 1981; Parks et al., 1994). On the other hand, double crossover recombination occurs between both the right and left FP9 HS and FP9 genomes, leading to the integration of the desired SIV sequences only. This type of recombination results in the generation of stable colourless recombinant viruses encoding the target insertion

cassette and lacks the marker and plasmid sequences. However, the frequency of double recombination events in FPV is very low (<0.007%) in comparison to the single crossover type (Parks et al., 1994).

Due to the fact that pCEFs are characterised by low transfection efficiency that can reduce the chances of HR, it has been recommended to transfect the cells first with the target transfer plasmid DNA, followed by infection (transfection-infection) (Chen et al., 2013). However, initial attempts to generate rFP9 following this order (transfection-infection) were unsuccessful. In this study, therefore, it was decided to infect the cells first with the FP9 (at 0.05 pfu/cell) and then follow with transfection of the FP9 transfer plasmid DNA into the FP9-infected cells (infection-transfection). By following this order, the frequency of recombinant virus construction was improved. Around 0.8% of rFP9 plaques from the infection/transfection mixture was generated (approximately two to four recombinant blue plaques were obtained per dish), and about 50% of these recombinant viruses could be recovered, most of which harboured the desired sequences. The transfection efficiency of the SuperFect® transfection reagent was assessed through transfecting 2 μg of purified RFP into pCEFs. The results demonstrate that the SuperFect reagent was highly efficient, and many cells (around 70% of cells per well of a 6-well plate) were transfected with the target fluorescent protein.

In contrast to the high frequency of rFP9 obtained in this study, Spehner et al. (1990) and Nazerian and Dhawale (1991) reported a low rate of recombinant FPV generation, ranging from 0.01 to 0.1%. Parks et al. (1994) demonstrated that the efficiency of HR in generating recombinant FPV can be affected by the length of FPV homologous flanking regions in the transfer plasmid vector. They found that the low rate of recombinant FPV formation may be improved by increasing the length of FPV homologous regions (Parks et al., 1994). Although the size of each FP9 homologous fragment used in this study

was small (around 0.5 Kb), the rate of rFP9 generation was high (0.8%). In Parks' study, the highest percentage of formation of recombinant FPV (0.62%) was only obtained by using the largest homologous fragments of FPV (4.5 Kb) (Parks et al., 1994). Using the modern SuperFect® transfection reagent, large amounts of transfer plasmid DNA, and requiring only single crossovers may explain the high proportion of rFP9 generation in this study.

4.2.2 Identification of positive rFP9

In the present study, the TCS screening strategy (Parks et al., 1994) was used to identify the positive rFP9 even when the chances of recombination events were low. It was not necessary to employ mycophenolic acid selection, which contrasts with the previously described method of Scheiflinger et al. (1998). The TCS method is based on using the bacterial LacZ gene as a chromogenic substrate marker encoding a β-galactosidase enzyme that is able to hydrolyse the X-gal to yield an intense blue-colour compound (Chakrabarti et al., 1985). Using this marker greatly improved the identification process of rFP9. However, it has been reported that the presence of selectable marker genes within a potential live vaccine can be associated with host-related side effects (Scheiflinger et al., 1998). For these safety concerns, it was decided to insert the marker gene outside the genomic flanking region in order to facilitate unstable integration of the marker cassette into the recombinant genome during the single recombination event. This marker integration leads to the generation of a blue plaque phenotype, indicating the preliminary construction of recombinant viruses encoding both the desired SIV sequences and the marker gene. Following multiple rounds of blue plaque purification, a subsequent intragenomic HR between the identical sequences within the recombinant genome leads to the elimination of the marker gene and the generation of pure recombinant viruses that appear as colourless plaques (Falkner and Moss, 1990; Parks et

al., 1994). However, these colourless plaques may indicate either a nonrecombinant FP9 or a pure recombinant virus that has lost the non-essential marker and plasmid-derived sequences. The selection of pure recombinant viruses was a challenge due to the fact that large numbers of recombinant viruses (blue plaques) have reverted to nonrecombinant viruses as a result of the intragenomic HR events. At the final stage of purification, PCR was used to distinguish between the pure rFP9 and nonrecombinant viruses, which both produce colourless plaques. Several sets of oligonucleotide primers have been designed specifically to confirm the presence of the inserted SIV expression cassettes, and to ensure the elimination of the plasmid and marker gene sequences. Each set was targeting various locations within the inserted sequence: one at 3' and the other at the 5' of the gene for optimal coverage of the whole desired sequence. Single and clearly isolated plaques were picked during each round of purification, in order to increase the chance of picking pure recombinants and hence reduce the rounds of plaque purification.

The marker gene was designed to be expressed as a viral late gene under control of the VACV late promoter (p11). Generally, blue colour development was observed within 18–24 h after staining (Prideaux et al., 1990). However, in some cases, this time was extended to 48 h until the blue colour developed. This delayed β-gal expression was only noted during the screening of the double rFP9 that encoded env.rev and tat.gag-pro insertion cassettes. The reasons for the delay are still unexplained, but two possible factors may contribute to the extended time of blue colour appearance: slow replication dynamics of FPV (Chaudhry et al., 2007) and inserting the β-gal marker gene under the transcriptional control of the VACV late promoter (p11). However, these factors are true of all recombinants, and yet, it was a feature only of the double env.rev/tat.gag-pro

recombinant. It was realised that the blue colour production in rFP9-infected cells was improved with longer incubation times (48–72 h post-staining).

Despite the different sizes of the insertion cassettes, all rFP9-produced plaques were roughly similar in size to those generated by the nonrecombinant FP9. However, there were slight differences in the viral titers (nearly two logs difference) between the recombinant viruses. This difference might be due to the considerable batch-to-batch variation of pCEFs in their ability to support the formation of rFP9-infected cell foci (Scheiflinger et al., 1998). Therefore, it can be concluded that the insertion of foreign exogenous DNA had no major influence on virus replication efficiency and morphogenesis, confirming previous studies (Soprana et al., 2011; Shouwen et al., 2015). However, these results still need to be practically confirmed, employing growth curve analysis to compare the growth kinetics of the nonrecombinant FP9 with the different rFP9s that have been constructed in this study.

A mixed population of blue and colourless plaques was observed during the first five rounds of purification. The genetic instability of the blue plaques was predicted due to the intragenomic HR event between the repeated sequences. Therefore, getting a yield of 100% blue plaques was very difficult or even impossible despite several cycles of purification. It was found that the ratio of blue to colourless plaques was increasing each cycle of purification until the fourth or fifth rounds of blue plaque purification, when the proportion of both plaques became nearly equal. At this point, screening for pure (markerless) recombinants started. Out of 24 plaques analysed in the first round of colourless plaque purification, only 3 to 5 retained the desired SIV sequences and lacked the marker gene. This small number of markerless recombinant plaques was improving each cycle of purification until only pure recombinant viruses were detected in the fifth round of colourless plaque purification. In the present study, it was noticed

that the proportion of unstable recombinant viruses that had been restored back to the parental phenotype was much higher than the pure recombinant viruses. In contrast to these findings, Falkner and Moss (1990) and Scheiflinger et al. (1998) demonstrated the rapid loss of the marker gene through using the transient dominant selection (TDS) technique for isolating recombinants. Falkner and Moss reported that, after three rounds of plaque purification, all the isolated plaques lost their marker genes, and half of them retained the target insertion sequences. Scheiflinger et al. found that 90–100% of the picked colourless plaques from the sixth rounds of purification were positive for the desired sequences and lacked both marker genes. However, this TDS strategy is based on employing mycophenolic acid selection and using double marker genes, which differ from the TCS technique used in this study (Falkner and Moss, 1990; Scheiflinger et al., 1998). In addition, using this type of selection marker (*E coli.* xanthine-guanine phosphoribosyl transferase [gpt] gene) was found to be associated with causing a second-site mutation in the recombinant genome (Rice et al., 2011).

Scheiflinger et al. (1992), Yao and Evans (2003), and Rice et al. (2011) used a new approach to insert foreign DNA into poxvirus vectors (VACV) without the need for any selection method or plasmid construction. This technique is based on using the helper virus system to clone and package the target sequences. These studies reported higher rates (5–10% by Scheiflinger et al., 30% by Yao and Evans, and 21–100% by Rice et al.) of recombinant generation than that obtained by the general *in vivo* HR technique (Scheiflinger et al., 1992; Yao and Evans, 2003; Rice et al., 2011). However, it has been reported that using this technique can introduce new unintended changes presumably caused by underlying mutations at the phenotypic levels in <25% of the generated recombinants, which can affect *in vivo* animal studies (Rice et al., 2011).

For confirming the construction of pure and stable rFP9, a total of 10–12 rounds of plaque purification were carried out in pCEFs (5–7 rounds for each blue and colourless purification). Blue plaque purification was performed to confirm the integration of the insertion cassette. However, colourless plaque purification was conducted to eliminate the non-essential marker and plasmid-derived sequences, and prove the stability of the target SIV sequences and their insertion sites. The genomic stability of the inserted DNA sequences was maintained over several continuous rounds of purification (10–12 times) in pCEFs as determined by PCR analysis. Repetitive screening of 14 randomly picked colourless plaques of the final pure markerless recombinant proved the purity and genetic stability of the encoded sequences and insertion sites.

4.3 SIV protein expression and characterisation in avian and mammalian cells using rFP9 vectors

In the present study, protein expression assays were limited due to the lack of specific antibodies representing all SIV sequences. Only four available monoclonal antibodies were supplied by NIBSC and used for the protein analysis and characterisation of specific SIVmac239 sequences: env, rev, gag, and tat genes. Three different protein expression assays were performed to detect and analyse the encoded proteins: WB, ICC, and ELISA.

4.3.1 WB assay

To confirm the ability of rFP9 to express the encoded transgenes in avian and mammalian cells, both pCEFs and Vero cells were co-infected with the target recombinant viruses. The specific sizes of SIVmac239 env, rev, and gag proteins were successfully detected and analysed using a WB assay. Only one of the target proteins (tat) was not recognised on the immunoblot because its primary antibody was not

suitable for detection in the denatured form of the protein in WB assay. Protein denaturation is one of the common problems related with the WB technique that may affect the natural state of the expressed proteins. On the immunoblot, native proteins are not fixed on the membrane and so can be denatured and lose their structural features (secondary, tertiary, and quaternary structures). Hence, some antibodies cannot recognise this form of protein unless they have been raised and tested for such an experiment (WB) (Burry, 2011). In the WB experiment, two types of negative controls (uninfected and nonrecombinant FP9-infected cells) were included. However, the assay was lacked an appropriate positive control, including the protein of interest. This unavailability of proper recombinant proteins was due to shipment delays and time constraints. A positive protein control is important to validate the procedures and the reagents used in the assay.

At the beginning of the assay, it was decided to evaluate the protein samples at four different time points (24, 48, 72, and 96 hpi) in order to determine the best time for optimal gene expression. It was noted that the highest protein expression levels by rFP9 occur between 48 and 72 hpi. Therefore, these two time points (48 and 72 h) were selected as the basis to analyse the expression of all the different protein samples by WB.

For SIV env protein detection, three different immunospecific bands with molecular masses of 160, 120, and 32 kDa were observed in pCEFs, while six various molecular weights (160, 140, 130, 120, 70, and 32 kDa) were detected in Vero cells using the characterised SIV env gp160/32 as a primary antibody (Kent et al., 1992). As expected, these specific protein bands were not observed with the two negative controls. Analysis of rFP9-infected pCEFs and Vero cell lysates revealed the presence of the SIV env main precursor (full-length uncleaved) protein (gp160) and the two cleaved forms of the env

protein, external surface glycoprotein (gp120) and truncated transmembrane protein (gp32), which match the expected sizes (Veronese et al., 1989; Khattar et al., 2013). It has been reported that the size of the transmembrane protein of SIVmac (32 kDa) is smaller than the same protein detected for HIV-1 (41 kDa). The difference between the two proteins is about 140 amino acids (Veronese et al., 1989). Detection of the env precursor protein in the infected cell lysates indicates that it was not efficiently processed and cleaved into mature proteins. In addition to these three main bands (160, 120, and 32 kDa) that were observed in the Vero cells, there were unexpected bands of three other proteins with molecular masses of approximately 140, 130, and 70 kDa. These proteins, which have not yet been characterised, probably represent proteolytically cleaved products of the main precursor env protein. The exact composition of these variants would require confirmation by comprehensive mass spectrometry.

The WB results demonstrated the successful expression of the target SIV env protein in pCEFs and Vero cells infected by rFP9. In agreement with previous studies, these findings proved the ability of rFP9 to express the encoded target sequences in mammalian (Vero) cells despite the fact that these cells are not permissive for FP9 replication (Britton et al., 1996). Somogyi et al. (1993) observed similar levels of early gene expression and genome replication of FPV in both pCEFs and Vero cells, suggesting that there is no major block to the replication cycle of FPV in this transformed type of mammalian cell. However, the expression of FPV late genes was found to be lower and delayed in Vero cells compared to that expressed in pCEFs (Somogyi et al., 1993). Although FPV can undergo a full replication cycle in Vero cells and hence produce new progeny viruses, the generated VLPs are not infectious (Somogyi et al., 1993; Zanotto et al., 2010). As can be seen from WB results, the

optimal env protein expression levels in Vero cells, which are non-permissive, were detected at 48 hpi. At 72 h, the expression was reduced and became lower than that expressed at 48 hpi. In contrast, the maximal expression levels in pCEFs, which are permissive cells, were observed at 72 hpi. These results are in line with previous findings demonstrating lower levels of FPV late gene expression in Vero than pCEFs cells (Somogyi et al., 1993). Another possible explanation for this expression variation could be that different amounts of proteins were loaded into each well of the gel through SDS-PAGE protein analysis. This is one of the study's limitations that may affect the outcome of protein expression results. Quantitative determination of the total protein concentration of each sample in this assay would ensure that an equal amount of proteins was added in each well.

For SIV rev protein detection, one specific band with a molecular mass of approximately 18 kDa was observed in rFP9-infected pCEFs and Vero cells. This protein size is consistent with the expected product of the SIV rev gene that was detected in a previous study by Cheng et al. (1992). However, no rev protein production was observed within the two negative controls, indicating the successful expression of the target SIV rev protein in infected avian and mammalian cells.

Two different monoclonal antibodies to SIV rev were used against the denatured rev protein, due to a shortage of reagents. The SIV rev EVA3072.4 antibody was used firstly for pCEFs, while the EVA3072.2 antibody was then used for Vero cells. Analysis of uninfected and rFP9-infected pCEFs lysates revealed the presence of high-frequency cross-reactions between the monoclonal EVA3072.4 antibody and the pCEFs cells used in the assay. The low specificity of the used rev antibody might be the reason for generating a high background of irrelevant proteins. Insufficient washing or inappropriate blocking conditions could be other reasons; however, the same WB

protocol was followed for analysing all the target SIV proteins, and this high background only happened once. Therefore, it can be concluded that commercially purified monoclonal antibodies may not always be specific for the detection of the target antigens.

For SIV gag protein detection, the WB analysis of rFP9-infected pCEFs and Vero cell lysates revealed the presence of the SIV gag main precursor protein with the expected molecular weight of approximately 55 kDa (p55) (Caravokyri et al., 1993). As can be seen from the results, only the uncleaved SIV gag protein (p55) was detectable in both infected cells using the specific gag-reactive monoclonal antibodies (SIV gag p27-ARP 396/397) isolated by Dr Szawlowski (Hanke et al., 1992). However, the expression of other mature capsid proteins (p27) could not be detected in both cells (pCEFs and Vero) despite the fact that the antibody used has a dual specificity for p55 and p27 proteins. It is known that cleavage of the gag precursor protein is entirely dependent on the presence of the viral protease, which is encoded by the structural pol gene (Accola et al., 1998; Owens et al., 2003). In this study, therefore, it was decided to construct rFP9 expressing the entire SIVmac239 gag gene and part of the pol gene (pro). The gag-pro polyprotein precursors were human codon-optimised and inserted into one of the designed FP9 insertion sites under the control of T7 promoter. However, the ribosomal slippage site of the pro gene was not codon-optimised in order to preserve the parent virus sequence. Although this structural design of rFP9 will facilitate the proteolytic cleavage of the gag precursor in the rFP9-infected cells, no cleaved gag proteins were observed. Two possibilities can be considered to explain the absence of mature capsid proteins in this expression study. First, the protease protein was either not expressed or was present at very low levels that were insufficient to cleave the main gag protein. Second, the encoded protease protein may be unable to process the gag precursor

protein for some unspecified reasons (Flexner et al., 1988; Mars et al., 1990). It has been reported that the expression of structural pol genes requires a ribosomal frameshift mechanism for the two overlapping ORFs (gag-pol) in order to reveal the pol ORF. This frameshifting event facilitates the synthesis of gag-pol precursor proteins and hence the expression of mature pol proteins (Jacks et al., 1988; Flexner et al., 1988; Karacostas et al., 1989). Therefore, low levels of translational shifting might be another possibility explaining the absence of processed gag proteins. The failure of expression of mature capsid proteins in this study is consistent with results obtained by previous studies that used VACV for expressing the full-length gag-pol region in human embryonic stem cell lines (H9), reported by Flexner et al. (1988). Flexner et al. also reported the absence of gag-processed proteins in monkey kidney fibroblast cell lines (CV-1) (Flexner et al., 1988). In contrast to these results, Mars et al. (1990) proved the proteolytic cleavage of gag precursor proteins in both CV-1 and Vero cells (Mars et al., 1990). In addition, Ourmanov et al. (2000) and Wyatt et al. (2004) demonstrated the successful synthesis and processing of gag proteins in BSC-1 (monkey kidney epithelial cell lines) using rMVA (Ourmanov et al., 2000; Wyatt et al., 2004).

4.3.2 ICC staining assay

An ICC assay was performed on rFP9-infected pCEFs and MRC-5 to visualise the cellular localisation of the expressed SIV proteins (env, rev, gag, and tat) using DAB peroxidase staining. As can be seen from the ICC results, all the target SIV proteins were successfully detected in both pCEFs (avian) and MRC-5 (mammalian) cells at 48 hpi.

It should be noticed that the env protein was the only protein that highly expressed and was clearly visualised within rFP9-infected cells compared to other SIV proteins. The

number of env-expressing cells was also higher than the number of positive cells expressing rev, gag, or tat proteins. This might be because of the different cellular localisation of the env and other SIV products. It has been reported that the synthesised env product can persist in the cytoplasm of infected cells over a long period of time due to the fact that the transcripts of this protein were found associated with membrane-bound polyribosomes (Zanotto et al., 2010). The high expression levels of the env protein could also be explained by the presence of different cleaved forms of the env protein processed by cellular enzymes, as demonstrated previously by WB assay. On the other hand, the expression level of gag proteins was low, and only a few positive gag-expressing cells were observed. The most likely explanation for this low protein level could be the unsuccessful proteolytic cleavage of the main gag precursor protein, as discussed previously. It might also be because the gag protein is one of the proteins that is synthesised on free ribosomes, which differs from the env product (Zanotto et al., 2010). These expression results of env and gag proteins are generally in agreement with the findings reported by Zanotto et al. (2010). In the same way, only small to moderate numbers of infected cells expressing the target SIV rev and tat proteins were identified, and they also showed faint staining of products. This result suggests that either there was a reduced expression level of these proteins or difficulty in determining their subcellular localisation. It has been reported that the SIV rev and tat proteins are concentrated within infected cells' nuclei/nucleoli (Cochrane et al., 1990; Dundr et al., 1995; Musinova and Sheval, 2015), which would make them difficult to identify using the ICC technique.

In both cell types (pCEFs and MRC-5), the expression levels of SIV rev, gag, and tat proteins were similar, and no obvious differences were observed. However, the env protein appeared to be expressed qualitatively at high levels in pCEFs compared to

MRC-5 cells. This result confirms previous observations of Somogyi et al. (1993), who observed higher levels of FPV gene expression and genome replication in pCEFs than in MRC-5 cells. The absence of both C7L and K1L host-range genes, which are required for VACV replication in most human cell lines (such as MRC-5), in the FPV genome could be responsible for the expression variations between pCEFs and MRC-5 (Perkus et al., 1990; Somogyi et al., 1993).

4.3.3 ELISA

In this study, a sandwich ELISA was performed to quantify the expression levels of SIV proteins in different cell lines at multiple time points. To analyse the expression efficiency of the rFP9 candidate in avian and mammalian cells, pCEFs/MRC-5/Vero cells were co-infected simultaneously with the target recombinants. MRC-5 and Vero cell lines, which are primate fibroblast-like cells, were used in order to prove the ability of rFP9 to express the encoded SIV protein in various mammalian hosts despite the fact that both are non-permissive cells for FP9 replication. Due to time constraints, it was difficult to use these two mammalian cell lines to examine the expression of all the target SIV proteins; therefore, it was decided to use the MRC-5 for env and rev and the Vero cells for gag and tat protein expression.

The preliminary ELISA results demonstrated that rFP9 can successfully express SIVmac239 env, rev, gag, and tat proteins in different cell types (pCEFs, MRC-5, and Vero). This confirms previous studies showing the ability of rFPV to express exogenous antigens in these various cell lines (Somogyi et al., 1993; Pacchioni et al., 2010; Zanotto et al., 2010; Pacchioni et al., 2013). In the present study, the expression levels of SIV env and rev proteins were followed over a period of four subsequent days after infection (24, 48, 72, and 96 h), whereas the SIV gag and tat protein levels were evaluated only at

48 and 72 h. As expected, the expression levels of the target SIV proteins were found to be generally stronger and achieved higher levels in avian cells than mammalian cells, even though the encoded sequences were human codon-optimised. Indeed, there were significant differences in the expression levels of SIV env and rev proteins between pCEFs and MRC-5 cells. However, the production amounts of SIV gag and tat proteins were not significantly different between pCEFs and Vero cells. Generally speaking, FPV replication was found to be more restricted in MRC-5 cells than in Vero cells, resulting in lower levels of gene expression in MRC-5 compared to the Vero cell lines (Somogyi et al., 1993). This previous finding may explain the variation of transgene expression levels that were detected in this study between the two mammalian cell types. It was noticed that the differences in the SIV protein expression levels between pCEFs and MRC-5 were greater than that observed between pCEFs and Vero cells. Hence, it can be concluded that the level of gene expression by rFP9 in mammalian cells is cell type-dependent; this is in concordance with FPV replication studies (Somogyi et al., 1993; Britton et al., 1996).

Moreover, preliminary data show that the expression patterns of the target SIV proteins by rFP9 were almost consistent. It can be noticed from the time course experiment that the minimum levels of all expressed SIV proteins were observed at 72 hpi in both avian and mammalian cells. However, the expression of the majority of SIV proteins peaked at 48 h among the different types of cells, with the exception of the env protein, which reached maximum levels in MRC-5 cells at 96 h. This result demonstrates the long-lasting expression of the env protein in MRC-5 cells by rFP9 in spite of its restricted replication in mammalian cells. It has been reported that using rFPV-T7 as an expression system in mammalian cells causes little CPE, and hence the infected cells can be maintained for long periods, allowing for high levels of transgene expression

(Das et al., 2000). In addition, the T7 RNA polymerase gene, which was responsible in the present study for directing the transcription of the encoded SIV sequences, was driven by an early/late p7.5 VACV promoter in order to ensure continuous and long-lasting expression of the target T7 gene. Using the T7 expression approach is also believed to produce higher levels of recombinant protein in mammalian cells than that obtained by using the conventional recombinant system (Fuerst et al., 1987; Kriajevska et al., 1993).

4.3.4 GFP expression for identification of double rFP9 encoding the DSIV genome and the T7 RNA gene

In the present study, the ability to construct a single rFP9 vector containing both the T7 RNA polymerase gene and the DSIV genome flanked with the T7 promoter was explored. The SIVmac239 genome was attenuated with a deletion of RT and int in the pol gene, a nef deletion, and mutated the active site of pro. In the absence of these viral structural and key accessory proteins, the DSIV genome cannot be replicated in FP9-infected cells. The deleted regions were substituted with the codon-optimised GFP gene and the encephalitis myocarditis virus's internal ribosome entry site (EMCV IRES). It was decided to insert the GFP marker gene within the middle of the DSIV genome in order to prove the expression efficiency of the T7 system within a single recombinant virus. To enhance the transgene expression of the full-length DSIV genome (around 8 kb), the EMCV IRES was inserted just downstream of the GFP that is located in the central region of the target gene. These IRES elements are capable of initiating an internal cap-independent translation process in the middle of the transgene transcripts (Elroy-Stein et al., 1989). It has been reported that inserting the EMCV IRES within the T7 expression system (rVACV-T7) between the T7 promoter and the target gene was

able to enhance the encoded protein expression levels (4–7 fold) (Elroy-Stein et al., 1989).

To construct a single rFP9 vector containing both the T7 gene and the target DSIV genome, a specific gene insertion order was followed in this study. The T7 polymerase gene was firstly integrated into the FP9 genome to construct a single rFP9. This rFP9 encoding only the T7 gene was then used as backbone to insert the DSIV genome cassette into a different insertion site to generate a double rFP9 encoding both components of the T7 expression system. This insertion order is in marked contrast to the findings reported by Kriajevska et al. (1993), who demonstrated the impact of the T7 gene insertion order on the efficiency of double recombinant generation. They found that inserting the target gene first, followed by the T7 gene, significantly improved the yield of construction of double recombinants, whereas the reverse gene insertion order may prevent the target gene integration into the recombinant genome. This gene restriction might be because of the transcription process of the inserted target gene by the phage T7 RNA already present in the recombinant genome. Initiating the transcription can influence the HR mechanism and restricts gene integration (Kriajevska et al., 1993).

The construction of a single recombinant virus encoding both the T7 gene and the reporter gene regulated by the T7 promoter has been described previously by Alexander et al. (1992) and Kriajevska et al. (1993). Using a single recombinant virus was found to be able to express higher levels of encoded proteins than that produced by two recombinant viruses (co-infection system) (Alexander et al., 1992). However, overexpression of the T7 gene as a result of using strong promoters can interfere and affect viral replication and transcription of the target genes. This uncontrolled expression was also found to be associated with recombinant virus instability

(Alexander et al., 1992). For these reasons, it has been suggested to regulate the expression of the T7 gene either by using a repressor gene (Alexander et al., 1992) or an early weak VACV promoter (P_F) (Kriajevska et al., 1993), in order to construct a stable single recombinant vector containing the whole T7 expression system. In contrast to these previous findings, the rFP9.T7pol.DSIV, which was constructed in the present study, was stable even after 10 rounds of plaque purification, despite the fact that a strong early/late p7.5 VACV promoter was used to direct the transcription of the T7 gene. In addition, no repressor gene or inducible promoters were introduced to regulate T7 expression. The DSIV genome, when transcribed and subsequently translated in the presence of T7 polymerase, is not expected to have any CPEs. This may explain why a viable recombinant vector was successfully constructed in this study.

As can be seen from the positive results of the GFP expressing-cells, the T7 RNA polymerase gene was able to drive the GFP gene expression in the same rFP9 vector. Due to the fact that GFP was inserted within the middle of the DSIV genome, its expression is considered linked to the expression of large gene clusters of the DSIV genome. This GFP expression suggests that it is likely that the T7 RNA polymerase system expresses all the DSIV genome sequences located downstream and upstream of the GFP. However, the expression of the reporter gene (DSIV genome) is still under investigation and needs to be practically confirmed using various protein expression assays.

4.4 Comparison of gene expression profiles between rFP9 and rMVA encoding env/rev SIVmac239 sequences under the control of T7 RNA polymerase

Gene expression profiles of rFP9 and rMVA encoding identical insertion cassettes (env and rev SIVmac239 sequences) were analysed in avian and mammalian cells. Vector combinations between the two recombinant viruses were also assessed. However, interpretation of protein expression levels with rFP9 against rMVA was difficult because different VACV promoters were used in the two expression systems (rFP9.T7pol and rMVA.T7pol).

In this protein analysis study, it was decided to employ a regulated T7 RNA polymerase to drive env/rev SIVmac239 sequences in rFP9 and rMVA viruses. The regulated T7 expression system was based on co-infection of target cells with two recombinant viruses (at an MOI of 2 pfu/cell for each virus): one encoding the target SIV sequences and the other one expressing the T7 RNA polymerase gene (Fuerst et al., 1987). Basically, four different groups of recombinant viruses were included in this comparative study: group one (rFP9.env.rev and rFP9.T7pol), group two (rMVA.env.rev and rMVA.T7pol), group three (rFP9.env.rev and rMVA.T7pol), and group four (rMVA.env.rev and rFP9.T7pol). The SIV protein levels expressed by these various recombinant viruses were analysed and compared using two different protein expression assays: ICC and ELISA.

4.4.1 ICC assay for qualitative comparative analysis between different virus combinations

By 48 hpi, all the different groups of recombinant viruses successfully expressed the target env and rev proteins, although at different levels, in pCEFs and MRC-5 cells. As

can be seen from the ICC results, the env protein expression levels by two rMVAs (group 2) was qualitatively higher than that expressed by two rFP9s (group 1) in both cell types. There are several factors that may contribute to the observed stronger expression level by rMVA over rFP9. During recombinant propagation in pCEFs cultures, it was noted that rFP9 replicates more slowly than rMVA despite the fact that this type of cell is highly permissive for both viruses. It is at least 5–6 days after pCEFs infection until rFP9 can induce visible CPE and plaques, whereas only 3–4 days were enough for replicating rMVA to produce larger plaques than those generated by rFP9 after 6 days of infection. This slow replication characteristic of FPV compared to MVA has been previously reported by Chaudhry et al. (2007) and Soprana et al. (2011). In pCEFs, MVA has also a strong ability to spread the infection to the surrounding cells quickly. Around 100 cells can be infected with MVA within 24 hpi using an MOI of 0.01 (Carroll and Moss, 1997). It could be possible that the high replication rate of rMVA in pCEFs gives them the ability to express higher levels of the encoded proteins than rFP9. However, it is difficult to exclude the possibility that rFP9 could still have the same expression efficiency as rMVA if the time of infection was extended to 72 and 96 h in an ICC assay. In the same way as pCEFs, rMVA appeared to express the target SIV env protein in MRC-5 cell line more efficiently than rFP9. Overall, the reduction in env expression by rFP9 compared with rMVA might be because blocks of FPV replication in non-permissive mammalian cells usually occurs at earlier stages than that observed for MVA replication. It has been reported that MVA replication is usually blocked after genome replication and late gene expression (Sutter and Moss, 1992). It also might be caused by FPV interfering with surface expression of env, which would selectively impair ICC results.

As a cross-infection of pCEFs with either rFP9/rMVA (group 3) or rMVA/rFP9 (group 4), it was found that the expression levels of the env protein were reduced compared to the co-infection with the same recombinant viruses. The reasons for this low expression level are unclear, but are likely to be due to competition between different recombinant viruses (rMVA and rFP9) to infect the target pCEFs cells and spread the infection. This competition can result in rapid cellular destruction and hence reduce the expression level of the target's antigens. On the other hand, cross-infection between rFP9 and rMVA appeared to work efficiently in MRC-5. The results show that high amounts of env protein were expressed in MRC-5 cross-infected with both rFP9.env and rMVA.T7pol (group 3), whereas no noticeable improvement was observed in cells that were cross-infected with both rMVA.env and rFP9.T7pol (group 4). It seems likely that the efficiency of env protein expression was enhanced when rFP9 was used to deliver the target SIV sequences under the control of T7 gene that was expressed by rMVA.T7pol. In fact, the difference in expression of the env protein in mammalian cells was more obvious by using rMVA.T7pol in comparison to rFP9.T7pol. Even though rMVA.T7pol has been reported to produce higher levels of toxicity in some primary mammalian cell cultures compared to the rFPV.T7pol (Das et al., 2000), the CPE of rMVA.T7pol in MRC-5 was found similar to that observed by rFP9.T7pol. Das et al. (2000) compared the expression efficiency of rMVA.T7pol and rFPV.T7pol in various cell lines. They found that both were able to achieve similar levels of the T7 polymerase despite their different cytotoxicity profiles. Therefore, the differences observed in this study between using rMVA.T7pol and rFP9.T7pol might be related to the poxvirus promoters directing the T7 gene. It is worth noting that different VACV promoters with varying transcriptional strengths were used to direct the transcription of the T7 RNA gene in FP9 and MVA recombinants. In rFP9.T7.pol, the expression of the T7 was

driven by an early/late p7.5 VACV promoter (Cochran et al., 1985), whereas in rMVA.T7pol, the expression was under the control of early/intermediate I3L VACV promoter (Santra et al., 2002). This promoter variation may affect the expression efficacy of the recombinant viruses. Different promoters were used because of concerns that too strong a promoter in MVA might be counterproductive.

The levels of SIV rev protein expressed by the different groups of recombinant viruses were generally indistinguishable using the ICC assay. This might be because only small numbers of rev-positive cells were identified in both infected pCEFs and MRC-5. In the same way, no improvement was observed in the rev protein levels in both cell types that were cross-infected with either rFP9/rMVA or rMVA/rFP9. These weak expression ICC results of the SIV rev protein were expected due to the difficulty in determining the subcellular localisation of the target SIV products, as discussed previously.

4.4.2 ELISA for quantitative comparative analysis between different virus combinations

The SIV env and rev expression profiles of four different groups of recombinant viruses were quantified in two cell lines (pCEFs and MRC-5) at multiple time points (24, 48, 72 and 96 hpi) using ELISA. A time course experiment was conducted to determine the best time for optimal gene expression by these various recombinants. Preliminary ELISA data show that no remarkable differences were observed in the expression levels of the env protein in avian cells between all the different virus combinations. Indeed, it was quite difficult sometimes to find out the most effective group in expressing the target SIV proteins due to the fact that low numbers of replicates were included in each assay.

While pCEFs are considered highly permissive cells for both FP9 and MVA replication, it was of interest in this study to have a combination between fast- and slow-replicating viruses in order to keep the infected cells intact for longer and hence improve the expression of the target proteins. Interestingly, cross-infected pCEFs with rFP9.env and rMVA.T7pol (group 3) expressed the highest levels of the env protein at 24 h. As expected, the optimal levels of the env protein were expressed in avian cells at 24 hpi. This might be due to the extensive replication of rMVA and rFP9 in this type of cell that supports high levels of poxvirus replication. Then, the expression levels started to decrease gradually, indicating that the vast majority of cells were infected and began to detach from the surface. It can also be seen that cross-infection with rMVA.env and rFP9.T7pol (group 4) enhanced the expression of the target env protein and produced higher levels than that generated by two rMVAs (group 2) over time. At 72 h time point, the results indicated that the production amounts of SIV env protein were significantly different between groups 4 and 3.

In terms of co-infection of pCEFs, it was found that two rFP9s seemed to express higher amounts of the env protein than two rMVAs at 48, 72, and 96 hpi. By monitoring virus-infected pCEFs over the subsequent four days, it was noted that rFP9 causes a slower cellular destruction compared to rMVA. Therefore, rFP9-infected pCEFs can survive for longer, allowing sustained and long-term expression of the encoded antigens. This could be one of the explanations why rFP9 was able to express the target protein in pCEFs more efficiently than rMVA.

In mammalian cells, however, there was a gradual increase in the env expression levels over time. The highest amounts of the env protein were expressed (by group 4) in MRC-5 at 96 hpi. This finding was also expected because MRC-5 is considered a non-permissive cell line for FP9 and MVA replication. Therefore, these recombinant

viruses, which cannot complete their replication cycle in MRC-5, showed reduced virulence and weak CPE, accompanied by slow cellular destruction, resulting in increased levels of protein production over time. Regarding MVA replication in mammalian MRC-5 cell lines, there is still some confusion. Some studies reported the ability of MVA to undergo a very limited replication in MRC-5 (Wyatt et al., 1998), whereas others claim that there is no evidence for replication (Blanchard et al., 1998). In 2013, Cottingham and Carroll reported that these studies demonstrated a very low rate of virus replication (less than 10 pfu/1 pfu input) in a limited number of cases. This weak amplification could be a result of laboratory contamination with the wild-type VACV (Cottingham and Carroll, 2013). Nevertheless, it was difficult in this study to prove either of these findings. However, it is still possible to report that there was a sign of MVA replication in MRC-5, as the infected cells showed very weak CPE and monolayer destruction that progressed slowly. Regardless of these debates, what is important to report in this study is that using two rMVAs (group 2) was the most effective among all the different groups of recombinant viruses in expressing the env protein in MRC-5 at 24, 48, and 72 hpi. Indeed, this group expressed significantly higher levels of the target env protein than that produced by other virus combinations at 48 h time point. The enhanced ability of rMVA might be attributable to the late stage of blockage of virus replication in mammalian cells, which occurred during virus assembly after genome replication (Sutter and Moss, 1992).

As a cross-infection of MRC-5, using rFP9.T7pol with rMVA.env (group 4) seemed to express significantly higher levels of the env protein than group 3 (rFP9.env and rMVA.T7pol). This finding shows strong correlation with the cross-infection results that were observed in avian cells. From these data, it seems likely that using rMVA as a viral vector to deliver the gene of interest under the control rFP9.T7pol would lead to

expressing high levels of the env protein in both pCEFs and MRC-5. However, these ELISA findings were just the opposite of those demonstrated by the ICC assay showing that using rFP9.env with rMVA.T7pol (group 3) was more efficient in MRC-5. In fact, relying on the ICC results to compare the expression efficiency between the recombinant viruses is often difficult because this technique is a qualitative research method compared to the ELISA. Nevertheless, these few conflicting results between the two assays can probably be explained by batch-to-batch variations of the cell density in supporting virus replication and hence target protein expression. It also might be due to the high SD that was observed between the replicates at particular time points. This high SD with a small sample size could affect the final ELISA results and lead to improper correlation between both assays.

The rev expression profile of different recombinant viruses was also assessed in both mammalian and avian cells. In mammalian cells, it was found that the peak of rev protein expression was produced by two rFP9s (group 1). Indeed, this group was the most effective in expressing the target rev protein in MRC-5 over time. This sustained level of expression until the fourth day post-infection confirms the strong late activity of the VACV p7.5 promoter (Wyatt et al., 1996; Becker et al., 2014) in directing the T7 RNA expression in MRC-5, since FP9 cannot replicate efficiently in these mammalian hosts. Furthermore, it can be noted that group 4 (rMVA.rev and rFP9.T7pol), which utilizes the rFP9.T7pol, expressed similar amounts of the rev protein to group 1 (rFP9.rev and rFP9.T7pol), particularly at 24 and 96 hpi. Hence, the rFP9.T7pol could be one of the main factors that were responsible for enhancing the expression levels of the target rev protein in MRC-5 by groups 1 and 4. Apart from these two groups, the production level of rev protein by other recombinant viruses was similar to each other, indicating that there are no major differences in expression efficacy between these

various recombinants. In pCEFs, however, the situation was different. It was found that vector combination between rFP9.rev and rMVA.T7pol (group 3) expressed the optimal levels of the rev protein. Interestingly, this group of recombinant viruses also produced the highest levels of the env protein in pCEFs, as discussed previously. At 48 and 96 hpi, significant differences were observed in the rev expression levels between group 3 and groups 2 and 4. In terms of co-infection of pCEFs, using two rFP9s (group 1) was able to express significantly higher levels of the rev protein than group 2 (two rMVAs) at 48 hpi. Generally speaking, two rFP9s appeared to express the target rev protein more efficiently than two rMVAs. Again, this finding shows a strong correlation with the co-infection results of pCEFs in expressing the env protein. However, marked differences were observed between the two proteins (env and rev) in the amount of production. It was found that the rev protein was expressed at levels substantially higher than the env protein. Previous studies reported the possibility that there may be an inverse correlation between the gene length and its expression level. Small genes appeared to have a higher expression level due to the high translation efficiency of their short mRNA (Castillo-Davis et al., 2002; Chiaromonte et al., 2003). Therefore, the high expression levels of the rev protein observed in this study could be related to its small gene length (324 bp) compared to the env gene (2640 bp).

5 Conclusions

Much research work is being carried out globally to develop a safe and effective HIV vaccine using viral vectors. Amongst them, poxviruses have been extensively studied as immunisation vehicles for the induction of protective immune responses against HIV. The highly attenuated host range-restricted FP9 strain is considered one of the promising poxvirus vectors that can be used as a vaccine candidate due to its proven advantages in gene transfer applications and safety profile in humans. rFP9 vectors offer the advantage of encoding a significant amount of exogenous antigens that can be successfully expressed in mammalian hosts, triggering specific immune responses to the recombinant proteins. This PhD project reports the preliminary construction of a model HIV vaccine candidate through generation of complex rFP9 encoding various genomic components of SIVmac239. It also describes the feasibility of using TCS for genetically manipulating silent retroviral ORFs and intergenic regions into five different recombination sites within the FP9 genome. To our knowledge, the present study is the first to use FP9 as a recombinant vector to genetically engineer multiple SIVmac239 sequences under the control of a T7 expression system.

In this project, five rFP9 encoding large and complex synthetic DNA sequences of SIVmac239 were constructed, and their genetic stability was also confirmed. Two of those were double recombinants encoding two independent expression cassettes and the other three recombinants were single vectors containing only one insertion. Employing the TCS technique using β-galactosidase marker cassette within FP9 transfer plasmid had a significant impact in facilitating the construction of these recombinants. Using this screening method has allowed the generation of rFP9 wholly employing pCEFs; producing pure recombinants containing solely the desired synthetic DNA with no extraneous material.

The resultant rFP9, which were constructed in this study, showed appropriate expression of the encoded SIV protein in both avian and mammalian cells, although at different levels. In line with our expectations, higher amounts of proteins were expressed in pCEFs in comparison to that detected in MRC-5 and Vero cell lines. The expression was regulated by T7 RNA polymerase system in a separate recombinant virus in order to control the production of the target SIV sequences in rFP9. Therefore, there will be encoded protein expression only in the presence of T7 polymerase. However, construction of a single rFP9 vector containing both components of the T7 expression system (T7 and reporter genes) was also demonstrated in this study using a GFP marker within a DSIV genome (reporter gene).

Gene expression profiles of rFP9 in pCEFs and MRC-5 were compared to another highly attenuated poxvirus vector, rMVA. Combinations of these recombinants were also assessed in the present study. Due to the fact that different poxvirus promoters were used in rFP9.T7pol and rMVA.T7pol, it was difficult to draw a final conclusion comparing the expression efficiency of these different groups of recombinants. Nevertheless, the preliminary ELISA data still indicate that the constructed rFP9 expressed the target env (in pCEFs) and rev proteins (in pCEFs/MRC-5) more efficiently than rMVA, whereas the constructed rMVA was more effective in expressing the env protein in MRC-5. It was also demonstrated that co-infection of mammalian cells with the same recombinant virus (either two rFP9 or two rMVA) has the ability to express higher levels of the encoded proteins than cross-infection with two different recombinants (either rFP9/rMVA or rMVA/rFP9). However, vector combination between rFP9 and rMVA seems to be more effective in avian than mammalian cells. Further studies with large number of replicates would be required in order to confirm these results.

6 Study Limitations and Recommendations

Certain study limitations have encountered during the course of this project. One of these constraints is that lack of confirmatory testing that was used to ensure the construction of pure rFP9 before it can be processed to the last step of protein expression studies. Although different sets of PCR primers were used to confirm the successful integration of SIV insertion cassettes, it was quite difficult to prove the presence of the whole desired DNA sequences without any mutation. For this reason, automated DNA sequencing should be planned as an important step toward the confirmation of pure recombinant generation.

The lack of appropriate positive controls in the WB assay is another limitation that could affect result validation. Furthermore, it would be interesting to quantify the total protein concentration of each sample before SDS-PAGE analysis. This would ensure an equal amount of protein is added in each well, and hence an appropriate comparison can be conducted between the different protein samples.

In addition, there were limitations associated with the statistical analysis in the present study. Due to time constraints, only small numbers of replicates were processed for each protein expression assay. This small sample size reduces statistical power and could adversely affect the comparative analysis between the different groups. Increasing the number of replicates should be taken into consideration in order to enhance the capacity of generalizing these data.

7 Future Work

To date no preventive HIV vaccine is available; and hence this study is aimed to generate a safe and effective live attenuated rFP9 based retroviral vaccine for SIVmac239 model of HIV infection. Despite the fact that significant progress has been made in this project towards the construction of retroviral vaccine, future work would still be needed to finalize the project scope.

It would be important to construct a triple rFP9 vector encoding three SIV gene expression cassettes. The functional RT and int proteins, which were deleted from the SIVmac239 genome (DSIV), would be provided in trans using the triple rFP9 construct, along with all structural (env, gag, and pol) and key accessory proteins except nef. Therefore, this triple packaging rFP9 (helper vector) would then be used together with the genomic rFP9 construct containing the DSIV genome and the T7 polymerase gene to generate replication restricted SIV like-particles through two cycles of replication (Figure 7.1). The resultant *in vivo* live attenuated vaccine candidate can then be used as immunogens to induce specific immune responses and hence hopefully confer protection against SIV infection in macaques. It is important to note that these two viral vectors (packaging and genomic rFP9) would be able to produce a replication-restricted retrovirus if they are mixed and simultaneously inoculated together into mammalian hosts. Used individually these recombinant poxviruses would not produce viable dual-cycle retroviruses.

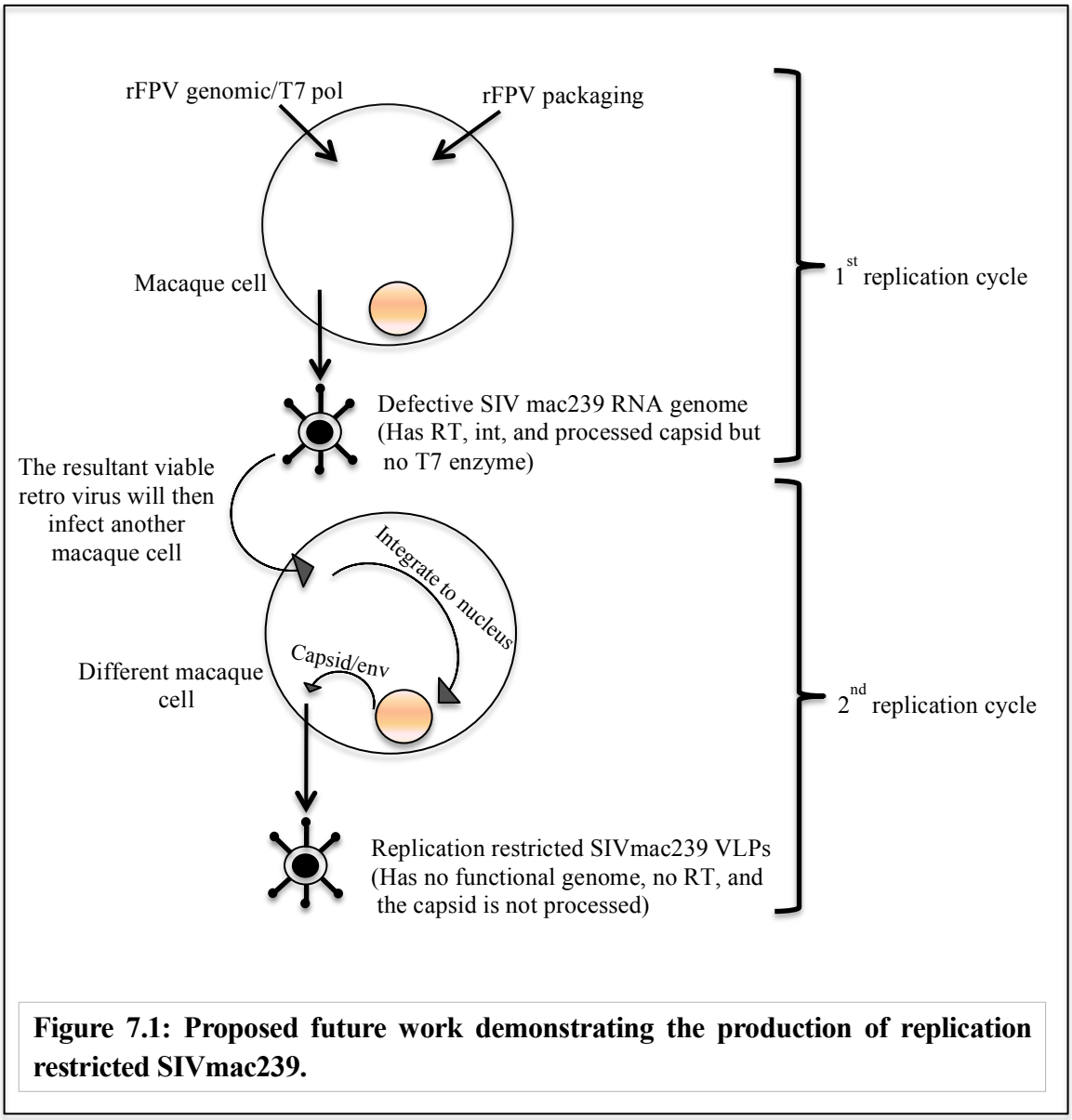

Figure 7.1: Proposed future work demonstrating the production of replication restricted SIVmac239.

For future work, SIV protein expression by rFP9, rMVA and vector combinations should also be carefully reassessed in pCEFs and MRC-5 cells. Further studies are necessary to conduct a large-scale quantitative comparative analysis between these various recombinants. In addition, expression of the DSIV genome and other encoded SIV sequences (such as RT, int, vpr, and vpx) in avian and mammalian cell lines would be another future consideration.

The following is a brief list of the main future work that needs to be carried out to finalize the project scope:

- Remove (purify) the marker gene from the single rFP9.RT.RNase.Int.Vpr.Vif construct.
- Prove the genetic stability of the double rFP9.Env.Rev/Tat.Gag-pro and the single rFP9.RT.RNase.Int.Vpr.Vif.
- Generate a triple packaging rFP9 by combining the double recombinant construct (rFP9.env.rev/tat.gag-pro) with the single rFP9.RT.RNase.Int vector.
- Sucrose cushion purification of both packaging and genomic rFP9s.
- Coinfect specific primate cells (Human Embryonic Kidney 293 cells [HEK293]) with packaging and genomic SIV/FP9 recombinants.
- Demonstrate that the first generation retroviral particles will successfully integrate and express in target $CD4^+$ T cells.
- Demonstrate that replication restricted SIVmac239 like-particles do not revert to replication competence *in vitro*.
- Mouse studies to investigate the proposed rFP9 expression system *in vivo*.

7.1 Animal studies

Animal models are considered an essential part for human vaccine development. Preclinical testing of new vaccine candidates in animals provides insight into how these vaccines can trigger host immune responses against the target pathogen (Gerdts et al., 2015). Therefore, using an appropriate animal model, which can mimic the pathology of the target disease in humans, is a key step toward the success of the vaccine candidate. However, it is sometimes difficult to develop an animal model that can perfectly provide all the important clues for understanding and evaluating the vaccine outcome in

humans (Gerdts et al., 2015).

For HIV/AIDS research, nonhuman primates (monkeys and apes) are considered the model of choice and the most relevant animals for assessing HIV vaccine candidates (Hatziioannou and Evans, 2012; Gerdts et al., 2015). However, housing and maintaining of these large primates in research laboratory are extremely expensive and difficult (Pandrea et al., 2009; Altevogt et al., 2011). For these reasons, murine models have been extensively used for evaluating the immune response generated by HIV vaccine candidates. In this project, we are aiming to use a mouse model to prove the principle of our split recombinant vector system (packaging and genomic rFP9) to generate replication-restricted retrovirus that can trigger both cellular and humoral immune responses.

7.1.1 Characterization of rFP9 vector system *in vivo* using a mouse model

Mouse studies are planned as future work to investigate how our vaccine candidates would work *in vivo*. Animal experiments need to be conducted according to UK Home Office legislation for animal experimentation. In addition, these experiments should have an animal licence, work under a project licence, and approve by the local ethical committee. Russell and Burch (1959) provide the three Rs "3Rs" as a guiding principle for using animals in research laboratory (Festing and Altman, 2002). The 3Rs include:

- **Replace:** use of mice in research should be avoided where possible and replaced by either the least sentient animals or *in vitro* characterization (not really possible in our project; but using mice in place of macaques).
- **Reduce:** use the smallest number of mice that can achieve the ultimate goals of the study.

- **Refine:** use experimental techniques to give maximum amount of data for smallest harm to mice.

7.1.2 Mice immunisation using the co-infection model with packaging and genomic rFP9s

To investigate our proposed rFP9 expression system, two rFP9 (packaging and genomic) would be used to vaccinate the mice. These two rFP9s need to be mixed in equal parts and simultaneously inoculated together into the mice. One rFP9 (packaging) encodes all the antigenic components of SIVmac239 except the nef gene, and the other rFP9 (genomic) contains the DSIV genome and the T7 polymerase gene that is responsible for directing the SIV sequences. This proposed vaccine candidate would be tested in a homologous prime-boost immunisation strategy in order to improve the host immune responses against the target SIV sequences.

Four groups of female BALB/C mice (6–8 weeks old) would be involved in the immunisation experiments, and each group consists of six mice. Three groups of these mice will be used as controls: group I- mice vaccinated with saline, group II- mice vaccinated with packaging rFP9 alone, and group III- mice vaccinated with genomic rFP9 alone. The last group (IV) of mice will be vaccinated with both packaging and genomic rFP9 constructs (Figure 7.2). A high dose of 10^8–10^9 pfu/ml of each construct will be used (intramuscular injection of virus-suspension per hind leg), in order to increase the chance of these two vectors to infect the same mammalian cell.

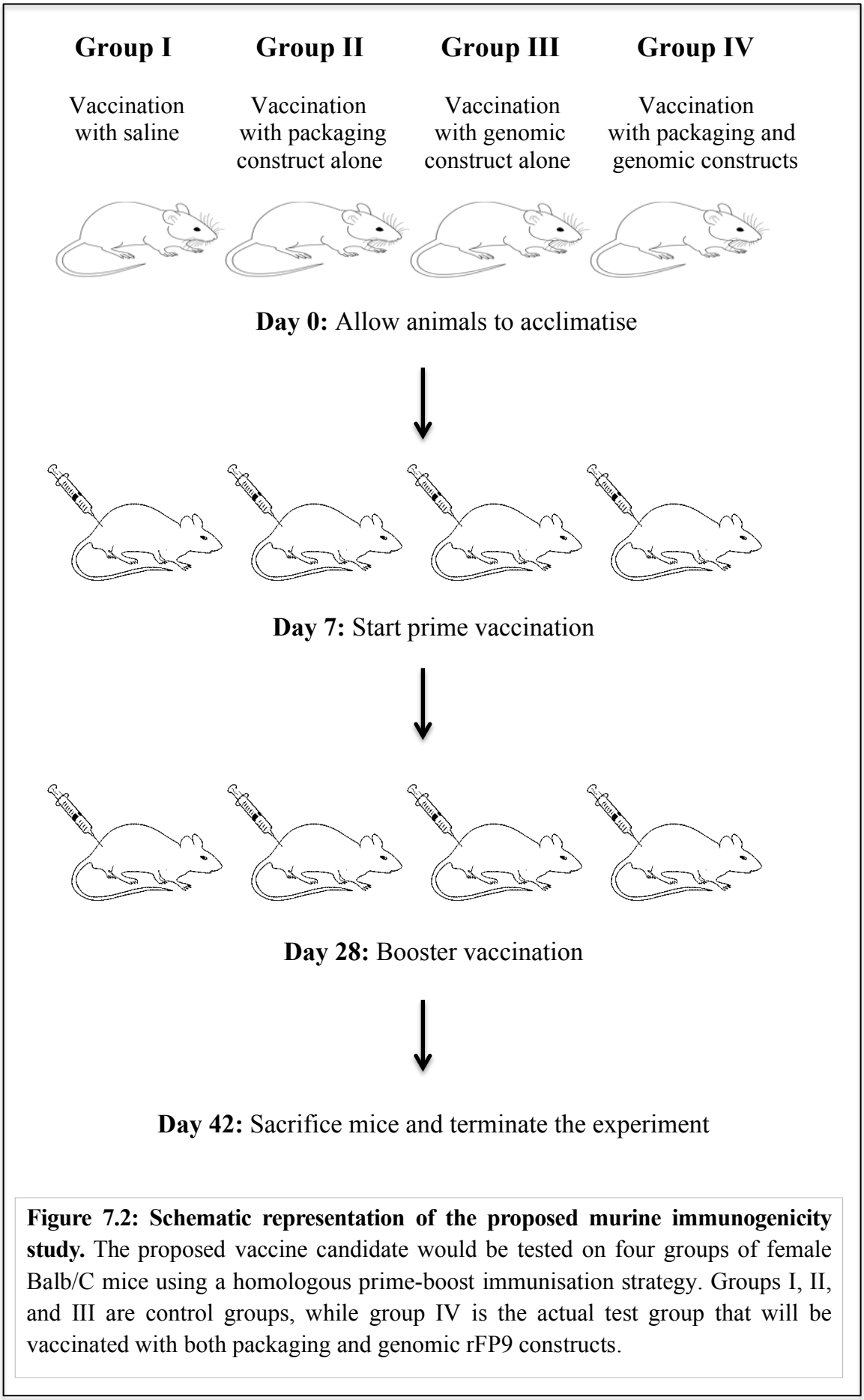

Figure 7.2: Schematic representation of the proposed murine immunogenicity study. The proposed vaccine candidate would be tested on four groups of female Balb/C mice using a homologous prime-boost immunisation strategy. Groups I, II, and III are control groups, while group IV is the actual test group that will be vaccinated with both packaging and genomic rFP9 constructs.

All mice should be housed at least seven days to settle and acclimatize prior to any procedure. On day 7, start prime vaccination followed by a booster dose on day 28 (a two-week interval). On day 42, the experiment needs to be terminated and all the immunised mice should be sacrificed by neck dislocation according to the terms of the UK Animals Scientific Procedures Act 1986 (ASPA) (Figure 7.2).

7.1.3 Sample collection

Two types of samples can be collected from the immunised mice: blood for determining humoral responses and immunoglobulin isotypes, and spleen for T cell response studies. Before sacrificing animals, the whole blood samples would be collected via tail bleeding into either an EDTA-treated tube to prepare PBMCs, or a plain tube for serum separation. After sacrifice, the blood sample can only be obtained from the heart (cardiac puncture) for serum isolation. In addition, the spleens can be extracted and harvested for splenocyte preparation.

7.1.4 Detection of anti-vector (rFP9) immune response by dot blot and WB assays

Several studies have demonstrated that vector-specific immunity induced by rFP9 in mammalian hosts is lower than responses generated to recombinant antigen. This low anti-vector immunity does not seem to affect the expression efficiency of the rFP9 vector, and might potentially increase anti-transgene responses (Webster et al., 2006). It is important in this project to detect the anti-FP9 antibodies two weeks after the booster immunisation, in order to confirm that all mice were successfully immunised. These types of immune responses can be measured by using the cell lysate of FP9-infected pCEFs as a detection antigen on a PVDF membrane. The membrane needs then to be incubated with immunised mice sera followed by anti-mouse HRP-conjugated secondary antibodies, in order to detect anti-FP9 antibodies.

7.1.5 Measurement of host immune response against the target SIV sequences

Different immunological parameters can be measured to prove the ability of our rFP9 vector system to induce specific immune responses (cellular and humoral) against SIV in vaccinated mice. These parameters include: SIV-specific antibodies, immunoglobulin isotypes, and T cell responses. However, relevant CD4 T cell count could be detected and measured only in immunised macaques.

7.1.5.1 Detection of SIV-specific antibodies by dot blot, WB, and ELISA assays

The ultimate goal of this project is generation of SIV like-particles that can be used as immunogens to induce specific humoral immunity and hence confer protection against SIV infection. Therefore, detection of env and gag-specific antibodies in the sera of the immunised mice should be one of the immunological parameters that need to be measured. These antibodies can be either detected using dot blot and WB assays or titrated using ELISA. Env and gag standard proteins (supplied by NIBSC) would be used as detection antigens in these screening assays.

7.1.5.2 Detection of env- and gag-specific IgG isotypes by an indirect ELISA assay

This assay is aiming to determine the antibody-mediated responses to SIV env and gag proteins by measuring antigen-specific IgG isotypes in the serum samples from immunised mice. Different specific antibodies for murine (anti-mouse IgG) IgG1, IgG2a, IgG2b, and IgG3 would be used to discriminate between each isotype. In addition, various ranges of concentrations of env and gag standard proteins should be used in the assay as a detection antigen in order to identify an optimal coating concentration of these SIV proteins. The final concentration of detected antibodies will be determined based on standard curves of each isotype.

In murine studies, it has been reported that mainly three subtypes of specific IgG antibodies against the SIV env protein can be detected: IgG1, IgG2a, and IgG2b. Interestingly, the detected levels of IgG3 responses were minimal and not comparable to other subclasses of antibodies (Skountzou et al., 2007; Cristillo et al., 2011).

7.1.5.3 Detection of cytokine profile (T cell response) by enzyme linked immune spot (ELISpot) assay

Murine ELISpot assay is one of the methods that can be used to assess the cellular immune responses within the splenocyte suspension. At two weeks after the booster immunisation, the spleens should be extracted and harvested in sterile condition for splenocyte preparation. In this experiment, murine IFN-γ, IL-4, IL-6, IL-10, IL-12, and TNF-α specific monoclonal antibodies would be used to coat an ELISpot plate at a concentration of 5 μg/ml. IFN-γ is generally considered the most relevant. After blocking and washing stages, the extracted mouse splenocytes need then to be cultured (1–2 X 10^6 cells/well) with the suitable re-stimulating SIVmac239 env and gag peptides. Thereafter, bound cytokines would be detected by using specific antibodies against mouse cytokines (such as biotinylated anti-mouse-IFN-γ) followed by streptavidin-alkaline phosphatase (AP) conjugate and AP substrate. An AID ELISpot reader should be used at the final stage of the experiment to measure the resultant spots (spot forming units per million cells [SFU/10^6]).

T cell responses in immunised mice can be evaluated using a mixture of specific re-stimulating peptides. These peptides representing env and gag genes of SIVmac239 strain are prepared either as 20 amino acid oligomers (20 mers) or 15 mers. The following peptide stimulants are examples that can be used in T cell response studies: SIVmac239 gag (from 309–323 amino acids) (QTDAAVKNWMTQTLL), SIVmac239

gag (from 313–327 amino acids) (AVKNWMTQTLLIQNA) (Siegismund et al., 2009), SIVmac239 env (from 211–230 amino acids) (CNTSVIQESCDKHYWDAIRF), and SIVmac239 env (from 231–250 amino acids) (RYCAPPGYALLRCNDTNYSG) (Skountzou et al., 2007).

Detection of IFN-γ and TNF-α-producing cells is considered as an indicator for the induction of Th1 immune responses. While IL-4, IL-6, and IL-10 are immunological parameters for the activation of Th2 cellular responses in immunised mice (Kang et al., 2003; Levy, 2007).

7.1.5.4 Intracellular cytokine staining (ICS)

Intracellular cytokine staining (ICS) is a flow cytometry-based assay that can be used to detect cytokine (IFN-γ, TNF-α, and IL-2) producing $CD4^+$ and $CD8^+$ T cells within extracted cells. This method is based on staining cell surface markers with anti-mouse fluorochrome-conjugated (fluorescein isothiocyanate (FITC)/Pacific Blue) antibodies, such as FITC conjugated mouse anti-CD8a, Pacific Blue conjugated mouse anti-CD4, and mouse anti-CD16/CD32. The stained cells need then to be fixed, permeabilised, and stained again for intracellular determinants with anti-IFN-γ allophycocyanin (APC), anti-TNF-α FITC or anti-IL-2 phycoerythrin (PE). At final stage, all stained cells are detected and analysed by flow cytometry instrumentation (Lovelace and Maecker, 2011; Jin et al., 2014).

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