Optimization of chimaeric HIV-1 virus-like particle (VLP) production and immunogenicity testing of VLPs in mice

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SOUTH AFRICAN AIDS VACCINE INITIATIVE

Abridged Contents

Declaration

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List of Abbreviations

aa	amino acid
AcMNPV	*Autographa californica* M (multiply-embedded) nuclear polyhedrosis virus
AIDS	Acquired immunodeficiency syndrome
ANOVA	analysis of variance
APC	antigen presenting cell
AZT	Azidodeoxythymidine
BEVS	Baculovirus Expression Vector System
BmNPV	*Bombyx mori* nuclear polyhedrosis virus
bp	base pair
CA	Gag p24 capsid protein
CD4	cluster of differentiation 4
CD8	cluster of differentiation 8
CT	Cholera toxin
CTL	cytotoxic T-lymphocytes
DC	Dendritic cells
DNA	deoxyribonucleic acid
dpi	days post infection
E.coli	Escherichia coli
ELISA	enzyme-linked immunosorbent assay
ELISPOT	enzyme-linked immunospot
Env gp	Envelope glycoprotein
FACS	Fluorescence-activated cell sorting
GagRT	HIV-1C Gag-RT polyprotein
GagTN	HIV-1C Gag-Tat-Nef polyprotein
GrtnC	p6-truncated Gag-RT-Tat-Nef HIV-1C polyprotein
HAART	Highly active anti-retroviral therapy
HIV-1C	human immunodeficiency virus 1 subtype C
hpi	hours post infection
hr	hour
HRP	horseradish peroxidase
IFN-γ	interferon gamma
Ig	immunoglobulin
IL-2	interleukin 2
IN	Pol integrase
IPTG	isopropyl-thio-β-galactosidase
Kb	kilobase(s)
kDa	kilo-Dalton
LA	Luria-Bertani agar
LI	Gag p6 linker protein
LTR	long terminal repeat
MA	Gag p17 matrix protein
MALT	mucosa-associated lymphoid tissue system
MHC	major histocompatibility complex
min	minute
MOI	multiplicity of infection
MU	micro- ultrafiltration
MVA	modified vaccinia virus Ankara
myr	myristylated
NAb	neutralizing antibody
NC	Gag p7 nucleocapsid protein
NK	natural killer cells
OD	optical density
ODGU	Optiprep density gradient ultracentrifugation
ORF	open reading frame
PAMP	Pathogen-associated molecular patterns
PBS	phosphate buffered saline
PCR	polymerase chain reaction
pfu	plaque forming units
pPolH	polyhedron promoter
PR	Pol protease
rAcMNPV	recombinant Autographa californica M nuclear polyhedrosis virus
RE	restriction enzyme
Rev	HIV viral expression regulator
rpm	revolutions per minute
RT	Reverse transcriptase
SDGU	sucrose density gradient ultracentrifugation
SDS-PAGE	sodium dodecyl sulphate-polyacrylamide gel electrophoresis
sec	seconds
Sf21 cells	*Spodoptera frugiperda* 21 cells
Sf9 cells	*Spodoptera frugiperda* 9 cells
sfu	spot forming units
SIV	Simian immunodeficiency syndrome
SP1	Gag spacer peptide 1
SP2	Gag spacer peptide 2
ss	single stranded
T.ni Pro cells	*Tirichoplusia ni* Pro cells
Tat	HIV transcriptional transactivator
TCID50	Tissue Culture Infectious Dose 50
TEM	transmission electron microscopy
Th	Helper T-cells
TN	TatNef fusion protein
TNF-α	tumour necrosis factor alpha
VLP	virus-like particle
VV	vaccinia virus
w/v	weight per volume

ABSTRACT

The devastating effect the HIV pandemic has had on the human population in the last twenty five years has highlighted the great need to develop a prophylactic HIV vaccine. The manufacture of a vaccine has proven difficult though, with a number of successful designs in animal models having little success in humans. In view of this, there has been a need for novel vaccine approaches that are able to elicit effective cellular and humoral immune responses, both of which are believed to be important in the eradication of the virus. One such approach is the use of HIV-1 Gag VLPs as vaccine candidates. In this study, the production of two chimaeric Gag VLP vaccine candidates (GagRT and GagTN) was optimized in insect cells, and their ability to enhance a murine immune response in a DNA prime-VLP boost vaccine strategy was evaluated.

The optimal conditions for maximized chimaeric VLP production were determined by evaluating the effects of four factors on the expression of the respective VLPs using factorial analysis of variance (ANOVA) and western blots. The factors investigated were insect cell line, cell density, multiplicity of infection (MOI) and infection time. The cell line and cell density used were the most important factors affecting the expression of both VLP constructs, while MOI had little to no effect. The optimal conditions for both candidates were similar, both demonstrating high yields of intact VLPs when produced in the *Sf*9 insect cell line, at a cell density of $1x10^6$ cells/ml, MOI of 5 and an infection time of 96 hours post infection.

Once these conditions were determined, large scale VLP production was investigated, and VLPs were purified using a micro-/ultrafiltration method. This method proved to be better than sucrose gradient density ultracentrifugation and Optiprep® gradient density ultracentrifugation in terms of the quality and quantity of VLPs that were purified.

The resulting VLPs were utilized in mouse immunogenicity experiments to evaluate the ability of the respective VLPs to enhance a cellular and humoral immune response after mice were vaccinated with the HIV-1 DNA vaccine, pVRCgrttnC. IFN-γ and IL-2

ELIspot assays were used to detect and assess the specificity of the cellular immune response stimulated, and the new LAV Blot 1 western blot kit was used to measure the humoral immune response induced.

Both sets of experiments demonstrated a dominant Gag CD4+ T-cell response and a strong boost effect was observed for mice inoculated with a 100 ng boost of the respective chimaeric VLPs. Although there was only a poor Gag CD8+ T-cell response to GagRT VLPs, GagTN VLPs stimulated a strong Gag-specific CD8+ T-cell response. In addition, there was a positive response to stimulation by a Tat CD4+ T-cell peptide in the GagTN experiment, and a strong RT CD4+ T-cell and CD8+ T-cell response for the GagRT experiments, indicating a broad cellular immune response was induced by both VLPs. No apparent Gag antibody immune response was elicited in either of the experiments.

In conclusion, while both chimaeric VLP constructs GagRT and GagTN demonstrated the ability to significantly enhance a cellular immune response which appeared to be broad and effective in mice, GagTN VLPs emerged as the more immunogenic vaccine candidate.

CHAPTER 1

Literature Review

1.1 Introduction

Human immunodeficiency virus (HIV) is a *Lentivirus*, from the subfamily *Orthoretrovirinae*, and family *Retroviridae* (Flint et al., 1999; MacGregor et al., 2005). HIV in humans can be either of two species, HIV-1 or HIV-2, based on the serological properties and sequence analysis of the viral genome (Luciw, 1996). Of the two species, HIV-1 is the most prominently found and is divided genotypically into three groups, namely the major (M), outlier (O) and non-M non-O (N) groups. The M group is the most dominant in the human population, with as many as 10 sub-types and 13 circulating recombinant forms (CRFs) identified to date (Young et al., 2006).

A HIV-1 infection results in the destruction of host CD4+ T-lymphocytes, an essential component of the immune system. Consequently, infected individuals are eventually diagnosed with Acquired Immune Deficiency Syndrome (AIDS), a lethal syndrome that has led to over 29 million deaths worldwide the last 25 years (UNAIDS, 2007). Third-world regions appear to be the most affected by HIV. In particular, HIV-1 subtype C is responsible for the majority of the infections throughout the world, and is the most common of the HIV subtypes in sub-Saharan Africa, the Indian continent and China, with approximately 39.5 million people infected in 2006 (UNAIDS, 2006). Consequently, there is a desperate need for the development of a safe, effective and affordable HIV vaccine.

1.2 HIV-1 structure and genomic organization

HIV-1 is a single stranded (ss) RNA virus with a genome size of 9.2 kb that is encapsulated in a cone-shaped nucleocapsid, within an enveloped, icosahedral protein shell (Fig. 1.1) (Flint et al., 1999). Infectious HIV virions contain two identical copies of the genome that have positive polarity with respect to translation (Luciw, 1996). The viral genome encodes for nine proteins which are divided into four distinct groups (Fig. 1.2) (Young et al., 2006):

(a) The structural proteins, Gag and Env, which are encoded for by the *gag* and *env* genes respectively. The *gag* gene encodes $Pr55^{gag}$, the precursor of the virion capsid proteins (Deml et al., 2005; Flint et al., 1999; Freed, 1998; Luciw, 1996). These proteins include the p17 matrix protein (MA), the p24 capsid protein (CA), the p7 nucleocapsid protein (NC), the p6 linker protein (LI) and small spacer peptides, p2 and p1 (SP 2 and SP1 respectively) (Deml et al., 2005; Freed, 1998; MacGregor et al., 2005).

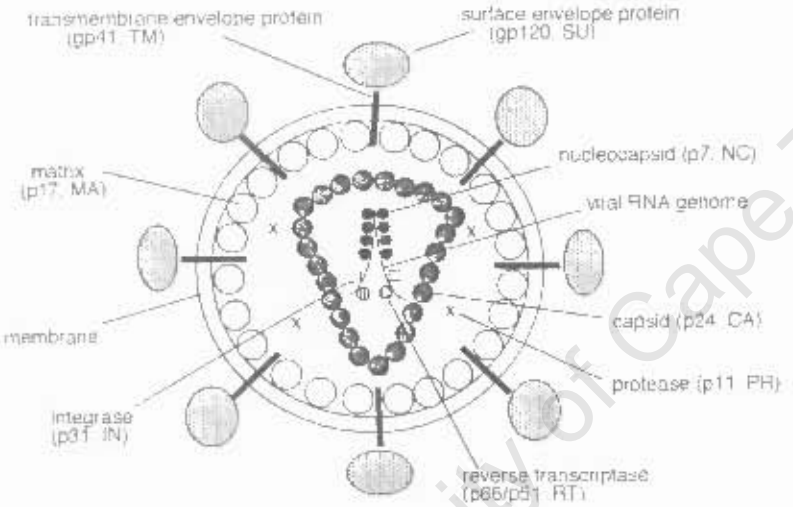


Figure 1.1 — The HIV-1 mature virion with the approximate locations of the labelled proteins (from (Freed, 1998)).

The *env* gene encodes a precursor, gp 160, for the surface (SU) and transmembrane (TM) envelope glycoproteins (Env gp), gp120 and gp41 respectively (Luciw, 1996; Wang, Lai, and Li, 1998).

(b) The enzyme proteins are encoded by the *pol* gene, which when translated, expresses the precursor, $Pr160^{gag\text{-}pol}$, for the virion enzymes: integrase (IN), protease (PR), reverse transcriptase (RT), and RNase-H.

(c) The regulatory proteins are the transcriptional transactivator (Tat) and viral expression regulator (Rev), both of which are encoded by overlapping exons, and are essential for virat replication.

(d) The final group are the accessory or auxiliary proteins (Vpu, Vpr, Vif and Nef), all of which are involved in viral replication and maturation.

1.3 Difficulties in combating HIV-1

In view of the growing number of HIV-positive individuals, many approaches have been looked at to eradicate the virus. In the last few years, highly active anti-retroviral therapy (HAART) has proved to be a successful method to control HIV infections, utilizing protease inhibitors and nucleoside analogs such as azidodeoxythymidine (AZT) to inhibit reverse transcription of HIV (Flint et al., 1999; Yoshizawa et al., 2001). However, HAART is an expensive method and therefore difficult to access for patients in developing countries (Chugh and Seth, 2004; Yoshizawa et al., 2001). Furthermore, while HAART has been able to reduce the viral infection in most individuals, it does not eliminate the virus (Luciw, 1996). In fact, drug-resistant virus has recently been detected, making HAART a poor long-term treatment (MacGregor et al., 2005; Yoshizawa et al., 2001).

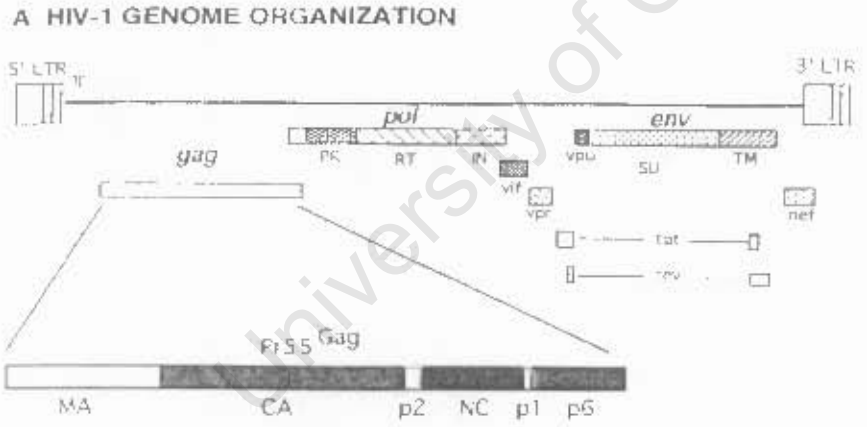


Figure 1.2 – The HIV-1 genome, highlighting the *gag* open reading frame (from (Freed, 1998)).

Thus, the development of a vaccine seems to be the best option to control an HIV-1 infection, and prevent its spread (Doan et al., 2005). A vaccine traditionally stimulates the host natural defenses against foreign particles, eliciting an immune response and thus providing protection. However, in the case of HIV, this is complicated by a number of factors. Firstly, HIV attacks the immune system directly, infecting T-lymphocytes as well as monocytes and macrophages (Luciw, 1996; Nabel, 2002). It slowly incapacitates the host defenses, and hence prevents protective immunity. Secondly, the HIV genome

integrates into the host genome, making it impossible to differentiate between host and viral DNA after integration, and thus it is difficult to eliminate the virus without harming the host cell as well (Luciw, 1996). Lastly, HIV is able to escape detection from the immune system when in its latent form because no viral proteins are displayed on the infected cell's surface for immune recognition purposes (Alcami et al., 2005). Furthermore, the HIV genome has a high mutation rate in certain regions, resulting in immune escape mutants; this makes it very difficult to design a broad spectrum HIV vaccine that provides immunity against all variants in humans (Paliard et al., 2000). Scientists have overcome some of these problems by looking at strategies that will enhance the host's initial cellular and humoral immune responses to HIV (Yao et al., 2003). To do this, the molecular and cellular basis for the host immune response, as well as various HIV immunogens, has been studied in detail (Nabel, 2002).

1.4 Gene targets for an HIV-1 vaccine

Traditional methods to develop a HIV-1 vaccine utilised live-attenuated viruses or chemically inactivated viruses as possible delivery vehicles (Nabel, 2002; Noad and Roy, 2003). There was some success in using whole killed HIV-1 in mice, however, there proved to be many safety concerns involved in using this method, because of the likelihood of incomplete inactivation of chemically inactivated viruses, or reversion to virulence by live-attenuated viruses (Noad and Roy, 2003).

A safer approach involved the incorporation of HIV antigens into vaccines, in an attempt to elicit broad, strong, adaptive immune responses against HIV-1. Some of the most commonly used antigens were those encoded for by the *env* gene (gp 120, gp 160 and gp 41), as these were the only proteins that demonstrated induction of both a cell-mediated immune response and neutralising antibodies (Nabs) in rodent and non-human primate trials (Deml et al., 2005; Gardiner et al., 2005; Leung et al., 2004; Yao et al., 2003; Young et al., 2006; Zanotto et al., 2005). NAbs are particularly important because they are believed to provide protective immunity against HIV-1 by effectively blocking its entry into host cells (Bojak, Deml, and Wagner, 2002; Doan et al., 2005; Yao et al.,

2003). However, due to the inability of the host immune system to access neutralizing Env epitopes* on primary HIV-1 isolates, and the high variability of the *env* gene, Env vaccines had disappointing results in human clinical trials (Pontesilli et al., 1998; Sandstrom and Wahren, 1999).

Expression products of the *pol* gene (Gag-Pol fusion protein, p160, RT, IN, PR) were also explored as potential vaccine targets, specifically because the *pol* gene is a conserved region within the HIV genome and has been shown to elicit cross-clade cytotoxic T lymphocytes (CTL) responses in infected HIV individuals (Betts et al., 1997). Zur Megede et al (zur Megede et al., 2003) demonstrated that a DNA vaccine expressing a *gag-pol* fusion gene was able to induce strong Pol-specific T- and B-cell responses. However, a concern when using *pol* expression products in vaccines is the potential deleterious enzymatic activity of IN, RT, and PR components. This has been avoided in recent studies by inactivating these proteins before they are used as vaccine components (Kong et al., 2003).

In most cases, accessory and regulatory HIV proteins were incorporated into various vaccine designs as additional components to Gag, Env or Pol, as they were able to enhance cell-mediated immune responses against HIV due to the presence of several CTL epitopes in the functional regions of these proteins (Yu et al., 2005). These proteins are also inactivated before use or shuffled to prevent toxicity problems that may arise as a result of their expression.

Of all the HIV-1 proteins, most current strategies are utilizing the Gag-based antigens because they appear to be the best potential vaccine candidates for T-cell responses. The *gag* gene has been identified as a highly conserved region in the genome. In addition, the organization of the Gag proteins within $Pr55^{gag}$ and within HIV virions is also highly conserved (Doan et al., 2005; Nabel, 2002). This suggests its use in a vaccine could provide protection against many HIV variants. Furthermore, the full length Gag polyprotein, $Pr55^{gag}$, is able to induce anti-HIV antibody production, along with long-lived helper T cell (T_h) and CTL responses (Doan et al., 2005; Yoshizawa et al., 2001).

* regions of an antigen that interact with the antigen binding site of an antibody or T-cell receptor (www.biotechshares.com/glossary.htm)

1.5 HIV vaccines using Gag-based antigens

$Pr55^{gag}$ is integrally involved in immature virion particle assembly, the incorporation of viral accessory proteins into virions and membrane-targeting functions (Deml et al., 2005; Nabel, 2002). Studies have shown that $Pr55^{gag}$ (499 amino acids) alone, in its unprocessed form, is sufficient to produce and release non-infectious virus-like particles (VLPs) of HIV when expressed in yeast, insect and mammalian cells (Doan et al., 2005; Yao et al., 2003). The resemblance of these VLPs to HIV virions means that a similar immune response, to that induced by infectious virions, could be induced by the VLPs (Deml et al., 2005; Noad and Roy, 2003). Hence, Gag antigens are of particular interest to vaccine researchers, and have been included in the design of a number of different HIV vaccine approaches.

1.5.1 Gene-based vectors to deliver Gag antigens

Gene-based vectors, both viral and non-viral, have proven to be successful transport vehicles for potential vaccines containing the *gag* gene or Gag antigens (Nabel, 2002).

1.5.1.1 Viral vectors

Viral vectors such as poxviruses [canarypox, fowlpox and modified vaccinia Ankara (MVA)], adenoviruses and adeno-associated viruses (AAV) have been employed to deliver Gag antigens to host cells for vaccine purposes (Nabel, 2002; Walther and Stein, 2000). These vectors either have viral replication genes that are replication-incompetent in humans, or these genes are deleted and replaced with genes encoding HIV protein/s (Nabel, 2002). They are generally able to produce large quantities of the protein of interest and to elicit strong host immune responses to the HIV proteins that they carry. Several studies have demonstrated success using viral vectors for vaccine purposes (Gherardi et al., 2004; Nabel, 2002; Xin et al., 2007), however there are safety concerns which remain an issue. In 2007, the Merck phase I human clinical trial vaccinating individuals with a recombinant, inactivated adenovirus-5 vector vaccine containing the *gag*, *pol*, and *nef* genes, failed to elicit protection against HIV infection, in addition to causing some subjects to become more susceptible to the disease (Ledford, 2008; Sekaly,

2008). The poor outcome of this trial highlighted the need for extensive safety testing and animal trials before any vaccine testing in humans.

1.5.1.2 DNA vaccines

Non-viral vectors such as DNA plasmids and liposomes provide another option for transportation of a vaccine to host cells (Deml et al., 2005). In particular, several DNA prototype vaccines have been used to carry the HIV-1 *gag* gene as well as other HIV-1 genes into host cells (including antigen presenting cells-APCs), where translation of the plasmids produces the desired viral protein/s. In this way, peptides of the recombinant protein expressed can be processed via both major histocompatibility complex (MHC) classes I and II, inducing humoral and cellular immune responses. These vaccines have demonstrated a safe, cost-effective and stable nature, in addition to being able to elicit both arms of the adaptive immune response in mice (Chugh and Seth, 2004; Yoshizawa et al., 2001). However, the immune responses elicited are not as strong as those induced by viral vectors in larger animals (Deml et al., 2005; Ramakrishna et al., 2004). Also, there are some possible dangers to using DNA vaccines in humans such as autoimmune responses due to anti-DNA antibody accumulation, and potential tumour formation due to chromosomal integration. Further research is therefore necessary before viral and non-viral vectors are used commercially.

Combinations of viral and non-viral vector vaccines have recently emerged as a method to improve the immune responses in animal models. In an interesting mix of viral vector DNA vaccine approaches, Zhang *et al.* (Zhang et al., 2004) illustrated the use of papillomavirus pseudoviruses to express Gag in mice, and elicit effective CTL and anti-HIV antibody responses, as well as develop memory immunity against Gag. Such strategies have demonstrated success in other similar studies, and will be discussed further in sections to follow.

1.5.2 Gag subunit vaccines

Subunit vaccines use recombinant proteins to present single viral antigens within the host, and stimulate an immune response. These vaccines are able to induce significant

immune responses, as was demonstrated in a recent study where a vaccine comprising of a HIV-1 Gag p24-Immunoglobulin A (Ig A) fusion protein successfully induced T-cell and antibody responses in mice (Obregon et al., 2006). While using protein subunits is a safer approach than live-attenuated vaccines and viral vectors, a major drawback of these vaccines is the large quantities of the antigen required to elicit a response. In many cases adjuvants must be used to enhance effects, making this a potentially expensive alternative (Nabel, 2002; Noad and Roy, 2003). However, new types of subunit vaccines using particulate antigens (such as immunostimulating complexes, virosomes and VLPs) have been shown to elicit effective adaptive immune responses using smaller quantities of vaccine (Deml et al., 2005; Doan et al., 2005).

1.5.3 Gag VLPs

VLPs are an attractive option for use as HIV-1 vaccines for several reasons. Firstly, they are analogous in size and morphology to immature HIV-1 viral particles, but without the RNA genome (Yao et al., 2003). This makes them non-infectious and therefore safe to use in repeated applications. Secondly, they are readily taken up into APCs, hence are able to stimulate strong CTL and anti-HIV antibody responses (Nabel, 2002; Noad and Roy, 2003). Thirdly, they are stable, replication-deficient and can be produced in large quantities (Noad and Roy, 2003).

The *gag* gene product, $Pr55^{gag}$, when expressed from an appropriate construct, is translated within the cytoplasm of the host cell and targeted to the plasma membrane where it accumulates (Flint et al., 1999; Yoshizawa et al., 2001). It then buds out of the cell in the form of VLPs (Fig. 1.3) (Deml et al., 2005). Upon release from the cell, VLPs are enveloped in a lipid bilayer derived from the host cell membrane. A number of eukaryotic cellular expression systems have been utilized to express HIV VLPs in this way for vaccine production purposes (Doan et al., 2005).

1.5.3.1. Gag VLP expression systems

One of the most commonly used viral expression systems utilized for Gag VLP production is the baculovirus expression system in insect cells (Doan et al., 2005; Noad

and Roy, 2003). It makes use of the baculoviruses, *Autographa californica* multiple nuclear polyhedrosis virus (AcMNPV) or *Bombyx mori* (silkworm) nuclear polyhedrosis virus (BmNPV) (GibcoBRL, 2001). The baculovirus expression system allows for the production of large amounts of VLPs in host insect cells. These cells can be cultured without mammalian-derived supplements, decreasing the possibility of opportunistic human pathogen contamination. Baculoviruses also have a restricted host range (no harmful effect on humans) but are usually chemically inactivated after the VLP production process (Noad and Roy, 2003). Insect cells are also easy to handle and are infected at a high multiplicity of infection (MOI), making this expression system very useful to generate high yields of recombinant proteins. However, one of the drawbacks of producing recombinant proteins in insect cells is that they have different glycosylation patterns to those of mammalian cells. It is unclear whether this is a problem yet, as the role of glycosylation varies for different proteins, and is therefore dependent on the nature of protein that is being expressed.

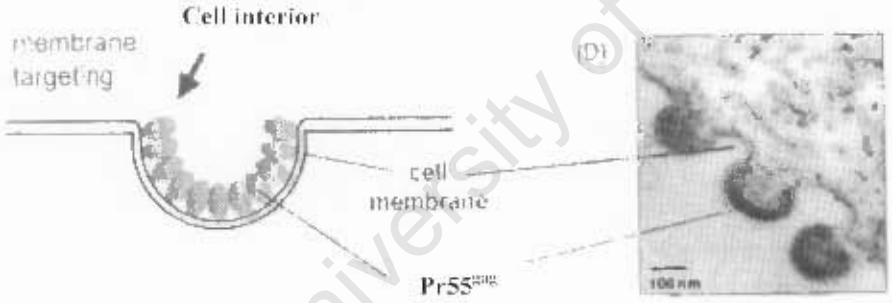


Figure 1.3 A diagram and electron micrograph of HIV-Gag VLPs budding from insect cells infected with Gag-recombinant baculovirus (from (Deml et al., 2005)).

Vaccinia virus recombinants have also been used to produce Gag VLPs in Hep2 and TK-143B cells (human B-cells) (Doan et al., 2005; Young et al., 2006). This expression system uses wild type vaccinia virus (VV) to infect cells, which are then transfected with a plasmid expressing the protein of interest. The gene of interest is incorporated into the viral genome, and VLPs are consequently secreted into the supernatant of cultured cells (Young et al., 2006). The VV expression system is not as common as the baculovirus system, because it produces lower Gag VLP yields and host cells are more difficult to handle (Doan et al., 2005).

Non-viral vectors such as DNA plasmids have also been used to transfer the *gag* gene into host cells *in vivo*, as mentioned previously. Furthermore, DNA vectors have been used to express Gag in yeast cells as well. These VLPs are surrounded by a yeast membrane, and bud from yeast spheroplasts (yeast cells lacking cell walls) (Doan et al., 2005; Sakuragi et al., 2002).

1.5.3.2 Derivations of Gag VLPs

In addition to full length $Pr55^{gag}$ self assembling into VLPs, specific truncated forms of $Pr55^{gag}$ are also able to form VLPs which are slightly smaller in size compared to the infectious virions, but have similar ultrastructure and biophysical properties (Deml et al., 2005; Doan et al., 2005; Luo et al., 1992; Royer et al., 1991; Wang, Lai, and Li, 1998). Deletions in the C-terminal protease-encoding region including certain parts of CA (amino acids 211-241) and LI (amino acids 436-471), can occur with little effect on VLP formation (Wang, Lai, and Li, 1998). In light of this, investigations have been made exploring the incorporation of foreign peptides into Gag VLPs.

There are two types of chimaeric VLPs that have been created in recent years. Type I VLPs include those which have foreign peptides integrated into or fused with the Gag polyprotein (either the truncated or full form of $Pr55^{gag}$), while type II VLPs include those which have foreign peptides associating with the outer surface of the VLP (Deml et al., 2005).

Type I VLPs can be subdivided into two groups, namely Type I frameshift VLPs and Type I in-frame VLPs. Type I frameshift VLPs occur as a result of inserting a gene of interest downstream of *gag* so that it is in the same frame as *pol*. Translation of the recombinant protein occurs through a (-1) ribosomal frameshift at the *gag-pol* frameshift signal, at a frequency of about 5 %, as would usually happen if Gag-Pol (p160) was being translated (Deml et al., 2005; Jacks et al., 1988). Therefore, the VLPs produced will carry the foreign protein on 5 % of the incorporated Gag molecules. It is believed that due to the low ratio of chimaeric VLPs to Gag VLPs, Type I frameshift VLPs are able to incorporate larger foreign protein inserts than Type I in-frame VLPs (Tobin et al., 1997).

The second group of Type I, Type I in-frame VLPs, involve inserting a gene of interest in place of non-essential components of $Pr55^{gag}$ or an in-frame fusion of the gene of interest to the C-terminus of a truncated form of Gag (Deml et al., 2005; Luo et al., 1992). Research has shown the inclusion of short polypeptides such as the gp120 V3 loop (Luo et al., 1992) into these VLPs (Wagner et al., 1994a; Wagner et al., 1996), but nothing larger than 200 amino acids (Deml et al., 2005).

The polypeptides chosen for Type I and II VLPs are commonly derived from other HIV antigens, such as Env (highly immunogenic), Nef (early phase protein) and Pol proteins, so as to elicit an enhanced immune response. Both VLP types are able to stimulate T_h and CTL responses.

Simian immunodeficiency virus (SIV) Gag polyprotein precursor, $Pr57^{gag}$, also forms VLPs and has been studied in some detail due to the similarities between SIV and HIV-1, the resemblance of SIV infections in rhesus macaques to the HIV-1 infections in humans, and the accessibility of the macaque model to study immune responses to Gag-based antigens (Doan et al., 2005). Furthermore, chimaeric SIV and HIV-1 VLPs (known as SHIV VLPs) have been investigated to augment the immune responses elicited in non-human primates (Notka et al., 1999). These VLPs contain an SIV backbone but have their *env*, *tat* and *rev* genes replaced by the respective HIV-1 genes (Dale et al., 2002; Doan et al., 2005).

1.6 Immunogenicity of Gag VLPs

The rate at which AIDS progresses in an individual is primarily dependent on the host immune response (Doan et al., 2005). Ideally, a HIV vaccine should induce an immune response which is able to protect against infection or lower the viral load and stop the progression of viral infection (Flint et al., 1999). Recent studies have shown that "elite controllers" (HIV-infected individuals who naturally control viral loads) generally display a more pronounced Gag-specific CD8+ T-cell response, whereas anti Env-specific responses tend to be associated with progression to AIDS (Kiepiela et al., 2007).

Furthermore, Gag VLPs appear to significantly stimulate cellular and humoral immune responses in non-human primate clinical trials. Nevertheless, there are still several areas of HIV-1 immunology that are not well understood and require further clarification. Thus, to develop an effective vaccine it is necessary to look at the molecular basis behind the immune responses in more detail.

1.6.1 Elicited immune response

An immune response to a viral agent is divided into two branches, the innate (non-specific) and adaptive (specific) responses (Flint et al., 1999). The innate response components function to immediately begin combating the infection, while also acting to stimulate the adaptive immune response. Gag VLPs are able to activate the innate immune response by acting as danger signals and eliciting cytokine and interferon production by dendritic cells (DCs) (Deml et al., 2005). Innate immune response activation also occurs in the presence of pathogen-associated molecular patterns (PAMPs) such as lipopolysaccharides and nucleic acids. It is thought that the response elicited by Gag VLPs is partly due to contaminating components from VLP preparations, as the most prominent VLP immune responses are those induced by yeast- and baculovirus-derived VLP preparations.

While the innate immune response is an important initial defense, the adaptive response is necessary for viral infection eradication and long-term protection (Flint et al., 1999). It is made up of two parts, namely the cellular and humoral responses. The induction of an adaptive immune response requires the presentation of antigens in MHC class I and II-peptide complexes (Deml et al., 2005; Flint et al., 1999). The interaction of a T_h-cell receptor with the MHC class II-peptide complex, in addition to the binding of co-receptors, activates T_h cells, so initiating the release of cytokines and T_h-cell proliferation and differentiation. The cytokines that are secreted establish which of the two responses occurs, as the cytokines are responsible for CTL and B-cell activation (Fig. 1.4) (Flint et al., 1999; Gherardi et al., 2004).

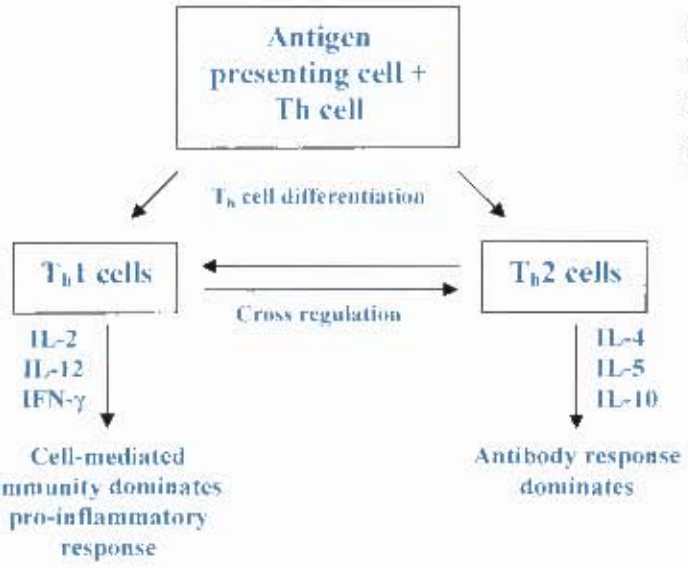


Figure 1.4 A simplified view of the process leading to the development of the Th1 and Th2 immune responses (adapted from (Flint et al., 1999)).

The cell-mediated or cellular response primarily involves the actions of T_h1 cells and killer T-cells (CTLs or CD8+ T cells). Th1 cell stimulation results in the release of the cytokines IL-2, IL-12 and IFN-γ by T_h1 cells, to activate CTLs (Flint et al., 1999). CTLs then interact with the MHC class I-viral peptide complexes on infected host cells, injecting enzymes into the infected cell to induce apoptosis. The CTL response is mainly responsible for controlling the viral infection and suppressing viraemia in HIV-infected individuals (Paliard et al., 2000). An increase in CTLs and T_h cells show an apparent decrease in virus load in HIV+ patients, confirming these cells' involvement in anti-HIV activity (Chugh and Seth, 2004; Deml, Wild, and Wagner, 2004). CTLs are also able to produce cytokines such as IFN-γ and TNF-α which have antiviral properties and are involved in blocking HIV-1 entry (Cocchi et al., 2000). Additionally, the T_h1 cell responses that are elicited are involved in preserving an effective CTL response (Chugh and Seth, 2004).

Exogenous antigens are usually processed and transported to MHC class II molecules (activates T_h2 cells) while endogenous antigens are displayed by MHC class I molecules (activates T_h1 cells and CTL responses) (Deml et al., 2005). In general, Gag VLPs were thought to be processed and complexed with MHC class II molecules only, as VLPs are exogenous antigens (Deml et al., 2005; Doan et al., 2005). However, recent studies have

shown cross-presentation of VLPs and other exogenous antigens, via the MHC class I pathway in DCs, suggesting that the MHC class I and II pathways are more flexible than what was thought (Doan et al., 2005). Hence, Gag VLPs are able to stimulate strong CTL and T_h cell responses *in vivo* because they can be processed and displayed by both MHC class I and II molecules, and they contain many T cell epitopes (Deml et al., 2005). Furthermore, new studies have shown that Gag VLPs are able to elicit a broad, cross-clade CTL response due to conserved target CTL epitopes, further demonstrating the promising potential of Gag as a vaccine candidate (Deml et al., 2005; Deml, Wild, and Wagner, 2004).

Induction of the humoral immune response requires T_h2 cells to recognize an antigen-MHC class II molecule and secrete cytokines (Fig. 1.4) that activate B cell proliferation to form antibody-secreting plasma cells and memory B cells (sent to the lymphoid tissue) (Flint et al., 1999).

Gag-only VLPs are able to induce the production of anti-Gag antibodies, but they are unable to elicit a NAb response, as has been shown in immunogenicity studies with rabbits and non-human primates (Deml et al., 2005). The function of anti-Gag antibodies has yet to be determined, but there appears to be a correlation between the decrease of anti-Gag antibodies and the progression of AIDS (Chugh and Seth, 2004). NAb stimulation is particularly important in fighting an HIV infection because it is thought to provide protection against the viral infection and remove cell-free virions from the host blood (Yao et al., 2003). However, the efficiency of the Nabs' effect against HIV is still unconfirmed (Yoshizawa et al., 2001). While some studies have demonstrated accelerated clearance of HIV virions in the blood of non-human primates when NAbs are present, others have shown NAbs to be ineffective due to rapid viral escape. Nevertheless, vaccines that only induce CTL responses are not effective enough to prevent viral escape either (McGettigan et al., 2003). Thus a combination of both CTL stimulation and antibody production are necessary to elicit a long-lasting response.

1.6.2 Enhancement of the immune response

Although there have been several attempts to develop the ideal HIV vaccine, there have been a number of drawbacks due to ineffective immune responses and the ability of HIV to avoid elimination by the immune system. To enhance the initial immune response elicited by Gag antigens, a few strategies have been explored.

Adjuvants have been used in conjunction with some potential HIV vaccines to improve the immune system induction of Gag VLPs and that of other sub-unit vaccines (Buonaguro et al., 2007; Doan et al., 2005). One of the more successful adjuvants has been the cholera toxin (CT), which has enhanced immune responses quite dramatically in animal models. However, CT has a high level of toxicity and cannot be used in humans (Guo et al., 2003). Several other adjuvants are now being investigated to augment immune responses, including hemagglutinin (the influenza virus surface glycoprotein), pro-inflammatory cytokines and co-stimulatory molecules (Young and Ross, 2003).

DNA sequence modifications such as codon optimisation and removal of cis-acting inhibitory sequences has also been suggested as a method to elevate the immune response elicited by Gag, through increasing the level of protein expression (Leung et al., 2004; Young and Ross, 2003). Genes that have been codon optimised for mammalian expression systems have demonstrated enhanced protein expression and stronger immune responses (zur Megede et al., 2000, Leung et al., 2004).

Vaccine immunization strategies have proven to affect the immune response elicited as well. Studies have found that prime-boost strategies are the most effective in stimulating strong cellular and humoral responses (Amara et al., 2005). These make use of DNA to prime the immune response, and a live-attenuated viral vector to boost the initial response (Smith et al., 2004). Alternatively, subunit or VLP vaccines could be used as boosting components. Some of the most common prime-boost systems are based on the use of recombinant poxviruses such as MVA as a booster (Gherardi et al., 2004). A recent study using Gag-Pol-Env DNA (to prime) and MVA (to boost) has shown that this is a very good way to attain high cellular immunity levels.

Even though some vaccines have successfully induced potent immune responses in animal models, the development of CTL escape mutants remains a major problem (Alcami et al., 2005). These escape mutants are the result of mutations in critical viral residues, which can mask the virus so that it is virtually undetectable to the immune system. To overcome this drawback, a vaccine needs to target as many CTL epitopes as possible, in order to elicit a broad, effective CTL response. Thus, as mentioned previously, the use of foreign epitopes inserted, fused (found in type I VLPs) or attached (found in type II VLPs) to the Gag VLP have been explored in some detail (Deml et al., 2005). Research has shown the use of a variety of different combinations making up recombinant VLP immunogens, many of which stimulate an increased immune response compared to naked Gag VLPs. Foreign epitopes have included gp160, V3 (the third variable of HIV-1 gp120) and CD4BR (CD4-binding region of HIV-1 gp120) to name a few. Non-structural proteins from the early phase of the HIV life cycle such as Tat, Nef and Rev have also been incorporated into Gag VLPs (Alcami et al., 2005). Buonaguro *et al.* (Buonaguro et al., 2002) successfully produced HIV-1A VLPs which packaged gp 120 and were able to elicit strong cellular and humoral immune responses. This demonstrated the ability of chimaeric Gag VLPs to enhance an immune response quite significantly.

Another aspect considered to be very important in combating HIV is the induction of a mucosal immune response. Systemic immunity is considered to provide the primary source of protection against a viral infection (Flint et al., 1999), however, in the case of HIV, mucosal immunity is essential for preventing transmission and enhancing viral infection control (Doan et al., 2005; Yoshizawa et al., 2001). The mucosal immune response is stimulated by exposure of immunogens at mucosal sites (Gherardi et al., 2004). Lymphoid tissues exist below the mucosal membranes in the digestive, respiratory and genito-urinary tracts (aka the mucosa-associated lymphoid tissue system – MALT). This system contains cells which carry B and CD4 T-lymphocytes (Flint et al., 1999; Gherardi et al., 2004). These lymphocytes secrete the antibody Ig A (responsible for preventing viral attachment and also believed to neutralise HIV-1) (Guo et al., 2003) and are able to move from the site of antigen presentation to lymphoid tissue elsewhere,

where an immune response can be elicited (Gherardi et al., 2004). Although the immune responses elicited are weaker than systemic responses, mucosal vaccination provides additional protection and also allows the vaccine candidate to overcome drawbacks of intravenous immunization (such as pre-existing systemic immunity or selective systemic immunosuppression). The site of mucosal immunization appears to play a large role in the extent of protection provided. Intranasal, intraperitoneal, intrarectal and intravaginal immunizations have been investigated, although intranasal immunization in mice has been found to be the best way to induce genital antibody responses (Gherardi et al., 2004).

1.7 Drawbacks of Gag VLPs in vaccines

In spite of the extensive research currently exploring the use of Gag VLPs for vaccine purposes, there are some disadvantages of VLPs which need to be considered (Doan et al., 2005). To begin with, the production of purified, enveloped VLPs has been a problem because it is difficult to find an expression system that is able to produce these complex particles with all the correct specifications (i.e. purity, yields, post-translational modifications). While the systems mentioned have performed more than adequately, each has its drawback. Also of some concern, is that Gag VLPs are able to associate with and incorporate random RNA material (in the absence of genomic RNA) from the cells they are expressed in (Khorchid et al., 2002). This is limited quite successfully by the removal of the ϕ-site upstream of *gag*, which prevents nucleic acid binding to NC, and reduces RNA content of VLPs by approximately 95 % (Persson et al., 1998). However, it is something to be aware of when using these VLPs as vaccines for use in humans. In terms of their potential as vaccines, VLPs have induced immune responses that are strong, but still weaker than those of replicating vectors. It is possible that due to the resemblance of the VLP structure to infectious virions, they might be inducing similar non-protective immune responses, but this remains to be established. Hence, further research is necessary before VLPs can be used as components of a human HIV-1 vaccine (Doan et al., 2005).

1.8 Conclusions and study objectives

Overall, the ability of Gag antigens and particularly VLPs to stimulate the immune system has allowed for the exploration of various ways to optimize HIV-1 vaccine design. However, while many attempts have had success in small animal models, testing these vaccines in humans could still render disappointing results due to differences in animal and human immune systems. Thus, further research is necessary to produce an effective HIV-1 vaccine that can induce broad, long-lived CTL and T_h cell responses, and potent neutralizing and anti-HIV-1 antibody responses (Deml et al., 2005).

In an attempt to make a novel HIV-1 vaccine, previous work in our laboratories resulted in the development of the multigene HIV-1 DNA vaccine candidate, pTHr.grttnC (Burgers et al., 2006). This vaccine design was based on HIV-1 subtype C, the primary subtype found in sub-Saharan Africa. It encodes a polyprotein (1224 amino acids) consisting of the HIV-1 proteins p6-truncated non-myristylated Gag, reverse transcriptase (RT), shuffled Tat and truncated Nef, all of which were inactivated for safety purposes as described previously (Burgers et al., 2006). In mouse immunogenicity studies, pTHr.grttnC was able to elicit significantly broad, strong cellular immune responses against several of the HIV proteins present, making it a promising vaccine candidate (Burgers et al., 2006). In view of this and the heightened cellular immune responses induced in mice when HIV-1 Gag VLPs were used to boost a pTHgagC DNA vaccine (Jaffray et al., 2004), several chimaeric HIV VLP DNA constructs were designed as possible 'boosts' to complement the pTHr.grttnC DNA vaccine (Halsey et al.). These VLP constructs encoded Type I in-frame VLPs comprised of either full length $Pr55^{gag}$ or p6-truncated Gag ($Pr50^{gag}$) fused to a functionally inactivated TatNef fusion protein, inactivated RT or a RT-Tat-Nef fusion protein.

Initially, the respective chimaeric VLPs were detectable when expressed in insect cells, but were not produced in sufficient quantities to properly explore their immunogenic characteristics, and thus their ability to elicit an effective anti-HIV immune response, in mouse studies. The objectives of this study were to optimise the production of selected chimaeric VLP constructs in insect cells, purify these VLPs, and finally determine the

ability of these VLPs to boost an immune response in mice. Two of the chimaeric VLP constructs created were selected for immunogenicity testing in mice. These constructs comprised of $Pr55^{gag}$ attached to the RT protein and a TatNef fusion protein respectively.

CHAPTER 2

The bulk production and quantification of recombinant baculovirus stocks

2.1 Introduction

Due to the growing utilization of HIV-1 VLPs in vaccine strategies, there has been a great need to express VLPs in large amounts. Recent research has examined several expression systems; however, the baculovirus-insect cell system has proved to be the most generally useful (Doan et al., 2005).

2.1.1 Baculovirus Expression Vector System (BEVS)

Baculoviruses are a diverse group of double-stranded DNA viruses that infect insects, particularly of the order *Lepidoptera* (Jorio, Tran, and Kamen, 2006; O'Reilly, 1994). The baculovirus virion consists of a rod-like protein capsid, approximately 200-400 nm in length, encasing a core. The core comprises of the condensed genome (80 – 200 kbp) associated with the protein, VP12 (O'Reilly, 1994). The capsid and core are collectively referred to as a nucleocapsid, and acquires an envelope once it buds from the plasma membrane of infected insect cells.

When they were first discovered, baculoviruses were found to be very useful as biopesticides, although in recent years they have been far more useful as versatile expression vectors (Jorio, Tran, and Kamen, 2006). Two of the most commonly used baculovirus vectors include *Autographa californica* M (multiply-embedded) nuclear polyhedrosis virus (AcMNPV) and *Bombyx mori* nuclear polyhedrosis virus (BmNPV). They have been used to express a number of recombinant proteins in insect cells, given their host specificity, potential for scaling up protein production, and high-level expression of complex proteins (Hunt, 2005; Kost, Condreay, and Jarvis, 2005).

The Baculovirus Expression Vector System (BEVS) uses recombinant baculovirus to express heterologous proteins via the infection of insect cells (GibcoBRL, 2001; Hunt, 2005). A recombinant baculovirus is created by replacing the non-essential polyhedron gene in wildtype baculovirus with a gene of interest. The most widely used system to create the recombinant baculovirus is the commercial Bac-to-Bac™ system, which uses site-specific transposition to transfer the gene of interest into bacmid DNA in DH10Bac

E.coli cells (method detailed in Appendix A1). Upon infection of insect cells by the recombinant baculovirus, the gene of interest is expressed instead of the polyhedron gene, thus creating recombinant baculovirus virions (late phase of infection) and large amounts of the recombinant protein (very late phase of infection).

The BEVS has been particularly useful for the production of VLPs, given the complexity of VLP formation. The system's ability to perform eukaryotic protein processing and post-translational modification (such as signal cleavage, phosphorylation, amidation and myristylation) has allowed research to investigate virion assembly processes in the absence of live virus, and has allowed the production of numerous VLP antigens for immunization purposes (Hunt, 2005; Kost, Condreay, and Jarvis, 2005). Some of these antigens include human papillomavirus VLPs, severe acute respiratory syndrome (SARS) VLPs and hepatitis C VLPs (Kost, Condreay, and Jarvis, 2005).

In this study, the BEVS was used to produce large quantities of chimaeric HIV VLPs for immunogenicity studies. Recombinant baculovirus stocks were amplified to accommodate optimization experiments, as well as bulk VLP production for the mouse immunological studies.

2.1.2 Chimaeric Gag VLPs

The two HIV chimaeric constructs chosen for optimization in this study were GagRT and GagTN (Fig. 2.1). They were chosen because Nef, Gag and RT were all previously identified as primary target regions for T-cell recognition in HIV-infected individuals in southern Africa (Masemola et al., 2004). This means they contain CTL epitopes that would be important to include in a multigene vaccine attempting to elicit a potent cellular immune response. In addition, RT, Tat and Nef are all non-structural proteins produced in the early phase of the viral life cycle (Luciw, 1996). Thus, their use in a prophylactic vaccine could stimulate an immune response that contributes to eliminating HIV in the critical early stages of infection (Hel et al., 2002; Scriba et al., 2005).

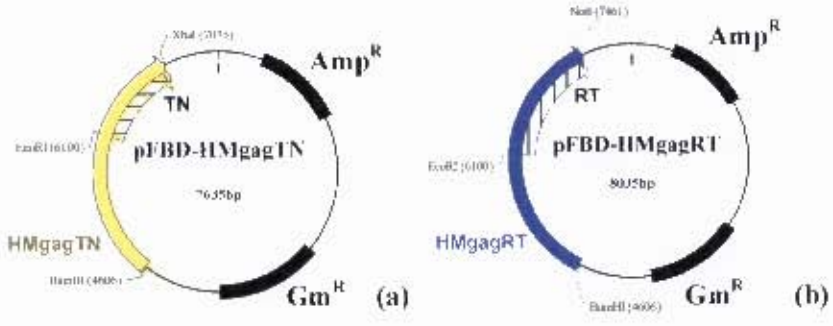


Figure 2.1 – Chimaeric Gag constructs used in this study. (a) GagTN construct - pFastBac Dual vector (see Appendix A2) with humanized, myristylated GagTN (HMgagTN) insert (yellow arrow), (b) GagRT construct - pFastBac Dual vector with humanized, myristylated GagRT (HMgagRT) insert (blue arrow). Black boxes indicate antibiotic resistance genes, Ampicillin (Amp) and Gentamycin (Gm).

Furthermore, each of the accessory proteins chosen to create the chimaeric VLPs have an essential function for viral propagation, so targeting cells expressing them could potentially cripple an HIV infection (Hel et al., 2002). RT is a vital enzyme of the HIV life cycle used to generate HIV DNA from the RNA genome (Luciw, 1996). It displays relatively low sequence variability and strong immunogenicity during natural infections, making it an important antigen to consider for viral containment (Pacheco et al., 2000). Nef is an accessory protein that downregulates CD4 and MHC class I expression of infected cells, influences T-cell activation and enhances virion infectivity (Hel et al., 2002; Scriba et al., 2005). It is known to induce strong CTL responses, despite its sequence variability, therefore making it a very useful target for vaccine purposes (Betts, Yusim, and Koup, 2002). On the other hand, Tat is involved in the upregulation of chemokine receptor expression and TNF-α overproduction among many other suspected functions (Scriba et al., 2005). It has a better conserved sequence than the *nef* gene, but does not contain epitopes that stimulate dominant immune responses (Ramakrishna et al., 2004; Scriba et al., 2005). Hence, the Tat and Nef proteins are fused together in the vaccine construct chosen, to complement each other.

The one major drawback of using accessory proteins such as these for vaccine purposes is the issue of safety, as the use of these proteins in past studies has proven lethal (Hel et al., 2002). Consequently, all these proteins were inactivated for vaccine purposes (Halsey et

al.). Briefly, the RT active site was mutated (YMDDL → YMAAL) to induce inactivation, three essential gene regions of Tat were shuffled in such a way as to preserve all potential epitopes but inhibit functionality, and Nef was inactivated by the removal of 30 base pairs (bp) at the 5′-end.

2.1.3 Quantification methods of recombinant baculovirus

For maximum and efficient protein production using BEVS, it is important to optimize the system accordingly (Janakiraman et al., 2006). To do this, it is necessary to know the concentration of infectious recombinant baculovirus particles in virus stocks. Two of the most common methods to quantify viral titres were chosen for this study, namely plaque assays and Tissue Culture Infectious Dose 50 ($TCID_{50}$) assays.

Plaque assays involve infecting insect cells with the recombinant baculovirus and then monitoring the lysis activity (demonstrated by the formation of plaques) of those cells infected (O'Reilly, 1994). By counting the number of plaques for a given virus dilution, the concentration of the initial viral stock can be determined. On the other hand, $TCID_{50}$ assays (also known as end-point dilution assays) involve the infection of several insect cultures using different dilutions of virus. The viral titre is then estimated by determining which dilution of the virus infects 50% of the cultures initially inoculated. While both methods have demonstrated success, both have drawbacks. Thus, it will be important to compare the assay results' to obtain a better indication of what the true viral titre is.

2.1.4 Chapter objectives

The aims of the work reported in this chapter were as follows:

(i) To produce large quantities of recombinant baculovirus stocks expressing GagRT and GagTN for use in optimization experiments,

(ii) To verify the content and integrity of the previously created recombinant AcMNPV (rAcMNPV) constructs after amplification of baculovirus stocks using PCR and DNA sequencing,

(iii) To determine the viral titre of the amplified recombinant baculovirus stocks.

2.2 Materials and Methods

2.2.1 Insect cell culture

Spodoptera frugiperda (*Sf*) 21 cells (Invitrogen) were used for the production of all baculovirus stocks. They were grown in tissue culture (TC) -100 insect medium (Sigma) supplemented with: 10% (v/v) foetal bovine serum (FBS, containing up to 50 mg/ml serum proteins) (Gibco), 50 μg/ml neomycin, 69.2 μg/ml penicillin G and 100 μg/ml streptomycin. Cells were grown in monolayer culture in sterile tissue culture flasks, and kept at a constant temperature of 27°C. Cells were maintained by seeding mid-log cells at $5x10^5$ cells/ml every 3-4 days, as detailed by the supplier (Invitrogen, 2002).

2.2.1.1 Measurement of cell viability

Insect cell viability was measured at regular intervals to confirm that cells were maintaining a healthy doubling rate and were not under unnecessary stress. This was done by staining cells (90 μl) with Tryptan Blue (10 μl), and placing them into a Neubauer counting chamber. The number of total cells and dead cells (cells that absorbed the blue stain) was counted in the four squares surrounding the central chamber, and an average count was obtained. The percentage of living cells was determined using the following calculation:

$$\frac{(\text{No. of total cells} - \text{no. of dead cells})}{\text{No of total cells}} \times \frac{100}{1} = \% \text{ cell viability}$$

2.2.2 Amplification of recombinant baculovirus stocks

2.2.2.1 Plaque purification

Recombinant bacmid DNA carrying chimaeric Gag constructs (GagRT and GagTN) and the respective recombinant baculovirus first and second supernatants, were kindly provided by Mr. R.J. Halsey (MCB, UCT). The baculovirus vector used to create recombinants was AcMNPV (method described in Appendix A1). Plaque purification using the second supernatants of the rAcMNPV stocks were carried out as described in

O'Reilly et al (O'Reilly, 1994). This was done to isolate pure recombinant baculovirus for amplification purposes.

2.2.2.2 rAcMNPV amplification

Purified virus from plaque purification was used (500 µl) to infect $2x10^6$ *Sf*21 cells in a sterile 2-ml TC well. Infected cells were incubated at 27 °C for 4 days before the supernatant was harvested (Passage 1). This process was repeated to create Passage 2 using 2 mls of Passage 1 as the infectant, and infecting $1x10^8$ *Sf*21 cells in sterile TC flasks. Passage 1 and 2 were stored at 4 °C.

2.2.3 Verification of recombinant baculovirus constructs

2.2.3.1 SDS-PAGE and western blotting

All buffers used for SDS-PAGE and western blots are detailed in Appendix B2. Sample loading buffer (5X) was added to samples of Passage 1 and 2 of the respective rAcMNPV stocks in a 1:5 ratio. The samples were then incubated at 90 °C for 5 min to lyse cells and denature protein, after which they underwent sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) (at constant current) on a 10 % SDS-polyacrylamide gel (Sambrook, Fritsch, and Maniatis, 1989), in order to separate proteins on the basis of molecular weight.

Once separated, western blotting was performed whereby proteins from the respective samples were transferred from the SDS-polyacrylamide gel onto nylon membrane using a Trans Blot® semi-dry transfer cell (Bio-Rad) and 1x transfer buffer. The conditions used for transfer were as follows: 400 mA, 15 V, 90 min. Membrane was then incubated in blocking buffer for 30 min, and a 1:2000 dilution of Gag p24 polyclonal primary antibody (Appendix D) (Reid) in blocking buffer overnight. It was subsequently washed three times for 15 min using washing buffer, then incubated in a 1:5000 dilution of Goat anti-rabbit Ig G secondary antibody (Appendix D) in blocking buffer for 1 hr. A second set of washes followed this, and then Gag protein bands were visualized using Nitro blue tetrazolium chloride/5-bromo-4-chloro-3-indolyl phosphate (NBT/BCIP, Roche).

Membrane was rinsed with water after 30 min to stop visual development reaction. The positive control used was HIV-1 B $Pr55^{gag}$ (Appendix D)

2.2.3.2 rAcMNPV DNA extraction

Passage 2 (500 µl) and DNA extraction buffer (Appendix B4) were combined in equal amounts and incubated at 95 °C for 10 min with agitation. The mixture was then incubated on ice for 2 min before being centrifuged at 2000 x *g* for 10 min. Resulting supernatant was removed, treated with 100 µg/ml of RNAse A, precipitated with 0.6 volumes of ice-cold isopropanol and further centrifuged at 2000 x *g* for 10 min. Sterile water (100 µl) was used to resuspend the pellet, and the resulting suspension was heated to 65 °C for 5 min. The sample was re-precipitated with 2 volumes of 95 % of ethanol and 0.1 volume of sodium acetate, and incubated at -20 °C for 2 hrs. It was then centrifuged at 2000 x *g* for 10 min, and the pellet finally resuspended in 10 µl elution buffer.

2.2.3.3 Polymerase Chain Reaction (PCR)

PCR was used to confirm that the genes of interest were present in the respective constructs. Primers were designed to amplify the *gag* gene, fused *tatnef* (*tn*) gene and the *rt* gene in the respective constructs (Table 2.1).

Table 2.1 Primers used for the PCR amplification of *hmgagC*, *tatnef* and *rt* genes

Name of primer	Sequence	Orientation
HMGAGF	5' ATGGACGGCCCCAAGGTGAAGC 3'	Forward
HMGAGR	5' ATTCTTGGCTGAGGGGGTCGC 3'	Reverse
GAGTNF	5' CATGGTCATCAGCTACG 3'	Forward
GAGTNR	5' TCAGTCCTTGTAGTACTCGG 3'	Reverse
GAGRTF	5' CATGGGTGCTCGCGCATCTATC 3'	Forward
GAGRTR	5' GATTCGAAAGCGGCCGCTGTTC 3'	Reverse

Three PCRs were carried out with the primer pairs HMGAGF/HMGAGR (*gag* gene), GAGTNF/GAGTNR (*tn* gene), and GAGRTF/GAGRTR (*rt* gene). Each PCR reaction of

50 µl contained 5 µl extracted rAcMNPV DNA, 10 pmol of the necessary primers, 10X Taq polymerase buffer, 0.25 mM dNTPs, 0.5 units SuperTherm Taq polymerase (Southern Cross Biotechnology) and sterile distilled H_2O. The HMGag and RT reactions required 1.5 mM $MgCl_2$, while the TN reactions required 2.0 mM $MgCl_2$. The PCR amplification cycle profile consisted of 1 cycle of 94 °C for 3 min, 30 cycles of 94 °C (30 sec each), 55 °C (except for TN – used 50 °C) (0.5 min each), 72 °C (40 sec each) and 1 cycle of 72 °C (5 min). Original recombinant bacmid DNAs were used as the positive PCR controls for each reaction to confirm that the experimental PCR products were the correct size (kindly provided by Mr. R.J. Halsey, MCB, UCT). The negative control contained sterile water in place of the template DNA. The resulting PCR products were run on a 0.8 % agarose gel at 100 V for 1 hr.

2.2.3.4 Subcloning

The PCR products were gel extracted (Qiagen gel extraction kit) and cloned into pGem®-T Easy vectors (Promega) according to the manufacturer's instructions (Promega, 1996-1999). The resulting plasmids were then transformed into competent *E.coli* DH5α cells and grown on Luria-Bertani agar (LA) medium with ampicillin, X-gal and isopropyl-thiogalactoside (IPTG) (Appendix B1). Blue-white colony selection was used to identify recombinant pGEM-T Easy clones. Clones were confirmed to be carrying the gene of interest via restriction endonuclease (R.E.) digestion using *Eco*RI. Selected clones were sequenced (UCT sequencing unit) using M13 forward and reverse primers to verify that the genes of interest were intact after rAcMNPV amplification.

2.2.4 Quantification of recombinant baculovirus stock

2.2.4.1 Plaque Assays

Under sterile conditions, *Sf*21 cells (cell density: $1.5x10^6$ cells/ml) were seeded into the desired number of TC 6-well plates (2 ml each), and were left to attach for 45 min. A log_{10} dilution series of Passage 2 (10^{-4} – 10^{-9}) was prepared using TC-100 medium. The medium was removed from each well and replaced sequentially with 1 ml of the diluted infectant series. Cells were incubated at ambient temperature for 2 hrs, after which time

the infectant was removed, and wells were overlaid with 3 ml Plaquing mix [Graces Insect Plaquing medium (Gibco), 4 % SeaPlaque Agarose (Adcock Ingram) and water in a 2:1:1 ratio]. Cells were stained 4 days post infection (dpi) with 100 μg/ml Neutral red dye (Sigma) for 5 hrs. The dye was then removed and the cells incubated for another day at 27 °C before plaques were counted.

2.2.4.2 $TCID_{50}$ assays

Under sterile conditions, Sf21 cells (cell density: $1.5x10^6$ cells/ml) were seeded in each well of a flat-bottomed 96 well TC plate using a Pasteur pipette, and were left to settle for 45 min. A log_{10} dilution series of Passage 2 (10^{-2} - 10^{-12}) was prepared using TC-100 medium, and each dilution was designated a row (8 wells) of the TC plate. Fifty µl of the appropriate dilution was then added to each well in the respective row. One row was designated the negative control (TC 100 medium added instead of infectant). After 10 days, each well was studied to determine if it was infected or not. The $TCID_{50}$ of the assay was calculated using the Karber formula (O'Reilly, 1994), and infectivity of the viral stock was calculated using the following equation:

Infectivity (pfu/ml) = 0.69 x $TCID_{50}$ (Dee and Shuler, 1997).

2.3 Results

2.3.1 Verification of recombinant baculovirus constructs

Recombinant baculovirus stocks for the chimaeric VLP constructs, GagRT and GagTN, were amplified for optimization experiment purposes. Approximately 100 ml of each virus stock was produced and stored at 4 °C. Western blots were used to confirm that the appropriate chimaeric proteins were produced by the respective baculovirus stocks (Fig. 2.2). Both GagRT and GagTN proteins were detected in the respective stocks, and appeared in greater quantities in the amplified stocks than in the initial stocks, as would be expected. It is important to note, that as has been seen in previous work, both chimaeric VLPs had a tendency to migrate slower than what would be expected for their molecular weight, implying they are slightly larger than what they actually are (Halsey et

al.). This is possibly related to their particulate structure, which may only denature partially after boiling.

Cleaved Gag products, p24 and p41 proteins were also present in the stocks, suggesting that there was some form of proteolytic processing of the chimaeric VLPs. This is further supported by previous studies which showed Gag cleavage products such as p49 (MA-CA-p2-NC), p41 (MA-CA), p25 (CA-p2) and p24 (CA) were present in insect cells expressing $Pr55^{gag}$ (Wagner et al., 1996). It has been suggested that proteolytic cleavage occurs due to partial degradation occurring inside and outside the insect cells caused by insect and baculovirus proteases (Cruz et al., 1999). Although this is not ideal, the chimaeric proteins were the most prominently detected in the respective samples, further suggesting that proteolytic cleavage was only partial.

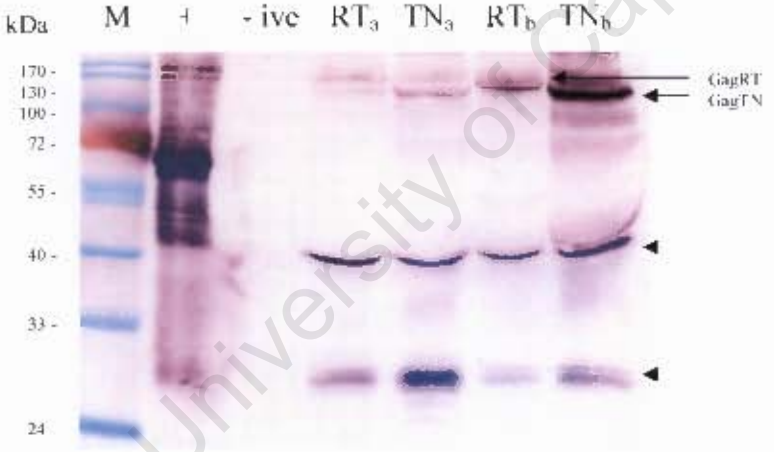


Figure 2.2 Western blots of the recombinant baculovirus stocks to be used for optimization experiments. Membranes were probed with p24-specific antiserum. Arrows (⟵) indicate the chimaeric protein bands. Arrowheads (◀) indicate supernatant bands corresponding to Gag p41 and p24 respectively. Abbreviations: M – molecular weight marker; + – $Pr55^{gag}$ (+ ive control); - ive – wildtype baculovirus - ive control; RT_a = GagRT infectant before amplification; TN_a = GagTN infectant before amplification; RT_b = GagRT infectant after amplification; TN_b = GagTN infectant after amplification. 2 µl of + ive control, and 40 µl of all other samples was loaded in each lane.

It is interesting to note that the intensity of the GagRT band is much less than that of the GagTN. This suggests that the insect cells seem to produce less GagRT than GagTN. It is probable that this difference in expression is related to the size of the respective

constructs, as GagRT (108 kD) is larger than GagTN (92 kD), however further experimentation is needed to verify this.

It was necessary to confirm that the integrity of the recombinant gene sequences had remained intact after baculovirus stock amplification was done. To verify the integrity of the recombinant constructs in the amplified stocks, PCR and sequencing of the respective genes was performed. Due to the large size of the full recombinant genes (GagRT – 2.854 kb, GagTN – 2.443 kb), sequence verification had to be done on the separate components of the recombinant genes rather than the full genes. Thus, the PCR was performed using primers complimentary to the end DNA sequences of *gag*, *rt*, and *tn* genes respectively (Table 2.1).

As expected, the PCR results confirmed that both constructs contained a *gag* gene of approximately 1.5 kb (Figure 2.3a). The GagRT construct contained an RT fragment of approximately 1.3 kb and the GagTN construct contained a TN fragment of approximately 0.9 kb (Figure 2.3b). No amplification occurred when using the baculovirus infectant stocks directly (without extracting DNA). This appears to be a difficult feat, and possibly requires the PCR to be optimized further. After PCR verification, the resulting PCR products were purified using a gel extraction kit (Qiagen) and cloned into pGem^{R}-T Easy vectors (Promega) in order to sequence the genes of interest. The sequencing results (Appendix C) verified that the correct gene sequences were present, with no mutations or truncations observed.

Once the integrities of the constructs were verified, the recombinant baculovirus stocks were quantified to determine the viral titre. This was done for two reasons. First, amplification of recombinant baculovirus stocks can result in the accumulation of mutant virus or defective virion particles (O'Reilly, 1994). This decreases the infectivity of the baculovirus stock as well as its ability to express the recombinant protein in insect cells. Thus, measuring the viral titre provides a good indication of the concentration of infectious particles within the stock tested (Dee and Shuler, 1997). Secondly, to optimize

the expression system, the MOI of the recombinant viral stocks has to be calculated, and this requires knowledge of the stock viral titre.

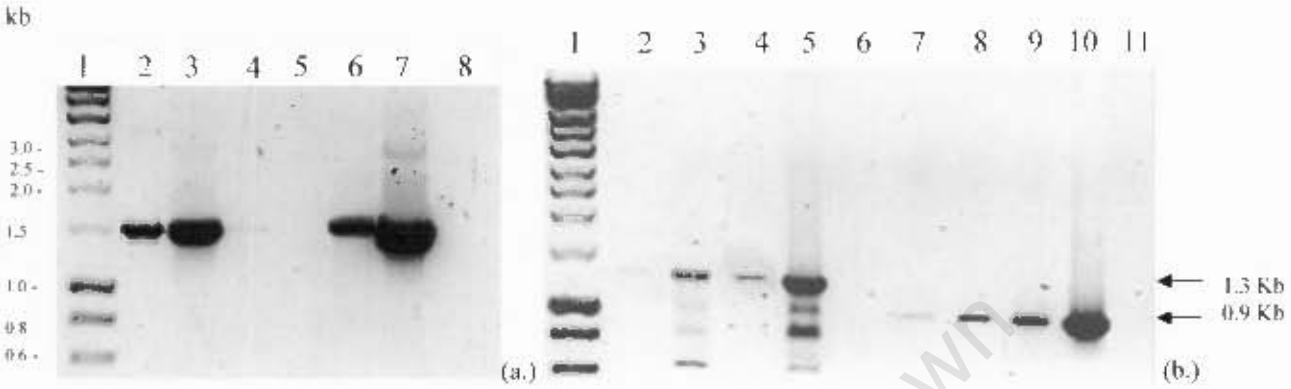


Figure 2.3 – PCR results confirming the presence of *gag*, *rt* and *tn* genes in the GagRT and GagTN constructs of recombinant baculovirus stocks. 11 µl of the PCR reactions were loaded onto a 0.8 % agarose gel. (a) PCR amplification of the *gag* gene: Lanes: 1- M, 2- extracted DNA from GagRT rAcMNPV amplified stock, 3- GagRT bacmid DNA (positive control), 4- GagRT rAcMNPV stock, 5- sterile water (negative control), 6- extracted DNA from GagTN rAcMNPV amplified stock, 7- GagTN bacmid DNA (positive control), 8- GagTN rAcMNPV stock. (b) PCR amplification of the *rt* and *tn* genes respectively: Lanes: 1- M, 2- GagRT rAcMNPV stock, 3- extracted DNA from R.J. Halsey's GagRT rAcMNPV stock (positive control), 4- extracted DNA from GagRT rAcMNPV amplified stock, 5- GagRT bacmid DNA (positive control), 6- sterile water with RT primers (negative control), 7- GagTN rAcMNPV stock, 8- extracted DNA from R.J. Halsey's GagTN rAcMNPV stock (positive control), 9- extracted DNA from GagTN rAcMNPV amplified stock, 10- sterile GagRT bacmid DNA (positive control), 11- water with TN primers (negative control). M = Hyperladder I DNA MW marker; kb = kilobase.

2.3.2 Quantification of recombinant baculovirus stocks

In this study, both plaque assays and $TCID_{50}$ assays were used to determine viral titre. Both assays were done in duplicate (Table 2.2). The results from the two assays were not in agreement with each other. While the plaque assays suggested that the titres were relatively low, the $TCID_{50}$ assays showed titres 10-100 fold greater than the plaque assays. In addition, the plaque assay results showed the average GagRT titre to be larger than the average GagTN titre, while the opposite was found in the $TCID_{50}$ assay. These discrepancies in the viral titres predicted by plaque assays, while problematic, are not uncommon. Previous research has shown that plaque assays do tend to underestimate the true viral titre, providing false information about viral stock infectivity (Janakiraman et al., 2006).

Given that the expected viral titre after amplification is about 10^9 pfu/ml (O'Reilly, 1994), both assays showed that the viral titres of the constructs were low (between 10^6-10^7 pfu/ml for GagRT and 10^6-10^8 pfu/ml for GagTN). However, the $TCID_{50}$ assays appeared to be more reliable in determining the viral titres because they provided reproducible results that were closer to the expected titre values.

Table 2.2 The average viral titre for rAcMNPV stocks (for constructs GagRT and GagTN), as determined using plaque and$TCID_{50}$ assays.

Assay type	**Viral titre (pfu/ml)**	
	GagRT	**GagTN**
Plaque assay 1	5.80×10^6	4.00×10^6
Plaque assay 2	4.10×10^6	5.70×10^6
Average	$\mathbf{4.95\times10^6}$	$\mathbf{4.85\times10^6}$
$TCID_{50}$ assay 1	1.40×10^7	8.70×10^7
$TCID_{50}$ assay 2	1.80×10^7	1.30×10^8
Average	$\mathbf{1.60\times10^7}$	$\mathbf{1.09\times10^8}$

2.4 Discussion

In this chapter, the integrity of *gagrt* and *gagtn* genes were verified by PCR and sequencing, sufficient recombinant baculovirus stocks were produced for optimization experiments to follow, and the viral titres of these stocks were determined.

It was important to confirm that the gene and protein sequences of GagRT and GagTN were not mutated in the process of amplifying recombinant baculovirus stocks, as a mutation or deletion could have resulted in no or poor VLP formation (Wang, Lai, and Li, 1998). Both the PCR and sequence data indicated that there was no alteration of the recombinant gene sequences. There was some proteolytic processing of the chimaeric VLPs though, as suggested by the presence of p41 and p24 cleavage products on the western blot. This could have been detrimental to VLP formation if the protease activity

was extensive. However, this does not appear to be the case here, as the chimaeric protein bands were strongly detected. Previous research has suggested that such protease activity can be minimized through expression system optimization, thus proteolytic cleavage should not be a major problem in the production of the chimaeric VLPs (Cruz et al., 1999).

The amplification of the recombinant baculovirus stocks was performed to carry out optimization experiments. This is usually a straightforward procedure in the baculovirus-insect expression system, which has been known to yield recombinant viral titres as high as $1x10^9$ pfu/ml after amplification (Jorio, Tran, and Kamen, 2006; O'Reilly, 1994; Sarafanov and Saenko, 2004). However, although both GagRT and GagTN rAcMNPV stocks were amplified to a certain extent, their titres ($1.60\ x10^7$ pfu/ml and $1.09\ x10^8$ pfu/ml respectively) were lower than the expected $1x10^9$ pfu/ml, even after repeated attempts at amplification. Many factors could have caused this, such as the cell line and cell passage number used, baculovirus infectivity, or inaccurate titre estimations.

Looking at the cell line chosen for the amplification, *Sf*21 cells have been used in the production of a variety of proteins. They are often used in transfections, plaque isolation and to produce high-titre stocks (Invitrogen, 2002; O'Reilly, 1994). In the experiments of this study, they displayed high viability and growth rate during the amplification process, and are unlikely to be the cause of the poor amplification. However, it is possible that the passage number could have had a negative effect on the ability of the cells to become infected and produce progeny virions. Studies have shown that in the late passages of insect cells, the cells lose their viability and grow at a slower rate (Invitrogen, 2002). Maruiak *et al* (Maruniak, Garcia-Canedo, and Rodrigues, 1994) showed that passage number was particularly important for plaque assays when he demonstrated that *Sf*9 cells at a low passage number produced a higher viral titre than those a higher passage number. Given the similarities between *Sf*9 and *Sf*21 cells, it is possible that an infection at a late passage could result in poorer viral yields than usual in *Sf*21 cells too. The cells used for the amplification procedures in these experiments were at passage 27, while those utilized for plaque assays were at passage 34. For these cells, this is not considered late,

as they have been known to survive over 100 passages. Nonetheless, had the cells been infected at an earlier passage, the results may have differed. This will be an important variable to explore in future studies.

Another factor which may have influenced the values observed for viral titre is the methods that were used for viral titre quantification. Several methods have been designed to quantify the infectivity of recombinant baculovirus stocks, however each has its drawbacks (Janakiraman et al., 2006).

Initially, plaque assays were used to quantitate the viral titres for the recombinant stocks. Although plaque assays are relatively less arduous than most methods, in this study there were many factors that had an effect on plaque formation such as precipitation of the dye, temperature and viscosity of the agarose, and cell density within the wells. If any of these factors were not exact, plaque formation would be deformed or absent. The plaque assays were also attempted a number of times before reproducible results could be obtained. In addition, the assays demonstrated low viral titres for both GagRT and GagTN stocks. This could have been due to the amplification being unsuccessful, or due to an underestimation of the true titre, as has been demonstrated by plaque assays previously (Janakiraman et al., 2006).

In view of this, it was necessary to do a second assay, namely, a $TCID_{50}$ assay, to determine which possibility was true. Unlike the plaque assays, the $TCID_{50}$ assay was a longer and more tedious method, and result determination is a subjective process (Janakiraman et al., 2006). However, results obtained in these experiments were reproduced without difficulty and were closer to the expected values than the plaque assay results, suggesting that the amplification, although not completely successful, did work. In view of this, the results of the $TCID_{50}$ assays were used for the optimization experiments that follow. In future, it may be of interest to look into alternative methods to determine viral titre such as the BakPAK Baculovirus^{TM} rapid titre kit (McCall et al., 2005).

On the whole, the rAcMNPV bulk stocks that were produced in this study were found to carry the full gene insert for both GagRT and GagTN, and stocks were produced in sufficient quantities to continue with optimization experiments, even though the viral titres were not as high as expected.

CHAPTER 3

The optimization of chimaeric HIV-1 VLP production in insect cell culture

3.1 Introduction

The expression of recombinant VLPs can be affected by several factors. Some of the major factors that have been identified when using an insect cell expression system include insect cell line employed, cell density, MOI, infection time, medium type, temperature, and shaking speed. Factors such as temperature and shaking speed are specific to the maintenance of healthy insect cells and are generally kept constant. However, the other factors mentioned above are dependent on the type of recombinant protein being expressed. To produce high chimaeric VLP yields for immunogenicity studies, it was necessary to explore the most favourable conditions for VLP production.

3.1.1 Factors affecting chimaeric VLP production

In this study, four factors were investigated as to their effects on VLP expression. One of these factors was the insect cell line used by BEVS. There are currently a number of possible insect cell lines that are being utilized for recombinant protein production; however, there can be a large variation in recombinant gene expression levels in different cell lines (Hink et al., 1991). This makes predicting which cell line would be ideal for production of a given recombinant protein difficult, and empirical evidence is thus necessary.

Two species of insects are most frequently used, namely *Spodoptera frugiperda* (fall army worm) and *Trichoplusia ni* (cabbage looper) (O'Reilly, 1994). Within the *S. frugiperda* species, the two cell lines, *Sf*9 and *Sf*21, have been identified as adequate hosts to AcMNPV vectors. These cell lines were the original cell lines used for baculovirus work, and were derived from the pupal ovarian tissue of the worm. Both *Sf*9 and *Sf*21 cells have similar cell shape, size and doubling time. However, *Sf*9 cells appear to be preferable for large scale recombinant protein expression, sometimes producing as much as double the quantity produced in *Sf*21 cells (O'Reilly, 1994). In addition, *Sf*9 cells are also able to grow in monolayer and suspension culture, while *Sf*21 cells tend to clump in suspension (in serum-free medium), growing best in monolayers. This means that large-scale protein production in *Sf*21 cells is both time-consuming and more expensive.

The *Trichoplusia ni* (*T.ni*) species is also exploited by BEVS as a potential host. These cells have a larger cell diameter and a faster doubling time than *Sf*9 cells, so the optimal time for recombinant-protein harvesting is likely to be less than that for *Sf*9 cells. They are also preferred for recombinant protein expression, especially secreted proteins (Maruniak, Garcia-Canedo, and Rodrigues, 1994). While High-Five™ cells are the most popularly utilized *T.ni* cell line, a new cell line called *T.ni* Pro™ cells has recently been produced for the purposes of more efficient recombinant protein expression. This cell line was used in this study, alongside *Sf*9 cells.

The second factor that was of interest to explore was the cell density used in the rAcMNPV infections. Cell density has previously been shown to be a dominant influencing factor of recombinant protein production, inhibiting production when the density is too high (Wickham et al., 1992). Insect cells generally require ample space, adequate aeration and fresh medium in order to survive, and are therefore healthier at a lower cell density (O'Reilly, 1994). At the same time, a greater number of healthy cells available and susceptible to a rAcMNPV infection could potentially lead to more recombinant protein being produced. Thus, a fine balance must be found in order to maximize yields.

The third factor that was evaluated, MOI, was an important factor to explore because it required a balance to be found between producing high protein yields and using the minimum quantity of rAcMNPV infectant. MOI describes the ratio of infecting baculovirus particles to the number of insect cells. Consequently, if the MOI is high, one assumes that recombinant protein production would also be high. However, high MOI values can be problematic for two reasons. Firstly, BEVS is a lytic expression system, leading to the eventual lysis of infected cells. This means that a MOI that is too high could be potentially detrimental to the system, killing cells before they have produced adequate amounts of recombinant protein. Secondly, using high MOI values requires greater quantities of infectant to be used, especially if the infection is done on a large scale. Given that the amount of chimaeric VLPs required to complete immunogenicity

studies is large, it is important to determine the optimal quantity of infectant necessary to produce maximum VLP yields using minimal infectant quantities, and this is possible if the optimal MOI can be determined.

The final factor considered here was infection time. Infection time in insect cells can vary quite substantially for recombinant proteins, depending on when the recombinant gene is expressed in the viral life cycle, and the stability of the cell line used. Both GagRT and GagTN genes are controlled by the polyhedron (pPolH) promoter, and are expressed late in the viral life cycle (72-96 hour post infection). Thus, the longer the infection time, the more likely that protein processing is going to be less efficient as cells near their death (Hu, 2005). In view of this, it is important to find the best time to harvest the chimaeric VLPs in terms of yield, while also maintaining the structural integrity of the VLPs.

3.1.2 VLP formation

The expression of the respective Gag chimaeras in insect cells results in the production of chimaeric VLPs and their eventual budding from the host cells. Thus, the majority of chimaeric VLPs are expected to be found intact in the cell culture supernatant. However, there has been evidence to indicate that Gag VLPs as well as chimaeric VLPs also bud into cytoplasmic vesicles and therefore could remain within cells (Royer et al., 1991). Hence, both the cell lysate and cell supernatant were analysed for the presence of chimaeric VLPs in this study.

3.1.2.1 Gag cleavage products

In addition to intact chimaeric VLPs, several studies have shown that specific as well as non-specific Gag cleavage products are also detected in extracted VLP samples, as seen in Chapter 2 (Cruz et al., 1999; Tobin et al., 1996). These products are likely to occur due to proteolytic activity which also appears to occur outside of the cells, as Gag products are detected in the cell culture supernatant. This is especially observable at later infection time points where cell lysis (and thus the release of cellular proteases) is more frequent. In general, it is very important to optimize the expression system, as this is one of the

only effective ways to reduce extracellular proteolytic activity significantly (Cruz et al., 1999).

3.1.3 Statistical analysis

Evaluating which conditions would be best suited for chimaeric VLP production is complicated by the investigation of several factors' effects as opposed to just two factors. The only way to determine optimal conditions effectively is to analyse the results using the statistical method, analysis of variance (ANOVA). ANOVA is used to analyze the variation occurring in a given experiment (Montgomery, 2005). This variation can arise from many sources, depending on the experimental design, and thus ANOVA allows one to identify the causes of variation and compare these sources using the relevant statistical tests. The tests in an ANOVA are based on an experimental F-ratio (the variation caused by an experimental treatment or effect, divided by the variation due to experimental error). The experimental F-ratio is compared to a null hypothesis F-ratio (equal to 1), and if the experimental F-ratio is large enough, that the possibility of it equalling 1.0 is smaller than a pre-assigned criteria (confidence level), the null hypothesis is rejected and the experimental factor's effect on the results is deemed significant. In other words, the hypothesised distribution is premised on the null hypothesis being true, but if the experimental F-ratio does not fit into the hypothesised distribution, then there is evidence that the null hypothesis is false, and that suggests that the investigated factor significantly affects the results. It is important to note that ANOVA is only appropriate if the data being tested has a constant variance and can be fitted to a normal distribution. If this variance is not constant or the distribution is not normal, the statistical model has to be transformed by means of a natural log application in order to account for the anomalies.

In this study, factorial ANOVA was used to determine the best VLP production conditions for the respective chimaeric constructs. The factorial experiment design involved the individual variation of each chosen factor, while the other factors remained constant (Montgomery, 2005). In this way, each factor's effect could be evaluated separately, and factor interactions could be determined. When factor-factor interactions occur, it is no longer meaningful to interpret the influence of the individual factor,

because the effect of an interaction is dependant on two conditions not one. Thus, the influence of an individual factor is overridden by the effect of an interaction. This experiment design makes the most efficient use of the optimization data and was therefore well suited for determination of the optimal VLP production conditions.

3.1.4 Chapter objectives

The aims of the work reported in this chapter were as follows:

(i) To optimize the production of chimaeric VLP production by evaluating the effect of four factors on chimaeric VLP expression.

(ii) To determine the optimal conditions for the expression of GagRT and GagTN through statistical analysis of the data obtained from optimization experiments.

3.2 Materials and Methods

3.2.1 Insect cell lines

*Sf*9 (Invitrogen) and *T.ni* Pro™ cells (Expression Systems) were utilized for the optimization experiments. The *Sf*9 cells were grown in SF-900 II insect medium (Gibco), and the *T.ni* Pro™cells were grown in ESF-AF medium (Expression systems). Both media were supplemented with 10 μg/ml gentamycin (Sigma). Cells were grown in sterile tissue culture flasks (Amersham), shaking at 120 rpm and kept at a constant temperature of 27°C. They were maintained by sub-culturing mid-log cells to $5x10^5$ cells/ml every 3 days.

3.2.1.1 Measurement of cell viability

Insect cell viability was measured at regular intervals to confirm that cells were maintaining a healthy doubling rate and were not under unnecessary stress. This was done as described in 2.2.1.1.

3.2.2 Recombinant infectant

The rAcMNPV infectants, GagRT and GagTN, made as described in 2.2.2, were used for all optimization experiments.

3.2.3 Optimization procedure

The optimization experiments were performed by infecting 10 ml cell cultures with the respective recombinant baculovirus infectant and incubating these cells at a constant temperature of 27 °C under shaking conditions (120 rpm). The four factors' parameters were varied as detailed in Table 3.1. Once the cells were infected, 1 ml samples were collected at the given time points (Table 3.1), and cell count and viability was evaluated to determine the effect the infections were having on cells. The optimization samples were then centrifuged at 1000 x *g* for 1 min using a bench top centrifuge, after which supernatant and cell pellet were separated. Both pellet and supernatant samples were stored at 4 °C for further use.

Table 3.1 The four factors and corresponding parameters which were tested in the optimization experiments

Factors	Parameters
1. Insect cell line	*Sf*9 cells *T.ni* Pro™cells
2. Cell density (cell/ml)	0.5×10^6 1.0×10^6 2.0×10^6
3. MOI	0.1 1.0 5.0
4. Infection time of the respective rAcMNPV (hours post infection)	48 72 96 120

The negative controls included cells that were infected with wild-type baculovirus (at a cell density of 1.0×10^6 cells/ml and MOI 5).

3.2.4 Protein analysis

3.2.4.1 Western blots

Western blots were performed on all optimization samples as described in 2.2.3.1. The positive control used was HIV-1 B $Pr55^{gag}$ (Appendix D) while the negative control was supernatant from cells infected with wild type baculovirus.

3.2.4.2 Gag p24 ELISA

Quantitative analysis of recombinant protein expression yields in the supernatant optimization samples was performed using the Vironstika® HIV-1 Antigen Microelisa system kit (Biomerieux), which recognizes the HIV-1 Gag p24 core antigen. The ELISA procedure was carried out as detailed in the manufacturer's instructions. ELISA absorbance readings were detected using the Bio-Tek® Powerwave XS at a wavelength of 450 nm. These results were then converted into concentration (pg/ml HIV p24) values using Kineticalc for Windows software (Bio-Tek® instruments Inc.). Each sample was tested in triplicate, and a mean value was used for further statistical analysis.

3.2.5 Statistical analysis

A Four-factor ANOVA was performed on the obtained Gag p24 ELISA results using R.2.3.1 software (R Development Core Team, 2006). Once data was obtained from the ANOVA analysis, its validity was tested by examining the residual values (The error as determined by summing the square of the group means) to determine the validity of the test. This was done by establishing if the data agreed with the basic assumptions of ANOVA; that is evaluating whether variance of the experiments was constant and whether the data fitted a normal distribution. Tukey tests (generic pair-wise T tests) were also performed to compare the parameters of significantly influencing factors and determine estimates of their confidence intervals.

3.3 Results

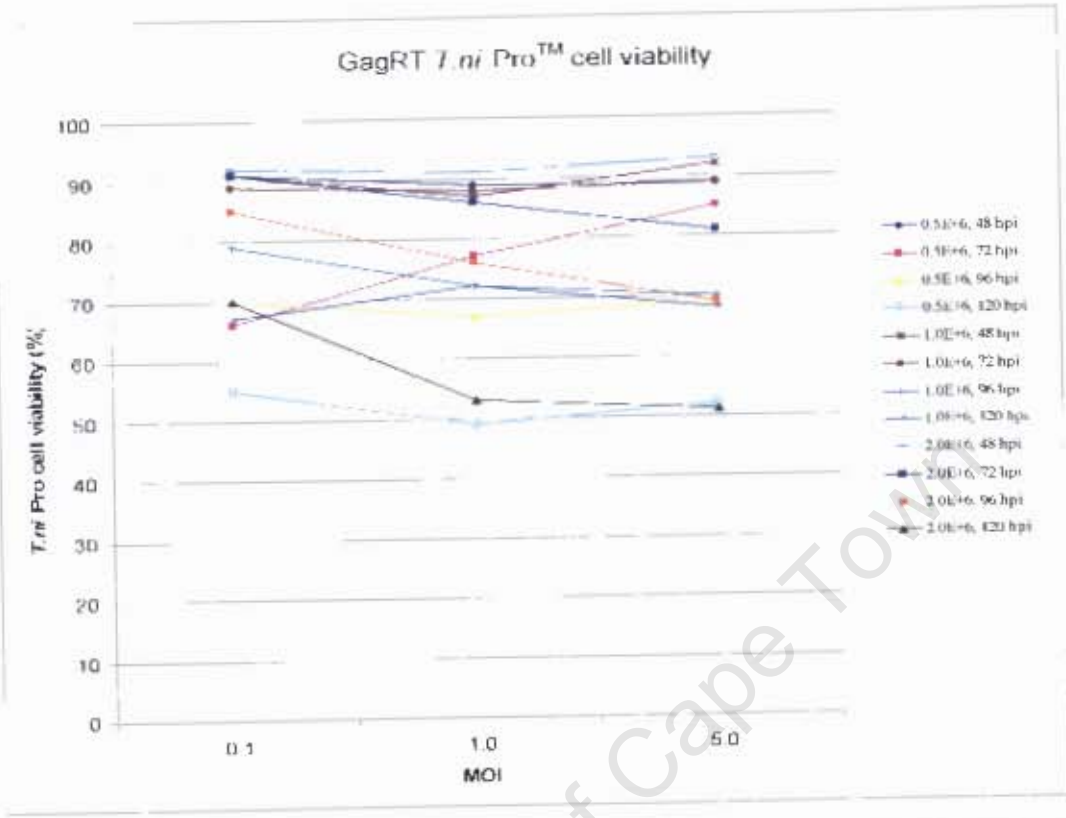
3.3.1 Cell viability

The optimization experiments involved varying the conditions and parameters detailed in Table 3.1. These parameters were initially selected using data from previous work done in our laboratory (pers. comm: Ms A. Lynch, UCT). Each experiment was performed twice for reproducibility purposes. Samples were taken at specific times, and the cell viability of infected cell samples was evaluated by calculating the percentage of living cells present in samples. This allowed the monitoring of the effect that the respective infections were having on the cells. In general, there was only a slight decline in cell viability as MOI increased, however infection time appeared to affect cell viability quite considerably (Fig. 3.1 and 3.2). As would be expected, cell viability generally decreased over time and cells appeared wrinkled and unhealthy at 120 hpi. This was probably as a result of both the baculovirus infection and depletion of nutrients.

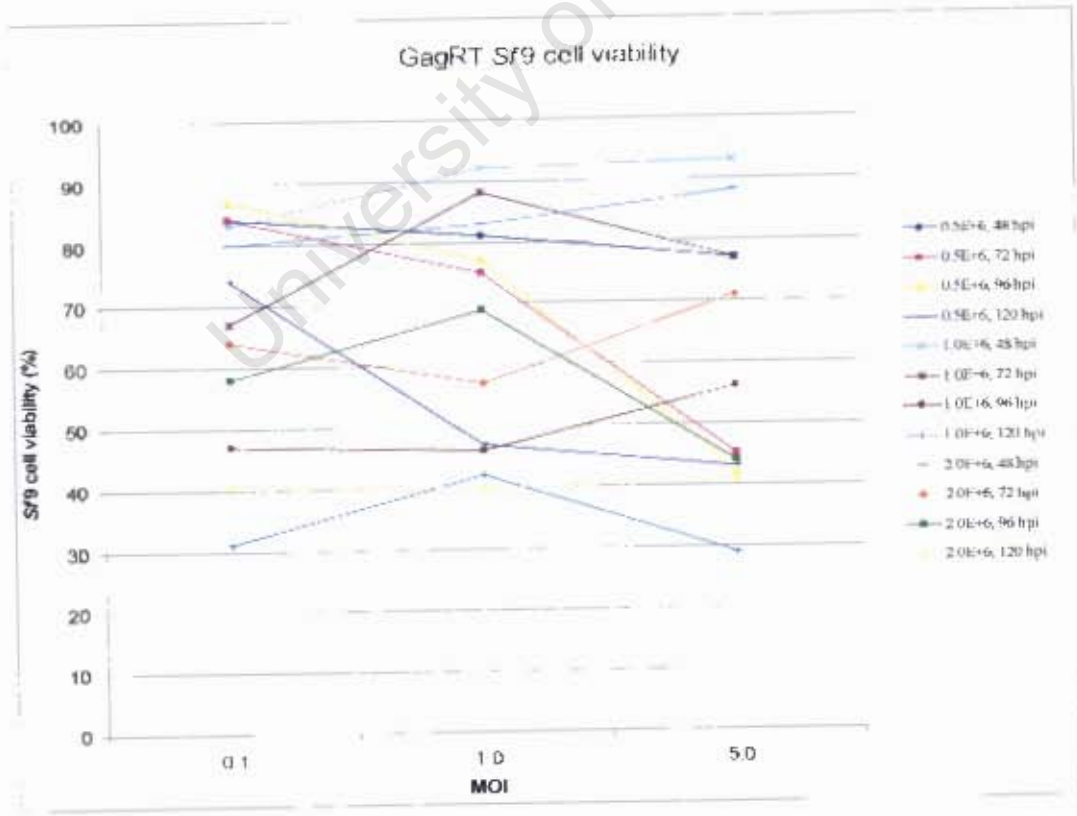
3.3.2 Western blots

Western blot data was used to confirm the production of the chimaeric proteins, and indicated that recombinant protein expression occurred at all time points measured and was found in both pellet and supernatant samples (detected by a Gag p24 primary antibody). No Gag products were detected in the negative controls. Antibodies specific to RT and Nef proteins were also used for confirmatory purposes, and also indicated corresponding chimaeric protein expression in the samples tested (data not shown). As was found in the previous chapter, both GagRT and GagTN migrated to positions a little higher than their molecular weight relative to the marker.

In particular, the western blot data for samples of cells infected with GagRT (Fig. 3.3 & 3.4) or GagTN (Fig. 3.5 & 3.6) (cell density $2x10^6$ cells/ml) illustrated that there appeared to be more of the chimaeric proteins in the cell pellets than supernatant samples, possibly as a result of aggregation of VLPs within the cell.

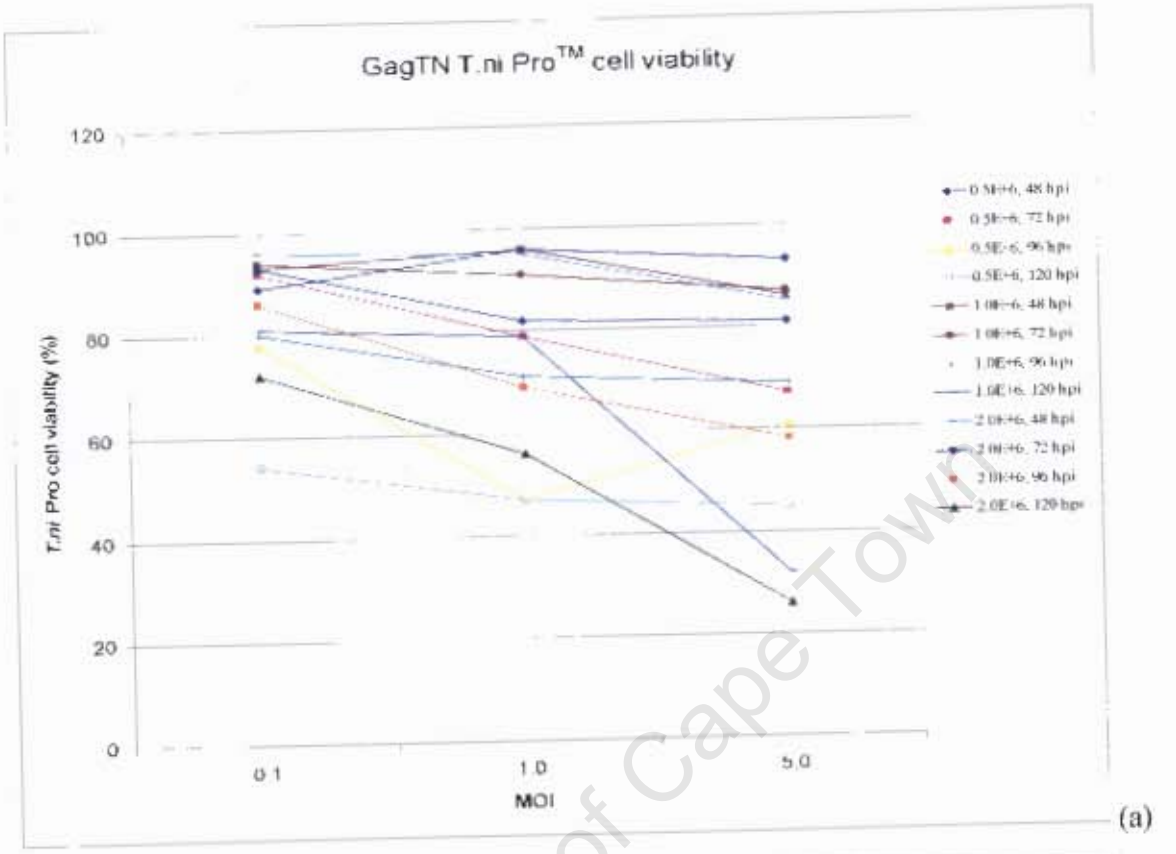


(a)

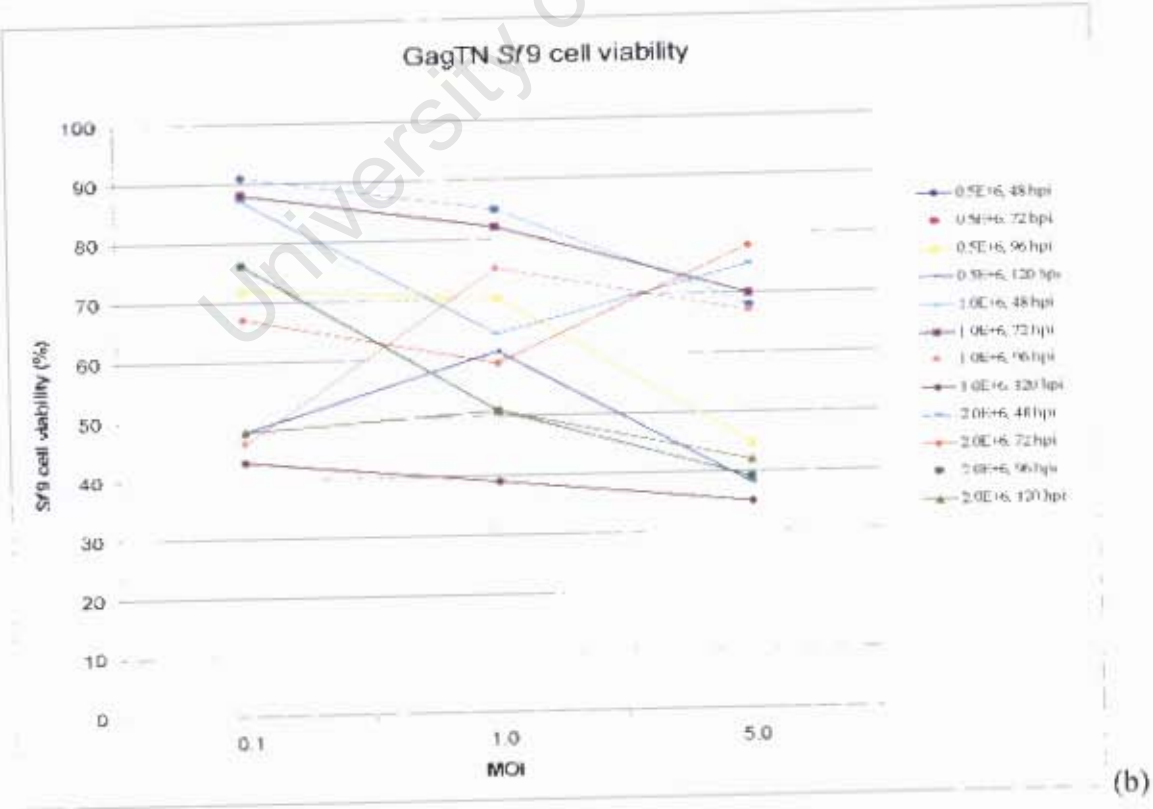


(b)

Figure 3.1 – The trend of cell viability after infection with GagRT using the designated MOI. (a.)*T.ni* Pro™ cell viability (b.) *Sf*9 cell viability. Hpi = hours post infection.



(a)



(b)

Figure 3.2 – The trend of cell viability after infection with GagTN using the designated MOI. (a.)*T.ni* Pro™ cell viability (b.) *Sf*9 cell viability. Hpi hours post infection.

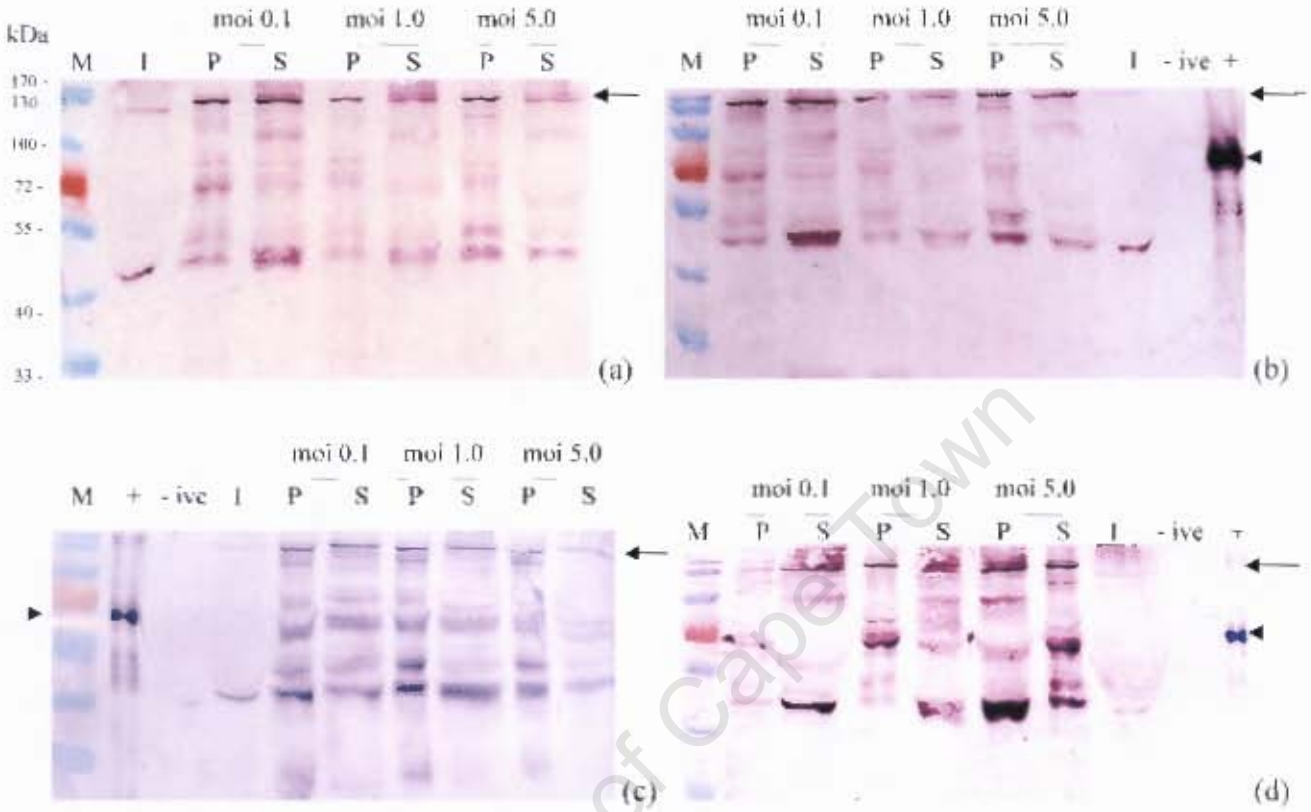


Figure 3.3 Examples of the optimization samples for GagRT VLPs produced in *T.ni* Pro™ cells at a cell density of $2x10^6$ cells/ml. Membranes were probed with HIV p24-specific antiserum. Samples analysed at 48 hours post infection (hpi), (a), 72 hpi (b), 96 hpi (c), 120 hpi (d). Abbreviations: M = molecular weight marker, I GagRT infectant, - ive = wildtype baculovirus, + HIV-1B Pr55gag, P cell pellet, S culture supernatant. Arrows (⟵) indicate where GagRT (108 kD) migrates to. Arrowheads (►) indicate where $Pr55^{Gag}$ migrates to.

Besides the chimaeric Gag proteins, Gag cleavage products were also detected in the collected samples, specifically $Pr55^{gag}$ and p41, but also several non-specific Gag products. Interestingly although not unpredictably, the cleavage patterns detected in the two cell lines were quite different. The p41 and $Pr55^{gag}$ bands were detected more prominently in *T.ni* Pro™ samples (Figs 3.3 and 3.6), while the *Sf*9 samples (Figs 3.4 and 3.5) contained two larger cleavage products (approximately 80-90 kD) in addition to $Pr55^{gag}$.

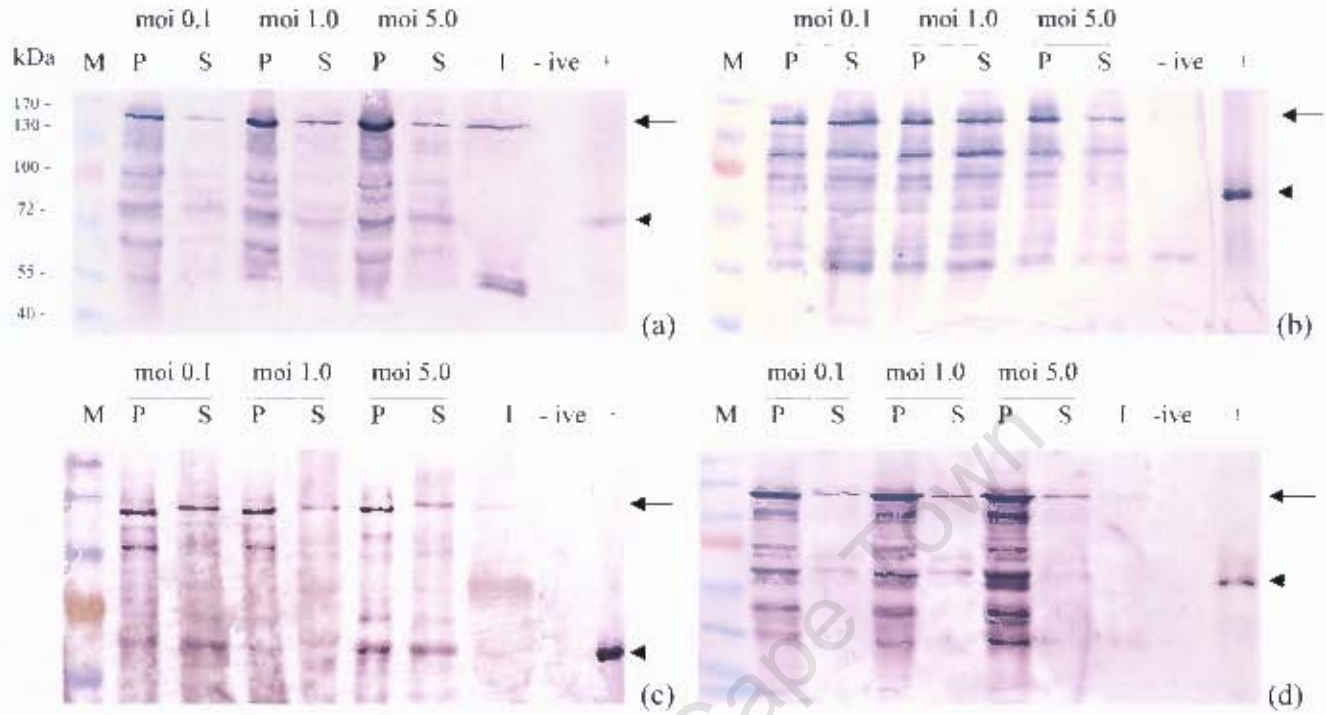


Figure 3.4 –Examples of the optimization samples for GagRT VLPs produced in *Sf*9 cells at a cell density of $2x10^6$ cells/ml. Membranes were probed with HIV p24-specific antiserum. Samples analysed at 48 hours post infection (hpi). (a), 72 hpi (b), 96 hpi (c), 120 hpi (d). Abbreviations: M = molecular weight marker, I= GagRT infectant, - ive = wildtype baculovirus, + = HIV-1B Pr55gag, P = cell pellet, S = culture supernatant. Arrows (⟵) indicate where GagRT (108 kD) migrates to. Arrowheads (►) indicate where $Pr55^{gag}$ migrates to.

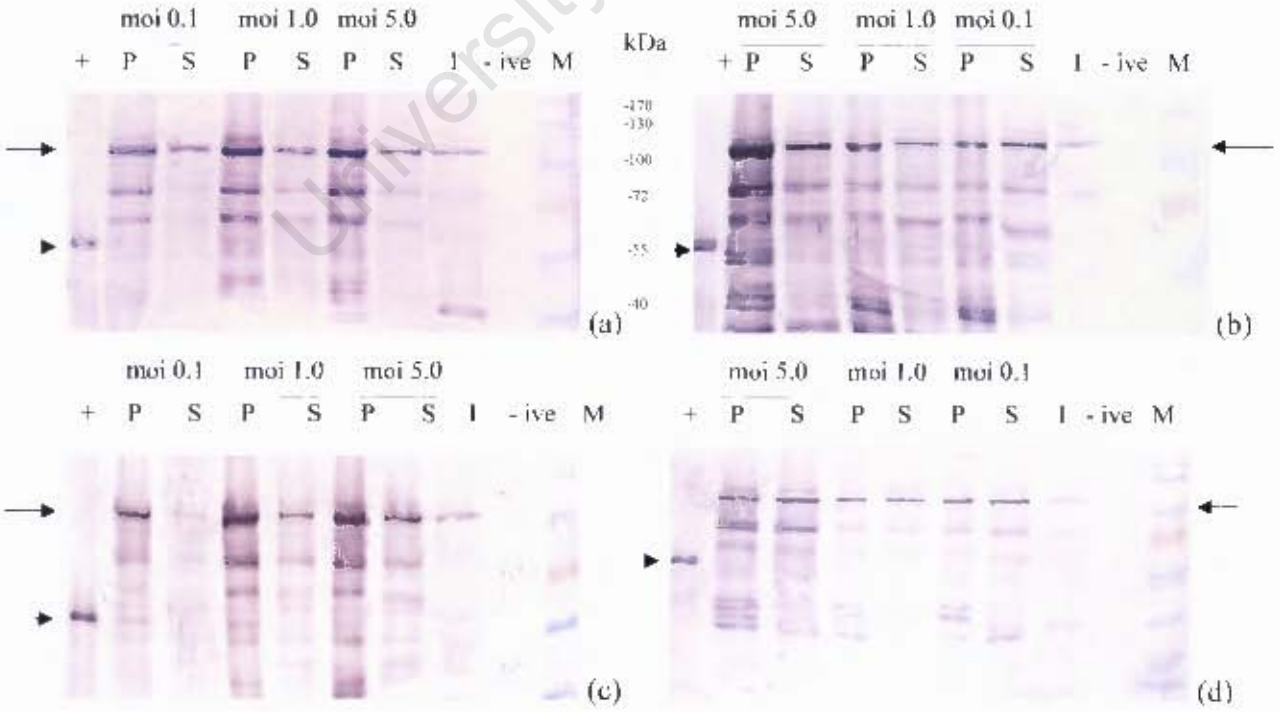


Figure 3.5 Examples of the optimization samples for GagTN VLPs produced in *Sf*9 cells at a cell density of $2x10^6$ cells/ml. Membranes were probed with HIV p24-specific antiserum. Samples analysed at 48 hours post infection (hpi), (a), 72 hpi (b), 96 hpi (c), 120 hpi (d). Abbreviations: M = molecular weight marker, I= GagTN, - ive = wildtype baculovirus, + = HIV-1B Pr55gag, P = cell pellet, S = culture supernatant. Arrows (⟵) indicate where GagTN (92 kD) migrates to. Arrowheads (◄ indicate where $Pr55^{gag}$ migrates to.

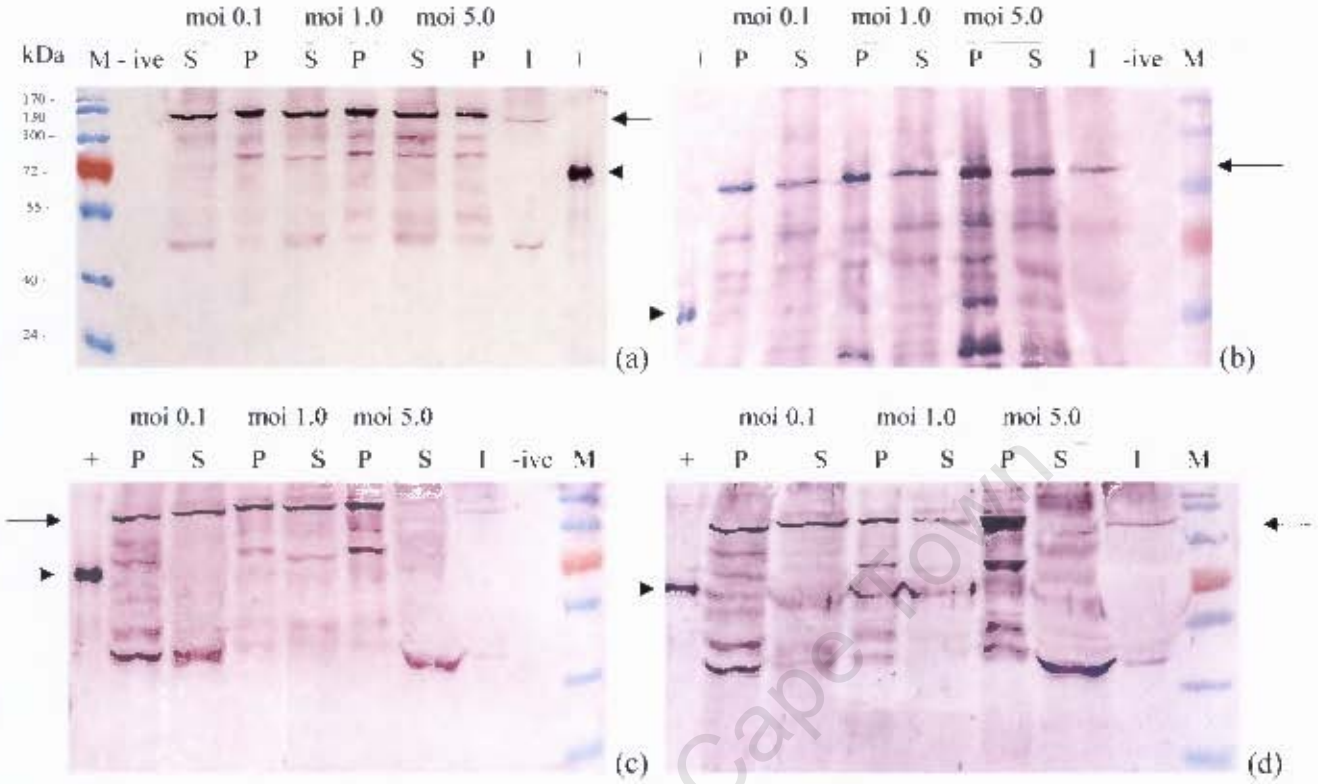


Figure 3.6 –Examples of the optimization samples for GagTN VLPs produced in *T.ni* Pro™ cells at a cell density of 2x10^6 cells/ml. Membranes were probed with HIV p24-specific antiserum. Samples analysed at 48 hours post infection (hpi), (a), 72 hpi (b), 96 hpi (c), 120 hpi (d). Abbreviations: M = molecular weight marker, I= GagTN, - ive = wildtype baculovirus, + HIV-1B Pr55gag, P cell pellet, S culture supernatant. Arrows (⟵) indicate where GagTN (92 kD) migrates to. Arrowheads (►) indicate where $Pr55^{gag}$ migrates to.

As was the case in the previous chapter, it was not unexpected to find Gag cleavage products when expressing Gag-derived VLPs. However, for the purposes of this study, it is not ideal, particularly when it leads to a decline of chimaeric protein yields. This seems to be the case for the 120 hpi samples, especially in the *T.ni* Pro™ cell samples (Figs 3.3 and 3.6).

3.3.3 Gag p24 ELISA and ANOVA

The ELISA was used as a means of measuring recombinant protein expression quantitatively, and was done in triplicate to determine a mean value for Gag protein expressed in each sample (Appendix E). Both pellet and supernatant samples were assayed for the presence of Gag p24, however, cellular matter in the pellet samples interfered with the ELISA detection method. Because of this, as well as the fact that only the culture supernatants will be used to obtain purified VLPs (Chapter 4), ELISA results

(Appendix E) obtained for supernatant samples were focussed on. The experiments showed that a maximum of 406.12 ng/ml HIV p24 was detected in the GagRT optimization experiments, when *Sf*9 cells were infected for 96 hours at a cell density of $1x10^6$ cells/ml and a MOI of 1.0 (Appendix E, Table E1). The maximum for GagTN experiments was 460.51 ng/ml HIV p24, detected when *T.ni* Pro cells were infected for 120 hours at a cell density of $1x10^6$ cells/ml and an MOI of 5.0 (Appendix E, Table E2). Generally, GagTN was expressed in moderately higher quantities than GagRT. This is possibly because of the smaller size of GagTN, which makes it easier to produce and process within cells.

Four-factor ANOVA was performed on the resulting ELISA data to determine, statistically, the best conditions for maximized VLP production. ANOVA also assisted in identifying the major factors and factor interactions that affected protein expression. The data (shown graphically in Figs 3.7 – 3.14) demonstrated that there were several similarities in the way in which the chosen factors affected the production of GagRT and GagTN. Specifically, cell density affected chimaeric protein expression appreciably. The highest cell density, $2x10^6$ cell/ml, did not produce the largest yields of chimaeric VLPs as would be expected; instead an optimal cell density of $1x10^6$ cell/ml was identified for both constructs (Figs 3.7, 3.8 for GagRT and Figs 3.11, 3.12 for GagTN).

Interestingly, MOI seemed to have no great effect on the production of either construct (Figs 3.9 for GagRT and Fig. 3.13 for GagTN). Recombinant protein expression was expected to increase as MOI was increased to a certain extent, given that MOI defines infectivity of the infectant. However, there was little to no effect observed as MOI increased in the experiments of both constructs. For *Sf*9 cells, a MOI of 0.1 produced lower yields, while yields produced by a MOI of 1 and 5 displayed similar levels of VLP expression.

The GagRT ANOVA results indicated that the factors which significantly affected recombinant protein expression were cell line, cell density and infection time, while

GagRT ANOVA graphical data

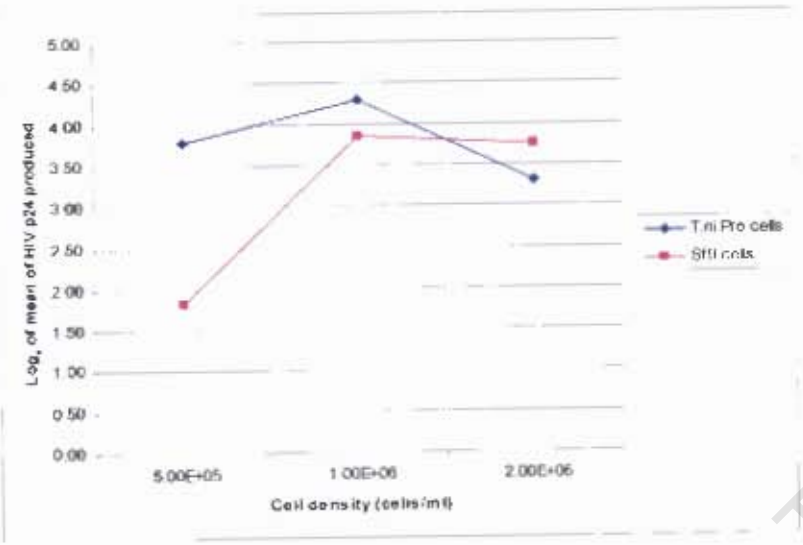


Figure 3.7 - The relationship between cell density and cell line for GagRT at a constant MOI and infection time.

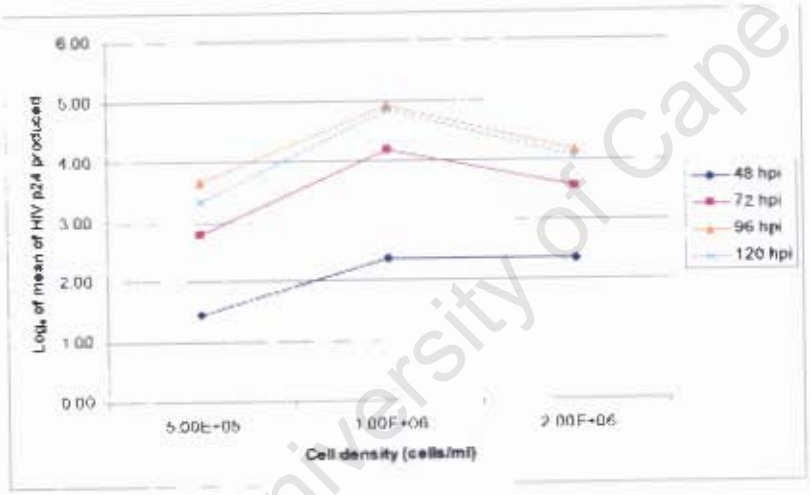


Figure 3.8 - The relationship between cell density and infection time for GagRT at a constant MOI and cell line.

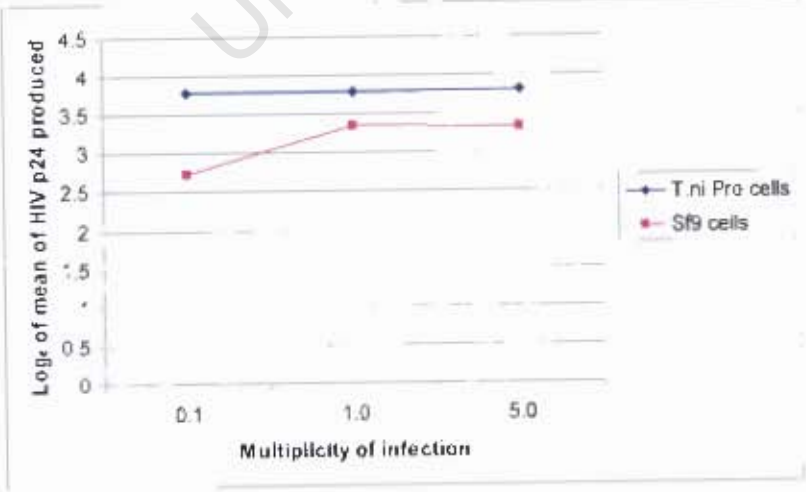


Figure 3.9 - The relationship between multiplicity of infection and cell line for GagRT at a constant infection time and cell density.

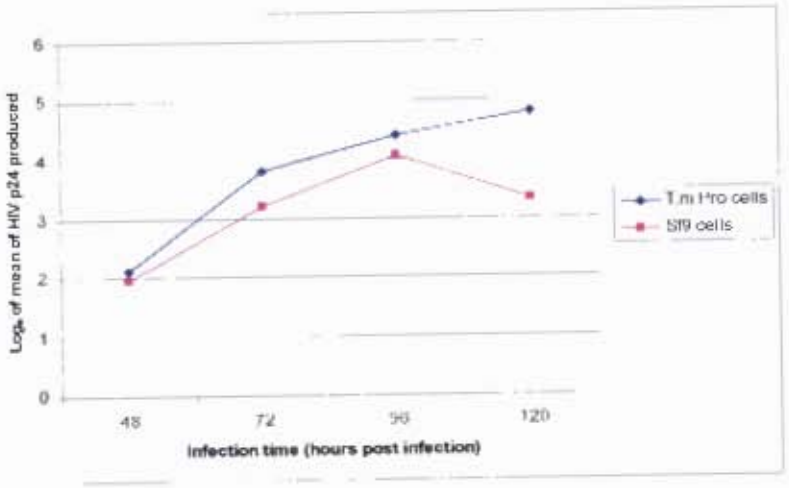


Figure 3.10 - The relationship between infection time and cell line for GagRT at a constant MOI and cell density.

GagTN ANOVA graphical data

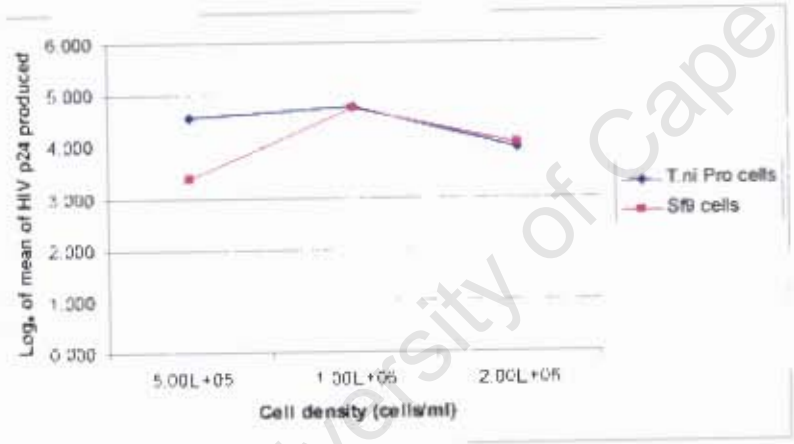


Figure 3.11 - The relationship between cell density and cell line for GagTN at a constant MOI and infection time.

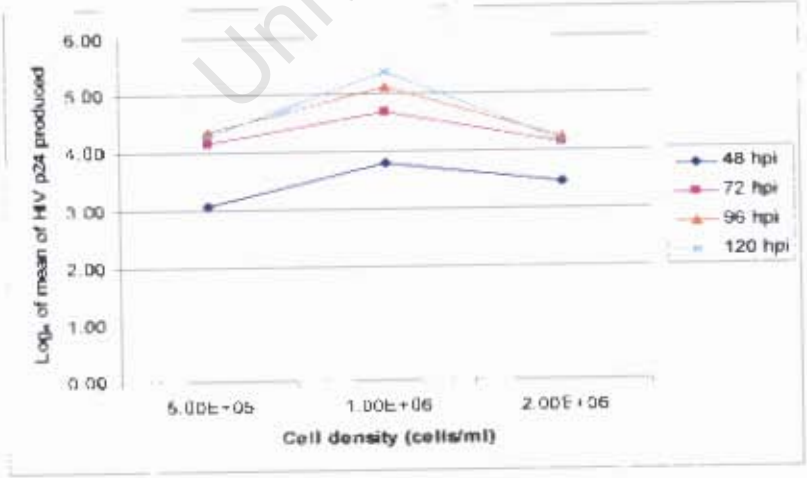


Figure 3.12 - The relationship between cell density and infection time for GagTN at a constant MOI and cell line.

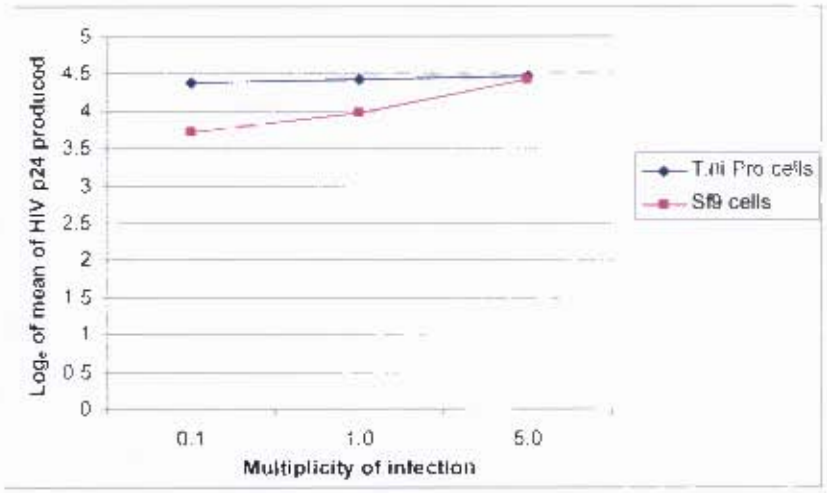


Figure 3.13 - The relationship between multiplicity of infection and cell line for GagTN at a constant infection time and cell density.

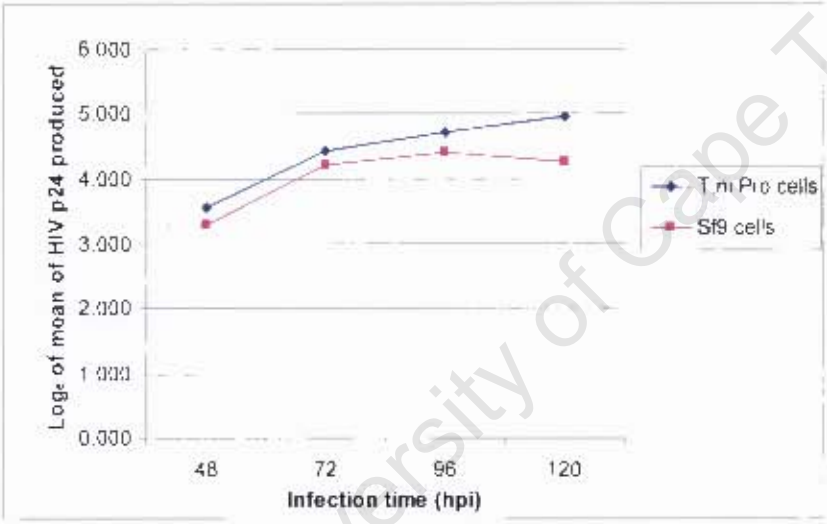


Figure 3.14 - The relationship between infection time and cell line for GagTN at a constant MOI and cell density.

factor interactions (which were implied by graphs within the same figure that differed in shape) occurred between cell line and cell density (Fig. 3.7), and cell line and infection time (Fig. 3.10). This means that cell line influenced or was dependent on both cell density and infection time. The *T.ni* Pro™ cell line seem to produce higher yields then the *Sf*9 cell line for the two lower cell densities and all infection times (Figs 3.7 and 3.10 respectively). There was a clear increase in protein expression over time as would be expected, although GagRT production began to plateau at the higher time points (Figs 3.8, 3.10), showing only a small variation in protein expression occurring at 96 and 120 hpi (Fig. 3.8).

The GagTN data was only slightly different to that of the GagRT data. The factors that seemed to have an effect on chimaeric protein expression were the same as that of GagRT, however there was only one factor interaction found, between cell density and cell line (Fig. 3.11). Unlike with GagRT, there was no obvious difference in protein production between the two cell lines, although the best infection time was also 120 hpi (Figs 3.12, 3.14). As with GagRT, a plateau was observed at the higher time points. The MOI data illustrated that a MOI of 5 was the favoured parameter for protein expression, but again the effect of MOI on protein production was not considerable (Fig. 3.13).

The ANOVA data was able to effectively identify the optimal conditions for chimaeric VLP production (Table 3.2). The optimal conditions were alike for the two constructs, probably because they are both Gag-based VLPs and are thus processed in similar ways in the cells.

Table 3.2 The optimal conditions to express the VLP constructs, GagRT and GagTN, in insect cells, as determined by ANOVA analysis of Gag p24 ELISA data

	***T.ni* Pro™cells**			***Sf*9 cells**		
Construct	**Cell density (cells/ml)**	**MOI**	**Time post infection (hr)**	**Cell density (cells/ml)**	**MOI**	**Time post infection (hr)**
GagRT	$1x10^6$	Any	120	$1x10^6$	Any	96
GagTN	$1x10^6$	5	120	$1x10^6$	5	120

Because of the complexity and cumulative effects that were generated by comparing four factors to each other, to determine how statistically significant the identified optimal conditions were, was not possible. Thus, to establish how significantly different protein expression was under the chosen parameters, estimated differences of the group means and pair-wise T-tests were performed on the data, under the assumption that all other factors were kept constant. This type of analysis is based on the assumption that there are no interactions between the factors, which we know to be false; nonetheless, it does provide a rough idea as to the magnitude of the significance of the optimal conditions determined. Parameters of the given factors were compared, and were deemed

significantly different to one another if the difference in their means resulted in the error bars crossing x=0. Fig. 3.15a showed that when comparing the 3 cell densities for GagRT to one another, none of the bars crossed 0, and therefore each cell density was significantly different to the other within a 95 % confidence level. The same could not be said for the infection time comparisons (Fig. 3.15b), which showed that 96 hpi and 120 hpi values were not significantly different in terms of protein expression.

In the case of GagTN, the pair-wise T-tests showed that while the two cell lines were significantly different to one another (Fig 3.16a), almost all infection times were not significantly different from each other (Fig. 3.16b). This suggests that using any of the three infection times (72, 96 and 120 hpi) would provide similar protein expression levels.

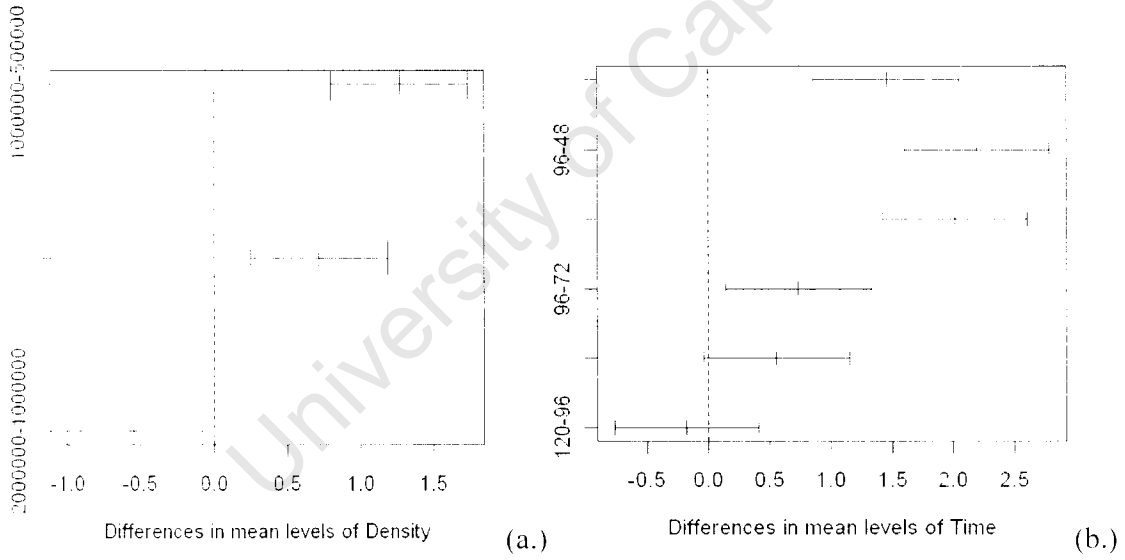


Figure 3.15 The results of pair-wise T-tests used to compare the group means of the given parameters in order to determine how significantly different these parameters are from one another. (a.) Comparison of cell densities for GagRT. (b.) Comparison of infection times for GagRT. All tests were done within a 95 % confidence interval, where factors were determined significantly different to one another if the error bars (representing standard deviation between two means compared) did not cross 0.

It is important to note that once the ANOVA analysis was completed, tests were done to determine if the ANOVA data fitted a normal distribution and had a constant variance (as

is required for an ANOVA analysis to be sound). It was found that the data had a non-constant variance because the residual values did not produce a random scatter plot when plotted against the fitted values, as is desired (Fig. 3.17a for GagRT data and 3.18a for GagTN data). In addition, the data did not conform to the expected linear normal Q-Q plot, instead forming a sigmoidal curve (Figs 3.17b and 3.18b). To remedy the problem, the raw data were transformed by applying a $\log_e$ function to all data (Figs 3.17c & d for GagRT data and Figs 3.18c & d for GagTN data). As can be seen, the transformed data demonstrates random scatter plots along y = 0, and linear normal Q-Q plots, indicating agreement with the ANOVA assumptions. This kind of transformation procedure is standard and deemed acceptable for statistical analysis.

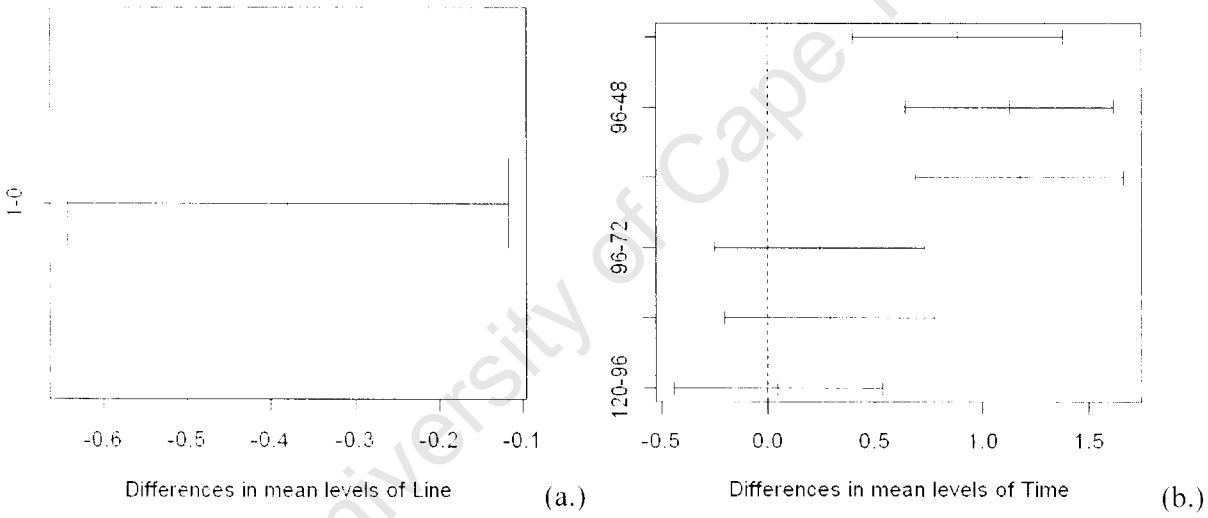


Figure 3.16 – The results of pair-wise T-tests used to compare the group means of the given parameters in order to determine how significantly different these parameters are from one another. (a.) Comparison of cell lines for GagTN, where 0 = *Tni* Pro™ cells, 1 = *Sf*9 cells. (b.) Comparison of infection times for GagTN. All tests were done within a 95% confidence interval, where factors were determined significantly different to one another if the error bars (representing standard deviation between two means compared) did not cross 0.

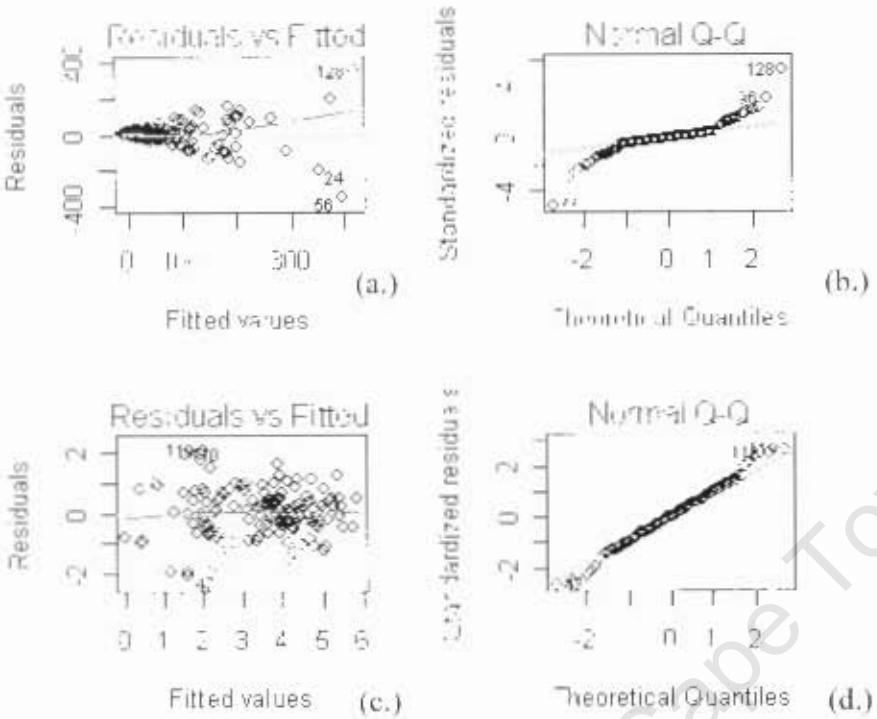


Figure 3.17 – Statistical tests done to confirm that GagRT data concurs with the basic assumptions of ANOVA. These tests make use of the residual values based on the p24 Gag ELISA data. The untransformed data is depicted in (a) and (b), while the transformed data is depicted in (c) and (d).

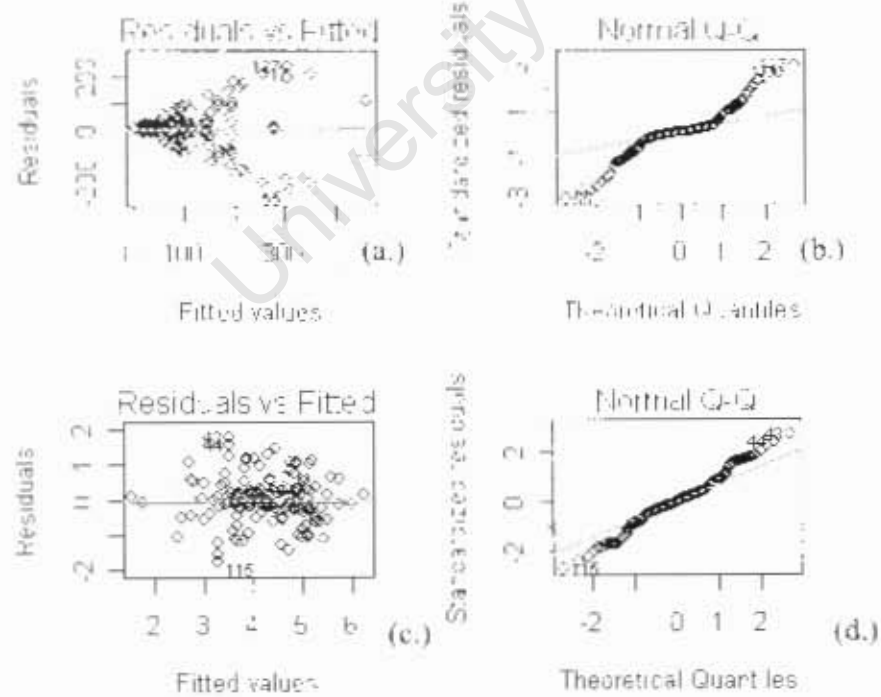


Figure 3.18 – Statistical tests done to confirm that GagTN data concurs with the basic assumptions of ANOVA. These tests make use of the residual values based on the p24 Gag ELISA data. The untransformed data is depicted in (a) and (b), while the transformed data is depicted in (c) and (d).

3.4 Discussion

The production of the chimaeric VLPs for immunogenicity studies required the optimization of VLP production to obtain sufficient VLP quantities and ensure that the predominant recombinant product would be the appropriate chimaeric VLPs. The maximum yields obtained (as determined by measuring Gag p24 levels) from optimization experiments were disappointing, with less than 0.5 µg/ml being obtained. Previous studies have shown that chimaeric VLP yields from insect cells can range from 5-20 µg/ml (Modrow et al., 1994). However, these yields were dependent on the position of the fused protein on Gag as well as the length of the fused protein (Luo et al., 1992). Both RT and the fusion protein, TN, are relatively large inserts (49 kD and 36 kD respectively), making both the chimaeric proteins fairly large (108 kD and 92 kD respectively). Thus, even though the proteins were being expressed by the cells, they may have been problematic to process given their size. Luo et al (Luo et al., 1992) found that the size of the fusion protein expressed was inversely proportional to VLP yields produced. This finding is supported in this study by the greater quantities of GagTN produced in comparison to the larger construct, GagRT.

Alternatively, because recombinant protein expression occurred close to cell lysis/death (late in the viral life cycle), protein processing was likely to be suboptimal because of 'compromised post-translational machinery and secretory pathways' (Hu, 2005; Ikonomou, Schneider, and Agathos, 2003). In this way, the time point of 120 hpi is probably not the best time to harvest intact VLPs, even though it was found to be optimal for both constructs when they were produced in *T.ni* Pro^{TM} cells. This was suggested by the cell viability data which showed that at 120 hpi, cells were unhealthy if not already dead. Furthermore, western blot data indicated that Gag cleavage products were more prominent at 120 hpi than at other time points, probably caused by the release of cellular proteases after cell death. Taking both these points into account, in addition to the fact that there is little to no significant difference in expression levels between 96 and 120 hpi times for both constructs, it would be preferable to harvest VLPs at 96 hpi to obtain higher yields of intact VLPs, rather than the suggested 120 hpi.

Generally, the optimal conditions that were determined by ANOVA showed several similarities for the two chimaeric constructs (Table 3.2), although, the chosen factors did affect recombinant protein expression in some surprising ways. In particular, cell density seemed to influence protein expression quite significantly, especially given that one cell density ($1x10^6$ cells/ml) was favoured in all experiments. In some studies, higher cell densities have led to better recombinant protein expression, but this was dependent of the cell line used (Jorio, Tran, and Kamen, 2006; Maruniak, Garcia-Canedo, and Rodrigues, 1994). Wickham et al hypothesised that high cell densities resulted in depletion of vital nutrients and/or accumulation of toxic by-products (Wickham et al., 1992). This appeared to be the case for both cell lines tested here, where the highest cell density generated poorer VLP yields. The cell density of $1x10^6$ cells/ml was the best option for chimaeric VLP production, providing enough space, aeration, and medium availability for the cells during the stressful time of infection.

Unexpectedly, MOI results showed that MOI had little effect on the production of either construct. In most cases, MOI is used to provide the means to control an infection, as the higher the MOI (within reason), the more viral particles there are available to infect one cell, and therefore the better the infection and recombinant protein production efficiency. However, in these experiments, once a threshold MOI was reached (which appeared to be 1.0), the MOI no longer had an effect on the amount of recombinant protein being produced, so that protein expression at MOI 1 and 5 did not differ extensively for either construct. It is possible that this was due to the range of MOI values investigated being too narrow, and perhaps using a MOI value of 10 might have produced better VLP yields. This will need to be investigated in future experiments.

The optimization data indicated that the two cell lines used in this study produced similar expression levels, according to the optimization data. This means that both cell lines are capable of producing chimaeric VLPs in comparable quantities, and as a result the cell line to be utilized for VLP production can be chosen based on factors such as availability and economic value, rather than protein production ability. On the whole, even though *Tni* Pro™ cells had a more rapid doubling time than *Sf*9 cells, they appeared to be more

sensitive to slight environmental changes. *Sf*9 cells were better at adapting to new media and had greater longevity, and consequently are more suitable for large-scale work.

Thus, the optimal conditions for large scale GagRT and GagTN VLP production were determined using the data from both the western blots and the ANOVA. It was found that using the *Sf*9 cell line at a cell density of 1 x 10^6 cells/ml, and infecting cells at an MOI of 1.0 for 96 hours, provided the highest yields of intact chimaeric VLPs.

While the optimization experiments seemed to work successfully, one drawback of the experiments was the use of a Gag p24 ELISA method to quantify VLP production. The ELISA measured Gag p24 concentrations as opposed to direct VLP measurements, and the results were thus influenced by the presence of the Gag cleavage products in the samples. From the western blot data, it seemed as though the dominantly produced protein was generally the chimaeric VLPs, thus they were assumed to be the main contributors to the detected Gag p24 in the ELISAs done. Nonetheless, it is of interest to explore other methods of quantification that could be more reliable.

The optimization experiments provided insight into the dynamics of the recombinant baculovirus infections, proving very useful in the determination of influencing factors and their effect on the expression of GagRT and GagTN. It was now important to begin large scale chimaeric VLP production and purification for the immunogenicity studies.

CHAPTER 4

The purification and analysis of chimaeric HIV-1 VLPs

4.1 Introduction

4.1.1 Purification of chimaeric VLPs

Chimaeric HIV VLPs, GagRT and GagTN, are targeted to the cell membrane of the host cell by the Gag N-terminal myristylation signal and bud from the cells (Halsey et al., 2008). In this way, their secretion into the culture medium allows purification to be made simpler, as the VLPs can be isolated without contamination by cellular proteins.

One of the most common methods employed to isolate HIV-1 Gag VLPs from clarified culture supernatant is sucrose density gradient ultracentrifugation (SDGU) (Doan et al., 2005; Gheysen et al., 1989; Jaffray et al., 2004). This method is a form of rate zonal centrifugation and employs a sucrose step gradient, comprising of a range of sucrose densities, to separate proteins on the basis of their buoyant density (Wiser, 2007). While there is some variance in the application of this purification method in the literature, it has been found to result in the successful purification of VLPs from both baculovirus and vaccinia virus expression systems (Doan et al., 2005). However, it does have its drawbacks in terms of the level of VLP purity and sterility of VLP preparations (Bess et al., 1997; Dettenhofer and Yu, 1999).

In view of this, Optiprep® density and velocity gradient ultracentrifugation have recently emerged as a new and improved method for HIV VLP purifications (Dettenhofer and Yu, 1999; Ford, Graham, and Rickwood, 1994). Optiprep® is a solution containing 60% of iodixanol. It is a non-toxic, non-ionic density gradient medium that was initially designed as an X-ray contrast solution (Segura, Garnier, and Kamen, 2006). Unlike sucrose, which increases in osmolality as density increases, iodoxanol is able to form iso-ismotic solutions at all densities. This provides better separation of proteins and maintenance of protein integrity (Dettenhofer and Yu, 1999).

In this study, SDGU and Optiprep® density gradient ultracentrifugation (ODGU) was compared in terms of their ability purify large amounts of intact, chimaeric VLPs. In addition, a microfiltration/ultrafiltration (MU) purification method was also evaluated.

The MU method has previously been used to successfully purify viral particles, but it has yet to be used for HIV VLP purifications (Segura, Garnier, and Kamen, 2006). It involves the use of membrane filtration columns to clarify and concentrate the culture supernatant, so that VLPs can be isolated from the resulting filtrate using ultracentrifugation. Although this method is less refined than the other methods, purified Gag-only particle yields were greater when isolated using the MU method compared to the other methods described (pers.comm: Ms A. Lynch, UCT.), and it was of interest to determine if this was true for the chimaeric VLPs.

4.1.2 Evaluation of VLP integrity

As mentioned previously, chimaeric HIV Gag constructs carrying additional epitopes such as gp160 or Nef, are able to form VLPs (Deml et al., 2005). These VLPs are generally stable and only slightly larger than the average Gag-only VLP; however, there is a size limitation that influences the formation of chimaeric HIV VLPs. Until recently, the size limit of the inserted epitope was believed to be approximately 200 amino acids (aa) (Deml et al., 2005), but recent work done in our laboratories has shown that this limitation can be extended to as much as 778 amino acids (Halsey et al.).

The chimaeric VLPs in this study, GagTN and GagRT, contain extra polypeptide fusions of 322 and 450 aa respectively, and have been previously shown to form intact VLPs (Halsey et al., 2008). However, given the requirement that these proteins be in particulate form to stimulate a potent immune response (Ellenberger et al., 2005), it was important to verify that both constructs expressed in insect cells form VLPs, and that these VLPs remained intact after purification. This is mainly because the immune response elicited by Gag in earlier mouse experiments was primarily attributed to the particulate nature of $Pr55^{gag}$. Thus, for GagRT and GagTN VLPs to elicit a similar or enhanced immune response, they must be in particulate form. Additionally, it was necessary to confirm that the VLPs budded out of the cells as expected, as they were extracted from the culture supernatant using the above mentioned methods.

4.1.3 Chapter objectives

The aims of the work reported in this chapter were as follows:

(i) To confirm intact VLPs are budding at the cell membrane of the insect cells expressing GagRT and GagTN.

(ii) To evaluate which is the best purification method for obtaining stable, intact, chimaeric VLPs in large quantities.

4.2 Materials and Methods

4.2.1 VLP Purifications

4.2.1.1 Sucrose density gradient ultracentrifugation (SDGU)

The appropriate optimal conditions (Table 3.2) were utilized to express the respective chimaeric VLPs in *Sf*9 cells (250 ml). At 96 hpi, infected cell culture was transferred to sterile 25-ml bottles (Sterilin) and centrifuged at 1000 x *g* for 20 min at room temperature to remove cellular matter. The supernatant was removed, layered onto a sucrose cushion comprised of 30 % sucrose (7 ml) and 70 % sucrose (2 ml) (both made up using 1x PBS). It was then ultracentrifuged at 120 000 x *g* in a Beckman SW 28 rotor for 90 min at 4 °C, and the resulting protein band at the 20 % / 70 % sucrose interface was aspirated using a syringe. The resulting aspirate was pipetted on top of a step gradient consisting of 20, 30, 40 and 50 % sucrose, and ultracentrifuged at 120 000 x *g* in a Beckman SW 28 rotor for 90 min at 4 °C. Once this centrifugation was complete, protein bands that were visible in the gradient were carefully removed using a syringe. The extracted bands were diluted up to 40 ml in 1x PBS (pH 7.4) and underwent a final centrifugation, performed at 110 000 x *g* for 60 min in a Beckman SW 55 Ti rotor at 4°C. The resulting pellet was resuspended in 500 µl PBS and stored at 4 °C. The negative control comprised of *Sf*9 cell culture infected with wild-type baculovirus and treated as described above.

4.2.1.1.1 Sucrose gradient fractionation

To confirm that the chimaeric VLPs were present in the visible aspirated bands on the sucrose gradient and to further characterize them, the gradient containing the protein bands of interest was fractionated into 1 ml aliquots. The sucrose density of each aliquot

was determined using a pocket PAL-3 refractometer (Atago). Aliquots were tested for the presence of chimaeric VLPs using western blot and Gag p24 ELISA analysis (Vironostika).

4.2.1.2 Optiprep® density gradient ultracentrifugation (OGDU)

As with the SDGU, infected cell cultures (250 ml per construct) were centrifuged after 96 hpi at 1000 x *g* for 20 min at room temperature. The cell pellet was discarded and the supernatant was ultracentrifuged at 120 000 x *g* in a Beckman SW 28 rotor for 90 min at 4 °C. The new pellet was then resuspended in 500 µl PBS, pipetted on top of a step gradient consisting of 10, 20, 30, 40 and 50 % Optiprep® (made using 1x PBS), and ultracentrifuged at 155 000 x *g* for 180 min at 4 °C. Once this centrifugation was complete, protein bands were visible at their buoyant densities on the gradient (differed depending on the construct), and were carefully removed using a sterile syringe. The extracted bands were treated as described for SDGU (4.2.1.1.). The negative control comprised of *Sf*9 cell culture infected with wild-type baculovirus and treated as described above.

4.2.1.3 Micro-/Ultrafiltration (MU)

As with the SDGU, infected cell cultures (250 ml per construct) was centrifuged after 96 hpi at 1000 x *g* for 20 min at room temperature. The supernatant was removed and microfiltered using a 0.45 µm CFP-4-E-4MA polysulfone membrane capsule filtration device (Amersham) under constant pressure (0.75 Bar) and tip speed (375 rpm). This step was necessary to remove excess baculovirus and cellular debris from the supernatant. The resulting clarified filtrate was subjected to ultrafiltration using a UFP-300-C-4MA polyethersulfone membrane filter (MWCO=300 kDa; Amersham) under a constant pressure (0.75 Bar) and tip speed (365 rpm). This filtration step concentrated the filtrate to 1/10 of the initial supernatant volume. It was then diluted in 1xPBS and centrifuged 120 000 x *g* in a Beckman SW 28 rotor for 60 min at 4 °C to pellet VLPs. The pellet was resuspended in 300 µl PBS and stored at 4 °C. Both filters were sterilized with two 0.1 M NaOH washes (one of which occurred overnight) and 3 ddH_2O washes (50 °C) prior to

their use. The negative control comprised of *Sf*9 cell culture infected with wild-type baculovirus and treated as described above.

4.2.2 Protein analysis

4.2.2.1 Coomassie-stained SDS-PAGE

All extracted VLP samples were run on SDS-PAGE gels as described in 2.2.3.1. The gels were incubated overnight in 10 ml Coomassie brilliant blue stain (Sambrook, Fritsch, and Maniatis, 1989) under shaking conditions (50 rpm) at room temperature. The gels were then destained the next day by incubation in Coomassie destain for 5 hrs, or until protein bands were detected visually.

4.2.2.2 Western blots

Western blots were performed on all samples as detailed in 2.2.3.1.

4.2.2.2 Gag p24 ELISA

All ELISA analysis was performed as described in 3.2.3.2.

4.2.3 Transmission Electron Microscopy (TEM) analysis

4.2.3.1 Negative staining

Extracted VLP samples of interest were adsorbed to glow discharged, carbon-coated copper grids for 15-20 min, followed by two water washes and finally staining with 2 % uranyl acetate for 90 sec. Grids were then viewed using a Zeiss S1109 electron microscope.

4.2.3.2 Cross-section TEM

To visualize VLPs budding from insect cells, cross sections of *Sf*9 cells (infected with the respective rAcMNPV stocks under optimal conditions – Table 3.2.) were prepared. At 72 hpi, cells were centrifuged at 1000 x *g* for 5 min, resuspended in 2.5 % (w/v) glutaraldehyde and stored overnight in the dark at 4ºC (to fix cells). Fixed cells were gently resuspended in 1 ml 1xPBS (pH 7.4) and pelleted at 1000 x *g* for 5 min. They were then washed twice in PBS and resuspended in PBS at 37 °C. Samples were post-fixed

with 1 % (w/v) osmium tetroxide (made in 2xPBS - pH 7.4) for 1 hr, followed by two 1xPBS and two ddH_2O washes. Subsequently, samples were dehydrated using several 5-min ethanol washes of increasing concentration (30, 50, 70, 80, 90, 95, 100 %). The samples were further dehydrated by resuspension in acetone, then embedded in Spurr's resin (Spurr, 1969), and baked at 60 °C (for setting purposes). Sections of approximately 100 nm thickness were prepared with a glass knife using a Leica Reichert Ultracuts microtome and mounted on copper grids. These sections were stained with 2 % uranyl acetate for 10 min at ambient temperature, rinsed 5 times with ddH_2O, post-stained with Reynolds' lead citrate for 10 min, re-rinsed with ddH_2O, and air dried. Grids were viewed using a Zeiss S1109 electron microscope. A negative control was prepared by infecting cells with wild-type baculovirus, instead of the rAcMNPV stocks.

4.3 Results

4.3.1 VLP budding

Cross sections of *Sf*9 cells infected with the respective rAcMNPV were examined using TEM analysis. Chimaeric VLP formation and budding from the cell membrane was confirmed (Fig. 4.1a-d), as VLP structures were morphologically similar to previously characterized Gag VLPs (Halsey et al., 2008; Jaffray et al., 2004). The VLPs were found to be at different stages of the budding process in the cells examined, with some VLPs being fully budded and others protruding slightly from the cell membrane. All VLP structures were round in shape, and demonstrated a large variation in the size, with GagTN VLP diameters ranging from 120-185 nm, and GagRT VLPs demonstrating a range of approximately 135-220 nm. As was expected, the majority of observed chimaeric VLPs were larger than what has been documented for wild type Gag VLPs (approximately 100-140 nm) (Jaffray et al., 2004).

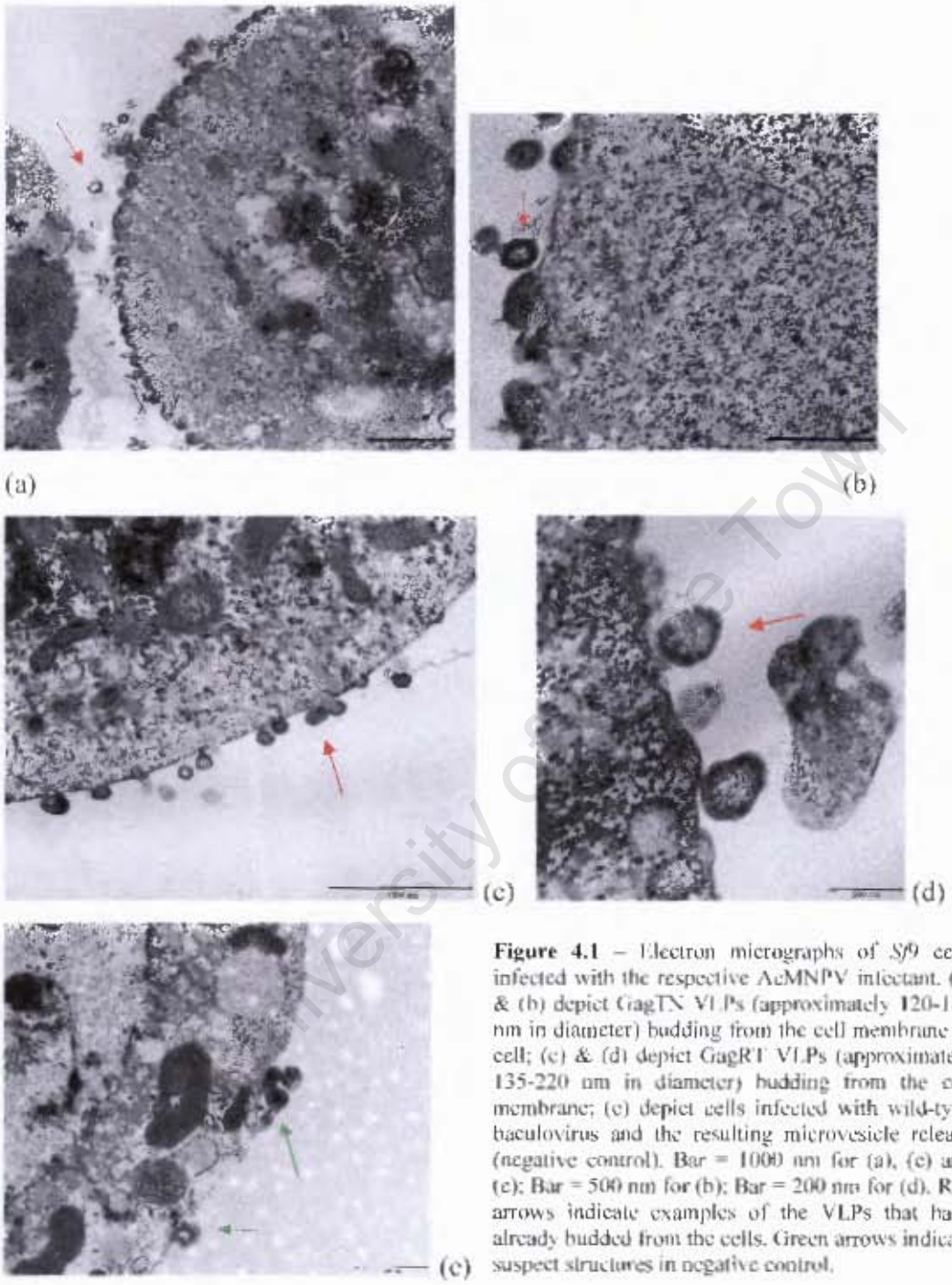


Figure 4.1 – Electron micrographs of *Sf9* cells infected with the respective AcMNPV infectant. (a) & (b) depict GagTN VLPs (approximately 120-185 nm in diameter) budding from the cell membrane of cell; (c) & (d) depict GagRT VLPs (approximately 135-220 nm in diameter) budding from the cell membrane; (e) depict cells infected with wild-type baculovirus and the resulting microvesicle release (negative control). Bar = 1000 nm for (a), (c) and (e); Bar = 500 nm for (b); Bar = 200 nm for (d). Red arrows indicate examples of the VLPs that have already budded from the cells. Green arrows indicate suspect structures in negative control.

The negative controls also contained what appeared to be budding structures associated the cell membrane (Fig. 4.1e); however these were not as regular-shaped and were bigger than the VLPs in the experimental samples (250-350 nm in diameter). Previous research

has indicated that these structures are very likely to be cellular microvesicles, given their variety in size, morphology and electron density (Bess et al., 1997).

4.3.2 SDGU purification

The first purification method investigated, SDGU purification, involved centrifuging infected cell supernatant at high speeds onto a 70 % sucrose cushion to obtain a protein band. This band was then placed onto a sucrose density gradient and ultracentrifuged for a given time, resulting in the formation of protein bands at their respective buoyant densities (differed depending on the proteins isolated) on the gradient. The bands were removed from the gradient and analysed for the presence of VLPs.

Both chimaeric VLP purifications resulted in the presence of more than one band on the density gradient, even after several repeated purifications (Fig. 4.2). The GagTN samples consistently produced three bands, while GagRT samples produced two bands. This was unexpected, as previous Gag-only VLP purifications had resulted in one protein band only at a density of about 1.15-1.17 g/ml of sucrose (Hammonds et al., 2007). Given this occurrence, it was necessary to isolate all bands and analyse them using western blots and TEM, so that we could establish which band contained intact, chimaeric VLPs. In addition, SDS-PAGE analysis was also performed to examine the purity of the samples obtained.

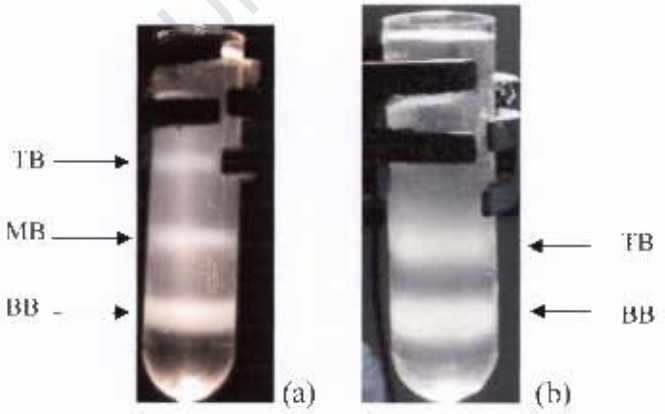


Figure 4.2 Sucrose density gradients (20-50 %) containing infected cell supernatant pellets after ultracentrifugation. (a) *Sf*9 cells were infected prior to purification with GagTN rAcMNPV (a) or GagRT rAcMNPV (b) as per the determined optimized conditions. Arrows indicate the protein bands that were isolated. Abbreviations: TB – top band, MB – middle band, BB = bottom band.

The Coomassie-stained SDS-PAGE gels illustrated that none of the isolated bands were pure, as several other protein products were still present in the samples (Fig. 4.3). Furthermore, the chimaeric VLPs did not appear to be the most prominent protein in any of the bands isolated, with only a faint detection of both chimaeric VLPs.

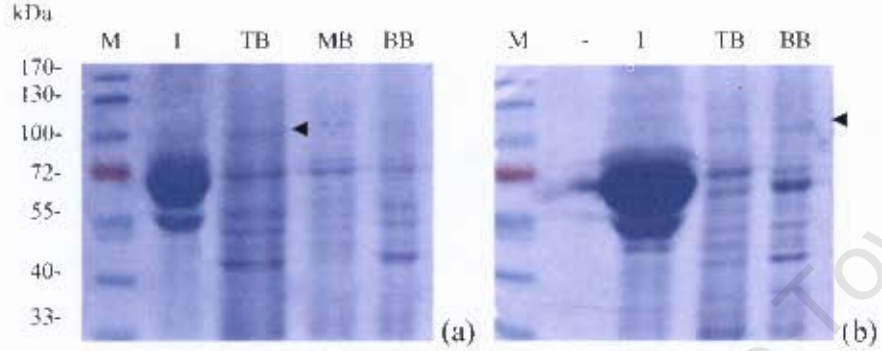


Figure 4.3 - Coomassie-stained SDS-PAGE gel photographs of the respective VLP samples after SDGU. (a) GagTN VLP band samples extracted from the sucrose density gradient; (b) GagRT VLP band samples extracted from the sucrose density gradient. Abbreviations: M = marker; I = respective baculovirus infectant; TB = top band isolated; MB = middle band isolated; BB = bottom band isolated. Arrow heads indicate the migration position of the respective chimaeric VLPs.

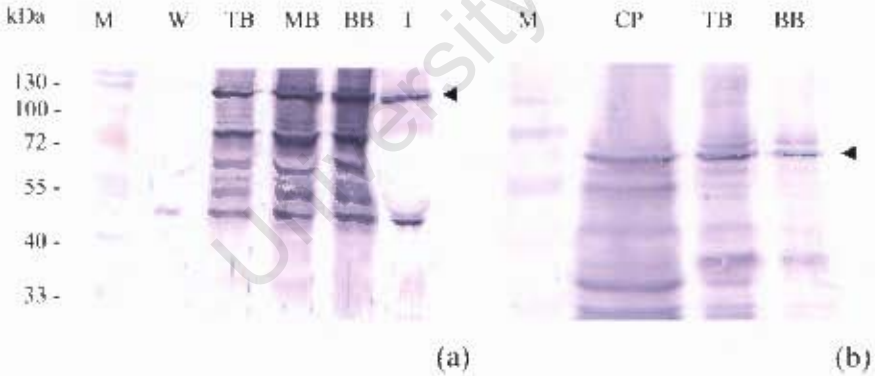


Figure 4.4 – Western blots of the respective VLP samples after SDGU, probed with Gag p24 antibody. (a) GagTN VLP band samples extracted from the sucrose density gradient. (b) GagRT VLP band samples extracted from the sucrose density gradient. M = marker; I = respective baculovirus infectant; W = discarded supernatant after final centrifugation; CP = sample of cell pellet; TB = top band isolated; MB = middle band isolated; BB = bottom band isolated. Arrow heads indicate the migration position of the respective chimaeric VLPs.

The impurity of the samples was confirmed by western blots, although the data showed all isolated bands contained chimaeric VLPs to some degree, despite their obvious differences in density. In particular, the GagTN sample bands could not be distinguished

from one another using the western blots (Fig. 4.4a), with all three appearing to contain GagTN and several other Gag products. The TEM data suggested that even though the top band of the GagTN sample seemed to contain larger quantities of VLPs, there were numerous unknown structures also present (Fig. 4.5a). The middle and bottom bands (Fig. 4.5b & c) were slightly cleaner samples, however intact VLPs were few and far between.

Similarly, the western blot of GagRT samples showed that GagRT was present in both bands (Fig. 4.4b). It was difficult to differentiate between the two GagRT bands using TEM (Fig .4.6), as both appeared to contain VLPs along with other structures. The origin of these structures was unknown, but could be cellular matter or contaminants from the sucrose used to form the gradient. Given that each band isolated from the gradient was comprised of several proteins, it was necessary to attempt to further characterize each band, thereby establishing which band/s would be the best to utilize for mice studies. Thus, sucrose gradient fractionations were performed for the respective VLP purifications, where 40 fractions of 1 ml were collected.

The bands from the GagTN purification were removed in the fractions 6-10 (bottom band), 15-17 (middle band) and 19-22 (top band), while the GagRT purification bands were removed in fractions 15-18 (bottom band) and 31-36 (top band). The sucrose density range of each band was determined (Table 4.1), and p24 ELISA was performed. The bottom bands of both constructs were closest to the density expected for Gag-only VLPs (1.15-1.17 g/ml) (Hammonds et al., 2007). The other bands occurred at lower densities, suggesting that their contents comprised more of the small, less dense proteins (such as Gag p24 and p41) than the bottom band. Alternatively, the chimaeric VLPs could be folding in such a way as to decrease their density, and therefore, despite their larger molecular weight, settle at a lower density than what was previously seen with Gag-only VLPs.

The ELISA data (Fig. 4.7) verified that the visible protein band fractions isolated from the gradient contained the majority of Gag p24 detected. In particular, the middle band of

the GagTN samples corresponded to the largest quantity of Gag p24 (Fig. 4.7a), while the top GagRT band contained the greatest p24 quantities (Fig. 4.7b). Interestingly, there was a third region (fractions 25-29) in the GagRT data that contained high Gag p24 concentrations but did not form a visible band. It is possible that the composition of these fractions included a large percentage of smaller Gag proteins (such as Gag p24), which were not particulate and thus unable to reflect light sufficiently to form a visible band.

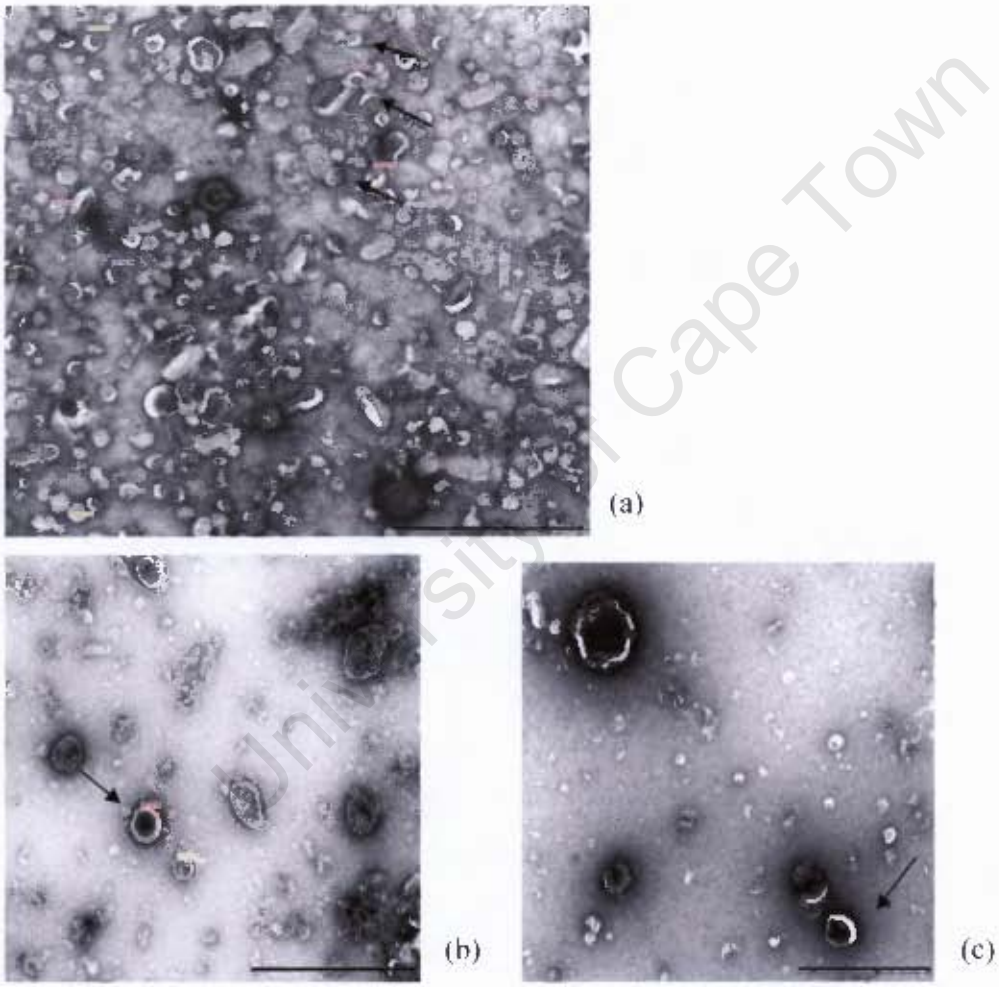
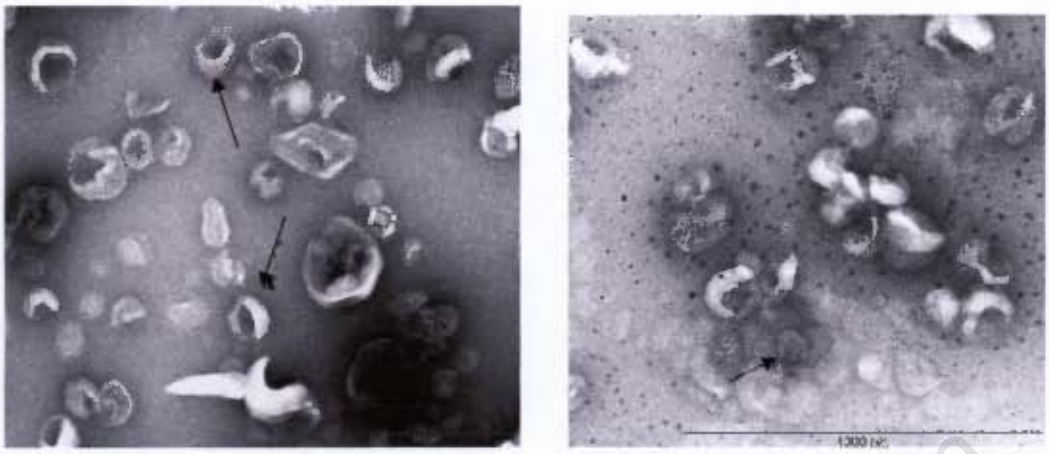


Figure 4.5 – Electron micrographs of extracted protein bands from the GagTN SDGU. (a) Top band; (b) Middle band; (c) Bottom band isolated. VLPs observed in all three extracts were between 125 – 170 nm in diameter. Bar = 1000 nm. Arrows depict examples of VLPs.



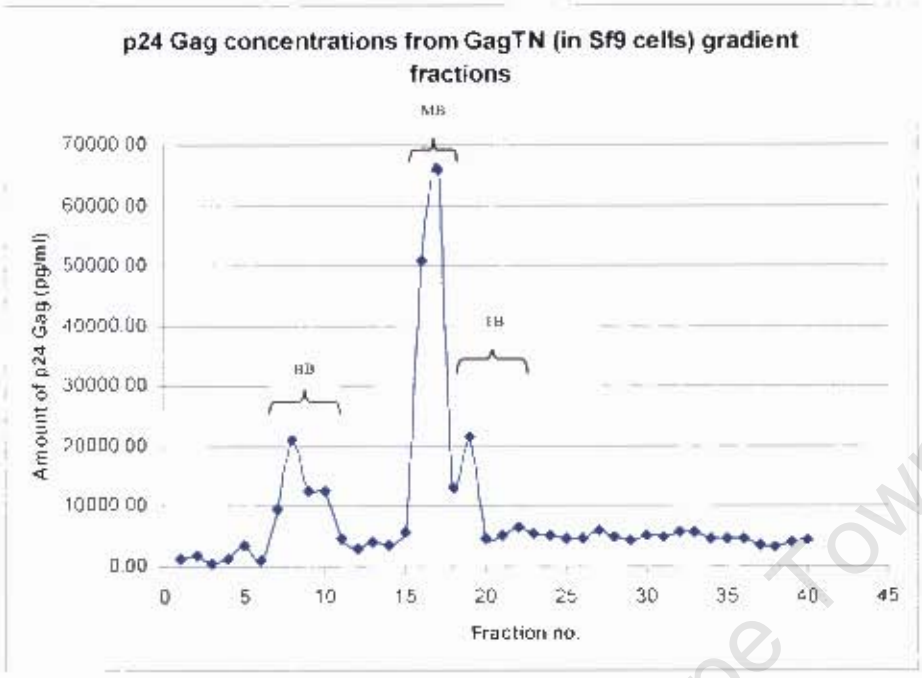
(a) (b)

Figure 4.6 - Electron micrographs of extracted protein bands from the GagRT SDGU. (a) Top band; (b) Bottom band. VLPs observed in all extracts were between 150 – 210 nm in diameter. Bar 500 nm for (a); bar = 1000 nm for (b). Arrows depict examples of VLPs.

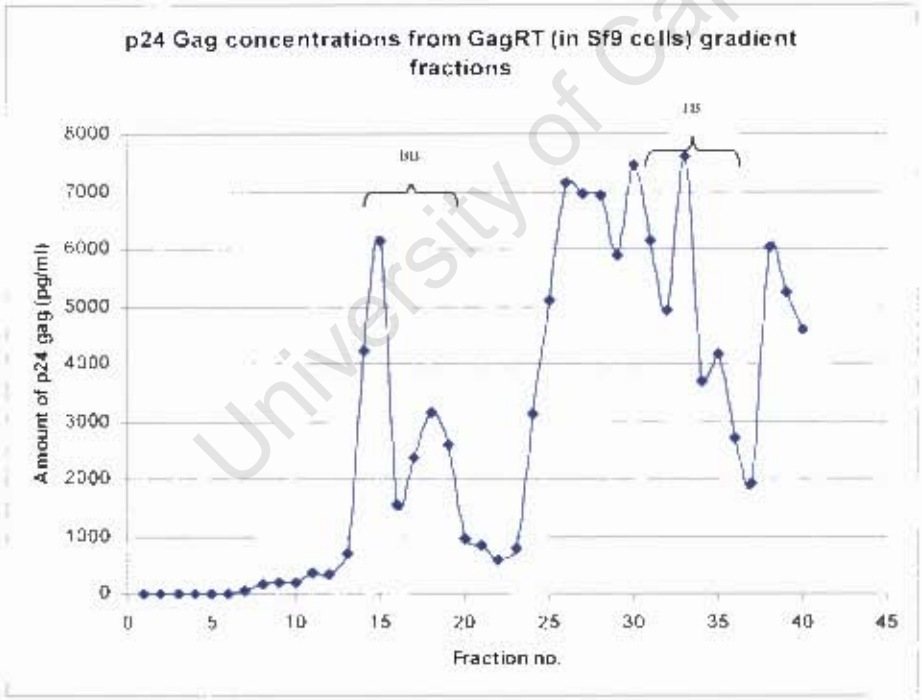
Table 4.1 - The density ranges of the protein bands isolated from the sucrose density gradient fractionation

Construct	Band isolated from gradient	Density range of fractions (g/ml of sucrose)	Density range of fractions (% w/v of sucrose)
GagTN	Bottom	1.146 – 1.176	34.2 - 40.2
	Middle	1.137 1.142	31.9 32.5
	Top	1.11 - 1.12	26.9 27.9
GagRT	Bottom	1.146 – 1.161	34.2 - 36.6
	Top	1.081	19.9 – 20.3

Because the ELISA data is a measurement of Gag p24 as opposed to $Pr55^{gag}$, it is difficult to conclude which band/s comprised of the majority of intact VLPs. In view of this, it would seem that SDGU requires all bands to be combined to obtain maximum VLP yields.



(a)



(b)

Figure 4.7 – Gag p24 ELISA data of the fractions obtained from a sucrose density gradient containing (a) GagTN-infected cell supernatant pellet; (b) GagRT-infected cell supernatant pellet. Brackets indicate the number of fractions that included in the respective protein bands. Abbreviations: TB – top band; MB – middle band; BB – bottom band.

It is important to note that the negative control (*Sf*9 cells infected with wildtype baculovirus) also displayed a protein band on the sucrose gradient (at approximately 35.5 % sucrose) after ultracentrifugation. This band was isolated and analysed. It was found to contain baculovirus particles amongst other unknown structures (TEM data not shown) but tested negative for the presence of Gag on western blots, as was expected.

4.3.3 ODGU purification

Similar to what was seen with the SDGU purification, the ODGU yielded more than one band on the density gradients of the respective VLP purifications (Fig. 4.8). The GagTN samples produced three bands and GagRT samples produced two bands. Given that both this purification method and SDGU employed density gradients to isolate bands, it was not surprising that all the isolated bands were found to carry the chimaeric VLPs, as was found for SDGU.

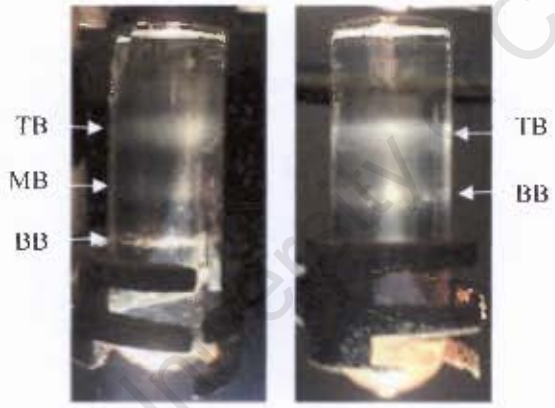


Figure 4.8 Optiprep® density gradients (20-50 %) containing infected cell supernatant pellets after ultracentrifugation at 26 000 rpm for 1.5 hrs. (a) Cells were infected prior to purification with GagTN rAcMNPV as per the determined optimized conditions. (b) Cells were infected prior to purification with GagRT rAcMNPV as per the determined optimized conditions. Arrows indicate the protein bands that were aspirated and analysed. Abbreviations: TB = top band, MB = middle band, BB = bottom band.

It was not possible to distinguish between the bands extracted using TEM (Figs 4.9 and 4.10 for GagTN and GagRT respectively), as all the bands contained intact VLPs. However, the electron micrographs did illustrate that the ODGU samples appeared relatively purer than what was seen with the SDGU purification, with fewer random structures. The increased purity of these samples was further demonstrated by both the

western blot (Fig. 4.11) and SDS-PAGE data (Fig. 4.12), with fewer Gag cleavage products detected. Also, the chimaeric VLPs, GagTN and GagRT, were the most prominent protein in their respective sets of samples, unlike the SDGU samples.

As was the case with SDGU, the negative control produced 1 band which tested negative for Gag, but did contain baculovirus particles (data not shown).

4.3.4 MU purification

The last of the purification methods investigated involved the use of membrane filters to isolate VLPs from other extracellular proteins. Once the clarified culture supernatant had been isolated, it was filtered through a 0.45 µm membrane to remove excess cellular debris. The resulting filtrate was then filtered through a 300 kDa membrane column to concentrate VLPs. Finally, the sample was centrifuged and the VLP pellet resuspended in PBS. The VLP samples were analysed as done with the other purification methods, using SDS-PAGE, western blot, and TEM data. Coomassie-stained SDS-PAGE (Fig. 4.13) showed the extracted VLP samples to be of similar purity to the ODGU VLP samples. Although there was a fair amount of Gag cleavage products in both constructs' samples, this can probably be explained by the concentration of all products being higher in the MU preparations, relative to the previous preparations analysed. Importantly, the cleavage products were undetectable when the VLP samples were diluted 1 in 10, while the chimaeric VLPs were still present at this dilution, confirming that these VLPs were the most prominent proteins in the preparations.

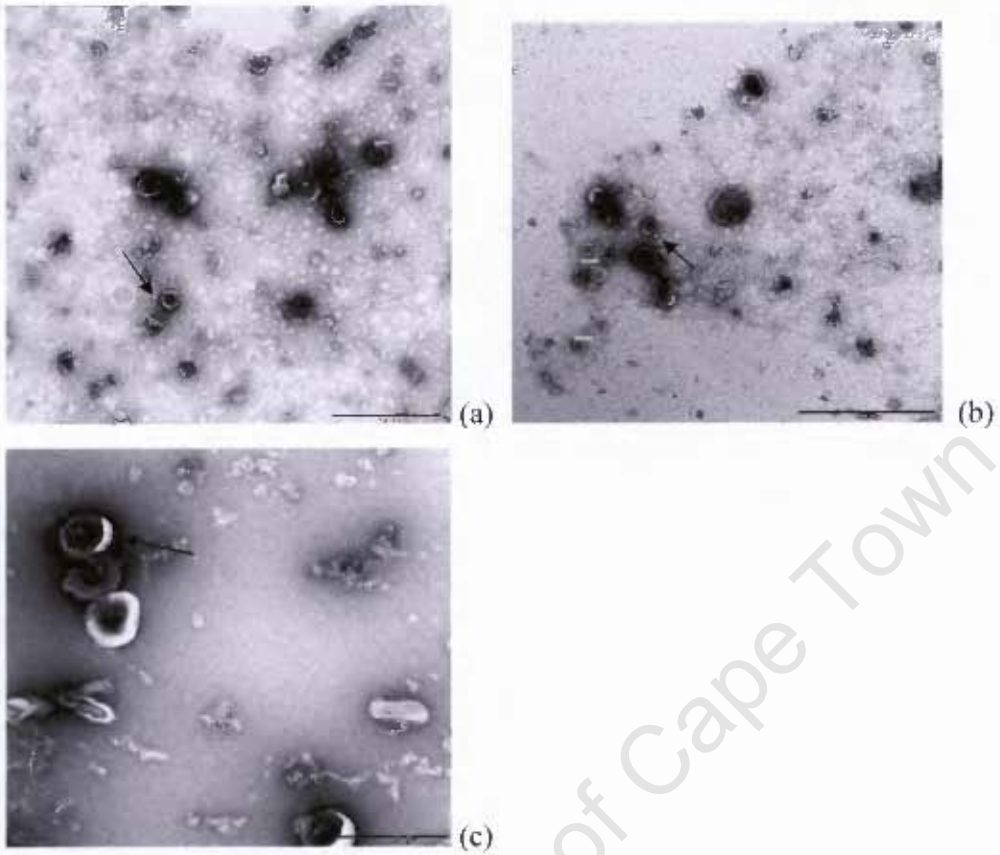


Figure 4.9 - Electron micrographs of extracted protein bands from the GagTN ODGU. (a) Top band, Bar = 1000 nm; (b) Middle band, Bar = 1000 nm; Bottom band, Bar = 500 nm. VLPs observed in all extracts were between 130 - 180 nm in diameter. Arrows indicate examples of chimaeric VLPs.

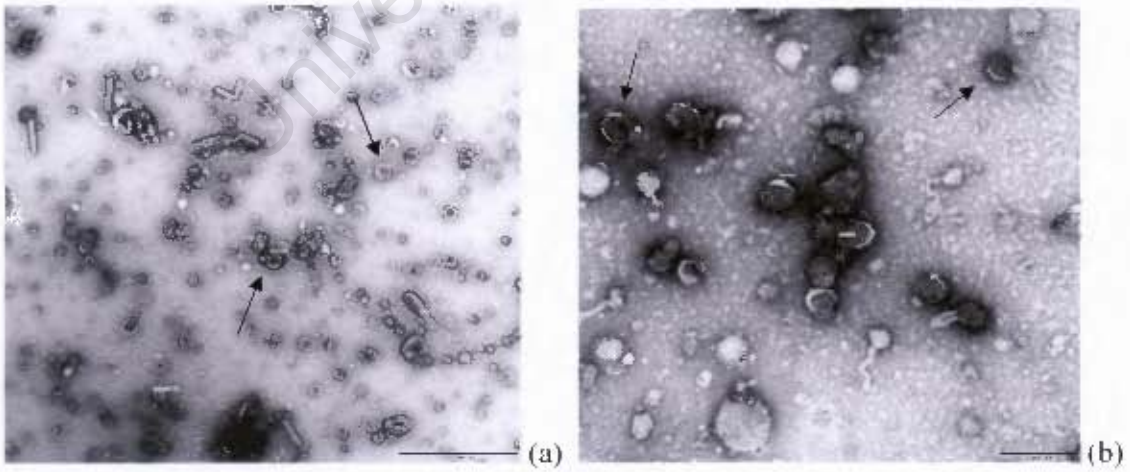


Figure 4.10 - Electron micrographs of extracted protein bands from the GagRT ODGU. (a) Top band; Bar = 1000 nm; (b) Bottom band; Bar = 500 nm. VLPs observed in all extracts were between 140 - 230 nm in diameter. Arrows indicate examples of chimaeric VLPs.

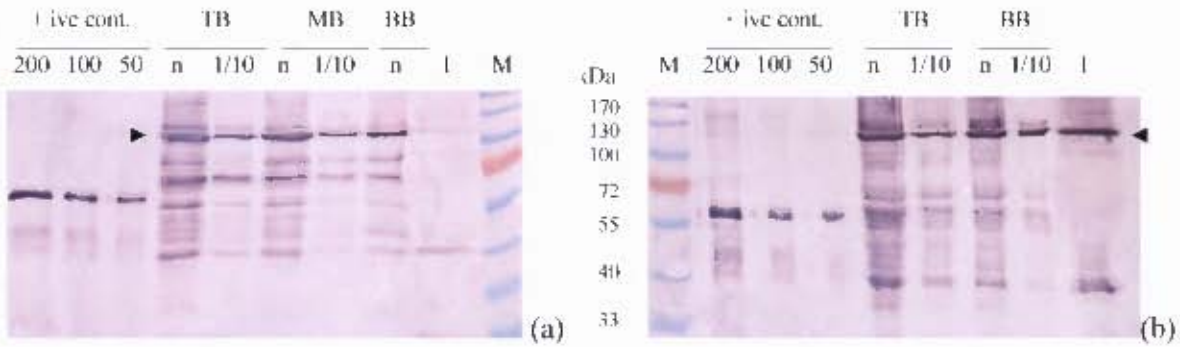


Figure 4.11 Western blots of the respective VLP samples after ODGU. (a) GagTN VLP band samples extracted from the Optiprep® density gradient. (b) GagRT VLP band samples extracted from the Optiprep® density gradient. Abbreviations: M = molecular weight marker; + ive cont. = HIV-1B $Pr55^{Gag}$ positive control of 200 ng or 100 ng or 50 ng; TB = Top band; MB = Middle band; BB = Bottom band; 1 = respective rAcMNPV infectant; n = neat sample; 1/10 = 1 in 10 dilution of sample. Arrow heads indicate the migration position of the respective chimaeric VLPs.

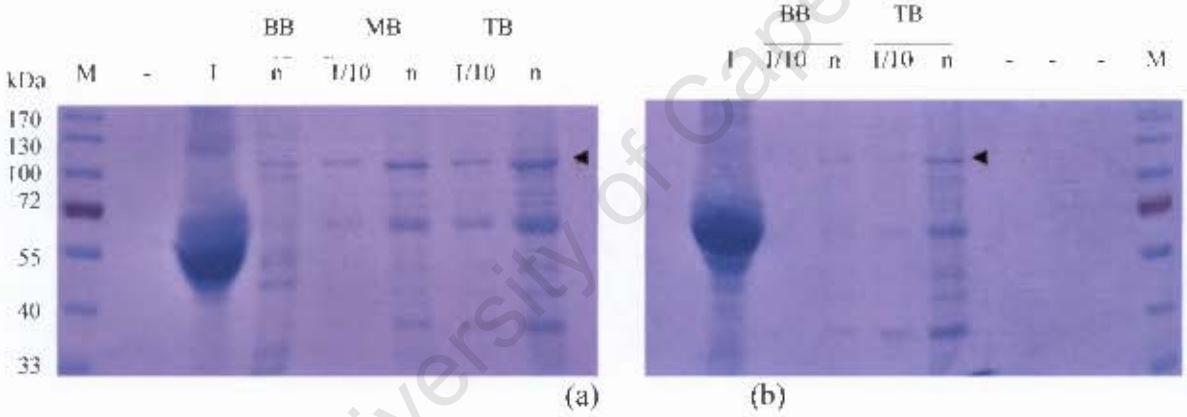


Figure 4.12 – Coomassie-stained SDS-PAGE gel photographs of the respective VLP samples after ODGU. (a) GagTN VLP band samples extracted from the Optiprep® density gradient. (b) GagRT VLP band samples extracted from the Optiprep® density gradient. Abbreviations: M = molecular weight marker; TB = Top band; MB = Middle band; BB = Bottom band; 1 = respective rAcMNPV infectant; n = neat sample; 1/10 = 1 in 10 dilution of sample. Arrow heads indicate the migration position of the respective chimaeric VLPs.

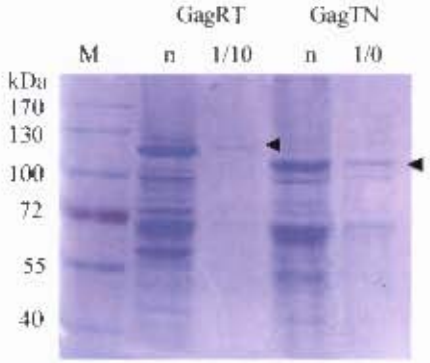


Figure 4.13 Coomassie-stained SDS-PAGE gel photographs of the respective chimaeric VLP samples after purification using MU purification. Abbreviations: M = molecular weight marker; GagRT = Extracted GagRT VLPs samples; GagTN = Extracted GagTN VLPs; n = neat sample; 1/10 = 1 in 10 dilution of sample. Arrow heads indicate the migration position of the respective chimaeric VLPs.

The western blot data (Fig. 4.14 and Fig 4.15) reiterated this point for both constructs, as well as implied that the presence of Gag cleavage products was less than what was found with the previous purification methods, because it was only faintly detected in samples.

In general, this purification method was suspected to be slightly harsher than the previous two methods because of the pressure exerted on the VLPs as they were filtered through the respective membranes. Therefore, Nef and RT-specific western blots were also performed to verify that the protein being detected on the Gag p24 blot also contained the respective fusion protein, and that these proteins were not being cleaved off the chimaeric protein (Fig. 4.14b and Fig. 4.15b). It was clear from these blots that the chimaeric nature of the respective VLPs was maintained after purification.

The TEM data (Fig. 4.16) showed that VLPs were being isolated in particulate form, and there appeared to be fewer random, unknown structures present, which were possibly removed during the ultrafiltration step. As was seen previously, the GagTN samples appeared to contain a larger quantity of chimaeric VLPs compared to GagRT VLP samples. This is probably related to the ability of the insect cells to express the respective proteins. Given that GagRT is slightly larger in molecular weight and size, it may be more difficult to produce. In addition, it might also fold in a higher energy conformation that renders it slightly less stable than the GagTN VLPs. TEM analysis was also done on the negative control to determine if similar structures were present (Fig. 4.17). Although there were some similar structures (spherical shapes were 88-110 nm in diameter), none were the same size as what would be expected for VLPs. Several of the smaller structures are therefore suspected to be microvesicles or cellular products.

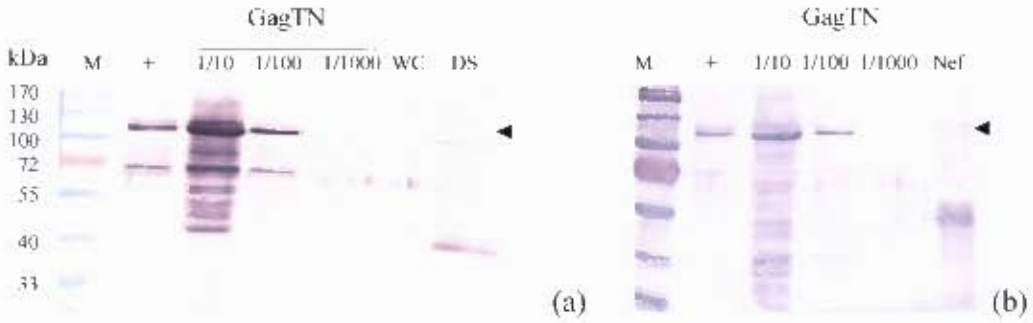


Figure 4.14 – Western blots of the respective GagTN VLP samples after MU purification. (a) VLPs samples were probed with Gag p24 primary antibody. (b) VLPs samples were probed with Nef primary antibody. Abbreviations: M = molecular weight marker; + = HIV-1C GagTN VLP positive control (100 ng); GagTN = Extracted GagTN VLPs; WC = Waste filtrate from column; DS = Discarded supernatant from final centrifugation; Nef = Nef positive control; 1/10 = 1 in 10 dilution of sample; 1/100 = 1 in 100 dilution of sample; 1/1000 = 1 in 1000 dilution of sample. Arrow heads indicate the migration position of the respective chimaeric VLPs.

All methods of purification demonstrated the ability to purify intact VLPs; however, as is seen in Table 4.2, there were obvious differences in the quantities of VLPs isolated, even though the conditions for VLP production were standardized. There was more Gag p24 present in the MU purifications for both constructs, with less isolated using ODGU, and almost four times less using SDGU. This suggested that the MU method was superior at producing better VLP yields than the other two methods, as was verified by the western blots and SDS-PAGE data.

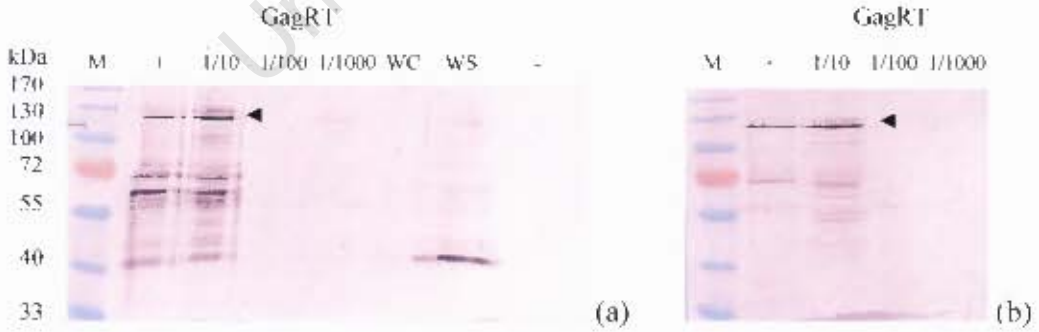
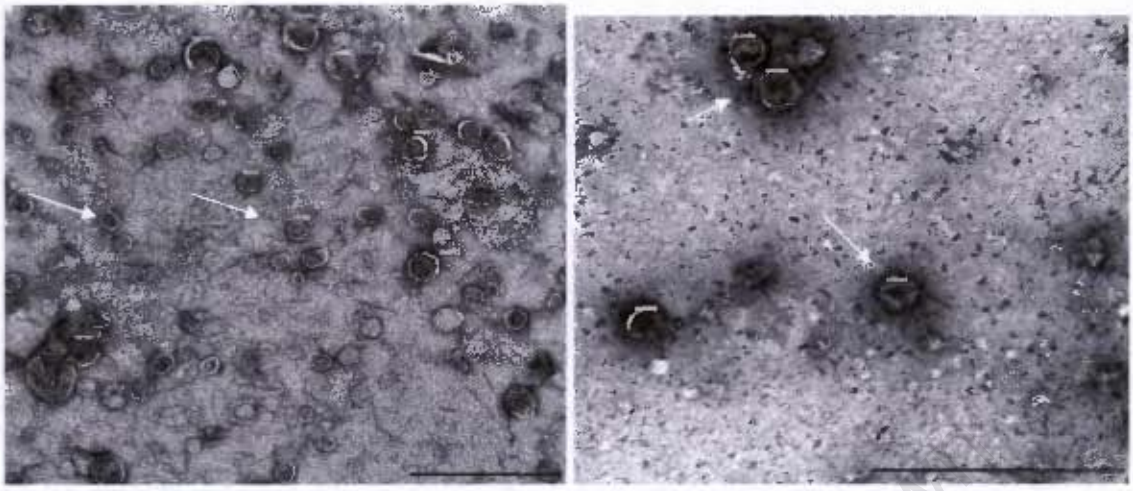


Figure 4.15 – Western blots of the respective GagRT VLP samples after MU purification. (a) VLPs samples were probed with Gag p24 primary antibody. (b) VLPs samples were probed with RT primary antibody. Abbreviations: M = molecular weight marker; + = HIV-1C GagRT VLP positive control (100 ng); GagRT = Extracted GagRT VLPs; WC = Waste filtrate from column; DS = Discarded supernatant from final centrifugation; 1/10 = 1 in 10 dilution of sample; 1/100 = 1 in 100 dilution of sample; 1/1000 = 1 in 1000 dilution of sample. Arrow heads indicate the migration position of the respective chimaeric VLPs.



(a) (b)

Figure 4.16 – Electron micrographs of the respective chimaeric VLPs extracted using ML purification. (a) GagTN VLPs; (b) GagRT VLPs. Bar = 1000 nm. GagTN VLPs observed in all extracts were between 120 – 180 nm in diameter, while GagRT VLPs ranged from 140-230 nm. Arrows indicate examples of chimaeric VLPs.

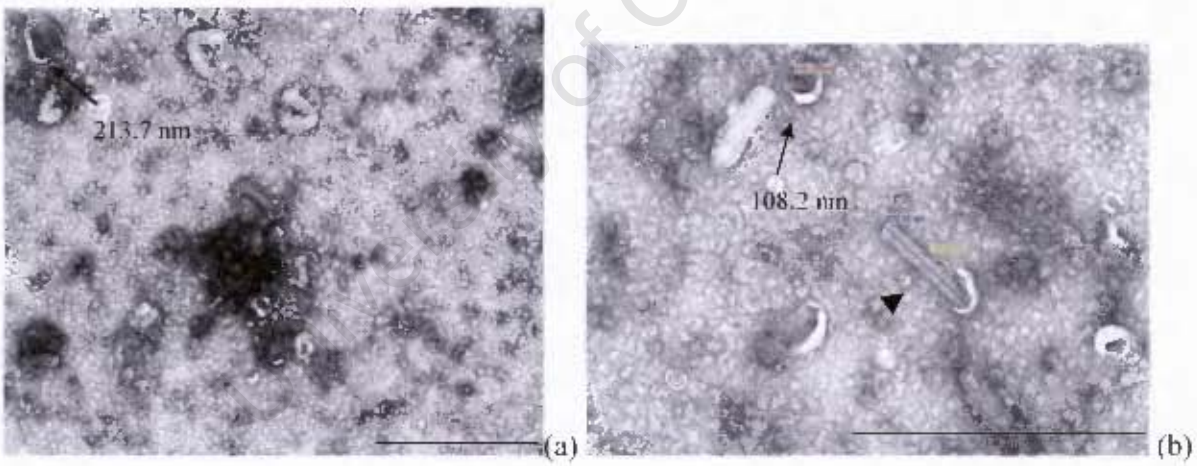


Figure 4.17 – Electron micrographs of the negative control sample extracted using ML purification. The negative control was made by infecting *Sf*9 cells with wildtype baculovirus, and harvesting proteins present in the culture supernatant using ML purification. Bar = 1000 nm. Arrows indicate structures resembling VLPs but that differ in size. Arrow head depicts a typical baculovirus particle (length: 370.75 nm; width: 54.1 nm).

Table 4.2 The Gag p24 ELISA data for the three purification methods

Construct	Method of Purification	VLP Samples	Gag p24 value (ng)
GagTN	SDGU	Band 1	99.65
		Band 2	169.06
		Band 3	21.27
		TOTAL	**135.98**
	ODGU	Band 1	109.18
		Band 2	216.63
		Band 3	46.29
		TOTAL	**372.10**
	Micro/ultrafiltration	Resuspended pellet	**428.78**
GagRT	SDGU	Band 1	49.76
		Band 2	27.32
		TOTAL	**77.08**
	ODGU	Band 1	91.55
		Band 2	38.11
		TOTAL	**129.66**
	Micro/ultrafiltration	Resuspended pellet	**254.80**

4.4 Discussion

The necessity to purify chimaeric VLPs before they are used in mouse immunogenicity studies is two-fold. Firstly, it is important that the immune responses elicited in mice are accurately attributed solely to the chimaeric VLPs. Secondly, if the VLPs are not extracted sterilely, the presence of endotoxins in the injected samples could cause the mice to suffer from toxic shock and die prematurely. Hence, the expression system used for VLP production and the method of purification is of great importance.

As mentioned previously, an insect cell expression system provides a novel approach to producing VLPs and has been used to successfully produce HIV VLPs, Human papillomavirus (HPV) L1 VLPs (Le Cann et al., 1995), and Hepatitis E VLPs (Li et al., 2005). One of the reasons for easy production and isolation of VLPs in insect cells is because VLPs can be targeted to bud out of the cells and into the culture supernatant. This means that a problematic and laborious purification procedure to isolate the protein

from inside cells is avoided, as VLPs can be isolated directly from the supernatant (Hink et al., 1991).

In this study, the chimaeric VLPs were myristylated, and therefore budded out of the insect cells after expression, as was demonstrated using TEM analysis. Although there was no definite verification that the budded structures were the Gag chimaeric VLPs, their absence in the negative control, and their similarity to previously characterized VLPs was convincing evidence (Halsey et al., 2008; Jaffray et al., 2004; Sakuragi et al., 2002). In addition, previous research on the GagRT and GagTN constructs in our laboratories used immunogold electron microscopy to show that similar structures as those depicted above contained Gag components (Halsey et al., 2008).

Once budding was established, three methods of VLP purification were explored to determine which method was able to isolate the highest VLP yields while still allowing chimaeric VLP stability and integrity to be maintained. Two criteria were used to evaluate the chosen methods, namely, the quality and quantity of VLP samples obtained in the respective purifications.

The quality of VLP preparations (in terms of VLP morphology, VLP size, composition and purity) was examined using western blots, Coomassie-stained SDS-PAGE gels and TEM analysis. The data indicated that all purified VLP samples contained intact chimaeric VLPs. As expected, these were larger and more irregular-shaped than the Gag-only VLPs (Jaffray et al., 2004), and varied widely in size, with GagTN VLPs having a diameter range of about 120 -185 nm, and GagRT VLPs having a range of 135 – 230 nm. This variation, while not ideal, was not unexpected for chimaeric VLPs, and has been seen previously (Halsey et al., 2008; Wagner et al., 1994a).

Although only one VLP band was expected to result from the density gradient ultracentrifugation purifications, there were multiple bands found for both constructs. This was surprising, but could be explained by the formation of VLP aggregates or the presence of Gag cleavage products, as both of these options would produce bands of

different densities. The formation of multiple bands complicated matters because it was difficult to differentiate between the bands formed, even after fractionation characterization. To use either SDGU or ODGU for bulk purification purposes, it would be necessary to combine all bands to accurately determine the VLP yield obtained by the respective purification methods, given that all bands contained chimaeric proteins of the correct molecular weight. However, combining the bands could potentially lower the purity of the extracted sample. In addition, the similar buoyant density of the band found in the negative control for SDGU suggests using this method to isolate pure VLPs would be a difficult task.

In terms of quality, the VLP samples obtained using the SDGU and ODGU purifications were quite different, despite the similarity of these methods. The VLP samples from the SDGU purification comprised of many more small Gag cleavage products than the ODGU samples, as demonstrated by western blot and SDS-PAGE analysis. While the presence of Gag cleavage products was found in all preparations and would not be ideal for the mouse studies, it was important to note that the ODGU and MU methods displayed strong chimaeric VLP bands and only a few prominent Gag cleavage products in the western blots and SDS-PAGE data. This was particularly noticeable for the diluted MU samples, which demonstrated detectable chimaeric VLP bands on the Coomassie-stained SDS-PAGE gels, which did not occur with the diluted samples of the other methods. In addition, the SDGU samples appeared to indicate that the respective chimaeric proteins were not the most prominent proteins isolated using this method.

No definitive conclusions could be made from the TEM analysis of the VLP samples; however, it was clear that there were several impurities found in the protein bands isolated via SDGU. These were also found in the preparations from the ODGU and MU, although not as frequently. Some of the structures found in the TEM analysis were baculovirus-related, which is not unexpected, but there were many unidentifiable structures that were possibly cellular matter (such as microvesicles), impurities from the sucrose utilized or damaged/unfolded VLPs (Bess et al., 1997). Such impurities could

produce high non-specific immune responses in experimental mice, therefore skewing results obtained in immunogenicity tests.

The quantity of VLPs within the given preparations was assayed using Gag p24 ELISA, in conjunction with the Coomassie-stained SDS-PAGE data. Both methods were used for this purpose to account for the fact that the ELISA measured only a component of Gag (p24) as opposed to quantifying the whole VLPs present in the respective samples. In this way, the ELISA results could have been skewed by the presence of Gag cleavage products, and therefore needed to be used alongside the SDS-PAGE data, so as to confirm they were reflecting what was being observed in the SDS-PAGEs.

Given that multiple protein bands were isolated from the SDGU and ODGU purifications, and that all bands contained chimaeric VLPs as well as other Gag products, it was difficult to make direct quantitative comparisons between methods without combining the amounts obtained for individual bands for SDGU and ODGU. The data illustrated that the MU yields were greater than the combined band yields of the other two methods. While the ELISA data was not a direct indication of the VLP yield, and was obviously influenced by the presence of Gag cleavage products, when used in addition to the Coomassie-stained SDS-PAGE data, it seemed likely that the MU purification method yielded the best VLP yields.

In conclusion, although SDGU has been used to extract HIV VLPs for previous mouse experiments in our laboratories as well as others, the results suggest that SDGU was not a good option for purification of the chimaeric VLPs. Both the quality and quantity of VLPs isolated using SDGU was poorer than what we found with ODGU. In terms of quality of VLPs, ODGU and the MU preparations were similar, as both methods yielded intact VLPs, with few cleavage products and impurities. However, the quantitative data favoured MU. The yields produced by this method were almost double that of the ODGU yields. MU was also less labour-intensive than the previous methods, making this purification method the better option for producing bulk VLP preparations for the mouse experiments.

Chapter 5

Mouse immunogenicity studies using chimaeric HIV-1 VLPs

5.1 Introduction

5.1.1 Prime-Boost vaccine strategy

There is an abundance of evidence to suggest that an effective cellular immune response against HIV-1 is able to control and suppress viraemia during primary and chronic HIV infections, in addition to providing long-lasting protection (Amara et al., 2005; Deml et al., 2005; Jaffray et al., 2004). Although several HIV vaccine attempts have illustrated the ability to induce cellular immune responses, many have been weak and lacked longevity in non-human primates and humans (Paliard et al., 2000). As mentioned previously, the use of a heterologous prime-boost vaccination is an effective method to enhance T-cell and humoral responses. The basic concept of a prime-boost vaccine is to prime an immune response against one or more target antigens using one vector, and then to enhance the specificity of this response by exposing the immune system to the same target antigens delivered by another vector (Woodland, 2004). Most of the recent prime-boost vaccinations have made use of a DNA vaccine prime and a viral vector vaccine boost, although, other boosting candidates have included subunit protein vaccines and VLP vaccines. VLPs have been of particular interest because they have been shown to elicit significant immune responses.

In this study, the chimaeric VLPs, GagRT and GagTN, were tested for their ability to act as vaccine boost candidates. The presence of multiple antigens in these VLPs reduces the likelihood of CTL escape, which often occurs when there is a single, dominant epitope (Deml et al., 2005; Ellenberger et al., 2005). Furthermore, multi-protein vaccine candidates are able to elicit broader and possibly more effective immune responses against HIV (Ellenberger et al., 2005; Nkolola et al., 2004). In particular, Tat, Nef and RT contain several prominent CTL epitopes. Tat and Nef-specific T-cell responses have shown a correlation with non-progression of HIV and possible protection (Scriba et al., 2005), while RT has induced potent Th1 responses in mice, when administered in low doses (Pacheco et al., 2000).

5.1.2 pVRCgrttnC DNA vaccine

In this study, the "prime" component for all vaccination experiments was the DNA vaccine, pVRCgrttnC (Fig. 5.1). This vaccine is one of the two plasmids that made up the SAAVI DNA-C2 vaccine, a second generation vaccine, created in the University of Cape Town SAAVI laboratories. It is based on the first generation vaccine, SAAVI DNA-C (pTHgrttnC), only differing in the vector backbone (Burgers et al., 2006). In particular, the plasmid, pVRCgrttnC, encodes a multigene DNA vaccine in the form of an HIV-1 subtype C polyprotein derived from Du422 and Du151 HIV isolates. All genes were optimized to reflect human codon usage and modified for safety purposes. The polyprotein, grttnC, comprised of four HIV proteins, namely, p6-truncated Gag (Myr^-), inactivated reverse transcriptase (RT), shuffled Tat and inactivated Nef (TN).

The backbone of the pVRCgrttnC plasmid was a pVRC vector provided by the Vaccine Research Centre of the National Institutes of Health, Bethesda, Maryland, USA. This backbone contained the kanamycin resistance gene as a selection marker, and a regulatory R region from the 5' long terminal repeat (LTR) of human T-cell leukaemia virus type 1 (HTLV-1), which acted as a transcriptional and posttranscriptional enhancer (Barouch et al., 2005). In general, CMV/R DNA vaccines elicit substantially higher HIV-1 specific cellular immune responses compared with the analogous CMV-only parental DNA vaccines in both mice and cynomolgus monkeys, hence its use in this vaccine (Barouch et al., 2005).

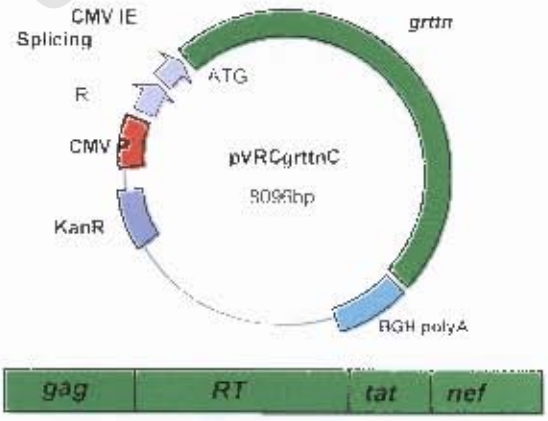


Figure 5.1 The pVRCgrttnC DNA vaccine used in mouse experiments. Abbreviations: CMV P: Cytomegalovirus promoter; KanR: Kanamycin resistance gene; grttn: Gag, RT, Tat and Nef fusion protein; BGH poly A: bovine growth hormone polyadenylation signal. (This figure was kindly provided by Prof. E. Shepard).

5.1.4 Chapter objectives

The aims of the work reported in this chapter were as follows:

(i) To utilize the extracted chimaeric VLPs as boost candidates in two DNA prime - VLP boost vaccine strategies in mouse studies.

(ii) To evaluate the cellular immune response elicited by the chimaeric VLPs in mice.

(iii) To evaluate the humoral response induced by the prime-boost vaccination of mice.

(iv) To investigate whether the use of two boost inoculations improves this elicited immune response against HIV.

5.2 Materials and Methods

5.2.1 Micro-/Ultrafiltration

The appropriate optimal conditions (Table 3.2) were utilized to scale up production of the respective chimaeric VLPs in *Sf*9 cells (2500 ml). At 96 hpi, infected cell culture was transferred to sterile 25-ml bottles (Sterilin) and spun at 1000 x *g* for 20 min to isolate the culture supernatant. Micro-/ultrafiltration were used to extract and purify VLPs from the culture supernatant, as described in 4.2.1.3.

5.2.2 Protein analysis

5.2.2.1 Quantitative western blots

To quantify the amount of purified chimaeric VLPs obtained, a dilution series of the respective samples was run parallel to a dilution series of a positive control [recombinant Gag p17/p24-C protein of known quantity (ARP 695.2 - FIT Biotech Oyj Plc, Tartu, Estonia)] on western blots (done as described in 2.2.3.1). The intensity of detected bands was measured using densitometry software, Genesoft (SynGene, Synoptics Ltd.), and the intensity data from the positive control samples was used to plot a standard curve. This standard curve was then employed to determine the concentration of the VLPs in the

purified stock solutions. These western blots were performed twice to determine an average value for the quantity of VLPs isolated.

5.2.2.2 Coomassie-stained SDS-PAGE

The purity of all VLP samples were analysed using SDS-PAGE (done as described in 4.2.2.1).

5.2.2.3 Negative staining TEM

All purified VLP samples underwent TEM analysis as described in 4.2.3.1, in order to verify VLP structural integrity.

5.2.4 Endotoxin tests

Endotoxin tests were performed using the QCL-1000® Limulus Amebocyte Lysate (LAL) kit (Cambrex Bio Science Walkersville Inc.). The procedure was carried out as detailed in the manufacturer's instructions. Samples were deemed acceptable for mouse experiments if endotoxin readings were equal to or below 0.125 Endotoxin Units/ml (EU/ml).

5.2.5 Vaccination of mice with the respective chimaeric VLPs

5.2.5.1 Preparation of mice

All mouse experiments were approved by the University of Cape Town Animal Ethics Committee. Female H-2^d Balb/c mice were used in vaccination experiments (South African Vaccine Producers Pty Ltd – Johannesburg, South Africa), and kept at the University of Cape Town Animal Unit for approximately 10 days before vaccination (to allow adaptation to surroundings). They were maintained here for the duration of the experiments.

5.2.5.2 Inoculation plan for experiments

The vaccination protocols for the four mouse experiments were performed by trained animal technologists according to the schedule described in Tables 5.1. The "prime" component of the vaccination entailed the administration of 100 μg of pVRCgrttnC DNA

(resuspended at 1mg/ml saline) (Aldevron, Fargo, ND, USA) per mouse. This was done via an injection of 50 μl into the quadriceps muscle of each mouse. The "boost" component of the vaccination was the administration of purified chimaeric VLPs (resuspended in 1x PBS). Mice were bled prior to inoculation, and at the end of the experiment, which was 12 days after the boost inoculation. The spleens of the infected mice were harvested to determine the cellular immune response induced by the respective inoculations.

Table 5.1 – The vaccines and vaccination protocols for the mouse experiments (pVRCgrttnC DNA prime - GagRT or GagTN VLP boost).

Group # mice	**Vaccine (prime) Day 0**	**Inoculum per mouse (µg)**	**Vaccine (boost) Day 28**	**Inoculum per mouse (µg)**
1	pVRCgrttnC	100	-	
2	pVRCgrttnC	100	pVRCgrttnC	100
3	pVRCgrttnC	100	Respective VLPs	0.05
4	pVRCgrttnC	100	Respective VLPs	0.10
5	pVRCgrttnC	100	Respective VLPs	0.20
6	-		Respective VLPs	0.05
7	-		Respective VLPs	0.10
8	-		Respective VLPs	0.20

There were five mice per group, all of whom received the respective vaccines via intramuscular injection. Blood sera samples were taken before inoculation and sacrifice. All mice were sacrificed on Day 40, and their spleens were removed and processed as described in 5.2.5.1.

5.2.6 Immunogenicity testing

5.2.6.1 Preparation of splenocytes for immunogenicity assays

The spleens of each group of mice within the given experiments were pooled. A single cell splenocyte suspension was generated by meshing the pooled spleens of each group through a metal sieve using RPMI medium (Gibco). Suspensions were transferred to 50 ml Sterilin^{TM} tubes and centrifuged at 1500 rpm for 5 min. The resulting cell pellet was re-suspended in 50 ml of RPMI and the centrifuge was repeated. Resulting pellets underwent two further washes, where fibrin clots were removed using a Pasteur pipette before the final centrifuge. They were once again resuspended in 50 ml RPMI medium, cell count and viability was determined and the required number of cells for the immunogenicity assays was removed. These cells were centrifuged at 1000 x *g* for 5 min. The red blood cells (RBC) of samples were lysed using a RBC lysis buffer (Gibco) when the splenocytes were used in IFN-γ and IL-2 assays.

Cells were centrifuged again at 1350 rpm for 7 min. The pellets were resuspended in complete RPMI medium (Appendix B5.1), and cells were re-counted, so that the required quantities of cells for the respective assays could be removed.

5.2.6.2 Determination of phenotype of splenocytes

Cell surface markers (CD3, CD4, CD8, CD19) on isolated splenocytes were investigated using flow cytometry. To prepare samples, 1 % blocking solution (in tris-buffered saline) was added to $1x10^6$ splenocytes, and incubated for 20 min in darkness. Cells were then washed with FACS buffer [PBS with 1 % FCS + 0.1 % NaN_3] and centrifuged at 1500 rpm for 5 min. The cell pellet was resuspended in residual FACS buffer, fluorescent antibodies specific to the respective cell surface receptors were added to samples, and they were incubated for 30 min in darkness. Cells underwent another FACS buffer wash and centrifuge, followed by the addition of a FACS Lyse solution (BD Bioscience) and a further incubation of 10 min at room temperature. The final step involved another FACS buffer wash, after which cells were resuspended in 900 µl FACS buffer. Labelled cells were acquired on a FACS Calibur flow cytometer (500 000 gated events acquired per sample) and analysed using Cellquest software (Becton Dickinson).

5.2.6.3 ELIspot assays

The isolated splenocytes were used in IFN-γ and IL-2 ELIspot assays to evaluate whether specific T-cell immune response had been induced in mice. These were performed using BD Biosciences kits, where the protocols were carried out as detailed in the manufacturer's instructions. Briefly, plates were coated with capture antibody (IFN-γ or IL-2 antibody at 5 µg/ml in PBS), sealed and incubated at 4 °C overnight. IL-2 plates were pre-wet with 70 % ethanol before the coating step, so that the spots created a sharper image when detected. The next day, wells were washed with blocking solution, and incubated in blocking solution for 2 hrs at room temperature. The blocking solution was then discarded and the respective stimulant peptides were plated in the necessary wells. The peptides antigens used as stimulants in these assays are detailed in Table 5.2. It is important to note that the Tat4 (NCYCKHCSYHCLVCFQTK) and Nef8 (VGAASQDLDKHGALT) peptides (final concentration: 4 µg/ml) were not referenced peptides. The MHC restriction of the Tat 4 peptide was unknown, while Nef 8 was an H-$2K^d$ binding peptide with a possible CD8 epitope in Nef. All peptides were prepared in R10 medium (Appendix B5.1), although the Tat4 stock contained 0.04 % DMSO (in final concentration).

A single cell suspension of splenocytes was prepared as described in 5.2.5.1, and splenocytes were plated in triplicate at 5 x 10^5/well in a final volume of 200 µl R10 culture medium. Concanavilin A (Con A) (Sigma MO, USA) was used as a positive assay control, and plated in the respective wells after the cell suspension to a final concentration of 0.5 µg/ml. Plates were then incubated in darkness at 37 °C, in a 5 % CO_2, humidified incubator. After 23 hrs, plates were washed 3 times with dH_2O, then 3 times with wash buffer I. Detection antibody [biotinylated anti-mouse IFN-γ or IL-2 diluted 1:250 in dilution buffer] was added to each well (100 µ l/well) and plates were incubated for a further 2 hrs at room temperature. The detection antibody was then discarded and plates were washed 3 times with wash buffer I. Avidin horseradish peroxidase (Avidin-HRP) (diluted 1:100 in dilution buffer) was added to the plates (100 µl/well) and incubated for 1 hr at room temperature. Plates were then washed with wash

buffer I and II as done before, and 100 µl/well of Nova Red substrate solution (Southern Cross) was added. Spot development was monitored for 5-10 min before reaction was stopped by washing wells with H_2O 5-6 times. Plates were air-dried at room temperature and stored in the dark until analysis.

Plates were scanned using an ELIspot CTL Analyser (Series 3B) and spots were detected and counted using a computerized Immunospot Image Analyzer (Cellular Technology Ltd, Cleveland, Ohio) with Immunospot software (v3.2). The mean number of spots was determined for triplicate wells and adjusted for one million splenocytes to provide data as spot forming units (SFU) per million splenocytes. The background responses were identified as the responses observed in the absence of peptide (stimulation with the medium only) or the presence of irrelevant peptide. For each group of mice, a response of ≥to the mean background response + 2 standard deviations (SD) was considered as a cut-off for a positive response.

Table 5.2 Stimulants used in IFN-γ and IL-2 ELIspot assays (final concentration: 2 µg ml)

Name of stimulant	Description	Amino acid sequence	References
R10 medium	Background control	-	-
Irrelevant peptide	H-2K^d binding peptide (Negative peptide control)	TYSTVASSL	(Burgers et al., 2006)
Gag CD8	H-2K^d binding peptide; CD8 epitope in Gag	AMQMLKDTI	(Burgers et al., 2006; Mata, 1999)
Gag CD4 (13)	MHC-II binding peptide; CD4 epitope in Gag	NPPIPVGDIYKR WIIGLNK	(Im, 2007; Mata, 1999)
Gag CD4 (17)	MHC-II binding peptide; CD4 epitope in Gag	FRDYVDRFFKT LRAEQATQE	(Im, 2007; Mata, 1999)
RT CD4	MHC-II binding peptide; CD4 epitope in RT	PKVKQWPLTEV KIKALTAI	(Casimiro, 2002)
RT CD8	H-2K^d binding peptide; CD8 epitope in RT	VYYDPSKDLIA	(Casimiro, 2002)

5.2.6.4 LAV blot western blot

A commercial New LAV Blot I (BioRad) was used according the manufacturers instructions to determine the specificity of the antibodies in mouse serum, and thus evaluate the humoral immune response induced by the respective vaccines. Mouse serum was used at a 1:40 dilution and antibody content detected with goat anti-mouse Ig G conjugated to alkaline phosphatase.

5.3 Results

5.3.1 Quantification of purified VLPs

VLP production was scaled up in order to generate the quantities necessary for mouse studies. Two batches were produced per VLP construct, so as to ensure there would be an adequate quantity of VLPs available for the immunogenicity experiments. Purification of the respective VLPs was performed using micro-/ultrafiltration, and once purified, VLPs were stored in PBS at 4 °C. Samples were analysed on western blots to confirm that chimaeric VLPs had been purified and isolated intact (Fig. 5.2). Although Gag p24 ELISA was previously used for quantitative purposes, it was found to underestimate the quantities of VLPs, possibly because disruption of the VLPs was not occurring optimally. Therefore, western blot densitometry was employed to quantify the Gag content of purified VLP samples for mouse experiments. A dilution series of a Gag p17/p24 positive control of known quantity was employed to create a standard curve that was used to determine the Gag concentration of chimaeric VLPs in the respective sample batches. As was observed in previous chapters, a prominent, intact chimaeric protein band was detected for the respective samples on the western blots, as well as less prominent Gag cleavage products (Fig. 5.2). The TEM data suggested the samples were fairly heterogeneous, but this was expected, given the data obtained in Chapter 4. More importantly, the TEM data illustrated that VLPs were structurally intact (Fig. 5.3).

Batch 2 of the respective chimaeric VLP stocks were selected for the mouse studies because they were more concentrated, were stored for less time than Batch 1, and had

lower endotoxin values (see Appendix G). Batch 2 of the GagTN VLPs contained about 50 µg of VLPs, collected from 2.5 litres of infected *Sf*9 cells. Batch 2 for GagRT VLPs contained approximately 38 µg of VLPs, isolated from the same quantity of cells. Quantification of these batches is given in Appendix F.

5.3.2 Analysis of purified VLPs

To analyse purity of the VLP samples, Coomassie-stained SDS-PAGE was used. This indicated that the samples were fairly pure, as cellular and baculovirus proteins were not prominently detected (Fig. 5.4).

Endotoxin test results (Appendix G) provided evidence of the sterility of the respective samples. The recommended level of endotoxins allowed for animal studies is one that is less than or equal to 0.125 endotoxin units per ml (EU/ml). Once VLP stocks had been diluted as was required for mouse inoculations, the endotoxin levels in the respective samples were below what was deemed unsafe for mice. These could therefore be used in the vaccination experiments.

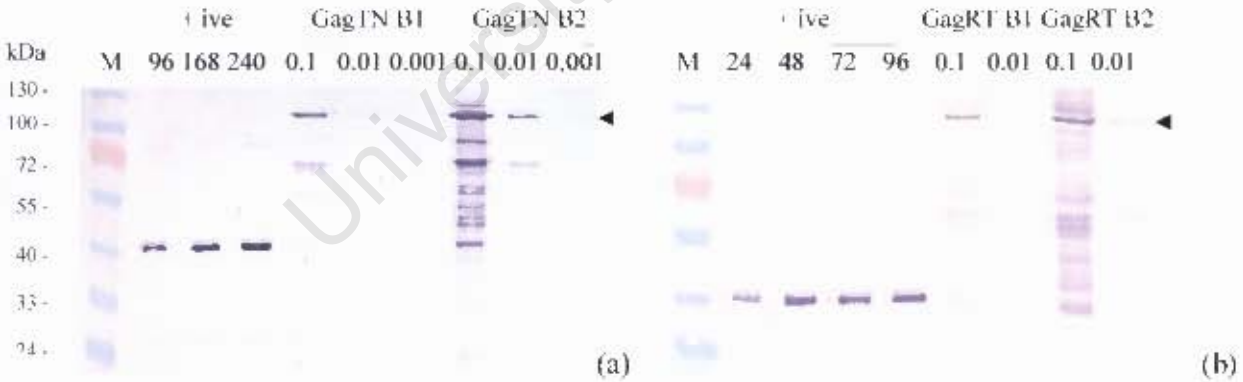
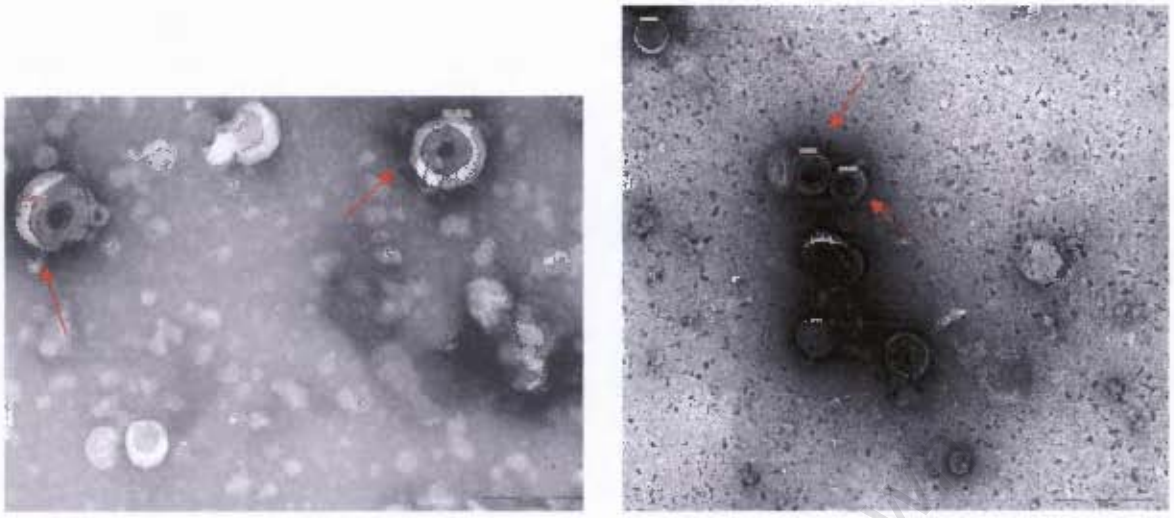


Figure 5.2- Western blots primed with HIV Gag p24 primary antibody were used to analyse and quantify purified VLP samples. (a) GagTN VLP samples; (b) GagRT VLP samples. Abbreviations: M = molecular weight marker; + ive = Gag p17/p24 (p41) positive control of known quantities (ng); B1 = batch 1; B2 = batch 2; 0.1 = 1 in 10 dilution; 0.01 = 1 in 100 dilution; 0.001 = 1 in 1000 dilution. Arrow heads indicate the migration distance of the respective chimaeric VLPs.



(a) (b)

Figure 5.3 - Electron micrographs of extracted chimaeric VLPs. (a) GagTN VLP sample; Bar = 200 nm (b) GagRT VLP sample; Bar = 500 nm. Red arrows depict examples of VLPs.

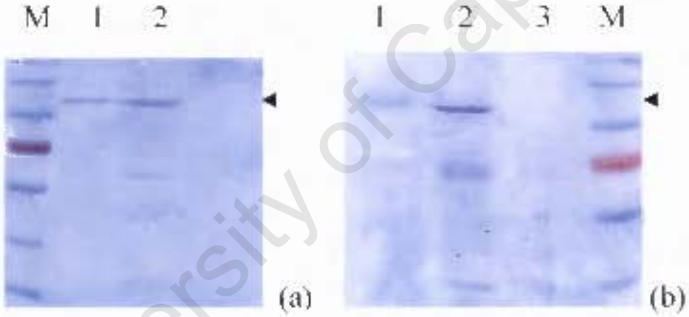


Figure 5.4 - SDS-PAGE gel photographs of the respective VLP samples after extraction using micro-/ultrafiltration. (a) GagTN VLP samples. Lanes: 1-molecular weight marker; 2-GagTN VLPs Batch 1; 3-GagTN VLPs Batch 2. (b) GagRT VLP samples. Lanes: 1-GagRT VLPs Batch 1; 2-GaRT VLPs Batch 2; 3-blank; 4- molecular weight marker. Arrow heads indicate the migration distance of the respective chimaeric VLPs.

5.3.3 Immunogenicity studies analysis

The prime-boost vaccine regimens were performed as detailed in Table 5.1. The experiments aimed to determine whether the chimaeric VLPs had potential to induce an enhanced cellular immune response when administered as a boost component of a prime-boost vaccine strategy. It entailed the use of a DNA vaccine prime (pVRCgrttnC) and one VLP boost (either GagTN or GagRT VLPs), administered 28 days after the initial inoculation. Mice were sacrificed after 40 days.

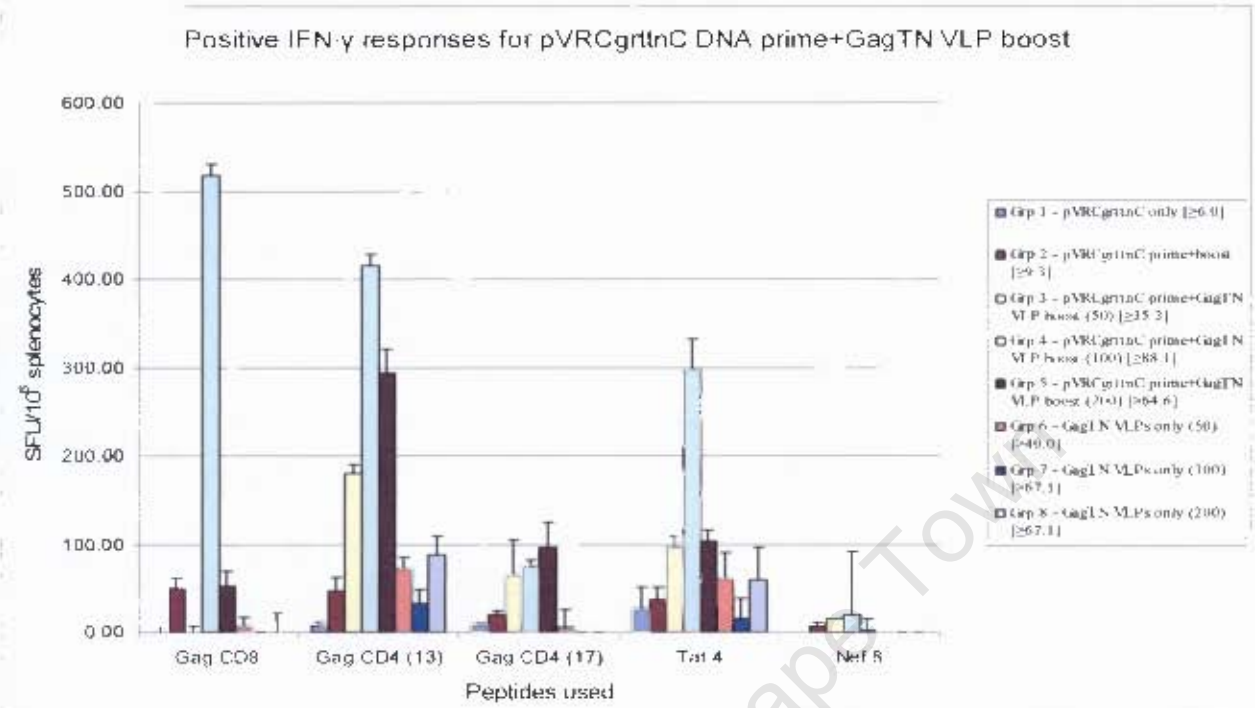
The spleens of the mice were removed and pooled for each group. The spleens were then processed to evaluate the cellular immune response that was elicited by the vaccines. Cell surface marker analysis of splenocytes was performed to establish cell phenotypes. The number of CD3, CD4, CD8, and CD19 cells and ratios of CD3/CD4, CD3/CD8 and CD4/CD8 was determined for each group. In order for the immune responses of groups of mice within a given experiment to be comparable, they needed to demonstrate similar lymphocyte ratios and T-cell numbers, as considerable differences could influence the interpretation of assay data to follow. As desired, the cell surface marker data indicated that group samples compared within each experiment had similar numbers and ratios of T-cells to each other (Appendix H).

CD4+ and CD8+ T cells in splenocytes pools secreting IFN-γ and IL-2 during *in vitro* restimulation with specific HIV-1 antigen peptides were measured in ELIspot assays. This was done to determine if specific cellular immune responses were elicited in the experimental mice, and if there was a prominent boost effect detected for mice that received VLP boosters. IFN-γ and IL-2 were chosen because both are central to the development of an adaptive immune response, specifically a cellular response (Janeway, 2005).

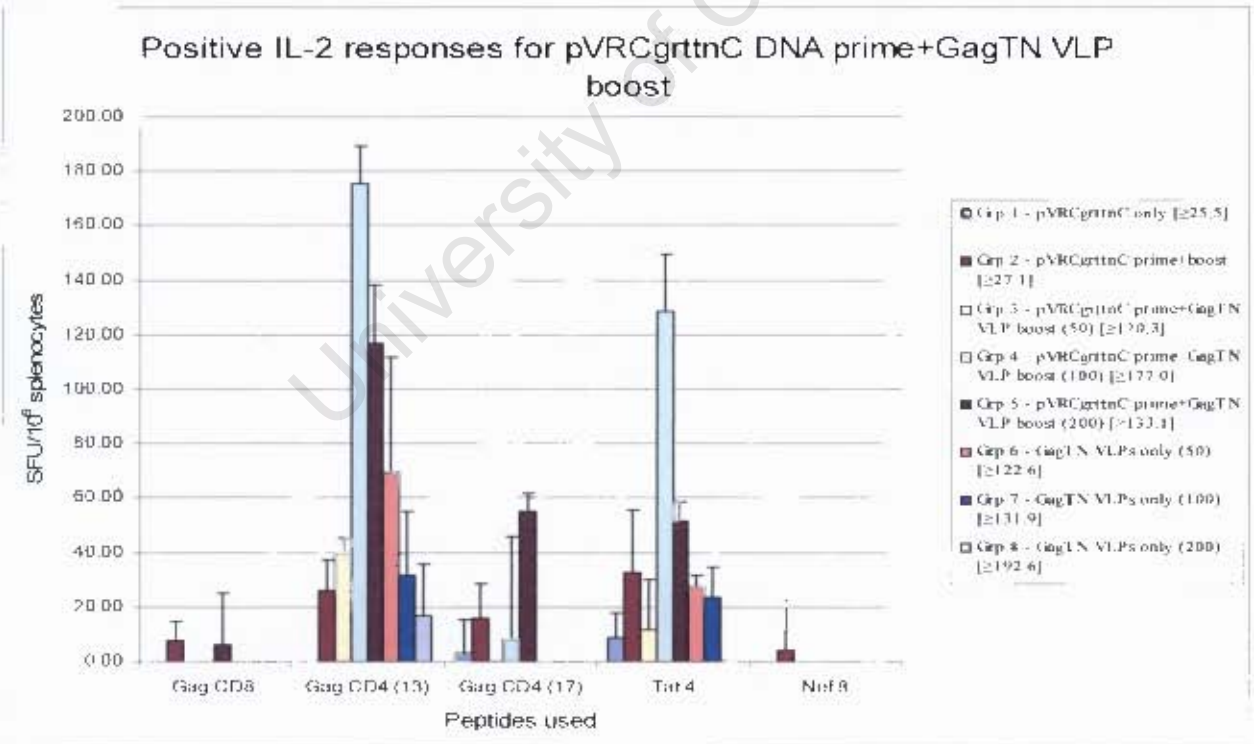
5.3.3.1 GagTN Experiment

In the experiment using the GagTN VLP boost, IFN-γ and IL-2 assay data demonstrated a similarity in the results obtained for the media and irrelevant peptide (Appendix I). These responses were due to non-specific stimulation of the cells, and were low as expected, however they were greater for those groups that received VLP inoculations, than those that only received DNA inoculations. There was also a strong, consistent, positive response stimulated by ConA (not shown) which verified that the assays had worked.

The IFN-γ data showed that, as has been seen in previous studies, mice that received a single DNA prime (Group 1) demonstrated weak responses to all peptide stimuli.



(a)



(b)

Figure 5.5 – The positive cellular immune responses elicited in mice vaccinated as per the prime-boost vaccine regimens detailed in Table 5.1(with Gag TN VLP boost), as determined by quantification of IFN- γ (a) and IL-2 (b) cytokines (SFU/10^6 splenocytes) secreted by pooled splenocytes after in vitro stimulation with HIV-specific peptides. The error bars represent 2 standard deviations above the mean number of sfu. Abbreviations. Med – medium, Irrel – irrelevant peptide. Dosage of VLP boosts (ng) are indicated in round brackets in the legend. The positive responses were defined as those responses that were above or equal to the values in the square brackets in the legend. These cut off values were taken as the highest responses observed for stimulation by the medium and irrelevant peptide (i.e. highest non-specific responses) per group of mice.

specifically Gag CD8 and CD4 peptides (Fig. 5.5a) (Jaffray et al., 2004). While the responses for Group 2 (DNA prime/DNA boost) were moderately better than Group 1, it was clear that the use of a GagTN VLP boost in addition to a DNA prime (Groups 3-5) was able to enhance the production of IFN-γ quite considerably.

Group 4 (DNA prime/100 ng VLP boost) developed the strongest IFN-γ response, specifically for the Gag CD8 peptide (518 ± 12.2 sfu), both Gag CD4 peptides (416 ± 12.2 sfu and 75 ± 8.1 sfu respectively) and the Tat4 peptide (298 ± 33.4 sfu) (Fig. 5.5a). The boost effect demonstrated by group 4 was particularly prominent for Gag CD8 and Gag CD4 (13) peptides, where a 90-fold and 40-fold increase (respectively) was observed when comparing these responses to those obtained for Group 1. This confirmed that the Gag response was a dominant one, as has been reported in other studies (Betts et al., 1997; Lichterfeld et al., 2005).

Groups that received VLP inoculations only (Groups 6-8) displayed relatively strong Gag CD4 responses, although these responses were still approximately 5-fold lower than those observed for Group 4. In particular, Group 7 (received the same VLP dose as group 4 but no DNA prime) generated the poorest IFN-γ responses amongst of the VLP-only groups, providing further evidence of the boost effect exhibited by Group 4.

Like Group 4, Groups 3 and 5 (received 50 and 200 ng GagTN VLP boosts respectively) also demonstrated a boost effect for the Gag CD4 peptides (180 ± 7.6 and 294 ± 26.9 sfu respectively) and the Tat peptide (97 ± 40.1 and 103 ± 13.1 sfu respectively). Group 5 appeared to induce stronger responses than Group 3, and also induced a Gag CD8 response, which was not displayed by Group 3. Overall, the data indicated that although a boost effect was developed by all three groups, the optimum dosage for an effective boost of the cellular immune response was clearly 100 ng GagTN VLPs (Group 4) in this experiment.

The IL-2 data (Fig. 5.5b) verified this to be true, as Group 4 also demonstrated a boost effect in this assay, specifically when stimulated by the Gag CD4 (13) (176 ± 13.6 sfu)

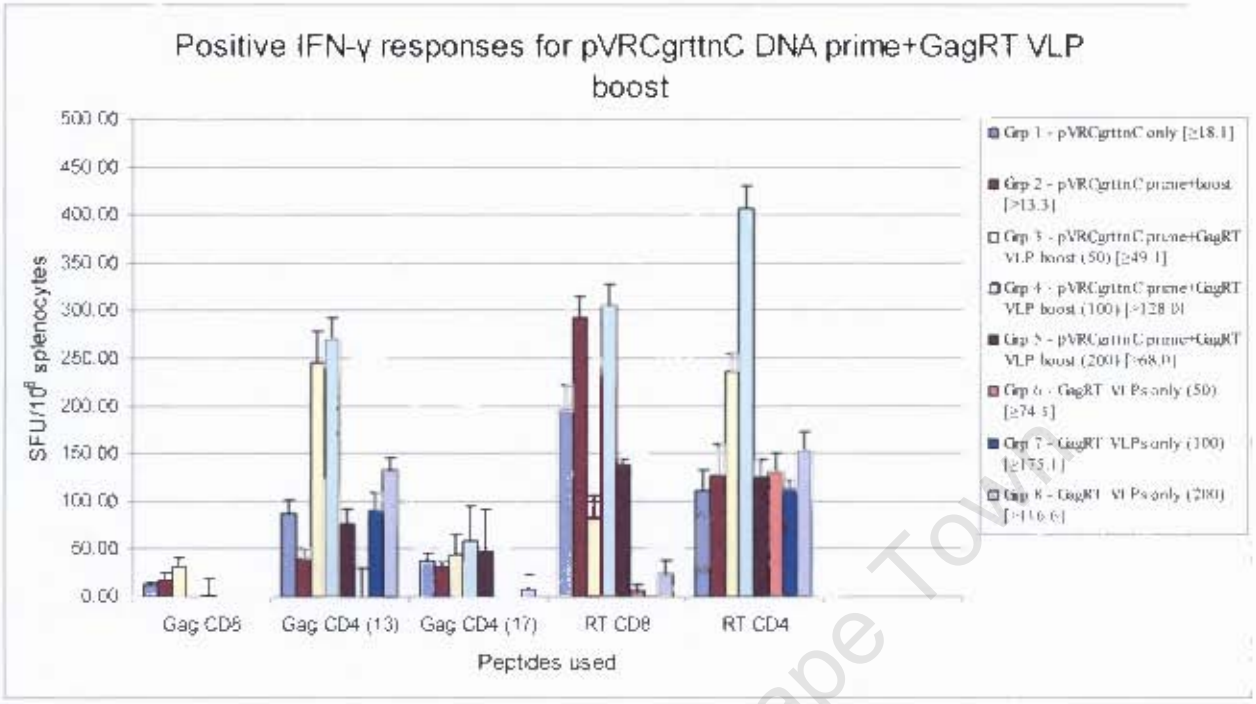
and the Tat (128 ± 21.2 sfu) peptides. There was no significant Gag CD8 IL-2 response for Group 4 as was seen with the IFN-γ data, which was not too surprising, given that CD4+ T-cells are mostly responsible for IL-2 production. Similar to what was seen with the IFN-γ data, Groups 6-8 (received VLP-only inoculations) showed the development of strong Gag CD4 (13) and Tat responses. Again, the DNA-only groups (Groups 1 and 2) demonstrated relatively poor IL-2 responses, corroborating that an enhanced immune response occurred when mice were boosted with 100 ng of GagTN VLPs (Group 4).

Unfortunately, the Nef responses in both the IFN and IL-2 assays demonstrated large variations (as indicated by the SD values) and were therefore deemed unreliable.

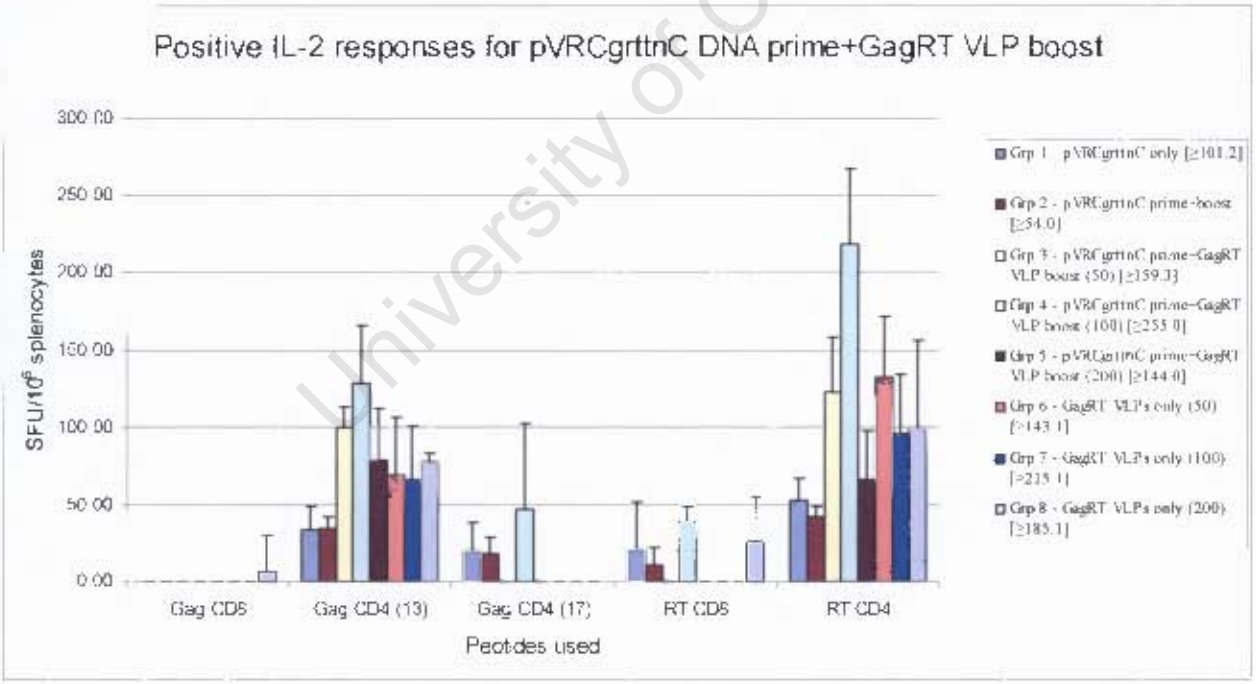
The LAV blot data indicated that there was no apparent humoral immune response elicited in the mice of any of the groups post vaccination (data not shown).

5.3.3.2 GagRT Experiment

The dose of 100 ng also proved to be the optimal VLP dose in the GagRT experiment, where Group 4 (received DNA prime/100 ng GagRT VLP boost) elicited the most effective IFN-γ and IL-2 responses (Fig. 5.6a and b). The highest frequency of IFN-γ producing cells from Group 4 were those recognising the Gag CD4 (13) peptide (271 ± 20.8 sfu) and RT CD4 and CD8 peptides (306 ± 22 sfu and 406 ± 25 sfu respectively) (Fig. 5.6a). These responses were much stronger than the DNA-only (Groups 1 and 2) or VLP-only (Group 6-8) responses, proving a boost effect did occur. The VLP dose used for Group 4 (100 ng) appeared to be the only one able to elicit a boost response, because although the results obtained from Group 3 also displayed an increased response to Gag CD4 (13) and RT CD8 peptides, this did not appear to be considerably greater than what was detected for the VLP-only groups. Group 5 generally produced relatively poorer IFN-γ responses compared to Groups 3 and 4. Interestingly, while Group 3 was also able to elicit a similar Gag CD8 response, Group 4 did not induce a positive Gag CD8 response.



(a)



(b)

Figure 5.6 – The positive cellular immune responses elicited in mice vaccinated as per the prime-boost vaccine regimens detailed in Table 5.1 (with Gag RT VLP boost), as determined by quantification of IFN- γ (a) and IL-2 (b) cytokines (SFU/10^6 splenocytes) secreted by pooled splenocytes after in vitro stimulation with HIV-specific peptides. Abbreviations: Med – medium, Irrel – irrelevant peptide. Dosage of VLP boosts (ng) are indicated in round brackets in the legend. The error bars represent 2 standard deviations above the mean number of sfu. The positive responses were defined as those responses that were above or equal to the values in the square brackets in the legend. These cut off values were taken as the highest responses observed for stimulation by the medium and irrelevant peptide (i.e. highest non-specific responses) per group of mice.

The IL-2 assay results (Fig. 5.6b) followed the same trend as the IFN-γ data, in that Group 4 demonstrated positive responses to both Gag CD4 peptides (128 ± 37 sfu and 47 ± 55.7 sfu respectively) and RT CD4 and CD8 (219 ± 48.5 sfu and 40 ± 9.2 sfu respectively). Similar to the GagTN VLP IL-2 data, there was no significant response to cells stimulated with Gag CD8, except for Group 8 (200 ng GagRT VLP dose only). Groups 6-8 appeared to be stimulated by Gag CD4 and RT CD8, but these IL-2 responses were not as strong as what was seen for Group 4; once again reaffirming a heightened cellular immune response occurred for mice receiving a GagRT VLP boost of 100 ng.

Although significant cellular responses were evidently induced by the vaccines, there was no apparent antibody response to Gag in the GagRT VLP experiment, as was the case with the GagTN experiment (data not shown).

5.4 Discussion

The importance of a cellular immune response against HIV has been highlighted in several animal vaccine trial studies (Amara et al., 2005; Buonaguro et al., 2006; Smith et al., 2004), in addition to the fact that long term non-progressor (LTNP) HIV patients have preserved HIV-1 specific CD4+ T cells, CD8+ T cells and memory T cells, all of which have been identified as a means to control viraemia (Pantaleo and Koup, 2004; Sadagopal et al., 2005). Currently, one of the more successful means to elicit a superior cellular immune response against HIV in animal models, is using a prime boost strategy, particularly with the use of HIV VLPs as a boost component (Jaffray et al., 2004; Paliard et al., 2000).

The immunology experiments in this study explored the potential of the chimaeric VLPs, GagRT and GagTN, to induce and possibly enhance a cellular immune response against HIV-1. This was evaluated by the enumeration of vaccine-specific IFN-γ and IL-2 producing cells in respective ELIspot assays.

Secretion of IFN-γ is believed to be indicative of the early stages of cellular immune response generation and therefore provides an idea of a vaccine's immunogenicity (Pantaleo and Koup, 2004). On the other hand, IL-2 is a typical indicator of antigen clearance and long-term memory T cells that demonstrate protection (Pantaleo and Koup, 2004; Sadagopal et al., 2005). It is believed that eliciting T-cells able to co-produce these two cytokines (also known as polyfunctional T cells) results in viral clearance and low antigen load, as is observed in LNTPs. Thus, these cytokines were used to indirectly gauge the type and specificity of the cellular immune response induced by the respective VLP vaccines.

The IFN-γ and IL-2 data clearly demonstrated a boosted cellular immune response occurring when the respective VLPs were administered as a boost vaccine after inoculation with the DNA prime. No such boost was observed when two doses of DNA was administered, or when only VLP inoculations were administered, verifying the success of using a heterologous prime boost strategy, as is demonstrated in the literature (Dale et al., 2002; Jaffray et al., 2004). Furthermore, an optimal VLP dose of 100 ng was found to induce the most potent immune responses in both chimaeric VLP boost experiments. Comparatively, this dose was lower than what has been published in other studies (Buonaguro et al., 2002; Halsey et al., 2008), but it was adequate to induce positive CD4+ and CD8+ T-cell responses.

A strong CD4+ T cell response specific to Gag was most prominently elicited in the GagTN and GagRT experiment for the group that received a DNA prime and VLP boost (100 ng) (Group 4). Although CD4+ T cells are the target of HIV-1, they play a key role in activation of both B cells and CD8+ T cells, and in controlling CTL responses (Sadagopal et al., 2005). In addition, they have been linked to the control of HIV-1 infection and replication (Jansen et al., 2006; Kalams et al., 1999). Thus, their induction by the respective VLPs is suggestive of the promising potential of these VLPs as future vaccine components.

The Gag CD8+ T-cell response elicited by Group 4 in the GagTN experiment was also considerable, although a much weaker Gag CD8 response was detected in the GagRT experiment. This was unexpected given that previous research showed significant CTL responses were stimulated when Gag VLPs were used as vaccination candidates in rhesus macaques (Paliard et al., 2000). Interestingly, there was a Gag CD8 response induced by the other two groups that received prime-boost vaccinations (VLP doses below and above that of Group 4) in the GagRT experiment, which suggests that the Gag CD8 IFN-γ data for Group 4 might be anomalous. Alternatively, the neligible Gag CD8+ T-cell response could be due to the fact that VLPs are exogenous antigens, and are therefore primarily presented to the immune system via MHC class II molecules, which stimulates the activation of T_h cells, rather than CTLs (Deml et al., 2005). There are occasions of cross presentation where exogenous antigens such as VLPs are processed and presented to the immune system via APC's using MHC class I molecules. In these cases, CD8+ T-cells are stimulated but this process is believed to be inefficient and influenced by many factors including dosage and time (Maecker et al., 2001). It will be important to further investigate the effect of dose on the elicited Gag CD8 response in future experiments, to confirm whether there was an error in the result here, or whether a larger quantity of VLPs is required to improve the Gag-specific CD8+ T-cell response.

The significance of a vaccine eliciting anti-Gag antibodies has yet to be determined in terms of combating HIV-1, but their disappearance has been linked to the progression to AIDS in HIV-1 infected individuals (Chugh and Seth, 2004). The lack of induction of a Gag-specific humoral response by the VLPs in these experiments could be related to the dose of VLPs used in these experiments, as was the case for the poor CD8 response in the GagRT experiment. Earlier VLP vaccine studies have used much higher VLP doses to achieve similar cellular immune responses as observed in this study, but they were also able to stimulate strong anti-Gag antibody responses (Buonaguro et al., 2002). It is possible that by increasing the dose of VLPs, or possibly incorporating a second VLP boost into the vaccine strategy, an improved humoral response could be induced. On the other hand, monitoring the mice over a longer period of time after the boost inoculation may show detectable Gag-specific humoral responses in vaccinated mice, because

humoral responses are commonly generated some time after vaccination (can be between 42 and 56 days after the initial inoculation (Young and Ross, 2006; Zhao et al., 2005)).

The multi-antigenic nature of the DNA and VLP vaccines in this study, allowed for the exploration of eliciting broader, cellular immune responses. Modifications to Gag have been known to affect particle formation and budding, as well as the immune responses that are induced (Young et al., 2006). This appeared to be true for the GagTN and GagRT experiments here, where a boost effect was observed for both Tat and RT peptides. In particular, potent CD4 and CD8 responses specific to RT were detected in the GagRT experiment, suggesting that a GagRT VLP boost was able to elicit an enhanced, broader immune response than using a HIV-1 DNA vaccine alone. The Tat data was slightly more questionable, as the Tat4 peptide used for *in vitro* stimulation in the ELIspot assays formed cyclic structures due to the presence of several cysteine amino acids (formed of sulphide bonds). This is believed to have a negative affect on the peptides' ability to elicit specific IFN-γ or IL-2 responses. Thus, further experimentation is needed to confirm the Tat data. Nevertheless, the apparent boosts for Tat and RT imply that an elicited cellular immune response can be manipulated by the boost component of a vaccine to influence the specificity of the response. In addition, the development of a broader cellular immune response is advantageous in that it decreases the possibility of T-cell escape (Sadagopal et al., 2005).

Comparing the ability of GagTN and GagRT VLPs to enhance the elicited immune response, GagTN appeared to induce stronger CD4 and CD8 T-cell responses than GagRT, specifically to Gag. There are a few possible reasons as to why this occurred. Firstly, GagTN is a slightly smaller particulate antigen than GagRT, which may have caused GagTN to be more easily taken up by the immune system. Alternatively, it is possible that the Gag portion of each VLP folds differently, to accommodate the different accessory proteins. Hence, different Gag epitopes are exposed to the immune system and elicit varied immune responses. This is corroborated by the varying sizes of the chimaeric VLPs (Chapter 4), which suggest they are perhaps not folded in the same way. Another possibility, and perhaps the most likely, is that the weaker Gag response elicited by

GagRT VLPs could have been as a result of the immunodominant VYY RT epitope, which might have been preferentially recognised by the mouse immune system (Larke et al., 2007). Thus, even though both GagRT and GagTN VLPs elicited a boosted cellular immune response, and appeared to be good candidates for use as a potential HIV vaccine, GagTN did appear to be better at inducing stronger cellular immune responses.

In general, the IFN-γ and IL-2 assay data displayed high non-specific responses for the groups that received VLP inoculations compared to those that only received DNA inoculations. This is not uncommon when using VLPs produced in insect cells using the BEVS, and has been linked to induction of the innate response through the presence of PAMPs derived from insect cell/baculovirus matter (Deml et al., 2005; Ludwig and Wagner, 2007). This can be beneficial in that the PAMP recognition encourages the development of potent and broad Th1 cellular and humoral immune responses; however it is unknown whether the non-specific responses had a masking effect on the specificity of the responses detected, and is something to keep in mind for future studies.

In conclusion, while all the experiments reported here are preliminary studies, they do provide vital insight into the ability of these novel chimaeric VLPs to elicit an improved cellular immune response when used to boost a response primed by a DNA vaccine. It will be necessary in future studies to determine the functionality of the T cell responses induced, as research suggests that only when polyfunctional T cells are elicited, is the cellular immune response effective in controlling an HIV-1 infection (Pantaleo and Koup, 2004; Sadagopal et al., 2005). For this purpose, no ELIspot is available, but intracellular cytokine staining in conjunction with flow cytometry can be used to identify those cells that are able to produce both cytokines after *in vitro* stimulation.

CHAPTER 6

Conclusions

In the world we live in today, the HIV/AIDS pandemic is likely to be one of the most serious crises humanity faces. About 2 million people die from HIV annually, there are 33.2 million people living with HIV, and as many as 2.5 million being newly infected every year (UNAIDS, 2007). With such devastating statistics, it is imperative for research to focus on the development and production of a suitable vaccine to eradicate HIV. In this study the production of two novel HIV chimaeric VLP vaccine candidates were optimized and their ability to elicit an effective immune response in mice, when they were used as boost components in a prime-boost vaccine strategy, was evaluated.

6.1 Optimization of production of the chimaeric VLP constructs

Before our laboratories began research into chimaeric VLP design, no other studies documented the formation of VLPs with fusion proteins as large as RT (450 aa) and TN (322 aa) incorporated into a type-I in-frame VLP (Halsey et al.). The novel, multi-antigenic nature of the chimaeric VLPs, GagRT and GagTN, meant their use in a vaccine could potentially induce broader, stronger cellular immune responses than those observed for Gag-only VLPs. However, their expression in insect cells was not previously adequate to evaluate their immunogenic abilities. Thus, it was of great interest to optimize the production of these VLPs in insect cells (using BEVS) for two reasons. Firstly, relatively large quantities of the VLPs were required for mouse immunogenicity studies, and therefore the best conditions for VLP expression had to be determined to obtain maximum yields. Secondly, it was of some relevance to analyse which factors investigated had the greatest effect on chimaeric VLP production, as this information

could possibly be useful when producing other chimaeric proteins in the same expression system, as well as for potential commercial production of these VLPs in the future, if they are used as HIV vaccines.

Although the optimal conditions to produce the respective VLP constructs were similar, several of the findings from the experiments done were different to what was expected. It was interesting to find that cell density was so influential in the production of VLPs by the cells, regardless of the cell line used. Even more surprising was that MOI had such a negligible effect on the quantity or quality of VLPs produced. Whether these characteristics are unique to the production of these recombinant proteins or not, remains to be investigated, but it was clear from these experiments that the value for empirical research should not be underestimated or substituted by theoretical predictions.

The expression of VLPs in insect cells using BEVS was slightly problematic in terms of the yields that were obtained. Previous studies have documented as much as 5-20 mg/L of recombinant protein production using BEVS (Wagner et al., 1994a). In this study, almost 1000-fold less of the respective VLPs were being expressed, even after optimization. This problem could have been the result of three possibilities. The first is that the large size of the VLPs probably contributed to their poorer production in insect cells. This is corroborated by the fact that other studies have shown an inverse correlation between chimaeric VLP yields and the size of the incorporated foreign proteins (Luo et al., 1992). A second possibility is that actual budding of the VLPs may have been inefficient, leading to a large percentage of VLPs remaining in the cell lysate during purification. The western blot data of Chapter 3 does demonstrate that a fair amount of VLPs do remain in the cells despite the myristylation signal on Gag, and these are likely to be VLPs that budded into cytoplasmic vesicles, as opposed to out of the cell (Royer et al., 1991). To what extent this occurs is unknown, but it may be of interest to evaluate this by extracting VLPs from the cell lysate in addition to the cell culture supernatant in future studies. The last possibility is that the use of the Gag p24 ELISA drastically underestimated the amount of VLPs being isolated. While the ELISA is a common method to quantify HIV Gag VLPs, it is flawed in that the efficiency of the disruption of

HIV VLPs (to expose p24 molecules for detection purposes) is unknown. In addition, results can be skewed by the presence of Gag cleavage products. Because proteolytic processing of the VLPs in insect cells was believed to be partial (Cruz et al., 1999), and the dominant product of the chimaeric VLP expression in insect cells was the chimaeric VLPs, the Gag p24 ELISA was deemed adequate to determine the optimization conditions for VLP production. However, alternative methods such as western blot densitometry, need to be explored for this purpose, because even though it is a more labour intensive method, it is likely to be more reliable.

6.2 Immunogenicity of the respective Gag VLPs

Research has favoured the use of VLPs as boosting components because of their particulate nature and ability to present protein epitopes to the immune system in their native conformation (Deml, Wild, and Wagner, 2004). Although using VLPs as the boosting components has only recently been investigated, it has proven to elicit strong cellular and humoral immune responses. Of note, a low dose of HIV-1C Gag VLPs was able to boost significant Gag-specific T-cell immune responses in mice (Jaffray et al., 2004).

In this study, an impressive boost effect in the cellular immune response was observed when the respective VLPs were used as boost components to complement the pVRCgrttnC DNA vaccine in mice. The GagTN VLPs seemed to induce better cellular responses than GagRT, especially with respect to Gag-specific responses, possibly because the two proteins folded differently, and therefore different epitopes were processed and displayed by MHC class I and II molecules. GagTN was also a smaller VLP than GagRT, and was perhaps more easily taken up by APCs.

A strong Gag-specific CD4+ T-cell response was induced by both VLP constructs, but the Gag CD8+ T-cell response was weak in the GagRT experiment. While a strong CD4 response was not surprising given the exogenous nature of VLPs, the poor CD8 response to GagRT was disappointing because of what has been documented in previous studies

(Paliard et al., 2000; Wagner et al., 1994b) and the importance of a CTL response in combating HIV (Koup et al., 1994). Nonetheless, potent CD4 responses are also important to induce, since their involvement in the secretion of cytokines (such as IFN-γ and TNF-α) induces protective immunity, and they have a major role in stimulating and maintaining CTL responses (Kalams et al., 1999; Rosenberg et al., 1997). To improve the CD8 response to GagRT VLPs in future studies, it may be necessary to explore the use of adjuvants, as these have been used to enhance cellular immune responses in animal trials previously (Buonaguro et al., 2007; Deml et al., 2005; Jiang et al., 2006). Alternatively, future vaccine studies should focus on the use of GagTN VLPs as the boost candidate.

A positive CD8+ T-cell response to RT was detected when GagRT VLPs were employed as the boost components, suggesting that there was some CTL stimulation occurring. In addition, the presence of CD4+ T-cell responses to RT and Tat suggested that the cellular immune response induced was broader than what was seen with using the DNA vaccine alone. This requires further affirmation, especially because of the questionable nature of the selected peptides in the ELIspot assays, but it is indicative of a potentially successful elicitation of a varied CD4 and CD8 response, which could possibly protect individuals against more than one HIV strain.

The lack of induction of a Gag humoral response in these experiments suggests that the prime-boost strategy may need to be optimized, or vaccinated mice will need to be monitored over a longer period of time after inoculations. Ideally, an HIV vaccine should elicit a potent cellular immune response and strong antibody response, with the particular induction of NAbs, as these are believed to be responsible for conveying protective immunity (Doan et al, 2005). Thus, in order to do that with the current chimaeric VLPs, Env proteins could be incorporated onto the surface of the VLPs to form Type II chimaeric VLPs. Type II VLPs are known to induce both the cellular and humoral arms of the immune response effectively, and are able to elicit Nabs as well (Ludwig and Wagner, 2007). This is something that should be explored at a later stage.

6.3 Perspectives for the future

The work reported here has detailed the determination of the optimal conditions to produce the respective chimaeric VLPs in insect cells, as well as demonstrated the ability of these VLPs to elicit strong cellular immune responses in mice. In doing so, it has identified a coherent method of optimizing the production of recombinant proteins in an insect cell-baculovirus expression system, and the findings could theoretically be used as a guide for similar future studies when attempting to maximize the expression of other recombinant proteins. In addition, this study has elements which have the potential to be expanded in several directions.

In terms of the optimization experiments that were done, other factors such as cell passage number and medium type could be investigated as to their ability to influence the expression of the chimaeric VLPs. In addition, more of the parameters of the factors that were already investigated could also be explored to confirm the trends observed here. The range of MOI could be extended to include MOIs of 10 and 15, and more cell lines such as High Five™ insect cells, could be investigated.

With regard to the immunogenicity studies performed, they were able to clearly illustrate the significance of using a chimaeric VLP vaccine boost to induce strong cellular immune responses, and both VLP candidates appeared to have the potential as effective HIV vaccine candidates. Future immunogenicity studies can now explore a number of approaches, including combining different chimaeric VLPs as boost components, investigating alternative immunization strategies such as intranasal or vaginal route of inoculation, and using adjuvants in addition to the chimaeric VLPs to enhance the CTL response elicited. Furthermore, the ability of these VLPs to induce strong cellular immune responses against HIV in non-human primate experiments is worth investigating, given the potential demonstrated here.

APPENDICES

Contents

Appendix A: Baculovirus Expression Vector System

A1: Bac-to-Bac® Baculovirus Expression System

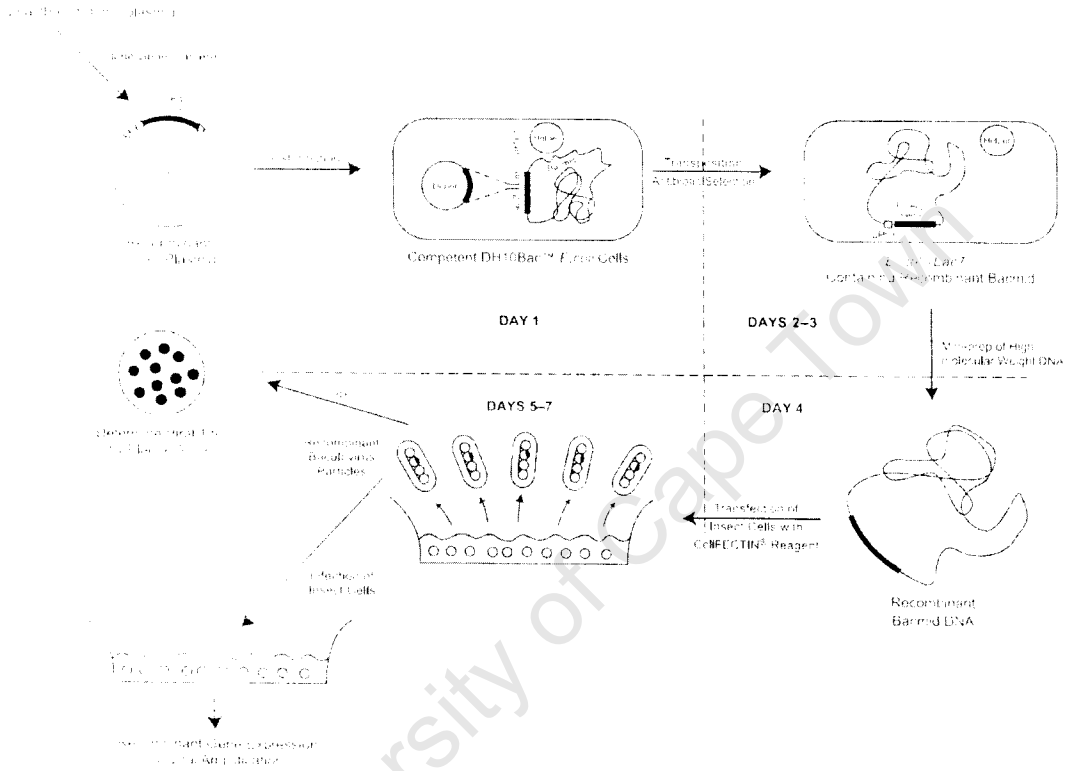


Figure A1 - The Bac-to-Bac® Baculovirus Expresion System (Invitrogen). The gene of interest is cloned into a pFastBac™ donor plasmid (Appendix A2) as a mini-Tn7 element controlled by the p10 or Polh promoter. The recombinant donor plasmid is transformed into competent *E. coli* DH10Bac™ cells containing a resident bacmid (with a mini-*att*Tn7 target site) and a helper plasmid. The bacmid *lacZα* gene compliments a *lac*Z delection on the bacterial chromosome to produce blue colonies in the presence of X-gal, and the inducer IPTG. The helper plasmid expresses transposition proteins (*in trans*) which assists the mini-Tn7 element in the donor vector to be transposed into the bacmid mini-*att*Tn7 target site. Successful transposition disrupts the *lacZα* gene and white colonies containing recombinant bacmids can be selected. Bacmid DNA is transfected into insect cells to generate recombinant baculovirus expressing the gene of interest (Invitrogen, 2004).

A2: pFastBac™ Dual vector map

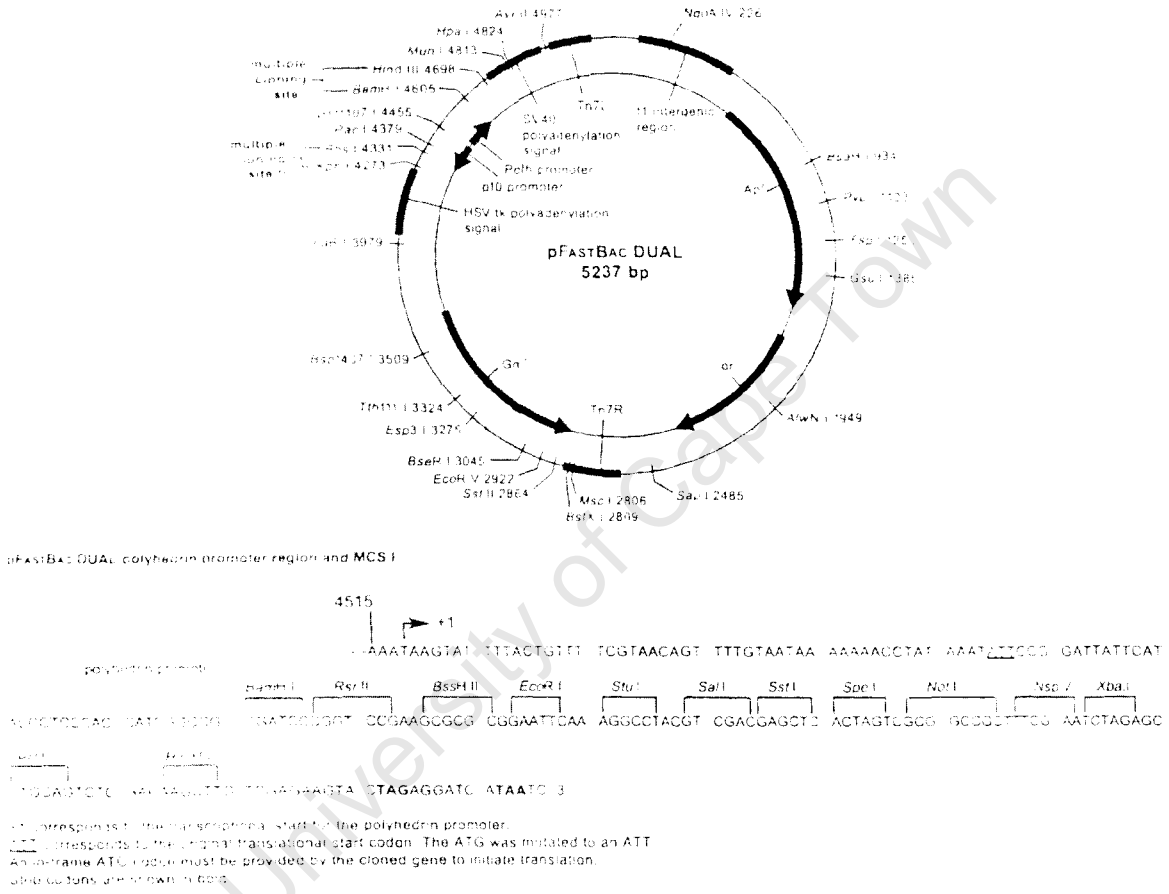


Figure A2 - pFastBac™Dual: vector map and pPolh MCS. This is a donor plasmid for the Bac-to-Bac® Baculovirus Expresion System, where the gene for a protein to be expressed by recombinant baculovirus in insect cell culture, is cloned into the MCS downstream of the Polh promoter.

Appendix B: Recipes for solutions and buffers used in this study

B1 – Bacterial strains and growth conditions

E. coli DH5α™ and DH10Bac™ (Invitrogen): Cultivated at 37 °C in Luria-Bertani (LB) broth under shaking conditions, and left to grow overnight.
LB agar plates: 1.5% Bacto Agar, Ampicillin (100 μg/ml) [IPTG (40 μg/ml) and X-gal (100 μg/ml) were added to the medium where necessary].

B2 - SDS-PAGE and Western blots

5x Sample loading buffer: 315 mM Tris-HCl, 10% (w/v) SDS, 40% glycerol, 40% 2-mercapto-ethanol, 0.025% bromophenol blue; pH6.8
1x Running buffer: 25 mM Tris-HCl, 200 mM glycine, 10 % SDS in dH_2O
Transfer buffer: 50 mM Tris base, 40 mM glycine, 20% (v/v) methanol; pH 9.2
Blocking buffer: 3% (w/v) BSA, 0.1% (v/v) Tween-20 in PBS
Washing buffer: 0.1% (v/v) Tween-20 in PBS

B3 – Coomassie Blue staining and destaining solutions

Coomassie brilliant blue staining solution: 40 % (w/v) Methanol, 7 % (w/v) glacial acetic acid, 0.1 g Coomassie Blue R in dH_2O
Detaining solution: 40 % (w/v) Methanol, 7 % (w/v) glacial acetic acid in dH_2O

B4 – rAcMNPV DNA extraction

DNA extraction buffer: 0.1 M Tris-HCl (pH 8.0), 10 mM EDTA, 1M KCl

B5 – Immunogenicity assays

B5.1 ELISPOT
Coating buffer: 1x PBS; pH 7.2
R10 medium: 88.9 % RPMI medium with Glutamax + 1 % Penicillin Streptomycin + 10 % FBS + 0.1 % 2-ME (Sigma)
Blocking solution: 88.9 % RPMI medium with Glutamax + 1 % Penicillin Streptomycin + 10 % FBS + 0.1 % 2-ME
Wash buffer I: 0.05 % Tween-20 in PBS; pH 7.4
Wash buffer II: 1x PBS; pH 7.4

Appendix C: Sequence data confirming intact genes in constructs

C1 – Alignment of DNA sequence for *gag* gene in GagTN construct

Forward sequence:

```
Upper line: pFBD-HMgagC, from 4438 to 5427
Lower line: gTN4_FM13, from 1 to 900
FBD-HMgagC:gTN4_FM13 identity= 93.11%(838/900) gap=9.09%(90/990)

4438  GGGTTGAATTAAAGGTCCGTATACTCCGGAATATTAATAGATCATGGAGATAATTAAAAT
      ||| |          |   ||||||  |  | |||     ||    ||    |  |   ||
1     GGGTTASMRGCCGTGATTGTATACGACTCACTATAGGGCGAATTGGGCCCGACGTCGCAT
4498  GATAACCATCTCGCAAATAAATAAGTATTTTACTGTTTTCGTAACAGTTTTGTAATAAAA
      |
61    GC............................................................
4558  AAACCTATAAATATTCCGGATTATTCATACCGTCCCACCATCGGGCGCGGATCCAAGCTT
                                       ||||  |  |   ||||  |  |  |
63    ..............................TCCCGGCCGCCATGGCGGCCGCGGGAAT
4618  GCCACCATGGGTGCTCGCGCATCTATCCTCAGAGGCGAAAAGTTGGATAAGTGGGAAAAA
       | |  ||||||||||||||||||||||||||||||||||||||||||||||||||||||
91    TCGATTATGGGTGCTCGCGCATCTATCCTCAGAGGCGAAAAGTTGGATAAGTGGGAAAAA
4678  ATCAGACTCAGGCCAGGAGGTAAAAAACACTACATGCTGAAGCATATCGTGTGGGCATCT
      ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
151   ATCAGACTCAGGCCAGGAGGTAAAAAACACTACATGCTGAAGCATATCGTGTGGGCATCT

4738  AGGGAGTTGGAGAGATTTGCACTGAACCCCGGACTGCTGGAAACCTCAGAGGGCTGTAAG
      ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
211   AGGGAGTTGGAGAGATTTGCACTGAACCCCGGACTGCTGGAAACCTCAGAGGGCTGTAAG

4798  CAAATCATGAAACAGCTCCAACCAGCCTTGCAGACCGGAACAGAAGAGCTGAAGTCCCTT
      ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
271   CAAATCATGAAACAGCTCCAACCAGCCTTGCAGACCGGAACAGAAGAGCTGAAGTCCCTT

4858  TACAATACCGTGGCAACCCTCTATTGCGTCCACGAGAAGATCGAGGTGAGAGACACAAAG
      ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
331   TACAATACCGTGGCAACCCTCTATTGCGTCCACGAGAAGATCGAGGTGAGAGACACAAAG

4918  GAGGCCCTGGACAAAATCGAGGAGGAGCAGAATAAGTGCCAGCAGAAGACCCAGCAGGCA
      ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
391   GAGGCCCTGGACAAAATCGAGGAGGAGCAGAATAAGTGCCAGCAGAAGACCCAGCAGGCA

4978  AAGGCTGCTGACGGAAAGGTCTCTCAGAACTATCCTATCGTTCAGAACCTTCAGGGGCAG
      ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
451   AAGGCTGCTGACGGAAAGGTCTCTCAGAACTATCCTATCGTTCAGAACCTTCAGGGGCAG

5038  ATGGTGCACCAAGCAATCAGCCCTAGAACCCTGAACGCATGGGTGAAGGTGATCGAGGAG
      ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
511   ATGGTGCACCAAGCAATCAGCCCTAGAACCCTGAACGCATGGGTGAAGGTGATCGAGGAG

5098  AAAGCCTTTTCTCCCGAGGTTATCCCCATGTTTACCGCCCTGAGCGAAGGCGCCACTCCT
      ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
571   AAAGCCTTTTCTCCCGAGGTTATCCCCATGTTTACCGCCCTGAGCGAAGGCGCCACTCCT

5158  CAAGACCTGAACACTATGCTGAACACAGTGGGAGGACACCAGGCCGCTATGCAGATGTTG
```

```
        ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
641     CAAGACCTGAACACTATGCTGAACACAGTGGGAGGACACCAGGCCGCTATGCAGATGTTG

5218    AAGGATACCATCAACGAGGAGGCAGCCGAATGGGACCGCCTCCACCCCGTGCACGCCGGA
        ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
691     AAGGATACCATCAACGAGGAGGCAGCCGAATGGGACCGCCTCCACCCCGTGCACGCCGGA

5276    CCTATCGCCCCCGGACAAATGAGAGAACCTCGCGGAAGTGATATTGCCGGTACTACCAGC
        ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
751     CCTATCGCCCCCGGACAAATGAGAGAACCTCGCGGAAGTGATATTGCCGGTACTACCAGC

5338    ACCCTTCAAGAGCAGATTGCTTGGATGACCAGCAACCCACCCATCCCAGTGGGCGATATT
        ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||| ||
811     ACCCTTCAAGAGCAGATTGCTTGGATGACCAGCAACCCACCCATCCCAGTGGGCGATATT

5398    TACAAAAGGTGGATTATTCTGGGGCTGAAC
        |||||||  |  |  ||||||||||||||
871     TACAAAAGKGGATTATTTCTGGGGCTGAAC
```

Reverse sequence:

```
Upper line: RC-pFBD-HMgagC, from 361 to 1382
Lower line: gTN4_RM13, from 10 to 896
RC-pFBD-HMgagC:gTN4_RM13 identity= 90.59%(809/893) gap=13.64%(141/1034)

352     TGTGAAATTTGTGATGCTATTGCTTTATTTGTAACCATTATAAGCTGCAATAAACAAGTT
              |  .|    || |    |||| ||  || ||   |  ||  |
1       AAARKCAMATGATTCGCCAGCTATTTAGGTGACACTATAGAATACTCAA...........
412     AACAACAACAATTGCATTCATTTTATGTTTCAGGTTCAGGGGGAGGTGTGGGAGGTTTTT

50      ..................................................................
472     TAAAGCAAGTAAAACCTCTACAAATGTGGTATGGCTGATTATGATCCTCTAGTACTTCTC
                                        |||    ||    | |        |||
50      ................................GCTATGCATCCAACGCGTTGGGAGCTC
532     GACAAGCTTGTCGAGACTGCAGGCTCTAGATTCGAAAGCGGCCGCGACTAGTGAGCTCGT
          | |  | ||||                                            ||   |
77      TCCCATATGGTCGACCTGC..............................AGGCGGC
592     CGACGTAGGCCTTTGAATTCTTATTGGCTGAGGGGGTCGCTACCAAAGAGGCTTTTGAGA
        ||      ||    ||| || ||||||||||||||||||||||||||||||||||||||||
103     CGCGAATTCACTAGTGATTATTCTTGGCTGAGGGGGTCGCTACCAAAGAGGCTTTTGAGA

652     CTGGTGAGAGGCTCTCTCTCAATTGGCTCCTGCTTTGGAGCGGGGGGTGGTCTCCTCAAAT
        ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
163     CTGGTGAGAGGCTCTCTCTCAATTGGCTCCTGCTTTGGAGCGGGGGGTGGTCTCCTCAAAT

712     CTGAAAGACTCAGCGGGGGGGGCGGTAGGCTCGGGTCTGTTTTGAAGGAAGTTGCCAGGG
        ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
223     CTGAAAGACTCAGCGGGGGGGGCGGTAGGCTCGGGTCTGTTTTGAAGGAAGTTGCCAGGG

772     CGGCCCTTATGACTGGGCCAAATCTTTCCCAGGAAGTTTGCTTGGCGCTCGGTGCAATCC
        ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
283     CGGCCCTTATGACTGGGCCAAATCTTTCCCAGGAAGTTTGCTTGGCGCTCGGTGCAATCC

832     TTCATCTGGTGGCCCTCTTTGCCACACTTCCAGCAGCCTTTCTTCCTGGGGGCGCGGCAG
        ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
```

```
343   TTCATCTGGTGGCCCTCTTTGCCACACTTCCAGCAGCCTTTCTTCCTGGGGGCGCGGCAG

892   TTCCTGGCAATGTGACCCTCCTTGCCACAATTGAAGCACTTGACGATTCTCCTGGGACCC
      ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
403   TTCCTGGCAATGTGACCCTCCTTGCCACAATTGAAGCACTTGACGATTCTCCTGGGACCC

952   TTAAAGTTACTCCTCTGCATCATGATATTGCCTGAGTTTGTCTGGCTCATGGCCTCAGCG
      ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
463   TTAAAGTTACTCCTCTGCATCATGATATTGCCTGAGTTTGTCTGGCTCATGGCCTCAGCG

1012  AGAACTCTGGCCTTGTGCCCAGGTCCTCCCACGCCTTGACATGCTGTCATCATTTCCTCA
      ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
523   AGAACTCTGGCCTTGTGCCCAGGTCCTCCCACGCCTTGACATGCTGTCATCATTTCCTCA

1072  AGGGTGGCACCTGGACCGAGAGCTCTCAAGATGGTTTTGCAATCGGGGTTAGCGTTTTGC
      ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
583   AGGGTGGCACCTGGACCGAGAGCTCTCAAGATGGTTTTGCAATCGGGGTTAGCGTTTTGC

1132  ACGAGCAGAGTATCTGTCATCCAGTTCTTAACCTCCTGAGTGGCTTGCTCAGCTCTAAGG
      ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
643   ACGAGCAGAGTATCTGTCATCCAGTTCTTAACCTCCTGAGTGGCTTGCTCAGCTCTAAGG

1192  GTTTTGAAGAATCTGTCCACGTAATCCCTAAAAGGCTCCTTGGGTCCTTGGCGGATGTCG
      ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
703   GTTTTGAAGAATCTGTCCACGTAATCCCTAAAAGGCTCCTTGGGTCCTTGGCGGATGTCG

1252  AGGATGGAGACGGGGGAGTACATTCTCACAATTTTGTTCAGCCCCAGAATAATCCACCTT
      ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
763   AGGATGGAGACGGGGGAGTACATTCTCACAATTTTGTTCAGCCCCAGAATAATCCACCTT

1312  TTGTAAATATCGCCCACTGGGATGGGTGGGTTGCTGGTCATCCAAGCAATCTG...CTCT
      ||||||||||||||||||||||||||||||||||||||||||||||||||     || |
823   TTGTAAATATCGCCCACTGGGATGGGTGGGTTGCTGGTCATCCAAGCAATCCTGCTCTTT
1369  TGAAGGGTGCTGGT
        | ||  ||||
883   GAAGGGKKGCTGGG
```

C2 – Alignment of DNA sequence for *gag* gene in GagRT construct

Forward sequence:

```
Upper line: pFBD-HMgagC, from 4532 to 5432
Lower line: gRT3_FM13, from 2 to 907
pFBD-HMgagC:gRT3_FM13 identity= 92.90%(838/902) gap=0.55%(5/907)

4531  TGTTTTCGTAACAGTTTTGTAATAAAAAAACCTATAAATATTCCGGATTAT....TCATA
      | ||  |   | ||  |   |||  |    |||||         |         ||  |
1     GGKTTYSGAMGCMGTGATTGTATACGACTCACTATAGGGCGAATTGGGCCCGACGTCGCA

4587  CCGTCCCACCATCGGGCGCGGATCCAAGCTTGCCACCATGGGTGCTCGCGCATCTATCCT
        ||||  |  |    ||||   |  |  | | |  ||||||||||||||||||||||
61    TGCTCCCGGCCGCCATGGCGGCCGCGGGAATTCGATTATGGGTGCTCGCGCATCTATCCT


4647  CAGAGGCGAAAAGTTGGATAAGTGGGAAAAAATCAGACTCAGGCCAGGAGGTAAAAAACA
      ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
```

```
121    CAGAGGCGAAAAGTTGGATAAGTGGGAAAAAATCAGACTCAGGCCAGGAGGTAAAAAACA

4787   CTACATGCTGAAGCATATCGTGTGGGCATCTAGGGAGTTGGAGAGATTTGCACTGAACCC
       ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
181    CTACATGCTGAAGCATATCGTGTGGGCATCTAGGGAGTTGGAGAGATTTGCACTGAACCC

4787   CGGACTGCTGGAAACCTCAGAGGGCTGTAAGCAAATCATGAAACAGCTCCAACCAGCCTT
       ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
241    CGGACTGCTGGAAACCTCAGAGGGCTGTAAGCAAATCATGAAACAGCTCCAACCAGCCTT

4827   GCAGACCGGAACAGAAGAGCTGAAGTCCCTTTACAATACCGTGGCAACCCTCTATTGCGT
       ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
301    GCAGACCGGAACAGAAGAGCTGAAGTCCCTTTACAATACCGTGGCAACCCTCTATTGCGT

4887   CCACGAGAAGATCGAGGTGAGAGACACAAAGGAGGCCCTGGACAAAATCGAGGAGGAGCA
       ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
361    CCACGAGAAGATCGAGGTGAGAGACACAAAGGAGGCCCTGGACAAAATCGAGGAGGAGCA

4947   GAATAAGTGCCAGCAGAAGACCCAGCAGGCAAAGGCTGCTGACGGAAAGGTCTCTCAGAA
       ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
421    GAATAAGTGCCAGCAGAAGACCCAGCAGGCAAAGGCTGCTGACGGAAAGGTCTCTCAGAA

5007   CTATCCTATCGTTCAGAACCTTCAGGGGCAGATGGTGCACCAAGCAATCAGCCCTAGAAC
       ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
481    CTATCCTATCGTTCAGAACCTTCAGGGGCAGATGGTGCACCAAGCAATCAGCCCTAGAAC

5067   CCTGAACGCATGGGTGAAGGTGATCGAGGAGAAAGCCTTTTCTCCCGAGGTTATCCCCAT
       |||||||||||||||||  ||||||||||||||||||||||||||||||||||||||||
541    CCTGAACGCATGGGTGAGGGTGATCGAGGAGAAAGCCTTTTCTCCCGAGGTTATCCCCAT

5127   GTTTACCGCCCTGAGCGAAGGCGCCACTCCTCAAGACCTGAACACTATGCTGAACACAGT
       ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
601    GTTTACCGCCCTGAGCGAAGGCGCCACTCCTCAAGACCTGAACACTATGCTGAACACAGT

5187   GGGAGGACACCAGGCCGCTATGCAGATGTTGAAGGATACCATCAACGAGGAGGCAGCCGA
       ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
661    GGGAGGACACCAGGCCGCTATGCAGATGTTGAAGGATACCATCAACGAGGAGGCAGCCGA

5247   ATGGGACCGCCTCCACCCCGTGCACGCCGGACCTATCGCCCCCCGGACAAATGAGAGAACC
       ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
721    ATGGGACCGCCTCCACCCCGTGCACGCCGGACCTATCGCCCCCCGGACAAATGAGAGAACC

5307   TCGCGGAAGTGATATTGCCGGTACTACCAGCACCCTTCAAGAGCAGATTGCTTGGATGAC
       ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
781    TCGCGGAAGTGATATTGCCGGTACTACCAGCACCCTTCAAGAGCAGATTGCTTGGATGAC

5367   CAGCAACCCACCCATCCCAGTGGGCGATATTTAC.AAAAGGTGGATTATTCTGGGGCTGA
       |||||||||||||||||||||||||||||||||| |||||  ||||||||||||||||||
841    CAGCAACCCACCCATCCCAGTGGGCGATATTTACAAAAAGKKGGATTATTCTGGGGCTGA

5426   ACAAAAT
       |  |||
901    AACAAAA
```

Reverse sequence:

```
Upper line: RC-pFBD-HMgagC, from 505 to 1377
Lower line: gRT3_RM13, from 16 to 886

RC-pFBD-HMgagC:gRT3_RM13 identity= 89.05%(789/886) gap=0.23%(2/888)
490   TACAAATGTGGTATGGCTGATTATGATCCTCTAGTACTTCTCGACAAGCTTGTCGAGACT
        |   | |  | ||    |||     |       |      ||         |   |
1     AAAKGACATGCATTMGCCAGCTATTTAGGTGACACTATAGAATACTCAAGCTATGCATCC

550   GCAGGCTCTAGATTCGAAAGCGGCCGCGACTAGTGAGCTCGTCGACGTAGGCCTTTGAAT
        |  |  ||               |         ||   | ||        ||    ||
61    AACGCGTTGGGAGCTCTCCCATATGGTCGACCTGCAGGCGGCCGCGAATTCACTAGTGAT

610   TCTTATTGGCTGAGGGGGTCGCTACCAAAGAGGCTTTTGAGACTGGTGAGAGGCTCTCTC
      | ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
121   TATTCTTGGCTGAGGGGGTCGCTACCAAAGAGGCTTTTGAGACTGGTGAGAGGCTCTCTC

670   TCAATTGGCTCCTGCTTTGGAGCGGGGGTGGTCTCCTCAAATCTGAAAGACTCAGCGGGG
      ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
181   TCAATTGGCTCCTGCTTTGGAGCGGGGGTGGTCTCCTCAAATCTGAAAGACTCAGCGGGG

730   GGGGCGGTAGGCTCGGGTCTGTTTTGAAGGAAGTTGCCAGGGCGGCCCTTATGACTGGGC
      ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
241   GGGGCGGTAGGCTCGGGTCTGTTTTGAAGGAAGTTGCCAGGGCGGCCCTTATGACTGGGC

790   CAAATCTTTCCCAGGAAGTTTGCTTGGCGCTCGGTGCAATCCTTCATCTGGTGGCCCTCT
      ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
301   CAAATCTTTCCCAGGAAGTTTGCTTGGCGCTCGGTGCAATCCTTCATCTGGTGGCCCTCT

850   TTGCCACACTTCCAGCAGCCTTTCTTCCTGGGGGCGCGGCAGTTCCTGGCAATGTGACCC
      ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
361   TTGCCACACTTCCAGCAGCCTTTCTTCCTGGGGGCGCGGCAGTTCCTGGCAATGTGACCC

910   TCCTTGCCACAATTGAAGCACTTGACGATTCTCCTGGGACCCTTAAAGTTACTCCTCTGC
      ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
421   TCCTTGCCACAATTGAAGCACTTGACGATTCTCCTGGGACCCTTAAAGTTACTCCTCTGC

970   ATCATGATATTGCCTGAGTTTGTCTGGCTCATGGCCTCAGCGAGAACTCTGGCCTTGTGC
      ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
481   ATCATGATATTGCCTGAGTTTGTCTGGCTCATGGCCTCAGCGAGAACTCTGGCCTTGTGC

1030  CCAGGTCCTCCCACGCCTTGACATGCTGTCATCATTTCCTCAAGGGTGGCACCTGGACCG
      ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
541   CCAGGTCCTCCCACGCCTTGACATGCTGTCATCATTTCCTCAAGGGTGGCACCTGGACCG

1090  AGAGCTCTCAAGATGGTTTTGCAATCGGGGTTAGCGTTTTGCACGAGCAGAGTATCTGTC
      |||||||||||||||||||||||||||||| ||||||||||||||||||||||||||||
601   AGAGCTCTCAAGATGGTTTTGCAATCGGGGTCAGCGTTTTGCACGAGCAGAGTATCTGTC

1150  ATCCAGTTCTTAACCTCCTGAGTGGCTTGCTCAGCTCTAAGGGTTTTGAAGAATCTGTCC
      ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
661   ATCCAGTTCTTAACCTCCTGAGTGGCTTGCTCAGCTCTAAGGGTTTTGAAGAATCTGTCC

1210  ACGTAATCCCTAAAAGGCTCCTTGGGTCCTTGGCGGATGTCGAGGATGGAGACGGGGGAG
      |||||||||||||| |||||||||||||||||||||||||||||||||| ||||||
```

```
721    ACGTAATCCCTAAAGGGCTCCTTGGGTCCTTGGCGGATGTCGAGGATGGAGAC.GGGGAG

1270   TACATTCTCACAATTTTGTTCAGCCCCAGAATAATCCACCTTTTGTAAATATCGCCCACT
       ||||||||||||||||||||||| |||||||||||||||||||||||||||||||||||
760    TACATTCTCACAATTTTGTTCAG.CCCAGAATAATCCACCTTTTGTAAATATCGCCCACT

1330   GGGATGGGTGGGTTGCTGGTCATCCAAGCAATCTGCTCTTGAAGGGTG
       |||||||| |||||||||||||||||||||||||||||||||| ||
839    GGGATGGGTGGGTTGCTGGTCATCCAAGCAATCTGCTCTTGAAAGGKK
```

C3 – Alignment of DNA sequence for *TN* gene in GagTN construct

Forward sequence:

```
Upper line: RC-HMgagTN, from 1 to 735
Lower line: GagTN3-M13R, from 116 to 850

RC-HMgagTN:GagTN3-M13R identity= 99.86%(734/735) gap=0.00%(0/735)
1      TCAGTCCTTGTAGTACTCGGGGTGCTTCTCGCGGGCCAGGTGGCGGCGGGCCAGGCTGCT
       |||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
116    TCAGTCCTTGTAGTACTCGGGGTGCTTCTCGCGGGCCAGGTGGCGGCGGGCCAGGCTGCT

61     GTCGAACACCCAGCGCAGCACCTCGCGGTCGGCGTCCTCCATGCCGTGCTGGCTCATGGG
       |||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
176    GTCGAACACCCAGCGCAGCACCTCGCGGTCGGCGTCCTCCATGCCGTGCTGGCTCATGGG

121    GTGCAGCAGGCAGTTGTTCTCGCCCTTGTTGGCCTCCTCCACCTCGCGGGGGTCCACGGG
       |||||||||||||||||||||||| |||||||||||||||||||||||||||||||||||
236    GTGCAGCAGGCAGTTGTTCTCGCCCTCGTTGGCCTCCTCCACCTCGCGGGGGTCCACGGG

181    CACCAGCTTGAAGCACCAGCCGAAGGTCAGGGGGTAGCGCACGCCGGGGCCGGGGGTGTA
       |||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
296    CACCAGCTTGAAGCACCAGCCGAAGGTCAGGGGGTAGCGCACGCCGGGGCCGGGGGTGTA

241    GTTCTGCCAGTCGGGGAAGTAGCCCTGGGTGTGGTACACCCACAGGTCCAGGATGTCCTG
       |||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
356    GTTCTGCCAGTCGGGGAAGTAGCCCTGGGTGTGGTACACCCACAGGTCCAGGATGTCCTG

301    GCGGCGCTTGCTGTGGATCAGGCCCTCCAGGCCGCCCTTCTCCTTCAGGAAGAAGCTCAG
       |||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
416    GCGGCGCTTGCTGTGGATCAGGCCCTCCAGGCCGCCCTTCTCCTTCAGGAAGAAGCTCAG

361    GTCGAAGGCGGCCTTGTAGGTCATGGGGCGCAGGGGCACCTGGGGGCGCACGGGGAAGCC
       |||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
476    GTCGAAGGCGGCCTTGTAGGTCATGGGGCGCAGGGGCACCTGGGGGCGCACGGGGAAGCC

421    CACGTCCTCCTCCTCCTCCTGGGCCTGCAGCCAGGCGCAGTCGGGGTTGTTGTGGGCGGT
       |||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
536    CACGTCCTCCTCCTCCTCCTGGGCCTGCAGCCAGGCGCAGTCGGGGTTGTTGTGGGCGGT

481    GTTGCTGCTGGTCAGGGCGCCGTGCTTGTCCAGGTCCTGGCTGGCGGGGCCCACGCCCTC
       |||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
596    GTTGCTGCTGGTCAGGGCGCCGTGCTTGTCCAGGTCCTGGCTGGCGGGGCCCACGCCCTC

541    GGCGGCGGGCTCGGTGCGGCGGATGCGCTCGCGCACGGCGGGCCAGCCCACCACGAGGCA
       |||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
```

```
656   GGCGGCGGGCTCGGTGCGGCGGATGCGCTCGCGCACGGCGGGCCAGCCCACCACGAGGCA

601   GTGGTAGGAGCAGTATTTGCAGTAGCACTTGTTGCAGGGGGTGTTGGGCTGGCTGCCGGG
      |||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
716   GTGGTAGGAGCAGTATTTGCAGTAGCACTTGTTGCAGGGGGTGTTGGGCTGGCTGCCGGG

661   GTGGTTCCAGGGCTCCAGGTTGGGGTCGATGGGCTCCATCCGTTTCTTGCGCCCGTAGGA
      |||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
776   GTGGTTCCAGGGCTCCAGGTTGGGGTCGATGGGCTCCATCCGTTTCTTGCGCCCGTAGGA

721   GATGCCCAGGCCCTT
      |||||||||||||||
836   GATGCCCAGGCCCTT
```

Reverse sequence:

```
Upper line: HMgagTN, from 1310 to 2271
Lower line: GagTN3-M13F, from 2 to 850

HMgagTN:GagTN3-M13F identity= 95.63%(809/846) gap=12.51%(121/967)
1429  CCTCTCACCAGTCTCAAAAGCCTCTTTGGTAGCGACCCCCTCAGCCAAGAATTCAAAGG.
                                 || |  || || ||  ||         ||
30    ........................TGCTCCCGGCCGCCATGGCGGCCGCGGGAATTCG

1488  ...CATGGTGGGCATCAGCTACGGCCGCAAGAAGCGCCGCCAGCGCCGCAGCACCCCGCC
         ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
64    ATTCATGGTGGGCATCAGCTACGGCCGCAAGAAGCGCCGCCAGCGCCGCAGCACCCCGCC

1545  CAGCAGCGAGGACCACCAGAACCCCATCAGCAAGCAGCCCCTGCCCCAGACCCGCGGCGA
      ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
124   CAGCAGCGAGGACCACCAGAACCCCATCAGCAAGCAGCCCCTGCCCCAGACCCGCGGCGA

1605  CCCCACCGGCAGCGAGGAGAGCAAGAAGAAGGTGGAGAGCAAGACCAAGACCGACCCCTT
      ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
184   CCCCACCGGCAGCGAGGAGAGCAAGAAGAAGGTGGAGAGCAAGACCAAGACCGACCCCTT

1665  CGACTGCAAGTACTGCAGCTACCACTGTCTGGTGTGCTTCCAGACCAAGGGCCTGGGCAT
      ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
244   CGACTGCAAGTACTGCAGCTACCACTGTCTGGTGTGCTTCCAGACCAAGGGCCTGGGCAT

1725  CTCCTACGGGCGCAAGAAACGGATGGAGCCCATCGACCCCAACCTGGAGCCCTGGAACCA
      ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
304   CTCCTACGGGCGCAAGAAACGGATGGAGCCCATCGACCCCAACCTGGAGCCCTGGAACCA

1785  CCCCGGCAGCCAGCCCAACACCCCCTGCAACAAGTGCTACTGCAAATACTGCTCCTACCA
      ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
364   CCCCGGCAGCCAGCCCAACACCCCCTGCAACAAGTGCTACTGCAAATACTGCTCCTACCA

1845  CTGCCTCGTGGTGGGCTGGCCCGCCGTGCGCGAGCGCATCCGCCGCACCGAGCCCGCCGC
      ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
424   CTGCCTCGTGGTGGGCTGGCCCGCCGTGCGCGAGCGCATCCGCCGCACCGAGCCCGCCGC

1905  CGAGGGCGTGGGCCCCGCCAGCCAGGACCTGGACAAGCACGGCGCCCTGACCAGCAGCAA
      ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
484   CGAGGGCGTGGGCCCCGCCAGCCAGGACCTGGACAAGCACGGCGCCCTGACCAGCAGCAA
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1965  CACCGCCCACAACAACCCCGACTGCGCCTGGCTGCAGGCCCAGGAGGAGGAGGAGGACGT
      ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
544   CACCGCCCACAACAACCCCGACTGCGCCTGGCTGCAGGCCCAGGAGGAGGAGGAGGACGT

2025  GGGCTTCCCCGTGCGCCCCCAGGTGCCCCTGCGCCCCATGACCTACAAGGCCGCCTTCGA
      ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
604   GGGCTTCCCCGTGCGCCCCCAGGTGCCCCTGCGCCCCATGACCTACAAGGCCGCCTTCGA

2085  CCTGAGCTTCTTCCTGAAGGAGAAGGGCGGCCTGGAGGGCCTGATCCACAGCAAGCGCCG
      ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
664   CCTGAGCTTCTTCCTGAAGGAGAAGGGCGGCCTGGAGGGCCTGATCCACAGCAAGCGCCG

2145  CCAGGACATCCTGGACCTGTGGGTGTACCACACCCAGGGCTACTTCCCCGACTGGCAGAA
      ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
724   CCAGGACATCCTGGACCTGTGGGTGTACCACACCCAGGGCTACTTCCCCGACTGGCAGAA

2205  CTACACCCCCGGCCCCGGCGTGCGCTACCCCCTGACCTTCGGCTGGTGCTTCAAGCTGGT
      ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
784   CTACACCCCCGGCCCCGGCGTGCGCTACCCCCTGACCTTCGGCTGGTGCTTCAAGCTGGT

2265  GCCCGTG
      |||||||
844   GCCCGTG
```

C4 – Alignment of DNA sequence for *RT* gene in GagRT construct

Forward sequence:

```
Upper line: HMgagRT, from 1561 to 2429
Lower line: GAGRT9_M13F, from 31 to 899

HMgagRT:GAGRT9_M13F identity= 91.88%(826/899) gap=0.00%(0/899)
1531  AACATCATCGGCCGGAACATGCTGACCCAGCTGGGCTGCACCCTGAACTTCCCCCATCAGC
                | | ||  |     |  ||    ||    ||  |    |
1     CGKTAMGAGGTCGTGATTGTATACGACTCACTATAGGGCGAATTGGGCCCGACGTCGCAT

1591  CCCATCGAGACCGTGCCCGTGAAGCTGAAGCCCGGCATGGACGGCCCCAAGGTGAAGCAG
        |   ||  |     ||       |||  |    ||||||||||||||||||||||||
61    GCTCCCGGCCGCCATGGCGGCCGCGGGAATTCGATTATGGACGGCCCCAAGGTGAAGCAG

1651  TGGCCCCTGACCGAGGTGAAGATCAAGGCCCTGACCGCCATCTGCGAGGAGATGGAGAAG
      ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
121   TGGCCCCTGACCGAGGTGAAGATCAAGGCCCTGACCGCCATCTGCGAGGAGATGGAGAAG

1711  GAGGGCAAGATCACCAAGATCGGCCCCGAGAACCCCTACAACACCCCCATCTTCGCCATC
      ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
181   GAGGGCAAGATCACCAAGATCGGCCCCGAGAACCCCTACAACACCCCCATCTTCGCCATC

1771  AAGAAGGAGGACAGCACCAAGTGGCGGAAGCTGGTGGACTTCCGGGAGCTGAACAAGCGG
      ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
241   AAGAAGGAGGACAGCACCAAGTGGCGGAAGCTGGTGGACTTCCGGGAGCTGAACAAGCGG

1831  ACCCAGGACTTCTGGGAGGTGCAGCTGGGCATCCCCCACCCCGCCGGCCTGAAGAAGAAG
      ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
301   ACCCAGGACTTCTGGGAGGTGCAGCTGGGCATCCCCCACCCCGCCGGCCTGAAGAAGAAG
```

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1891  AAGAGCGTGACCGTGCTGGACGTGGGCGACGCCTACTTCAGCGTGCCCCTGGACGAGGGC
      ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
361   AAGAGCGTGACCGTGCTGGACGTGGGCGACGCCTACTTCAGCGTGCCCCTGGACGAGGGC

1951  TTCCGGAAGTACACCGCCTTCACCATCCCCAGCATCAACAACGAGACCCCCGGCATCCGG
      ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
421   TTCCGGAAGTACACCGCCTTCACCATCCCCAGCATCAACAACGAGACCCCCGGCATCCGG

2011  TACCAGTACAACGTGCTGCCCCAGGGCTGGAAGGGCAGCCCCGCCATCTTCCAGGCCAGC
      ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
481   TACCAGTACAACGTGCTGCCCCAGGGCTGGAAGGGCAGCCCCGCCATCTTCCAGGCCAGC

2071  ATGACCAAGATCCTGGAGCCCTTCCGGGCCAAGAACCCCGAGATCGTGATCTACCAGTAC
      ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
541   ATGACCAAGATCCTGGAGCCCTTCCGGGCCAAGAACCCCGAGATCGTGATCTACCAGTAC

2131  ATGGCCGCCCTGTACGTGGGCAGCGACCTGGAGATCGGCCAGCACCGGGCCAAGATCGAG
      ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
601   ATGGCCGCCCTGTACGTGGGCAGCGACCTGGAGATCGGCCAGCACCGGGCCAAGATCGAG

2191  GAGCTGCGGGAGCACCTGCTGAAGTGGGGCTTCACCACCCCCGACAAGAAGCACCAGAAG
      |||||||||||||||||||| ||||||||||||||||||||||||||||||||||||||
661   GAGCTGCGGGAGCACCTGCTGGAGTGGGGCTTCACCACCCCCGACAAGAAGCACCAGAAG

2251  GAGCCCCCCTTCCTGTGGATGGGCTACGAGCTGCACCCCGACAAGTGGACCGTGCAGCCC
      ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
721   GAGCCCCCCTTCCTGTGGATGGGCTACGAGCTGCACCCCGACAAGTGGACCGTGCAGCCC

2311  ATCCAGCTGCCCGAGAAGGACAGCTGGACCGTGAACGACATCCAGAAGCTGGTGGGCAAG
      ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
781   ATCCAGCTGCCCGAGAAGGACAGCTGGACCGTGAACGACATCCAGAAGCTGGTGGGCAAG

2371  CTGAACTGGACCAGCCAGATCTACCCCGGCATCAAGGTGCGGCAGCTGTGCAAGCTGCT
      ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
841   CTGAACTGGACCAGCCAGATCTACCCCGGCATCAAGGTGCGGCAGCTGTGCAAGCTGCT
```

Reverse sequence:

```
Upper line: RC-HMgagRT, from 1 to 758
Lower line: GAGRT9_M13R, from 123 to 880

RC-HMgagRT:GAGRT9_M13R identity= 99.60%(755/758) gap=0.00%(0/758)
1     CTAGATTCGAAAGCGGCCGCTGTTCACGAACTCCCACTCGGGGATCCAGGTGGCCTGCCA
      |||||||||||||||||| ||||||||||||||||||||||||||||||||||||||||
123   CTAGATTCGAAAGCGGCCGTTGTTCACGAACTCCCACTCGGGGATCCAGGTGGCCTGCCA

61    GTAGTCGGTCCACCAGATCTCCCAGGTCTCCTTCTGGATGGGCAGCCGGAACTTGGGGGT
      |||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
183   GTAGTCGGTCCACCAGATCTCCCAGGTCTCCTTCTGGATGGGCAGCCGGAACTTGGGGGT

121   CTTGCCCCAGGTCACGATGCTCTCCAGGCTGATCTTCTGCACGGCCTCGGTCAGCTGCTT
      |||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
243   CTTGCCCCAGGTCACGATGCTCTCCAGGCTGATCTTCTGCACGGCCTCGGTCAGCTGCTT

181   CACGTCGTTGGTGTGGGTGGTCCGCCGCTTGGCGTACTTGCCGGTTTTCAGGTTCTTGAA
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303   CACGTCGTTGGTGTGGGTGGTCCGCCGCTTGGCGTACTTGCCGGTTTTCAGGTTCTTGAA

241   GGGCTCCTGGTAGATCTGGTAGGTCCACTGGTCGTCGCCCTGCTTCTGGATCTCGGCGAT
      ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
363   GGGCTCCTGGTAGATCTGGTAGGTCCACTGGTCGTCGCCCTGCTTCTGGATCTCGGCGAT

301   CAGGTCCTTGCTGGGGTCGTAGTACACGCCGTGCACGGGCTCCTTCAGGATCTCCCGGTT
      |||| ||||||||||||||||||||||||||||||||||||||||||||||||||||||
423   CAGGKCCTTGCTGGGGTCGTAGTACACGCCGTGCACGGGCTCCTTCAGGATCTCCCGGTT

361   CTCGGCCAGCTCCAGCTCGGCCTCCTCGGTCAGGGGCACGATGTCGGTCAGGGCCTTGGT
      ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
483   CTCGGCCAGCTCCAGCTCGGCCTCCTCGGTCAGGGGCACGATGTCGGTCAGGGCCTTGGT

421   GCCCCGCAGCAGCTTGCACAGCTGCCGCACCTTGATGCCGGGGTAGATCTGGCTGGTCCA
      ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
543   GCCCCGCAGCAGCTTGCACAGCTGCCGCACCTTGATGCCGGGGTAGATCTGGCTGGTCCA

481   GTTCAGCTTGCCCACCAGCTTCTGGATGTCGTTCACGGTCCAGCTGTCCTTCTCGGGCAG
      ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
603   GTTCAGCTTGCCCACCAGCTTCTGGATGTCGTTCACGGTCCAGCTGTCCTTCTCGGGCAG

541   CTGGATGGGCTGCACGGTCCACTTGTCGGGGTGCAGCTCGTAGCCCATCCACAGGAAGGG
      ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
663   CTGGATGGGCTGCACGGTCCACTTGTCGGGGTGCAGCTCGTAGCCCATCCACAGGAAGGG

601   GGGCTCCTTCTGGTGCTTCTTGTCGGGGGTGGTGAAGCCCCACTTCAGCAGGTGCTCCCG
      |||||||||||||||||||||||||||||||||||||||| ||||||||||||||||
723   GGGCTCCTTCTGGTGCTTCTTGTCGGGGGTGGTGAAGCCCCACTCCAGCAGGTGCTCCCG

661   CAGCTCCTCGATCTTGGCCCGGTGCTGGCCGATCTCCAGGTCGCTGCCCACGTACAGGGC
      ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
783   CAGCTCCTCGATCTTGGCCCGGTGCTGGCCGATCTCCAGGTCGCTGCCCACGTACAGGGC

721   GGCCATGTACTGGTAGATCACGATCTCGGGGTTCTTGG
      |||||||||||||||||||||||||||||||||||
843   GGCCATGTACTGGTAGATCACGATCTCGGGGTTCTTGG
```

Appendix D: Positive controls and antibodies used for western blots

Table D1- Positive control proteins used in SDS-PAGE and western blotting

Protein	Details	Size (kDa)	Source
Gag	HIV-1 BH10 $Pr55^{gag}$	55	Commercial[1]
Gag	HIV-1 p17 p24 protein	41	Commercial[2]
Nef	Recombinant HIV-1 Bru Nef (*E.coli*)	27	Mikrogen[3]
RT	$HIV\text{-}1_{HXB2}$ Reverse Transcriptase dimer (*E. coli*)	51, 66	D. Stammers[3]

[1] Quality Biological, Inc, Gaithersburg, USA.
[2] FIT Biotech Oyk Plc Eesti Filiaal, Tartu, Estonia
[3] National Institute for Biological Standards and Control (NIBSC), Centralised Facility for AIDS Reagents, Medical Research Council (MRC), United Kingdom (UK).

Table D2 - Primary antibodies used in western blotting

Antigen	Host	Dilution	Details	Designation	Source
p24	Rabbit	1:2000	Polyclonal antiserum to recombinant $HIV\text{-}1_{HXB2}$ p24 GST (*E.coli*).	ARP432	G. Reid [1]
Nef	Mouse	1:1000	Monoclonal antibody (IgG1) to HIV-1 Nef	01-003	FIT Biotech [2]
RT	Sheep	1:2000	Antiserum to Recombinant HIV-1 LAV Reverse Transcriptase	ARP428	M. Page [1]

[1] National Institute for Biological Standards and Control (NIBSC), Centralized Facility for AIDS Reagents, Medical Research Council (MRC), United Kingdom (UK).
[2] FIT Biotech Oyj Plc, Tampere Finland.

Table D3 - Secondary antibodies used in western blotting

Antigen	Host	Dilution	Details	Source
Mouse Ig G	Goat	1:5000	Affinity purified alkaline phosphatase conjugate	Sigma
Rabbit Ig G	Goat	1:5000	Affinity purified alkaline phosphatase conjugate	Sigma
Sheep Ig G	Goat	1:5000	Affinity purified alkaline phosphatase conjugate	Sigma

Appendix E: Gag p24 ELISA and ANOVA raw data

Table E1 –Gag p24 ELISA results of **GagRT** optimization samples

Cell line	Cell density (cells/ml)	MOI	Time post infection (hrs)	Mean of HIV p24 produced (ng/ml)	ANOVA mean of HIV p24 produced
T.ni pro insect cells	5.00E+05	0.1	48	22.53	2.3900824
T.ni pro insect cells	5.00E+05	1.0	48	21.40	2.1231516
T.ni pro insect cells	5.00E+05	5.0	48	7.24	1.9495538
T.ni pro insect cells	5.00E+05	0.1	72	137.78	4.08069235
T.ni pro insect cells	5.00E+05	1.0	72	31.99	3.43027495
T.ni pro insect cells	5.00E+05	5.0	72	43.02	3.60117475
T.ni pro insect cells	5.00E+05	0.1	96	61.13	3.8003357
T.ni pro insect cells	5.00E+05	1.0	96	84.61	4.4246475
T.ni pro insect cells	5.00E+05	5.0	96	99.79	4.59948755
T.ni pro insect cells	5.00E+05	0.1	120	178.31	5.09310765
T.ni pro insect cells	5.00E+05	1.0	120	130.04	4.79631835
T.ni pro insect cells	5.00E+05	5.0	120	174.95	5.00552585
T.ni pro insect cells	1.00E+06	0.1	48	7.92	1.9305321
T.ni pro insect cells	1.00E+06	1.0	48	8.60	2.1357406
T.ni pro insect cells	1.00E+06	5.0	48	10.83	2.23156025
T.ni pro insect cells	1.00E+06	0.1	72	192.15	5.0557533
T.ni pro insect cells	1.00E+06	1.0	72	108.05	4.4531088
T.ni pro insect cells	1.00E+06	5.0	72	56.93	4.0257577
T.ni pro insect cells	1.00E+06	0.1	96	196.30	4.8388818
T.ni pro insect cells	1.00E+06	1.0	96	212.58	5.28774595
T.ni pro insect cells	1.00E+06	5.0	96	187.77	5.23124355
T.ni pro insect cells	1.00E+06	0.1	120	277.61	5.5882136
T.ni pro insect cells	1.00E+06	1.0	120	188.54	5.02382345
T.ni pro insect cells	1.00E+06	5.0	120	***362.85**	5.68309415
T.ni pro insect cells	2.00E+06	0.1	48	8.22	1.95658215
T.ni pro insect cells	2.00E+06	1.0	48	10.50	2.22652185
T.ni pro insect cells	2.00E+06	5.0	48	10.45	2.1488563
T.ni pro insect cells	2.00E+06	0.1	72	32.13	2.9068367
T.ni pro insect cells	2.00E+06	1.0	72	34.35	3.5363479
T.ni pro insect cells	2.00E+06	5.0	72	31.70	3.1769042
T.ni pro insect cells	2.00E+06	0.1	96	55.07	3.9907626
T.ni pro insect cells	2.00E+06	1.0	96	42.74	3.75334405
T.ni pro insect cells	2.00E+06	5.0	96	46.36	3.82695645

T.ni pro insect cells	2.00E+06	0.1	120	39.07	3.66360775
T.ni pro insect cells	2.00E+06	1.0	120	67.75	4.12883745
T.ni pro insect cells	2.00E+06	5.0	120	72.71	4.280622
Sf9 insect cells	5.00E+05	0.1	48	1.84	0.21302585
Sf9 insect cells	5.00E+05	1.0	48	16.51	1.35327785
Sf9 insect cells	5.00E+05	5.0	48	3.27	0.60430105
Sf9 insect cells	5.00E+05	0.1	72	3.24	0.65836495
Sf9 insect cells	5.00E+05	1.0	72	11.65	2.25302895
Sf9 insect cells	5.00E+05	5.0	72	26.19	2.67505675
Sf9 insect cells	5.00E+05	0.1	96	14.16	2.36735085
Sf9 insect cells	5.00E+05	1.0	96	18.71	2.51503105
Sf9 insect cells	5.00E+05	5.0	96	74.70	4.27717095
Sf9 insect cells	5.00E+05	0.1	120	4.21	1.42720245
Sf9 insect cells	5.00E+05	1.0	120	29.26	1.7982546
Sf9 insect cells	5.00E+05	5.0	120	25.31	1.79065825
Sf9 insect cells	1.00E+06	0.1	48	5.88	1.66496525
Sf9 insect cells	1.00E+06	1.0	48	19.78	2.98419825
Sf9 insect cells	1.00E+06	5.0	48	25.67	3.2416561
Sf9 insect cells	1.00E+06	0.1	72	47.21	3.73174215
Sf9 insect cells	1.00E+06	1.0	72	70.66	3.94641985
Sf9 insect cells	1.00E+06	5.0	72	47.52	3.825866
Sf9 insect cells	1.00E+06	0.1	96	105.55	4.312652
Sf9 insect cells	1.00E+06	1.0	96	***406.12**	5.20374045
Sf9 insect cells	1.00E+06	5.0	96	103.08	4.50820845
Sf9 insect cells	1.00E+06	0.1	120	99.79	4.08597425
Sf9 insect cells	1.00E+06	1.0	120	112.64	4.51914025
Sf9 insect cells	1.00E+06	5.0	120	60.61	4.09899365
Sf9 insect cells	2.00E+06	0.1	48	14.17	2.3304273
Sf9 insect cells	2.00E+06	1.0	48	9.10	2.1625555
Sf9 insect cells	2.00E+06	5.0	48	29.02	3.2017371
Sf9 insect cells	2.00E+06	0.1	72	45.30	3.29897105
Sf9 insect cells	2.00E+06	1.0	72	102.94	4.57216015
Sf9 insect cells	2.00E+06	5.0	72	47.49	3.83340875
Sf9 insect cells	2.00E+06	0.1	96	144.86	4.5758228
Sf9 insect cells	2.00E+06	1.0	96	196.34	4.8890508
Sf9 insect cells	2.00E+06	5.0	96	50.12	3.90534985
Sf9 insect cells	2.00E+06	0.1	120	63.50	4.1118265
Sf9 insect cells	2.00E+06	1.0	120	60.80	4.065883
Sf9 insect cells	2.00E+06	5.0	120	52.00	3.93724605

* - denotes the highest Gag p24 values detected for the cell line tested

Table E2 Gag p24 ELISA results of **GagTN** optimization samples

Cell line	Cell density (cells/ml)	MOI	Time post infection (hrs)	Mean of HIV p24 produced (ng/ml)	ANOVA mean of HIV p24 produced
T.ni pro insect cells	5.00E+05	0.1	48	19.15	2.861345
T.ni pro insect cells	5.00E+05	1.0	48	82.77	4.3001145
T.ni pro insect cells	5.00E+05	5.0	48	37.09	3.549313
T.ni pro insect cells	5.00E+05	0.1	72	103.54	4.6397985
T.ni pro insect cells	5.00E+05	1.0	72	146.10	4.981695
T.ni pro insect cells	5.00E+05	5.0	72	92.64	4.460348
T.ni pro insect cells	5.00E+05	0.1	96	148.10	4.9784215
T.ni pro insect cells	5.00E+05	1.0	96	115.25	4.746394
T.ni pro insect cells	5.00E+05	5.0	96	152.39	5.0134145
T.ni pro insect cells	5.00E+05	0.1	120	221.46	4.9946745
T.ni pro insect cells	5.00E+05	1.0	120	125.08	4.8092445
T.ni pro insect cells	5.00E+05	5.0	120	196.05	5.271171
T.ni pro insect cells	1.00E+06	0.1	48	77.90	3.7518545
T.ni pro insect cells	1.00E+06	1.0	48	97.03	3.910509
T.ni pro insect cells	1.00E+06	5.0	48	84.98	3.7650365
T.ni pro insect cells	1.00E+06	0.1	72	103.86	4.617969
T.ni pro insect cells	1.00E+06	1.0	72	257.33	5.0129435
T.ni pro insect cells	1.00E+06	5.0	72	46.82	399652.9456
T.ni pro insect cells	1.00E+06	0.1	96	304.79	5.4744205
T.ni pro insect cells	1.00E+06	1.0	96	153.03	4.9403805
T.ni pro insect cells	1.00E+06	5.0	96	145.26	4.9706615
T.ni pro insect cells	1.00E+06	0.1	120	351.02	5.6443245
T.ni pro insect cells	1.00E+06	1.0	120	194.38	5.161949
T.ni pro insect cells	1.00E+06	5.0	120	**460.51**	6.105239
T.ni pro insect cells	2.00E+06	0.1	48	20.40	2.858372
T.ni pro insect cells	2.00E+06	1.0	48	35.89	3.5479845
T.ni pro insect cells	2.00E+06	5.0	48	35.60	3.571962
T.ni pro insect cells	2.00E+06	0.1	72	44.99	3.8051655
T.ni pro insect cells	2.00E+06	1.0	72	55.41	3.930003
T.ni pro insect cells	2.00E+06	5.0	72	97.67	4.579258
T.ni pro insect cells	2.00E+06	0.1	96	75.28	4.3141805
T.ni pro insect cells	2.00E+06	1.0	96	49.87	3.844521
T.ni pro insect cells	2.00E+06	5.0	96	67.28	4.2088545
T.ni pro insect cells	2.00E+06	0.1	120	95.56	4.553692
T.ni pro insect cells	2.00E+06	1.0	120	44.67	3.79794

T.ni pro insect cells	2.00E+06	5.0	120	76.54	4.3152755
Sf9 insect cells	5.00E+05	0.1	48	5.16	1.6400265
Sf9 insect cells	5.00E+05	1.0	48	17.47	2.656394
Sf9 insect cells	5.00E+05	5.0	48	32.28	3.422503
Sf9 insect cells	5.00E+05	0.1	72	29.11	3.3704675
Sf9 insect cells	5.00E+05	1.0	72	60.08	3.3861225
Sf9 insect cells	5.00E+05	5.0	72	92.81	4.0260635
Sf9 insect cells	5.00E+05	0.1	96	101.31	3.3997215
Sf9 insect cells	5.00E+05	1.0	96	83.70	3.397586
Sf9 insect cells	5.00E+05	5.0	96	114.47	4.4893515
Sf9 insect cells	5.00E+05	0.1	120	23.92	2.584555
Sf9 insect cells	5.00E+05	1.0	120	64.36	3.7028115
Sf9 insect cells	5.00E+05	5.0	120	73.90	4.2811575
Sf9 insect cells	1.00E+06	0.1	48	21.87	3.080956
Sf9 insect cells	1.00E+06	1.0	48	41.72	3.560146
Sf9 insect cells	1.00E+06	5.0	48	202.76	4.6002895
Sf9 insect cells	1.00E+06	0.1	72	151.84	4.442265
Sf9 insect cells	1.00E+06	1.0	72	214.95	5.003286
Sf9 insect cells	1.00E+06	5.0	72	184.24	5.138867
Sf9 insect cells	1.00E+06	0.1	96	308.33	5.3087525
Sf9 insect cells	1.00E+06	1.0	96	168.97	4.8803145
Sf9 insect cells	1.00E+06	5.0	96	197.78	5.1518245
Sf9 insect cells	1.00E+06	0.1	120	190.40	4.8474795
Sf9 insect cells	1.00E+06	1.0	120	169.04	4.961018
Sf9 insect cells	1.00E+06	5.0	120	**278.43**	5.6291475
Sf9 insect cells	2.00E+06	0.1	48	28.75	3.220281
Sf9 insect cells	2.00E+06	1.0	48	41.83	3.723669
Sf9 insect cells	2.00E+06	5.0	48	43.63	3.747812
Sf9 insect cells	2.00E+06	0.1	72	48.43	3.8223315
Sf9 insect cells	2.00E+06	1.0	72	68.61	4.2271355
Sf9 insect cells	2.00E+06	5.0	72	94.61	4.449823
Sf9 insect cells	2.00E+06	0.1	96	113.30	4.7187775
Sf9 insect cells	2.00E+06	1.0	96	60.67	4.1022305
Sf9 insect cells	2.00E+06	5.0	96	63.72	4.1540155
Sf9 insect cells	2.00E+06	0.1	120	59.77	4.0897105
Sf9 insect cells	2.00E+06	1.0	120	60.18	4.093679
Sf9 insect cells	2.00E+06	5.0	120	61.37	4.1090395

Table E3 - ANOVA summary for **GagRT** data

Factors/factor interactions tested	Df	Sum Sq	Mean Sq	F value	Pr(>F)	
Line	1	15.256	15.256	12.0387	0.00089	***
Density	2	38.697	19.349	15.2683	3.05E-06	***
Multiplicity	2	3.165	1.583	1.2489	0.29306	
Time	3	106.796	35.599	28.0916	4.36E-12	***
Line:Density	2	34.951	17.475	13.7902	8.71E-06	***
Line:Multiplicity	2	2.794	1.397	1.1026	0.33764	
Density:Multiplicity	4	0.879	0.22	0.1734	0.95132	
Line:Time	3	9.441	3.147	2.4833	0.06776	.
Density:Time	6	2.632	0.439	0.3462	0.90984	
Multiplicity:Time	6	0.819	0.136	0.1077	0.99529	
Line:Density:Multiplicity	4	2.018	0.504	0.3981	0.80937	
Line:Density:Time	6	3.937	0.656	0.5178	0.79297	
Line:Multiplicity:Time	6	2.305	0.384	0.3032	0.93328	
Density:Multiplicity:Time	12	7.326	0.61	0.4817	0.91905	
Line:Density:Multiplicity:Time	12	3.521	0.293	0.2316	0.99612	
Residuals	71	89.974	1.267			

Table E4 - ANOVA summary for **GagTN** data

Factors/factor interactions tested	Df	Sum Sq	Mean Sq	F value	Pr(>F)	
Line	1	5.224	5.224	6.073	0.016149	*
Density	2	18.9	9.45	10.9851	6.97E-05	***
Multiplicity	2	4.119	2.06	2.3943	0.09857	.
Time	3	32.327	10.776	12.5263	1.16E-06	***
Line:Density	2	11.832	5.916	6.877	0.001862	**
Line:Multiplicity	2	2.416	1.208	1.4044	0.252247	
Density:Multiplicity	4	1.65	0.413	0.4796	0.750574	
Line:Time	3	1.312	0.437	0.5084	0.677769	
Density:Time	6	3.249	0.542	0.6295	0.706144	
Multiplicity:Time	6	5.009	0.835	0.9704	0.451617	
Line:Density:Multiplicity	4	2.263	0.566	0.6576	0.623499	
Line:Density:Time	6	0.481	0.08	0.0932	0.99684	
Line:Multiplicity:Time	6	1.476	0.246	0.2861	0.941741	

Density:Multiplicity:Time	12	3.039	0.253	0.2944	0.988466
Line:Density:Multiplicity:Time	12	0.967	0.081	0.0937	0.999962
Residuals	71	61.078	0.86		

Significant codes:

*** - p ≤0.0001
** - p ≤0.001
* - p ≤0.05
. - p ≤0.1
no code - p ≤1.0

Those groups with 1 or more * are deemed as major factors or factor interactions.

Appendix F: Quantification of VLP stocks

Table F1 Quantification of GagTN and GagRT VLP stocks, batches 1 and 2, using Western blot densitometry μβ

VLP stocks	Quantitation 1 (μg/ml)	Quantitation 2 (μg/ml)	Average amount of VLPs in stocks (μg/ml)	Std deviation (μg/ml)
GagTN Batch 1	14.36	17.8	16.08	2.43
GagTN Batch 2	22.74	17.54	20.14	3.68
GagRT Batch 1	1.13	3.97	2.55	2.01
GagRT Batch 2	17.16	13.18	15.17	2.81

Appendix G: Endotoxin data

Table G1 The endotoxin results of the respective VLP stock solutions and the mouse inoculations

Construct	Batch	OD405 mm	Endotoxin levels of stock solution (EU/ml)	Dilution for mouse inoculation	Final endotoxin levels of mouse inoculations (EU/ml)*
GagTN	1	0.116	79.992	1/322	0.248
GagTN	2	0.101	50.047	1/403	0.124
GagRT	1	0.104	60.048	1/51	1.177
GagRT	2	0.094	34.32	1/302	0.114

* Acceptable Endotoxin level for mice: < 0.125 EU/ml

Appendix H: Cell surface marker data

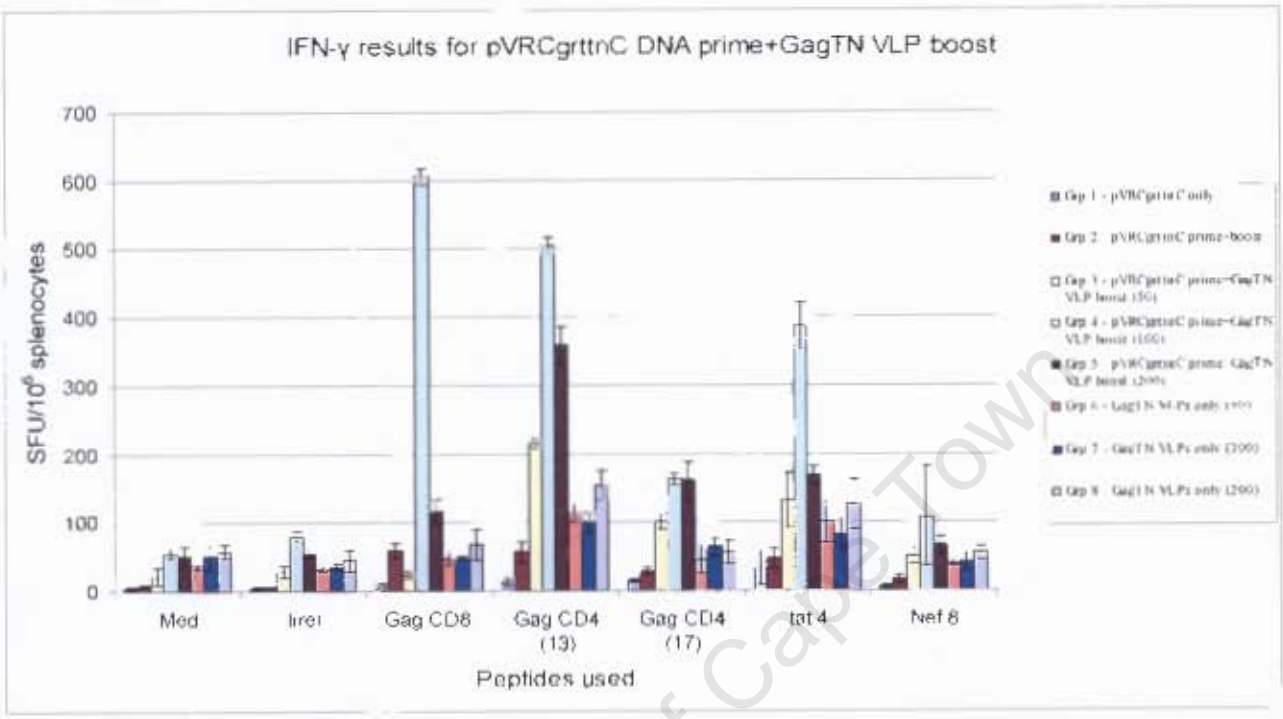
Table H1 – The proportion (as % of events in the lymphocyte gate) of B and T cells in the spleens of the mice groups vaccinated with GagTN VLP experiment 1 vaccines.

Vaccine Group	CD3/CD19 Negative	CD3+	CD3+/CD4+	CD3+/CD8+	CD4+/CD8+ ratio	CD19+
1	12.7	40.5	35.9	12.5	2.9	46.8
2	15.1	38.2	27.3	12.1	2.3	46.7
3	13.5	37.6	27.5	12.8	2.1	48.9
4	14.6	43.8	34.1	13.5	2.5	41.6
5	17.1	41.1	29.7	13.5	2.2	41.8
6	15.5	43.0	31.4	14.2	2.2	41.6
7	17.4	37.0	27.2	12.5	2.2	45.6
8	14.6	40.4	31.2	12.9	2.4	45.0

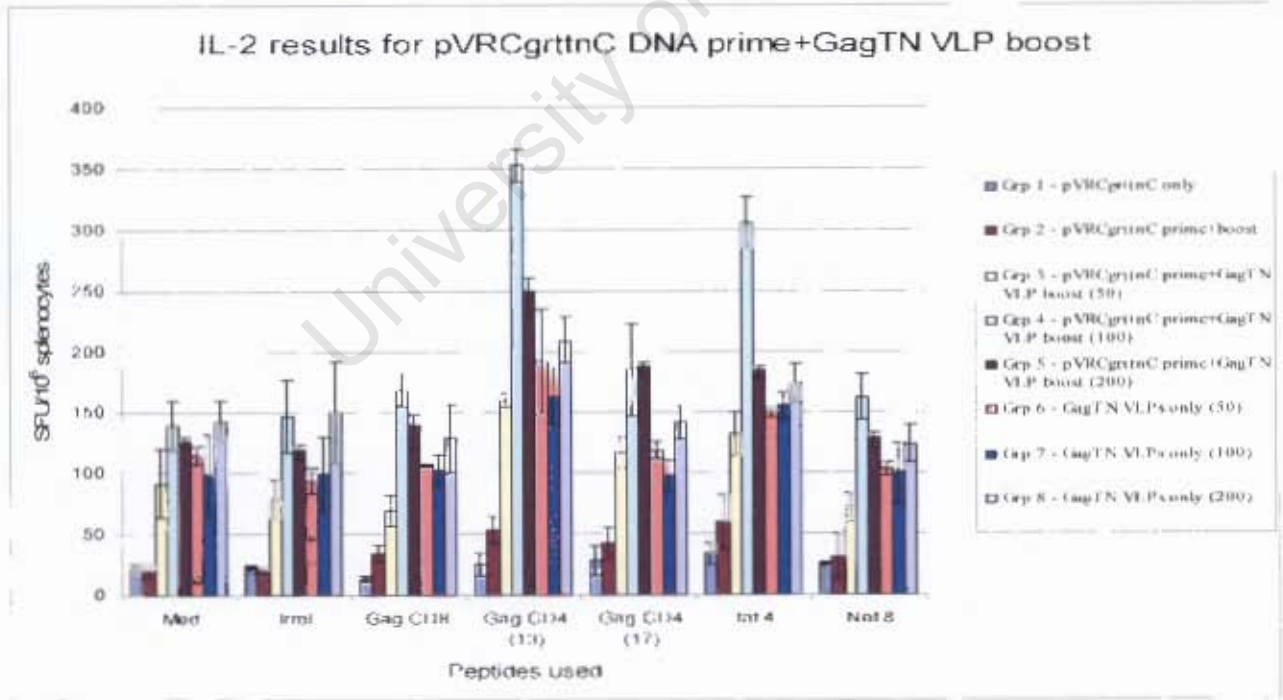
Table H2 The proportion (as % of events in the lymphocyte gate) of B and T cells in the spleens of the mice groups vaccinated with GagRT VLP experiment 1 vaccines.

Vaccine Group	CD3/CD19 Negative	CD3+	CD3+/CD4+	CD3+/CD8+	CD4+/CD8+ ratio	CD19+
1	13.7	47.6	32.8	13.6	2.4	38.7
2	16.5	46.5	31.3	14.3	2.2	37.0
3	16.5	43.5	29.0	13.7	2.1	40.0
4	15.2	48.5	33.2	14.7	2.3	36.3
5	15.1	44.5	29.5	14.4	2.0	40.4
6	15.7	47.7	32.4	15.2	2.1	36.6
7	18.7	42.0	29.1	13.7	2.1	39.3
8	15.0	44.7	29.9	13.9	2.2	40.3

Appendix I: IFN and IL-2 data (before positive responses were determined)

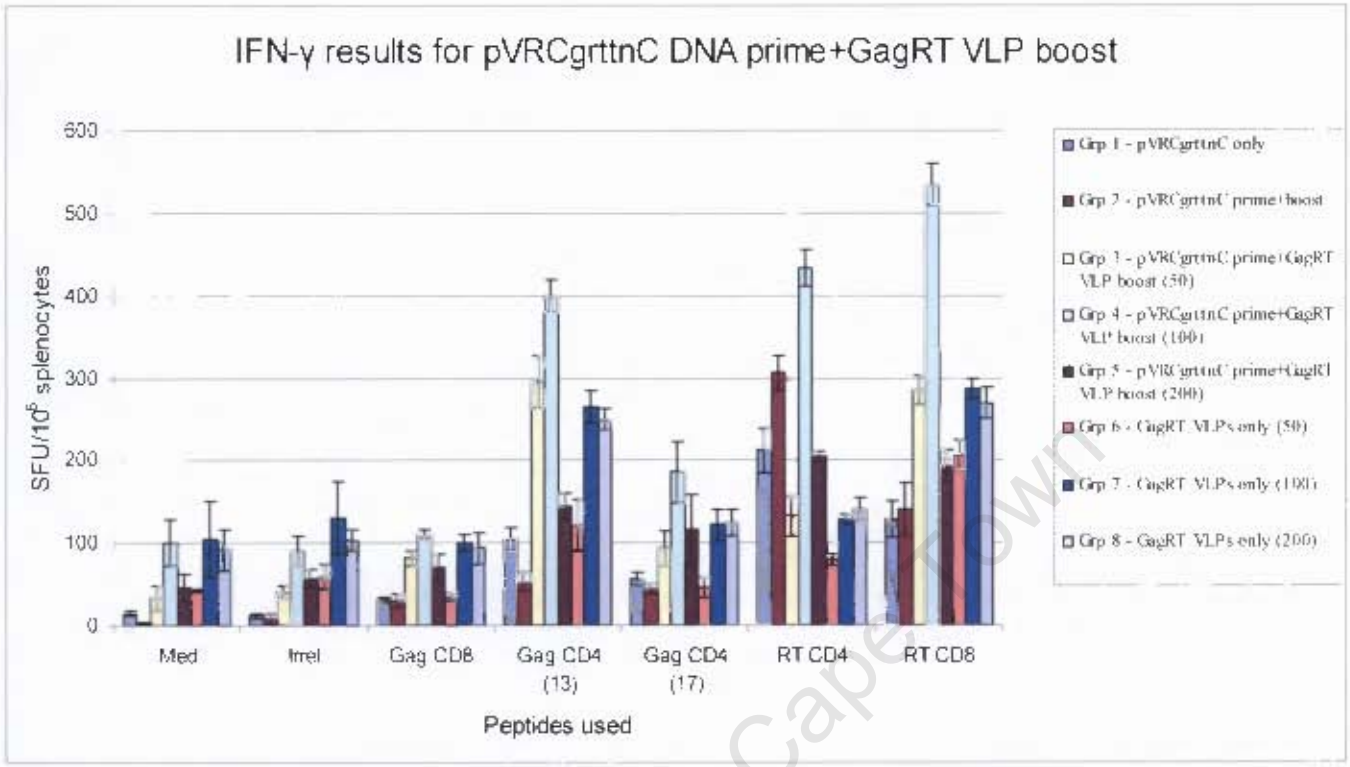


(a)

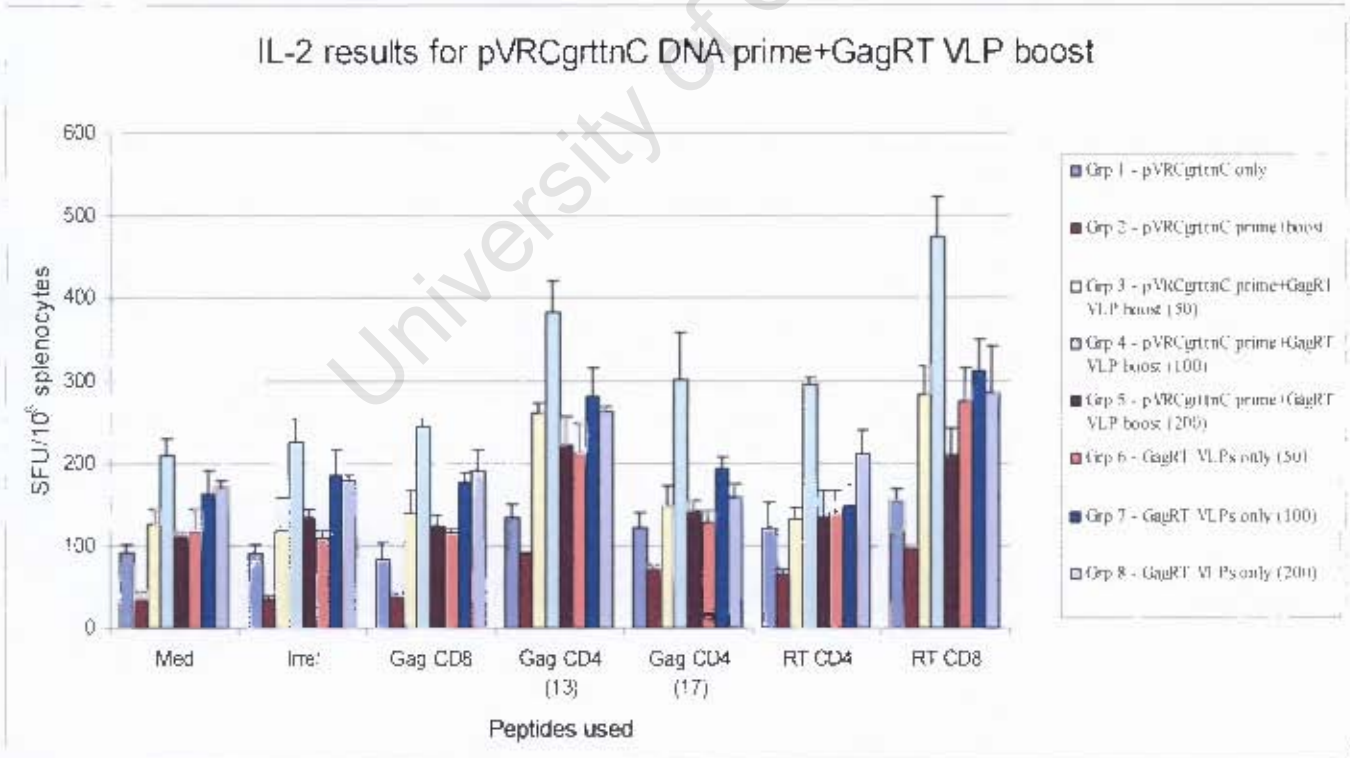


(b)

Figure II – Evaluation of the cellular immune response elicited in mice vaccinated as per the prime-boost vaccine regimens detailed in Table 5.1 (with Gag TN VLP boost), as determined by quantification of IFN- γ (a) and IL-2 (b) cytokines (SFU/10^6 splenocytes) secreted by pooled splenocytes after in vitro stimulation with HIV-specific peptides. Abbreviations: Med – medium; Irrel – irrelevant peptide. Dosage of VLP boosts (ng) are indicated in brackets in the legend.



(a)



(b)

Figure 12 – Evaluation of the cellular immune response elicited in mice vaccinated as per the prime-boost vaccine regimens detailed in Table 5.1 (with Gag RT VLP boost), as determined by quantification of IFN- γ (a) and IL-2 (b) cytokines (SFU/10^6 splenocytes) secreted by pooled splenocytes after in vitro stimulation with HIV-specific peptides. Abbreviations: Med – medium; Irrel – irrelevant peptide. Dosage of VLP boosts (ng) are indicated in brackets in the legend.

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