Assessment of BTV VP7-169 as a vector for the display of foreign peptides

By

Debora Bolton

Submitted in partial fulfilment of the requirements for the degree MSc in the Faculty of Natural and Agricultural Sciences

University of Pretoria

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[31]"Julle moet julle dus nie bekommer en vra: 'Wat moet ons eet of wat moet ons drink of wat moet ons aantrek?' nie. [32] Dit is alles dinge waaroor die ongelowiges begaan is. Julle hemelse Vader weet tog dat julle dit als nodig het. [33] Nee, beywer julle allereers vir die koninkryk van God en vir die wil van God, dan sal Hy julle al hierdie dinge gee.

Mattheus 6:33

[1]Luister, so se die Here wat jou geskep het, Jakob, wat jou gevorm het, Isreal: Moenie bang wees nie, Ek verlos jou, Ek het jou op jou naam geroep, jy is Myne. [2]As jy deur water moet gaan, is Ek by jou, deur riviere, hulle sal jou nie wegspoel nie; as jy deur vuur moet gaan, sal dit jou nie skroei nie, die vlamme sal jou nie brand nie, [3] want Ek is die Here jou God, die Heilige van Israel, jou Redder. Ek gee Egipte as losprys vir jou, Kus en Seba in jou plek. [4] Omdat jy vir My kosbaar is, omdat Ek jou hoog ag en jou liefhet, gee Ek mense in jou plek, volke in ruil vir jou lewe.

Jesaja 43: 1-4

[11] Ek weet wat Ek vir julle beplan, se die Here: voorspoed en nie teespoed nie; Ek wil vir julle 'n toekoms gee, 'n verwagting! [12] Dan sal julle My aanroep, tot My kom bid, en Ek sal julle gebed verhoor. [13] Julle sal vra na my wil en julle sal dan my wil ken as julle met julle hele hart daarna vra.

Jeremia 29:11-13

[17] Ek het my daarop toegele om te verstaan wat wysheid en kennis is, en wat dit is om 'n gebrek aan wysheid en kennis te he. Ek het vasgestel: ook dit is 'n gejaag na wind. [18] Baie wysheid stel hoe eise, wie kennis versamel, versamel smart.

Prediker 2:17-18

DECLARATION

I, Debora Bolton, hereby declare that the thesis/dissertation that I herewith submit for the degree Magister Scientiae to the University of Pretoria contains my own work and has not been previously submitted by me for a degree to this or any other tertiary institution.

______________________________ ____________________________

Debora Bolton Date

ACKNOWLEDGEMENTS

- Prof H. Huismans, for all the help and patience with the dissertation, for teaching me how to receive criticism but also how to give criticism and finally for teaching me what it means to never give up
- Dr. Wilma Fick, for all your help, encouragement and for becoming a friend
- Mnr. Flip Wege, for all your help with the cell culture
- Mnr. A Hall, for all the help with the fluorescence and confocal microscopy
- Fellow students and friends, Charl Hechter, Lorraine Barnes and Marnie Potgieter
- Girls at the Ampath Genetics Laboratory, in particular Elizka, for all your support and motivation
- NRF and Biopad for providing the funds
- My parents and Anelle, for all your love, support and for always believing in my abilities
- Tannie Magda, for all your interest, support and motivation
- Marius, for being the driving force behind completing this, for turning dreams into reality and being a part of my life
- God, for giving me the ability to complete a dissertation, giving me strength and perseverance throughout and for teaching me valuable lessons through this experience

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Summary

African horsesickness virus (AHSV) belongs to the *Orbivirus* genus in the family *Reoviridae.* This non-enveloped virus consists of an outer capsid formed by two structural proteins, VP2 and VP5, and an inner core formed by structural proteins VP7 and VP3. Three additional structural proteins associated with viral replication, as well as ten dsRNA molecules responsible for replication, are found inside the core. VP7 is the smallest of the structural proteins and each monomer consist of two domains, a hydrophilic top and hydrophobic bottom domain. Upon expression of VP7, the protein spontaneously assembles into trimers. Recombinant expression of the core protein VP7 results in large hexagonal structures formed by a double layer of these VP7 trimers with the hydrophobic bottom domains on the inside and hydrophilic top domains on the outside. The use of these crystal structures as a general display system for the display of foreign peptides/epitopes is being investigated in our group. In this regard, sites for the insertion of foreign peptides/epitopes were constructed at amino acid positions 177, 144 and 200 of the top domain of the VP7 protein and the resultant proteins named vectors AHSV-9 VP7-177, AHSV-9 VP7-144 and AHSV-9 VP7-200. Various inserts ranging from the HIV-1 ELDKWA epitope and FMDV VP1 epitopes to the eGFP peptide were inserted and subsequently analysed for immunogenicity. Results showed that a significant immune response was only elicited if the soluble trimer component of a chimeric VP7 protein was used for inoculation purposes. The crystal particles initially investigated as a display system did not result in any immune response. These results emphasized the importance of protein solubility for eliciting a significant immune response.

The importance of solubility prompted an investigation into the use of the Bluetongue virus (BTV) VP7 protein as a vaccine display system. This protein is inherently more soluble than AHSV VP7 and does not result in crystal hexagonal structures if recombinantly expressed. An insertion site analogous to that of the AHSV-9 VP7-177 vector, located at amino acid 177 within an RGD loop in the top domain of VP7 was constructed. This new BTV VP7 vector, BTV-10 VP7-169, was characterised with regard to solubility and the ability to form trimers. In order to investigate the effect on solubility and trimerisation, FMDV VP1 epitope and eGFP were inserted into the BTV-10 VP7-169 vector. Results showed that following the construction of the insertion site, the vector was largely insoluble compared to the AHSV VP7 vectors and that insertion of the abovementioned peptides/epitopes did not have a significant effect on solubility. Although trimers were present, the yield was low compared to that of the AHSV chimeric VP7 proteins.

Methods of improving the solubility of the chimeric VP7 proteins were investigated by treatment with solubilisation agents, sarkosyl and L-arginine. The results indicated that a strong denaturant such as sarkosyl can solubilise the particulate component of all chimeric VP7 proteins whereas L-arginine had limited effect. The effect of these agents on the folding of the proteins were evaluated

using fluorescence, since the ability to fluoresce is regarded as an indicator of correct folding. A comparison of the different VP7-eGFP proteins treated with these solubilisation agents showed that the sarkosyl solubilised proteins were not necessarily correctly folded. These results combined with the previously performed solubility assays indicated that a large proportion of correctly folded chimeric VP7 proteins associate with the particulate fraction. Investigation showed that expression of a large amount of correctly folded chimeric proteins results in the aggregation of these proteins within the expressing host cell. Once harvested these proteins remain associated with the insoluble fraction but can be solubilised by arginine treatment, or in some cases mere resuspension in a low-salt buffer, and used for vaccination purposes.

In conclusion, the comparative analyses of solubility and trimerisation for the display vectors indicated that AHSV-9 VP7-144 vector may be most suitable for the display of foreign epitopes/peptides as it consistently yielded the largest component of correctly folded proteins. Furthermore, considering that large amounts of correctly folded chimeric VP7 proteins occurred in the insoluble component of each the VP7 display proteins, this study emphasize that use of solubility assays alone does not provide adequate information regarding the potential of a display vector for vaccination purposes.

List of abbreviations

µl	microliter
µg	microgram
aa	amino acid
ABA	N-terminal-AHSV-BTV upper domain region-AHSV-C-terminal
AHSV	African horsesickness virus
AHS	African horsesickness
Ala	L-alanine
Arg	L-arginine
BAB	N-terminal-BTV-AHSV upper domain-BTV-C-terminal
BHK	Baby hamster kidney cell line
BLV	Bovine leukemia virus
bp	base pair
BTV	Bluetongue virus
CLP	core like particle
CPE	cytopathic effect
CTL	cytotoxic T cell
DNA	deoxyribonucleic acid
dsRNA	double stranded ribonucleic acid
E. coli	*Escherichia coli*
EDTA	ethylenediaminetetraacetic acid
eGFP	enhanced green fluorescent protein
ELISA	Enzyme-linked immunosorbent assay
FMD	Foot and mouth disease
FMDV	Foot and mouth disease virus
GFP	green fluorescent protein
Gly	glycine
GdnHCl	guanidine hydrochloride
h	hour
HA	hemagglutinin
HBcAg	core antigen of the Hepatitis-B-Virus
HBsAg	surface antigen of the Hepatitis-B-Virus
HBV	hepatitis B virus
HCV	hepatitis C virus
HIV	Human immunodefiency virus
HPV	Human papilloma virus
IFNα	interferon alpha
IgG	immunoglobulin G
IPTG	isopropyl-β-D-thiogalactopyranoside
KDa	kilo Dalton
LB	Lauria-Bertani
M1	Matrix protein 1
MAPs	multiple antigen peptides
ml	milliliter
mM	millimolar
MOI	multiplicity of infection
MW	molecular weight
NS#	non-structural protein denoted with a number
nt	nucleotide
NtPE	*Pseudomonas aeruginosa* Exotoxin A
OD	optical density
pfu	plaque forming units

PCR	polymerase chain reaction
pH	negative decimal logarithm of the hydrogen ion activity in a solution
p.i.	post infection
RE	restriction enzyme
RGD	arginine-glycine-aspartic acid
RNA	ribonucleic acid
RNase	ribonuclease
SDS	sodium dodecyl sulphate
SDS-PAGE	sodium dodecyl sulphate polyacrylamide gel electrophoresis
SEM	scanning electron microscope
Ser	serine
Sf-9	*Spodoptera frugiperda* clone 9 insect cell line
SIV	simian immunodeficiency virus
TEMED	N,N,N',N'-tetramethylethylenediamine
TRIS	Tris(hydroxymethyl)-aminomethane
TRIS-HCl	Tris(hydroxymethyl)-aminomethane hydrochloric acid
Tyr	tyrosine
UV	ultraviolet
VLP	virus-like particle
VP#	viral protein denoted by number
VP7	viral protein 7
Wt	wild-type
w/v	weight per volume
X-gal	5-bromo-4-chloro-3-indolyl-D-galactopyranoside

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Chapter 1

Literature Review

1.1 Introduction

Vaccine technology has come a long way since Edward Jenner developed the first human vaccine for smallpox in the late 18th century (Jenner, 1798; 1800). Early vaccines were limited to live vaccines which are based on the use of the live pathogen responsible for infection. These vaccines resulted in the eradication of smallpox and the control of a number of other diseases such as yellow fever (Querec *et al*., 2006) and polio (Brennan and Dougan, 2005). Vaccination with live pathogens does, however, have drawbacks and many vaccination-related deaths have been recorded over the years for a number of these vaccines (Amanna *et al*., 2009). The need for safer alternatives therefore fuelled vaccine development toward inactivated/subunit vaccines which involves the use of either a whole inactivated pathogen or parts of a pathogen for vaccination purposes. Although these vaccines are safer with regard to pathogen infectivity, drawbacks relating to the protective immunity conferred remain an issue (Storni *et al*., 2005).

In theory, subunit vaccines are the safest vaccine alternative as only proteins or immunogenic peptides/epitopes of the pathogen are present. It is also the most effective as the specific protein products responsible for a neutralizing antibody response can be isolated and used. Subunit vaccines have become very diverse as molecular technology progressed. Vaccine types range from using single proteins for vaccination purposes to using a carrier protein or protein particles to display immunologically important epitopes/peptides to the immune system (Hansson, 2000). Details of these will be discussed below.

The research group directed by Prof. Henk Huismans in the Genetics Department at University of Pretoria is involved in developing a general vaccine display system based on the use of the soluble component of African horsesickness virus (AHSV) protein VP7 as a carrier protein for the display of immunologically important epitopes/peptides. In this review the rationale for using VP7 as a carrier protein as well as results from immunisation trials following display of immunologically important epitopes, such as those of human immunodeficiency virus (HIV) and foot and mouth disease virus (FMDV), on the protein surface of AHSV VP7 will be discussed. Several obstacles associated with presenting the proteins or immunogenic peptides/epitopes to the immune system in a conformation that provides the best display for eliciting an immune response, have been identified. In this regard, previous results show that

the solubility of the chimeric VP7 protein is pivotal in providing a strong immune response upon inoculation (Rutkowska *et al.*, 2011). Solubility of the carrier protein is therefore of considerable importance.

Bluetongue virus (BTV) is the prototype virus of the genus *Orbivirus* to which AHSV belongs (Fenner, 1976). Investigation have shown that the VP7 protein of AHSV and BTV are structurally similar (Basak *et al.*, 1996) although at sequence level these two proteins only show 44% homology (Roy *et al.*, 1996a). Unlike AHSV VP7, BTV VP7 is an inherently soluble protein when recombinantly expressed. As solubility is of such importance for eliciting a good immune response, this inherent solubility of BTV VP7 can be an advantage when developing a general display system that relies on the production of soluble recombinant protein. The assumption is that BTV VP7 may already provide a soluble backbone for insertion of immunologically important epitopes/peptides. BTV VP7 has not yet been characterised as a carrier protein for the display of foreign peptides/epitopes. The focus of the research group lies in the development of a viable VP7 display system using either BTV or AHSV VP7 proteins as general vaccine display systems. A brief introduction on these viruses and in particular the VP7 protein will follow.

1.2 BTV and VP7 structure

AHSV is a dsRNA virus that belongs to the genus *Orbivirus* in the family *Reoviridae* (Verwoerd *et al.*, 1979). Viral composition and VP7 structure will generally be described according to results for BTV as it is the prototype member of the orbiviruses. Knowledge of this structure will provide background when the construction of the VP7 vaccine delivery system is discussed later in the chapter.

Similar to other members of the *Reoviridae* family, orbiviruses are non-enveloped with two protein shells and a genome consisting of dsRNA segments (Verwoerd, 1969; Verwoerd *et al.*, 1972). The outer shell, known as the outer capsid, consists of two structural proteins: VP2 and VP5 whereas the innershell, known as the core, consists of two other structural proteins: VP3 and VP7. Three additional structural proteins, VP1, VP4 and VP6, as well as ten dsRNA genome segments are enclosed within the core (Huismans, 1979; Mertens *et al.*, 1987). The ten dsRNA segments each encode a single protein except for segment 10 which encode two non-structural proteins (Mertens *et al.*, 1984). Four of these genome segments encode structure-related proteins, namely VP2, VP3, VP5, and VP7 whilst three other segments codes for the proteins associated with the transcription complex, VP1, VP4 and VP6. The remaining three dsRNA genome segments code for the four non-structural proteins NS1, NS2, NS3 and

NS3a that are involved in virion assembly and egress (Reviewed in Roy, 1996a). Figure 1.1 shows a schematic representation of the BTV virion.

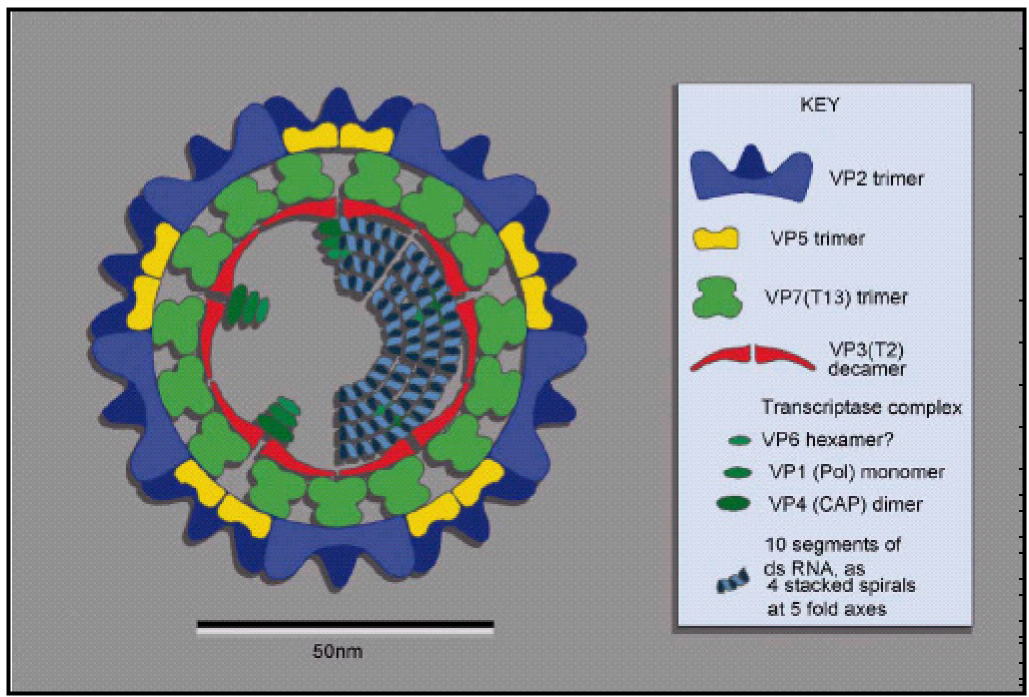


Figure 1.1 A schematic representation of BTV virion (Mertens, 2004)

Before discussing the VP7 display vector, various subunit vaccines will be reviewed to provide background on the strategy for a vaccine display system.

1.3 Subunit vaccines

Subunit vaccines were first introduced in the early 1980s. Although most of the licensed vaccines that are currently commercially available are inactivated, new generation vaccines are moving in the direction of protein- and peptide-based subunit technology. Commercial subunit vaccines include vaccines for diseases such as diphtheria and hepatitis B (Liljeqvist, 1999). The basic strategy involved in the development of such a vaccine relies on using part of the infectious pathogen to elicit an immune response. One of the main advantages of using a subunit vaccine, rather than a vaccine based on the whole pathogen, is that the risk of pathogenicity is eliminated as it cannot replicate in the host (Hansson, 2000). However, as these vaccines do not resemble a natural pathogen infection, the immune response is limited to a humoral response (Storni *et al.*, 2005) but can be amplified by addition of adjuvants (Perrie *et al.*, 2008). Another drawback related to the immunogenicity of this vaccine category is that multiple, as well as prime-booster doses, have to be administered to confer long-term protection (Ramshaw and Ramsay, 2000). Presently, the advantages of these vaccines however outweigh these drawbacks. Increased safety due to inability to revert to a

pathogenic form in addition to well defined composition makes these vaccines the most likely candidates to be licensed (Babiuk, 1999). Subunit vaccines can be categorized into those based on single proteins and those based on epitopes or peptides of immunogenic proteins.

1.3.1 Protein-based subunit vaccines

This class of subunit vaccines is based on using whole pathogen proteins for vaccination purposes. Within this group, distinction can be made between using single proteins and using single proteins that assemble into particles such as virus-like particles (VLPs) or core-like particles (CLPs). In each of these subgroups, proteins can be recombinantly expressed in an appropriate expression system such as yeast, bacteria, mammalian or insect cells and subsequently harvested for vaccine purposes. Alternatively, the genes encoding the relevant protein can be incorporated into a viral genome and expressed *in vivo* as part of the genome. The most commonly used viral genome vectors include the vaccinia virus, canarypox virus and various adenovirus strains and alphavirus (Brave *et al*., 2006; Dertzbaugh, 1998).

1.3.1.1 Single proteins as immunogens

Rather than using the whole pathogen for vaccination purposes, single proteins usually located on the surface of the pathogen is used in this approach. This vaccine strategy is based on the fact that the immune response elicited to a whole pathogen is raised against specific proteins that are in contact with the aqueous surroundings of the cell. In fact, neutralising epitopes are usually found in one or more proteins located on the surface of the pathogen (Hansson *et al*., 2000).

A great deal of research has focussed on the development of AHSV and BTV vaccines using the outer capsid protein VP2, which is not only the determinant of viral serotype, but also contains the major antigenic region (Martinez-Torrecuadrada *et al*., 2001). Recombinantly expressed BTV VP2 elicits full protection against a virulent homologous BTV challenge with no clinical signs in the sheep following inoculation (Inumaru and Roy, 1987; Roy *et al*., 1990). Furthermore, it has been shown that purification of VP2 from the BTV virion induces serotype-specific neutralising antibodies capable of protection against virulent homologous BTV challenge (Huismans *et al*., 1987). These studies were the first to indicate that a subunit vaccine may be a viable option for AHS. Burrage *et al*. (1993) were the first to show that serotype-specific neutralising antibodies could be raised following inoculation of neonatal mice with AHSV-4 VP2. Roy *et al*. (1996b) later claimed complete protection against a fatal viral challenge using recombinantly expressed AHSV-4 VP2. Although the use of VP2 as a subunit vaccine seems viable, recombinant expression of VP2 is complicated by protein aggregation.

Subsequent studies showed that when baculovirus expressed AHSV-5 VP2 was used for inoculation in horses, only partial protection against viral challenge was observed (Du Plessis *et al*., 1998). The authors contributed the induction of only partial protection to the protein being present predominantly in insoluble aggregates, incapable of inducing neutralizing antibodies. Vreede & Huismans (1994) and Martinez-Torrecuadrada *et al.* (1995) postulated that the low levels of soluble VP2 may be explained by a toxic effect induced by soluble VP2 on insect cells. Although VP2 would be a convenient candidate for an AHSV or BTV vaccine, producing high yields of soluble VP2 using recombinant DNA technology remains an obstacle (Maree, 2000). As an alternative, the AHSV *L2* gene segment, coding for VP2, was cloned into a vaccinia virus expression system that yields live recombinant viruses, and these were used for inoculation intradermally. Subsequent immunizations revealed that AHSV VP2 was sufficient for protection against a virulent challenge, but the level of protection was similar to currently used attenuated vaccine preparations (Stone-Marchat *et al*., 1996).

Antigenic proteins which are insoluble upon recombinant expression are not limited to orbivirus vaccines. In foot-and-mouth disease (FMD), the predominantly insoluble FMDV VP1 protein only elicited an immune response following solubilisation prior to inoculation (Shire *et al*., 1984). Wang *et al*. (2003) showed protection against a FMDV challenge but also only when extensively solubilised using sodium dodecyl sulphate (SDS) prior to inoculation. Although solubilisation was successful in this case, the neutralizing antibody titre was low compared to the current vaccine used.

As shown in these examples, solubility of immunogenic proteins poses a problem for vaccine development. This is however not surprising considering that they are located on the surface of the virion and that these proteins are predominantly hydrophobic and inherently insoluble. Furthermore, according to Liljeqvist (1999), a common problem associated with these protein-based vaccines is proteolytic degradation of the introduced protein. Although various strategies including increasing the protein solubililty (Murby *et al*., 1995) have been employed to improve the production of these single protein subunit vaccines, it remains a problem for this vaccination strategy.

1.3.1.2 Proteins that assemble into particles

Virus-like particles (VLPs) mimic the structure of the virus, whilst lacking the genetic component required for infection. The major advantage of using this vaccine strategy is that it resembles a natural infection and therefore results in a broader immune response, including humoral and cellular responses (Noad and Roy, 2003). A number of examples of VLPs will be discussed below.

Hepatitis B surface antigen (HBsAgs)

A prime example of single protein VLPs as an immunogen, is the hepatitis B surface antigen (HBsAg). The first hepatitis B vaccine involved the purification of HBsAgs from the plasma of infected individuals (Mcaleer *et al*., 1984) followed by inoculation. Due to safety concerns relating to the use of plasma, a subunit vaccine was developed for hepatitis B prophylaxis (Valenzuela, 1982). This vaccine, developed by expressing the hepatitis B surface antigen in a yeast expression system, is still used commercially today (Thanavala and Lugade, 2010).

Human papillomavirus (HPV)

Another example is that of human papillomavirus (HPV) VLPs which were produced by expressing the HPV capsid protein gene L1 in either insect cells or *Saccharomyces cervisiae*. The VLP vaccine was used in phase I clinical trials against HPV and results from this study showed that the incidence of HPV differed from 3.8% in the placebo group to 0% in the vaccinated group after inoculation with three doses of HPV VLPs (Koutsky *et al*., 2002). Currently two licensed HPV VLP vaccines are commercially available, the HPV 16/18/6/11 quadrivalent and a 16/18 bivalent HPV L1 VLP vaccines (Zhang *et al*., 2010).

Bluetongue virus (BTV)

As an alternative to using BTV VP2 as subunit vaccine, researchers turned their attention to BTV VLPs. Initial studies showed that VLPs were highly immunogenic compared to VP2 alone and that less VP2 protein provided protection against a virulent challenge when presented on a VLP compared to VP2 alone (Roy *et al*., 1990; Roy *et al*., 1992). Later, Roy *et al*. (1994) showed long-term protection against homologous viral challenges as well as partial protection against heterologous challenges. These results indicated that VLPs has the potential to be used as a vaccination strategy, although the yield of VLPs will have to be scaled up to an industrial level to be commercially viable (Roy *et al*., 2009).

A successful example of *in vivo* expression of VLPs were shown when horses were vaccinated using a canarypox virus vector co-expressing synthetic genes of both the outer capsid proteins, AHSV VP2 and VP5, resulting in an AHSV VLP (Guthrie *et al*., 2009). The inoculated horses developed specific neutralizing antibodies and were protected against

infection with virulent serotype 4 of AHSV. Research will now focus on developing a polyvalent AHSV vaccine using the canarypox virus vector (Guthrie *et al*., 2009).

Although VLPs have proved to be effective vaccine candidates, core-like particles (CLPs) have also been shown to result in a significant immune response (Wright *et al*., 2001). As an example, Roy *et al*. (1994) inoculated sheep with BTV-10 VP7 CLPs expressed in insect cells and these provided partial protection against a homologous viral challenge. Similarly, Maree *et al*. (1998) investigated AHSV-9 CLPs by co-infecting insect cells with recombinant baculoviruses expressing core proteins VP3 and VP7 respectively. A low CLP yield was however observed and it was found that VP7 was expressed at a much higher level due to suspected gene-specific properties leading to the accumulation of excess VP7 (Maree *et al*., 1998). Instead of incorporation into CLPs, accumulated VP7 formed distinctive crystal aggregates, similar to the previously described VP7 crystals of AHSV-4 (Chuma *et al.,* 1992).

Foot and mouth disease virus (FMDV)

Although a chemically inactivated vaccine preparation as well as animal movement restrictions provide reasonable control of FMD, high-containment manufacturing facilities, which are very expensive, are required for vaccine production (Grubman *et al*., 2005). Safer alternative vaccines, such as FMD VLPs, are therefore investigated as a possible solution. A FMD VLP consists of the three capsid proteins, VP0, VP1 and VP3. Most researchers have expressed the VLPs by incorporating the P1 gene coding for the three capsid proteins into an adenoviral vector (Grubman *et al*., 2005) but baculovirus expression as well as bacterial expression (Li *et al*., 2008; Lee *et al*., 2009) have recently also been accomplished. Swine showed complete protection one day after inoculation with adenovirus-vectored VLP vaccine, but only if administered in combination with adenovirus-vectored IFNα (Grubman *et al*., 2005). Baculovirus-expressed FMD VLPs also resulted in complete protection in 80% of animals following a virulent homologous challenge (Li *et al*., 2008). Although these results are promising, no subunit FMDV vaccine is available yet.

1.3.2 Peptide-based subunit vaccines

The identification of specific epitopes responsible for eliciting a neutralizing antibody response in the body, has led to the development of this novel vaccine approach that is non-pathogenic whilst still capable of inducing a sufficient immune response. Epitopes used for peptide-based vaccines are usually confined to one or more pathogen surface protein (Hansson *et al*., 2000) but are typically only 6-20 amino acid residues in length resulting in a low immunogenicity when used on their own (Ellis, 2001). Synthetic peptides are usually also rapidly degraded before these are recognised by the immune system (Olive *et al*., 2001). For this reason, peptides are either genetically fused or chemically conjugated to carrier molecule which would then form a large particle displaying the neutralizing epitope. When conjugated, bacterial proteins commonly encountered by humans, such as T toxoid, are used. Alternatively the epitope/peptide can be fused to a carrier protein at the N-terminal, C-terminal or internally, depending on the optimum display of the peptide/epitope while maintaining the structure of the carrier protein (Ellis, 2001). Once fused, the recombinant carrier protein is expressed in an appropriate expression system. By identifying both B-cell and T-cell epitopes, a humoral as well as a cellular immune response can be elicited making this vaccine approach potentially very attractive (Ellis, 2001).

There are several advantages to using peptide-based vaccines rather than protein-based vaccines (Purcell *et al*., 2007). Peptide-based vaccines can include multiple epitopes from the same pathogen or even multiple epitopes from different pathogens. Peptides can also be produced cost effectively on a large scale. Also, deleterious sequences from full-length proteins which can be oncogenic or responsible for autoimmune reactions can be excluded.

1.3.2.1 Peptides conjugated to particles

Peptides-based vaccines have limitations such as an inherent inability to cross the mucosal membrane, as well as rapid enzymatic degradation resulting in an insufficient immune response (Malik *et al*., 2007). Strategies for improving vaccine efficiency include the use of various epitope/peptide delivery systems. These include lipopeptides, branched polypeptides such as multiple antigen peptides (MAPs) (Sette *et al*., 2003) and more recently microspheres and various nano- and microparticles (Malik *et al.,* 2007).

Greenwood *et al*. (2008) used FMDV VP1 epitopes conjugated to nanobeads to inoculate sheep and found that these particles induced cell mediated as well as humoral immune responses. Results showed that although a single peptide induced an immune response, the

use of multiple peptides conjugated individually or together on one nanobead induced a significantly better response.

1.3.2.2 Peptides fused to carrier proteins

A prime example of such a particle carrier protein is the hepatitis B core protein (HBcAg) which, by itself, results in significant T-cell responses as well as high antibody titres upon inoculation. This 21kDa protein self-assembles into a CLP and can be expressed in large quantities in both a prokaryote or eukaryote system. Foreign epitopes can be inserted into HBcAg at various amino acid positions and are then efficiently presented on core particles that consist of between 180-240 HBcAg subunits. Mihailova *et al*. (2006) inserted hepatitis C B- and T-epitopes into the HBcAg and results from vaccination studies showed significant humoral responses with low, but evident cellular responses. Recently, the highly immunogenic epitopes of Hepatitis B (HBV) and C (HCV) were inserted at the C-terminal of HBcAg (Sominskaya *et al*., 2010). Mice immunized showed insert-specific T-cell responses, high antibody titres against the HBV epitope as well as cytotoxic T-lymphocyte (CTL) response.

The hepatitis B surface protein (HBsAg) VLP has also been used to display epitopes/peptides to the immune system. Netter *et al.* (2001) constructed a restriction enzyme site within the hydrophilic A determinant region and inserted peptides of various lengths containing epitopes of hepatitis C virus E2 protein. Mice were inoculated with these recombinant HBsAg VLPs and strain specific antibody responses were elicited.

The RNA bacteriophage PP7 which assembles into a VLP upon expression has also been used as a display system for a neutralizing epitope from the major capsid protein of human papilloma virus (HPV-16) (Do Carmo Caldeira *et al*., 2010). Here, large quantities of insert-specific antibodies were produced resulting in the protection of mice when infected with pseudoviruses HPV-16 and HPV-45.

Another example of a VLP presenting epitopes, is the HPV L1 protein. As mentioned earlier, HPV L1 expressed in a baculovirus expression system assembles into VLPs morphologically and immunologically identical to wild-type (Wt) virions (Koutsky *et al*., 2002). These VLPs have been investigated as carrier proteins by a number of researchers. HPV L1 has previously been modified by deleting 25 amino acid of the C-terminal and inserting peptides/epitopes. Dale *et al*. (2002) inserted fragments of SIV gag p27, HIV-1 tat and HIV-1 rev proteins but only partial protection was elicited. The bovine papilloma virus (BPV) L1 was recently modified to present repeats of a cross-neutralising HPV L2 epitope at an internal BPV

L1 site and upon inoculation in rabbits resulted in partial neutralisation as well as L2-specific antibodies (Slupetzky *et al*., 2007).

This vaccine strategy has also been employed for FMD vaccines. The major antigenic site of FMDV VP1 was expressed after the coding region was inserted into non-toxic *Pseudomonas aeruginosa* Exotoxin A (ntPE) (Challa *et al*., 2007). Intranasal inoculation with this chimeric protein resulted in mucosal as well as systemic immune responses against not only ntPE, but also against the FMDV VP1 major antigenic site. FMDV VP1 epitopes have also been fused to the N-terminal of the Cholera toxin B subunit. This recombinant carrier protein showed significant protection in mice and pigs against a FMDV challenge (Song *et al*., 2005). Recently, VP1 major antigenic site was inserted into one of the four extended loops on the infectious bursal disease virus protein VP2 and elicited a neutralising response upon inoculation of mice (Remond *et al*., 2009). However, large-scale evaluation of peptide-based vaccines in cattle showed that only 40% protection could be afforded indicating that the number of antigenic sites or T-cell epitopes presented to the immune system was too limited (Rodriguez *et al*., 2009).

This strategy for presenting foreign epitopes to the immune system lies at the centre of the research that will be presented here. The use of orbivirus proteins as carrier proteins will be discussed leading to the use of the capsid protein, VP7.

1.4 BTV proteins as carrier proteins

Using BTV proteins as carrier proteins for epitope/peptide display are mostly based on dual baculovirus-mediated expression of both VP7 and VP3 which results in the formation of core-like particles (CLPs). Immunogenic epitope/peptides are inserted into either the VP7 or VP3 genes and subsequently expressed using an appropriate expression system. Belyaev and Roy (1992) inserted a hepatitis B virus preS2 – epitope into the N-terminus of VP7 but could only identify recombinant core particles when unmodified VP3 and VP7 was co-expressed in the same expression system. Immunoelectron microscopy of recombinant CLPs confirmed the presence of the hepatitis B virus preS2- epitope on the surface of the particle. In another study, researchers inserted a glycoprotein from Bovine leukemia virus (BLV) into the C-terminal of the VP3 protein and the resultant CLP raised insert specific antibodies (Tanaka *et al*., 1995). Adler *et al*. (1998) inserted a T-cell epitope of the M1 Influenza A virus into the VP7 protein. A T-cell response was elicited when the recombinant VP7 and VP3 were dual expressed and the resulting core-like particles used for immunization.

Another BTV peptide/epitope display system that has been investigated is the non-structural protein, NS1. The unique feature of this protein is that it forms tubules during infection (Hewat *et al*., 1992) which have been implicated in the transport of the newly synthesized virions to the cell membrane (Owens et al., 2004). Once recombinantly expressed, tubular structures similar to those observed during infection can be identified in cells (Urakawa and Roy, 1988). Monastyrskaya *et al.* (1995) localized the antigenic site of the NS1 protein to the C-terminal which is exposed on the surface of the protein. This site has been used for displaying epitopes from *Clostridium difficile* toxin A, hepatitis B virus preS2 region, bovine leukemia virus p15 protein (Mikhailov *et al*., 1996), HA of Influenza A, VP1 of FMDV (Ghosh *et al*., 2002a) and the lymhocytic choriomeningitis virus nucleoprotein (Ghosh *et al.,* 2002b). In each case the tubular structure of NS1 was retained and each recombinant protein elicited a cellular as well as a humoral response. NS1 is expressed at a high level, can be isolated easily and accommodate large inserts of up to 60kDa without affecting tubule formation (Murphy and Roy, 2008).

1.5 AHSV protein VP7 structure and use as carrier protein

One of the two core proteins of AHSV namely VP7 has also been investigated for vaccine purposes. Unlike BTV VP7, recombinant expression of AHSV VP7 in insect cells produces large, disk-shaped, hexagonal crystals (Chuma *et al*., 1992, Maree *et al*., 1998). Wade-Evans *et al*. (1997) injected these AHSV-9 VP7 crystal aggregates, purified from BHK cells, into Balb/c mice. After inoculation with the crystals in three Freund's adjuvant doses, complete protection against heterologous AHSV-7 challenge was elicited. As a result of the inability of passively transferred antibodies to confer protection to a subsequent generation, the authors hypothesized that a T-cell response was elicited by vaccination. These results illustrate the plausibility of using VP7 as a subunit vaccine against AHS and also form the basis of our investigations into using VP7 as an epitope/peptide display system. A brief description of the VP7 protein structure follows to aid in understanding of the display system.

VP7 is one of the smallest of the orbivirus proteins with a size of 38,548 kDa and is rich in hydrophobic amino acids (Roy, 1996a). Although the complete crystal structure of AHSV VP7 has not been resolved, Basak *et al*. (1996, 1997) have determined the crystal structure of the upper domain of AHSV VP7. Comparisons with BTV VP7 indicated that the structure and composition of these two proteins are very similar, with exceptions on sequence level. Each VP7 monomer consists of two distinct domains, an upper and a lower domain. Upon expression, these monomers spontaneously assemble into trimers (Figure 1.2) such that the upper domain of one monomer rests on the lower domain of another monomer in a clockwise

direction. The smaller upper domain is folded into an antiparallel β sandwich whilst the lower domain is composed of 9 α-helices as well as extended loops that are formed by both the N- and C-terminus (Roy, 1996a). These two domains are joined by a single Lys residue which, when mutated, results in defective core formation (Le Blois and Roy, 1993).

Furthermore, the upper domains, forming the head region of the trimer, have a characteristic Arg-Gly-Asp (RGD) motif that has been shown to be exposed to the aqueous surroundings of the host cell located at amino acid residues 168-170. In AHSV, the Arg residue is substituted by an Ala residue, but an RGD motif is nonetheless located at amino acid residues 178-180. The RGD motif is a ligand site that interacts with host proteins from the integrin family (Basak *et al.*, 1996). In this regard, researchers have shown that the BTV VP7 RGD motif plays an integral role in cell entry (Tan *et al.*, 2001).

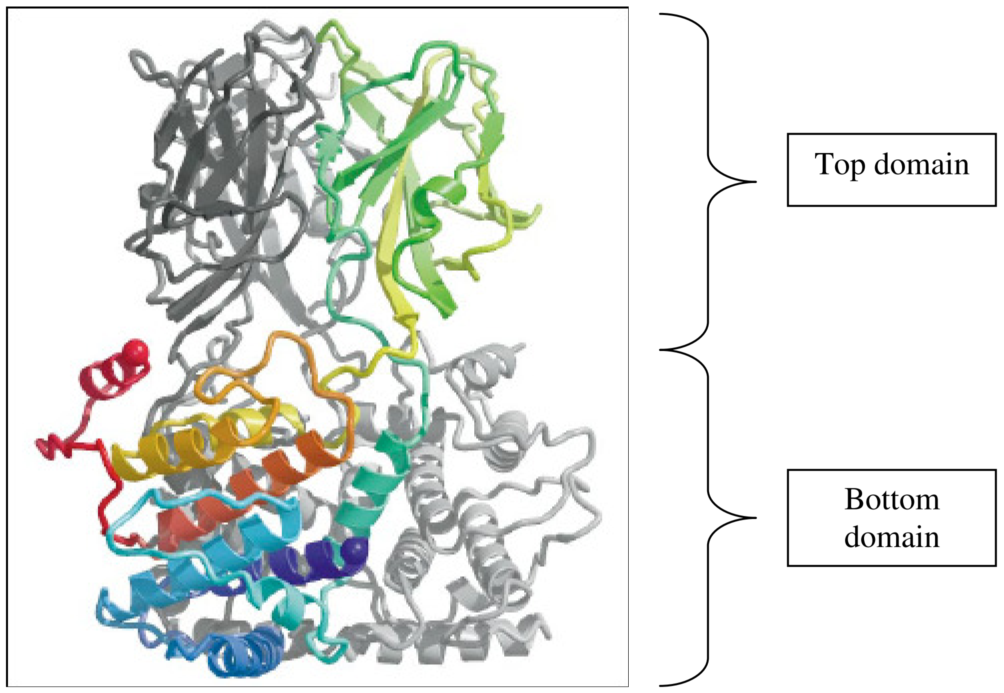
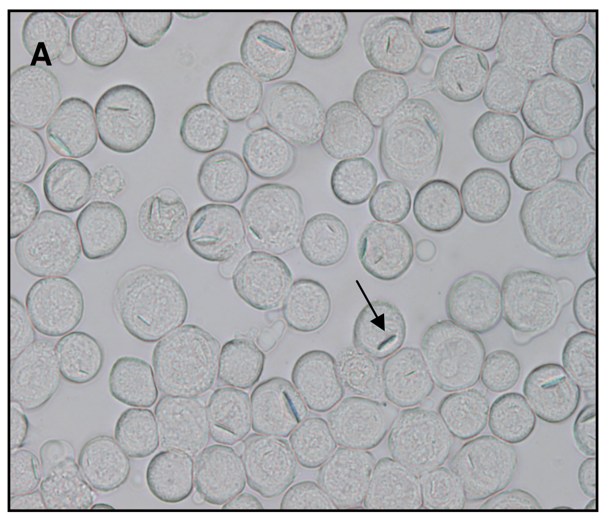


Figure 1.2 A computer generated representation of the BTV-10 VP7 trimer crystal. One VP7 monomer of the trimer is represented in color (Basak *et al.*, 1997).

The trimers interact via hydrophobic patches on their connecting surface, but binding seems to be largely unspecific, although residues 271 and 255 are closely associated with trimer-trimer interactions (Limn *et al.*, 2000). In the virus particle the trimers are arranged in six- and five-membered rings (at the vertices of the icosahedrons). A total of 760 VP7 molecules forms 260 trimers which arrange around 132 aqueous channels that can be grouped into three types; type I channels running along the icosahedral fivefold axes, type II channels that surround the fivefold axes and type III which are located around the threefold axes. A number of these channels penetrate through the inner layer enabling the extrusion of newly synthesized mRNA after transcription has been activated (Roy, 2005). The lower domains of

VP7 are pressed down on six corners of a single VP3 molecule, such that four trimers (12 molecules) are associated with one VP3 molecule (Roy, 2005).

As mentioned, a feature unique of Wt AHSV VP7 is its ability to form crystal aggregates when recombinantly expressed by a baculovirus expression system. These not only vary in size (between 1 - 25μm in diameter), but also in number per cell (varying between one to three). Figure 1.3 shows the VP7 crystals expressed in insect cells and also a scanning electron micrograph of a crystal.



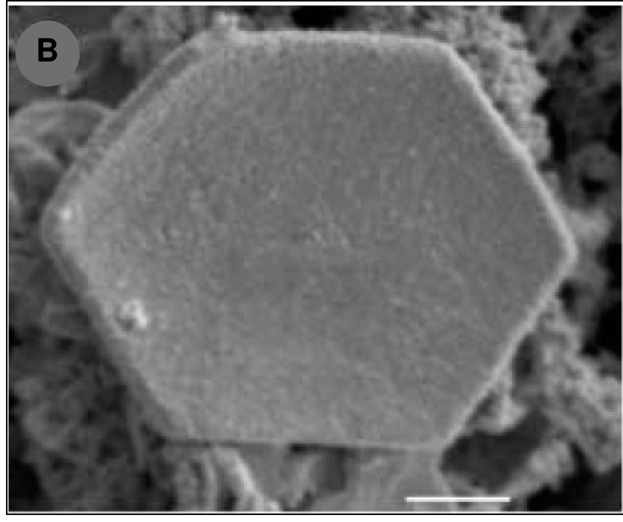
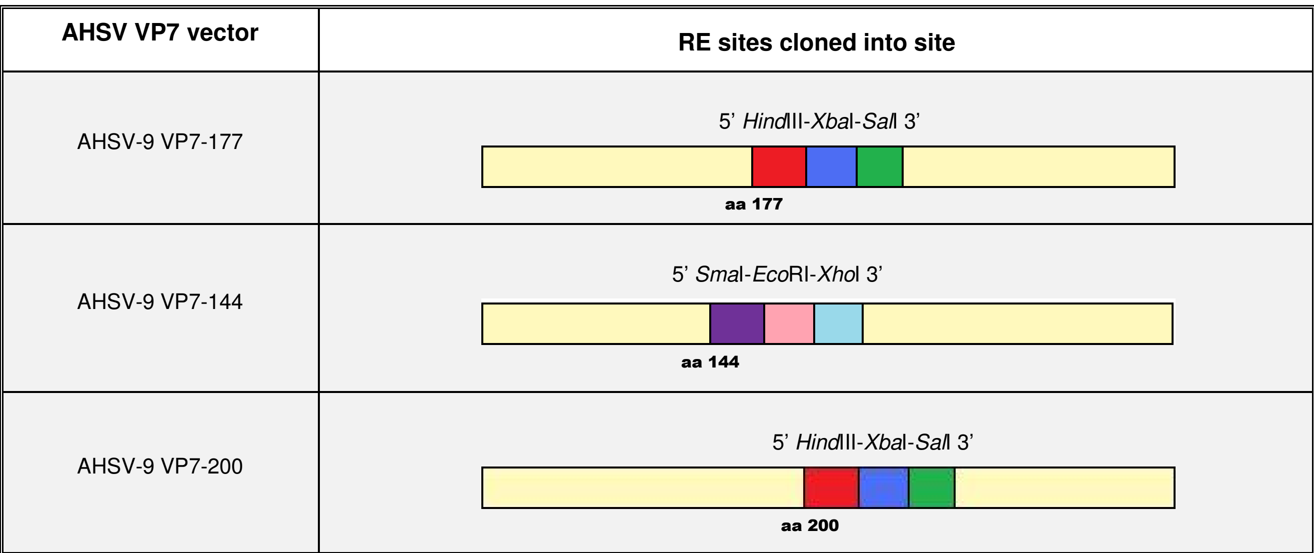


Figure 1.3 **(A)** Light microscopy photos of 10x magnification of Sf-9 cells infected with recombinant baculoviruses expressing Wt AHSV-9 VP7 (Meyer, 2003). The arrow indicates a VP7 crystal. (**B)** Scanning electron micrographs show the VP7 crystal (Maree, 2000).

These crystals form a lattice consistent with VP7 monomer structure with a direct structural relation to the ring-shaped capsomers on the core (Burroughs *et al*., 1994; Basak *et al*., 1997; Grimes *et al*., 1995). They are suspected to consist of a double layer of VP7 trimers, with their hydrophobic bottom domains on the inside and their hydrophilic top domains exposed to the surroundings (Maree, 2000). As mentioned earlier, Wade-Evans *et al*. (1997) found that inoculating mice with these VP7 crystals resulted in complete protection following a lethal heterologous challenge. These results initiated the idea of using these crystals for the display of epitopes/peptides to the immune system. This VP7 particulate display system is advantageous as expression in insect cells results in high yields of crystal particles which can be purified in a single step by ultracentrifugation (Maree, 2000).

Maree (2000) investigated the AHSV-9 VP7 protein to identify sites suitable for epitope/peptide insertion. Hydropathic profiles indicated that both the C- and N-terminal were hydrophobic whilst the central region of VP7 had alternating hydrophobic and hydrophilic regions. Subsequent hydrophobicity plots showed one large and four smaller

hydrophilic regions. Using computer software, a three-dimensional model of VP7 trimer was generated to identify amino acids located on the surface of the trimer. Three of the four hydrophilic regions were found to be accessible on the VP7 surface of which two sites showed the most promise: amino acid position 177/178 (within a hydrophillic RGD loop) as well as position 200/201, which are both hydrophillic areas on exposed loops of the VP7 protein. Once identified, these sites were modified by insertion of three restriction enzyme sites, *Hind*III, *Xba*I and *Sal*I (Maree, 2000). The third site, at position 144/145, also located within the same RGD loop, was later also modified by insertion of three restriction enzyme sites *Sma*I, *Eco*RI and *Xho*I (Riley, 2003). The three constructed VP7 vectors were named in accordance with their insertion site, for example modified VP7 with restriction enzyme sites at amino acid position 177 was named the AHSV-9 VP7-177 vector. Figure 1.4 provides a summary of the restriction enzyme sites inserted at each position within the VP7 protein, and the vector name.



AHSV VP7 vector	RE sites cloned into site
AHSV-9 VP7-177	5' *Hind*III-*Xba*I-*Sal*I 3' aa 177
AHSV-9 VP7-144	5' *Sma*I-*Eco*RI-*Xho*I 3' aa 144
AHSV-9 VP7-200	5' *Hind*III-*Xba*I-*Sal*I 3' aa 200

Figure 1.4 A schematic representation of the three different AHSV-9 VP7 display vectors. The different RE sites inserted into each AHSV-9 VP7 vector at the specific aa position is indicated above each construct.

Figure 1.5 shows the location of all three insertion sites on AHSV-9 VP7 top domain. All the sites seem to be on the surface of the trimer and would therefore likely be in contact with the aqueous environment within the cell.

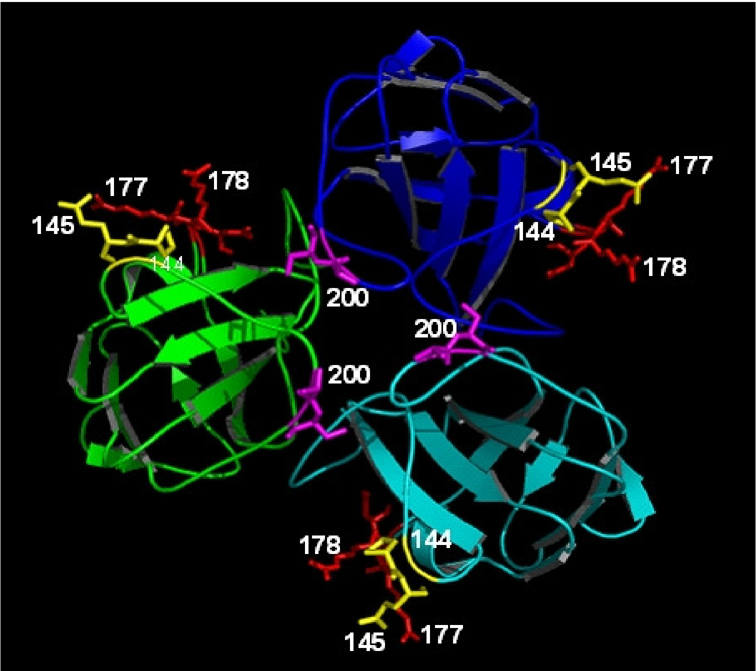


Figure 1.5 A schematic representation of the crystallographic structure of the top view of AHSV VP7 trimer. Each monomer is represented in a different color. The hydrophilic sites targeted for mutagenesis are shown in red at position 177-178, yellow at position 144-145 and magenta at position 200 (Maree,2000)

To evaluate the immune response against these mutant proteins, several epitopes/peptides were inserted into either AHSV-9 VP7-177 or AHSV-9 VP7-144 or both. Meyer (2003) inserted varying repeats of the linear HIV-1 epitope (ELDKWA) into both AHSV-9 VP7-177 and AHSV-9 VP7-144. The insertion resulted in a decrease in the solubility of each VP7 mutant protein and large, characteristic crystal structures could be seen upon expression.

Full length FMDV VP1 as well as a peptide containing a region of conserved linear B-cell epitopes of FMDV VP1 were inserted into AHSV-9 VP7-177 (Rutkowska *et al*., 2011). Serotype-O of FMDV contains five antigenic sites of which only one site located within site A of the G-H loop, is continuous. Site A consists of the major antigenic site, the G-H loop located between residues 141 and 161, and the minor antigenic site, the C-terminal residues located between residues 200-213. This G-H loop has also been shown to contain a linear B-cell epitopes (Challa *et al*., 2007). The B-cell epitope that was inserted into AHSV-9 VP7-177 consists of amino acid residues 129-164 of VP1, from the Sat2 vaccine strain Zim 7/83/2 (Aggarwal and Barnett, 2002). The insertion of this epitope into AHSV-9 VP7-177, subsequently referred to as AHSV-9 VP7-177FMDVEpi, resulted in a decrease in the solubility of the AHSV-9 VP7-177 vector. SEM analysis showed that AHSV-9 VP7-177FMDVEpi did form characteristic hexagonal crystals but the surface was uneven.

Martinez-Torrecuadrada *et al*. (1995), investigated antigenic regions on AHSV-4 VP2 and identified a region they called fragment H between amino acids 285-413. Later, they isolated two distinct epitopes within this region, epitope “a” (aa 318-342) and “b” (aa 374-403) which showed an increased capacity to induce neutralizing antibodies when they were used in combination for immunization (Martinez-Torrecuadrada *et al*., 2001). Rutkowska *et al*. (2011) inserted a 110aa region spanning these two epitopes (aa 298-408) into AHSV-9 VP7-144 and found that this insertion also decreased the solubility of the protein.

Although initially the crystals were investigated as peptide display systems, this strategy was later combined with the use of the soluble component of the recombinant VP7 proteins as it was shown that changes in solubility did not affect trimer formation (Rutkowska *et al*., 2011). A poor immune response was observed when animals were inoculated with the chimeric VP7 crystals. Immune responses assessed by enzyme-linked immunosorbent assay (ELISA) tests, neutralization assays and western blot analyses showed that in each case low insert-specific immune responses were elicited. Rutkowska *et al*. (2011) found that the response could be significantly improved if the AHSV-9 VP7-177FMDVEpi was in soluble trimer form. Following inoculation with the soluble VP7 chimeric proteins strong insert-specific immune responses resulted, as well as neutralising antibodies. In fact, it was consistently found that chimeric VP7 proteins with a soluble fraction of 20% of the total protein or more were more immunogenic. However, when this fraction dropped below 5% of the total protein, no insert specific immune response was ever elicited (Rutkowska *et al*., 2011). These results clearly reiterate the importance of solubility in eliciting an immune response and therefore shunted investigations into increasing the solubility of the chimeric VP7 proteins.

1.5.1 Soluble AHSV VP7 trimers as vaccine display systems

Since the soluble AHSV-9 VP7 trimers resulted in a significant immune response compared to VP7 particles, research in our group is currently directed towards developing a vaccine display system based on soluble VP7 trimers. Advantages of this display strategy include that the physical constraints associated with trimers may provide some uniformity required for adequate immune display. Secondly, the flexibility of the individual VP7 trimers may increase the relative exposure of the inserted peptide to the immune system compared to a single CLP. Also, the humoral immune response against 260 chimeric VP7 trimers should also be greater than against a single particle (Rutkowska *et al*., 2011).

As mentioned, research on the insertion of peptides/epitopes of different sizes and hydrophobicities into the different VP7 insertion sites has been conducted. These ranged from various AHSV-4 VP2 epitopes, FMDV peptides and gp41 HIV-1 epitopes to reporter genes such as full length enhanced green fluorescent protein (eGFP) (Riley, 2003; Maree, 2000; Mizrachi, 2008; Rutkowska *et al.*, 2011). These peptides/epitopes sizes range from as little as 19 amino acids, in the case of AHSV-4 VP2 epitopes, to approximately 240 amino acids in the case of eGFP. Once expressed and used for inoculation, the extent of solubility of these trimers seemed to be the most important factor in eliciting an immune response, as chimeric proteins with larger soluble fractions were consistently more immunogenic and induced antibodies measurable by ELISA and western blot analyses (Rutkowska *et al.*, 2011). Solubility assays of these constructs have also shown that, depending on the insert and the particular display vector used, the solubility of the chimeric VP7 protein could be manipulated within the range of 3% to 70% of the total amount of VP7 expressed (Rutkowska *et al.*, 2011).

eGFP has been inserted into AHSV-9 VP7 vectors AHSV-9 VP7-144 and AHSV-9 VP7-177 (Mizrachi, 2008; Seameco, unpublished results). Green fluorescent protein (GFP) is a 238 amino acid protein found in the jellyfish, *Aequorea* and was first discovered by Shimomura *et al.* (1962). The protein is responsible for the chemiluminescence associated with jellyfish and has an excitation peak at 395nm giving emission peak at 508nm. The valuable application for this protein however, was only discovered when Chalfie *et al.* (1994) and Inouye and Tsuji (1994) showed that the protein also fluoresces in other organisms upon expression. This implied that GFP requires no jellyfish-specific proteins for fluorescence (Tsien, 1998).

Structurally, the GFP chromophore is a *p*-hydroxybenzylideneimidazolinone which is formed by residues 65-67 encoding amino acids Ser-Tyr-Gly. Chromophore formation comes about by the folding of GFP into semi-native conformation, followed by nucleophillic attack of the amide bonds between residues 65 and 66. Dehydration and subsequent dehydrogenation finally results in incorporation of the aromatic group on residue 66 into the imidazolinone. Only once this stage is reached does the chromophore acquire fluorescence. Crystal structure resolution shows that this chromophore is attached to an α-helix which is imbedded within an 11-stranded β-barrel (Tsien, 1998). Also, discrepancies concerning the aggregation of GFP have in some instances shown it as crystallizing into a dimer (Yang *et al.* 1996), whereas other studies have shown that GFP can also crystallize as a monomer (Brejc *et al.*, 1997). The ability to dimerize has been attributed to high protein concentration and high ionic strength (Cubitt *et al.*, 1995).

Wild-type GFP folds efficiently when expressed at or below room temperature but at higher temperatures folding efficiency declines. Researchers have thus introduced mutations that increase the number of molecules that fold correctly under adverse conditions, like temperatures above 25˚C. One of these mutations, F64L, helps GFP to fold properly but also gives greater brightness at higher temperatures (Cormack *et al*., 1996). Other mutations cause alterations of wavelength frequencies, such as the S65T mutation which causes ionization of the phenol of the chromophore (Heim *et al*., 1995), resulting in rapid chromophore formation. This holds several advantages like a six-fold increase in brightness compared to the wild-type protein as well as faster oxidation which results in faster chromophore formation (Cubitt *et al*., 1995). Enhanced GFP (eGFP) is a derivative of GFP that has been mutated at sites F64 and S65 (Heim *et al*., 1995).

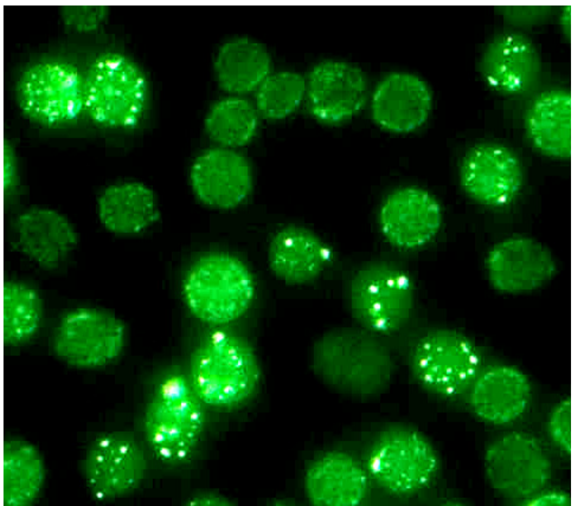


Figure 1.6 A 40x magnification of Sf-9 insect cells expressing AHSV-9 VP7-177eGFP viewed by fluorescent microscopy (Mizrachi, 2008).

An important feature of a chimeric VP7-eGFP protein is that its expression can be visualised by fluorescence microscopy (Figure 1.6). Furthermore, the visualisation does not only show that the protein is expressed, but can also give an indication of correct folding (Tsumoto *et al*., 2003). Solubility assays AHSV-9 VP7-177eGFP showed that approximately 19% of proteins were in the top half of the gradient resenting the soluble, trimeric form and almost half the total amount of protein was completely insoluble (Mizrachi, 2008). Quantifying the fluorescence values between the soluble and particulate fraction showed fluorescence/unit was approximately 7 times higher for the soluble trimers than the particulate fraction. These results indicated improved exposure of eGFP to the cellular environment when inserted in the soluble trimers compared to the particles.

Research involving the AHSV-9 VP7 vectors has provided information regarding the factors important for producing a soluble chimeric VP7 protein. The size of the foreign insert seems less important for efficient display, providing that after insertion of the peptide/epitope, VP7 is still able to fold correctly. This was illustrated by the fact that a large peptide such eGFP resulted in a considerable soluble fraction, whereas a small insert such as a gp41 HIV-1 epitope resulted in a completely insoluble protein (Meyer, 2003; Mizrachi, 2008). The hydrophilicity of an insert is important only with respect to the secondary structure, ie correct folding of the chimeric protein. The most important aspect, however, is the display of the insert to the aqueous environment and although correct folding of the peptide is important, display is mostly dependent on the insertion site (Rutkowska *et al*., 2011). Space constraints associated with the proximity of the insertion sites in a VP7 trimer can be a determining factor in epitope display. For instance, the insertion sites for AHSV-9 VP7-177 and AHSV-9 VP7-144 are located far apart once the trimer has been formed, whereas the AHSV-9 VP7-200 sites are in close proximity (Figure 1.5). In the case of AHSV-9 VP7-144 and AHSV-9 VP7-177, there was an increase in the solubility of Wt AHSV VP7 following introduction of the RE sites in the top domain, whereas insertion of these sites into position 200 did not have an effect on the solubility. It did however have an effect on the formation of the chimeric protein trimers (Rutkowska *et al*., 2011).

As solubility is important for eliciting an immune response, it was reasonable to hypothesize that the most soluble VP7 vector for peptide/epitope display would likely result in the most soluble chimeric protein. Wt BTV VP7 is inherently more soluble than AHSV VP7, and therefore the possibility of developing a BTV VP7 display system is the focus of the research presented in this study.

1.5.2 Inherent solubility of AHSV and BTV VP7

A novel BTV-10 VP7 display vector, referred to as BTV-10 VP7-169, was recently constructed for display of immunologically important peptides/epitopes (Dr. W. Fick, University of Pretoria). The merit for the construction of this vector lies in the difference in solubilities between AHSV VP7 and BTV VP7. This difference in solubility was well demonstrated in a study by Monastyrskaya *et al.* (1997) which investigated the effects of domain-switching between BTV-10 VP7 and AHSV-4 VP7. In the study, the central upper domain regions of VP7 were switched between the two orbivirus proteins resulting in an N-terminal-AHSV-BTV upper domain region-AHSV-C-terminal (ABA) and N-terminal-BTV-

AHSV upper domain-BTV-C-terminal (BAB). Results showed that both chimeric proteins formed trimers, however, the solubilities of the chimeric proteins differed substantially. ABA was as soluble as Wt BTV VP7, whereas the majority of BAB was insoluble. This result indicated that the insolubility of AHSV VP7 can be attributed to the upper domain of the protein.

For AHSV VP7, the RGD domain is located at a position downstream from that of BTV VP7. The Arg residue of the RGD domain of BTV VP7 is substituted by an Ala residue, and although this substitution was initially implicated in contributing to AHSV VP7 insolubility (Basak *et al*., 1996), further investigations showed that mutation of this site had no effect on solubility (Monastyrskaya *et al*., 1997). Another residue was identified as possible contributing to AHSV VP7 insolubility, namely Arg-345. This Arg residue, located at position 345 of the C-terminal of BTV VP7, has been replaced by Leu residue in AHSV VP7 (Monastyrskaya *et al*., 1997). Investigations involving mutation of this specific site, however, only showed a marginal increase in solubility (Meyer, 2003).

Although no specific sequence differences between BTV and AHSV VP7 could be identified that would contribute to the increased BTV VP7 solubility, the fact remains that BTV VP7 is inherently soluble and could provide a more soluble vector for peptide/epitope display compared to AHSV VP7. This study therefore aims to characterise the BTV-10 VP7-169 protein with regards to solubility, and subsequently investigates the effects on this solubility when inserting peptides/epitopes of various lengths into this vector.

1.6 Aims

The aim of this study was to investigate the potential of using the BTV-10 VP7-169 vector as a vaccine display system, and to compare it to the available AHSV-9 VP7 display vectors.

Specific short term objectives included:

- The characterisation of a vector based on the major core protein VP7 of bluetongue virus (BTV), BTV-10 VP7-169, with regards to solubility and the ability to form trimers as part of assessing this vector for the display of foreign peptides.
- Constructing chimeric proteins in which a FMDV VP1 epitope and the full length eGFP protein are displayed in the BTV-10 VP7-169 vector
- Exploring aspects related to the display potential of chimeric BTV-10 VP7-169 protein including the solubility and ability to form trimers

References:

Adler S, Reay P, Roy P, Klenk H-D (1998) *Medical Microbiology and Immunology*, **187**, 91-96.

Amanna IJ and Slifka MK (2009) Wanted, dead or alive: New viral vaccines. *Antiviral Research*, **84**, 119–130.

Aggarwal N, Barnett PV (2002) Antigenic sites of foot-and-mouth disease virus (FMDV): an analysis of the specificities of anti-FMDV antibodies after vaccination of naturally susceptible host species. *Journal of General Virology,* **83**, 775-782.

Babiuk LA (1999) Broadening the approaches to developing more effective vaccines *Vaccine*, **17**, 1587±1595.

Basak AK, Gouet P, Grimes J, Polly R, Stuart D (1996) Crystal Structure of the top domain of African horse sickness virus VP7: comparisons with Bluetongue virus VP7. *Journal of Virology*, **70**, 3796-3806.

Basak AK, Grimes JM, Gouet P, Roy P, and Stuart D I (1997). Structures of orbivirus VP7: implications for the role of this protein in the viral life cycle. *Structure,* **5**(7)**,** 871-83.

Birnboim HC, Dolly J (1979) A rapid alkaline extraction procedure for screening recombinant plasmid DNA. *Nucleic Acid Research,* **7**, 1513-1523.

Belyaev AS, Roy P (1992) Presentation of hepatitis B virus preS2 epitope on bluetongue virus core-like particles. *Virology*, **190**, 840-844.

Brave A, Ljungberg K, Wahren B, Liu MA (2006) Vaccine delivery methods using viral vectors. *Molecular pharmaceutics*, **4**, 18-32.

Brejc K, Sixma TK, Kitts PA, Kain SR, Tsien RY, *et al.* (1997) Structural basis for dual excitation and photoisomerization of the *Aequorea victoria* green fluorescent protein. *Proceedings of the National Academy of Science*, **94**, 2306–2311.

Brennan FR, Dougan G (2005) Non-clinical safety evaluation of novel vaccines and adjuvants: new products, new strategies. *Vaccine*, **23**, 3210-3222.

Burrage, T. G., and Laegreid, W. W. (1994). African horsesickness: pathogenesis and immunity. *Immunology and Microbiology of Infectious Diseases*, **17**, 275-85.

Burroughs JN, O'Hara RS, Smale CJ et al. (1994) Purification and properties of virus particles, infectious sub-viral particles, cores and VP7 crystals of African horse sickness virus serotype 9. *Journal of General Virology*, **75**, 1849-1857.

Campbell MK (1999) **Biochemistry**. Third edition. Harcourt college Publishers, Orlando, Florida.

Chalfie M, Tu Y, Euskirchen G, Ward WW, Prasher DC (1994) Green fluorescent protein as a marker for gene expression. *Science*, **263**, 802-805.

Cha HJ, Shin HS, Lim HJ, Cho HS, Nimish ND, Pham MQ, Bentley WE (2005) Comparative production of human interleukin-2 fused with green fluorescent protein in several recombinant expression systems. *Biochemical Engineering Journal*, **24**, 225-233.

Charbonnier F, Kohler T, Pechere J-C, Ducruix A (2001) Overexpression, refolding and purification of the histidine-tagged outer membrane efflux protein OprM of *Pseudomonas aeruginosa*. *Protein Expression and Purification*, **23**, 121-127.

Challa S, Barrette R, Rood D, Zinckgraf J, French R, Silbart L (2007) Non-toxic Pseudomonas aeruginosa exotoxin A expressing the FMDV VP1 G-H loop for mucosal vaccination of swine against foot and mouth disease virus. *Vaccine*, **25**, 3328-3337.

Chassy BM, Mercenier A, Flickinger J (1988) Transformation of bacteria by electroporation. *Trends in Biotechnology*, **6**, 303-309.

Cohen SN, Chang ACY, Hsu L (1972) Nonchromosomal antibiotic resistance in bacteria: genetic transformation of Escherichia coli by R-factor DNA. *Proceeding of the National Academy of Science*, **69**, 2110-2114.

Cormack BP, Valdivia RH, Falkow S (1996) FACS-optimized mutants of the green fluorescent protein (GFP). *Gene*, **173**, 33–38.

Cubitt AG, Heim R, Adams SR, Goyd AE, Gross LA, Tsien RY (1995) Understanding, improving and using green fluorescent proteins. *TIBS*, **20**, 448-455.

Dale CJ, Liu XS, De Rose R, Purcell DFJ, Anderson J, Xu Y et al. (2002) Chimeric Human Papilloma Virus–Simian/Human Immunodeficiency Virus Virus-like-Particle Vaccines: Immunogenicity and Protective Efficacy in Macaques. *Virology*, **301**, 176-187.

Demirev AV, Lee C-H, Jaishy BP, Nam D-H, Ryu DDY (2006) Substrate specificity of nonribosomal peptide synthetase modules responsible for the biosynthesis of the oligopeptide moiety of cephabacin in *Lysobacter lactamgenus*. *FEMS Microbiology Letters*, **255**, 121-128.

Dertzbaugh MT (1998) Genetically engineered vaccines: An overview. *Plasmid*, **39**, 100-113.

Do Carmo J, Medford A, Kines RC, Lino CA, Schiller JT, Chackerian B, Peabody DS (2010) Immunogenic display of diverse peptides, including a broadly cross-type neutralizing human papillomavirus L2 epitope, on virus-like particles of the RNA bacteriophage PP7. *Vaccine*, **28**, 4384-4393.

Du Plessis M, Cloete M, Aitchinson H, Van Dijk AA (1998) Protein aggregation complicates the development of baculovirus-expressed African horsesickness virus serotype 5 VP2 subunit vaccines, *The Onderstepoort Journal of Veterinary Research*, **65**, 321-329.

Ellis RW (2001) Technologies for the design, discovery, formulation and administration of vaccines. *Vaccine*, **19**, 2681-2687.

Fenner F (1976) Classification and nomenclature of viruses; second report of the International Committee on Taxonomy of Viruses. *Intervirology*, **7**, 1-115.

Fink AL (1998) Protein aggregation: folding aggregates, inclusion bodies and amyloid. *Folding and Design*, **3**, R9-R23.

Garcea RL, Gissmann L (2004) Virus-like particles as vaccines and vessels for the delivery of small molecules. *Current Opinion in Biotechnology*, **15**, 513-517.

Garcia-Fruitos E, Gonzalez-Montalban N, Morell M, Vera A, Ferraz RM, Aris A, Ventura S, Villaverde A (2005) Aggregation as bacterial inclusion bodies does not imply inactivation of enzymes and fluorescent proteins. *Microbial Cell Factories*, **4**, 1-6.

Garcia-Mata R, Bebok Z, Sorscher EJ, Sztul ES (1999) Characterization and Dynamics of Aggresome Formation by a Cytosolic GFP-Chimera. *The Journal of Cell Biology*, **146**, 1239-1254.

Ghosh MK, Deriaud E, Saron M-F, Lo-Man R, Henry T, Jiao X, Roy P, Leclerc C (2002a) Induction of protective antiviral cytotoxic T cells by a tubular structure capable of carrying large foreign sequences. *Vaccine*, **20**, 1369-1377.

Ghosh MK, Borca MV, Roy P (2002b) Virus-derived tubular structure displaying foreign sequences on the surface elicit CD4-Th cell and protective humoral responses. *Virology*, **302**, 383-392.

Gonzalez-Montalban N, Garcia-Fruitos E, Villaverde A (2007) Recombinant protein solubility—does more mean better? *Nature Biotechnology*, **25**, 718-720.

Greenwood DLV, Dynon K, Kalkanidis M, Xiang S, Plebanski M, Scheerlinck J-P Y (2008) Vaccination against foot-and-mouth disease virus using peptides conjugated to nano-beads. *Vaccine*, **26**, 2706-2713.

Grimes J, Basak AK, Roy P, Stuart D (1995) The crystal structure of bluetongue virus VP7. *Nature*, **373**, 167-169.

Grubman MJ, Baxt B (2005) Foot-and-Mouth Disease. *Clinical Microbiology Reviews*, **17**, 465-493.

Guthrie AJ, Quan M, Lourens CW, Audonnet J-C, Minke JM, Yao J et al. Protective immunization of horses with a recombinant canarypox virus vectored vaccine co-expressing genes encoding the outer capsid proteins of African horse sickness virus. (2009) *Vaccine*, **27**, 4434-4438.

Hansson M, Per-Ake N, Stahl S (2000) Design and production of recombinant subunit vaccines. *Biotechnology and Applied Biochemistry*, **32**, 95-107.

Heim R, Cubitt AB, Tsien RY (1995) Improved green fluorescence. *Nature,* **373**, 663–664.

Huismans H (1979) Protein synthesis in bluetongue virus infected cells. *Virology*, **92**, 385-396.

Huismans H, Van der Walt NT, Cloete M, Erasmus BJ (1987) Isolation of a capsid protein of bluetongue virus that induces a protective immune response in sheep. *Virology*, **157**, 172-179.

Inouye S, Tsuji FI (1994) Aequorea green fluorescent protein: Expression of the gene and fluorescence characteristics of the recombinant protein. *FEBS Letters*, **341**, 277-280.

Inumaru S, Roy P (1987) Production and characterization of the neutralization antigen VP2 of bluetongue virus serotype 10 using a baculovirus expression vector. *Virology*, **157**, 472-479.

Ishibashi M, Tsumoto K, Tonkungaga M, Ejima D, Kita Y, Arakawa T (2005) Is arginine a protein-denaturant? *Protein Expression and Purification*, **42**, 1-6.

Jenner, E., (1799). Further observations on the Variolae vaccinae. Sampson Low, London.

Jenner, E., (1800). A continuation of facts and observations relative to the Variolae vaccinae, or Cowpox. Sampson Low, London.

Kar A, Iwatani N, Roy P (2005) Assembly and intracellular localization of the Bluetongue virus core protein VP3. *Journal of Virology*, **79**, 11487-11495.

Kopito RR (2000) Aggresomes, inclusion bodies and protein aggregation. *Trend in Cell Biology*, **10**, 524-530.

Koutsky, L.A. *et al.* (2002) A controlled trial of a human papillomavirus type 16 vaccine. *New England Journal of Medicine*, **347**, 1645–1651.

Kudou M, Yumioka R, Ejima D, Arakawa T, Tsumoto K (2011) A novel protein refolding system using lauroyl-l-glutamate as a solubilizing detergent and arginine as a folding assisting agent. *Protein Expression and Purification*, **74**, 46-54.

Le Blois H and Roy P (1993) A single point mutation in the VP7 major core protein of bluetongue virus prevents the formation of core-like particles. *Journal of Virology*, **67**,355 359.

Li Z, Yi Y, Yin X, Zhang Z, Liu J (2008) Expression of Foot-and-Mouth Disease Virus Capsid Proteins in Silkworm-Baculovirus Expression System and Its Utilization as a Subunit Vaccine. *PLoS*, 3, e2273

Liljeqvist S, Stahl S (1999) Production of recombinant subunit vaccines: protein immunogens, live delivery systems and nucleic acid vaccines. *Journal of Biotechnology*, **73**, 1-33.

Limn C-K, Staeuber N, Monastyrskaya K, Gouet P, Roy P (2000) Functional dissection of the major structural protein of Bluetongue virus: Identification of key residues within VP7 essential for capsid assembly. *Journal of Virology*, **74**, 8658-8669.

Luckow VA (1993) Baculovirus systems for the expression of human gene products. *Current opinion in Biotechnology,* **4**, 564-572.

Mahler H-C, Friess W, Grauschopf U, Kiese S (2009) Protein aggregation: Pathways, induction factors and analysis. *Journal of Pharmaceutical Sciences*, **98**, 2909 -2934.

Malik DK, Baboota S, Ahuja A, Hasan S, Ali J (2007) Recent advances in protein and peptide drug delivery systems. *Current Drug Delivery*, **4**, 141-151.

March JC, Rao G, Bentley WE (2003) Biotechnological applications of green fluorescent protein. *Applied Microbiology and Biotechnology*, **62**, 303-315.

Maree FF (2000) Multimeric protein structures of African horsesickness virus and their use as antigen delivery systems. PhD thesis

Maree S, Durbach S, Huismans H (1998) Intracellular production of African horsesickness virus core-like particles by expression of the two major core proteins, VP3 and VP7, in insect cells. *Journal of General Virology*, **79**, 333-337.

Martinez-Torrecuadrada J, Langeveld JPM, Meloen RH, Casal JI (2001) Definition of neutralizing sites on African horse sickness virus serotype 4 VP2 at the level of peptides. *Journal of General Virology*, **82**, 2415-2424.

Martinez-Torrecuadrada J and Casal JI (1995) Identification of a linear neutralizing domain in protein VP2 of African horsesickness virus. *Virology*, **210**, 391-399.

Mcaleer WJ, Buynak EB, Maigetter RZ, Wampler DE, Miller J, Hilleman (1984) Human hepatitis B vaccine from recombinant yeast. *Nature*, **307**, 178-180.

Mertens PPC, Burroughs JN, Anderson J (1987) Purification and properties of virus particles, infectious subviral particles, and cores of bluetongue virus serotypes 1 and 4. *Virology*, **157**, 375-386.

Mertens PPC, Brown F, Sangar DV (1984) Assignment of the genome segments of bluetongue virus type 1 to the proteins which they encode. *Virology*, **135**, 207-217.

Mertens PPC, Diprose J (2004) The bluetongue virus core: a nano-scale transcription machine. *Virus Research*, **101**, 29-43.

Meyer Q (2003) The presentation of HIV-1 neutralizing epitopes on the surface of major structural protein VP7 of African horse sickness virus. MSc. Thesis

Mihailova M, Boos M, Petrovskis I, Ose V, Skrastina D, Fiedler M *et al*. (2006) Recombinant virus-like particles as a carries of B- and T-cell epitopes of hepatitis C virus (HCV), *Vaccine*, **24**, 4369-4377.

Mihailov M, Monastyrskaya K, Bakker T, Roy P (1996) A new form of particulate single and multiple immunogen delivery system based on recombinant Bluetongue virus-derived tubules. *Virology*, **217**, 323-331.

Mizrachi E (2008) Solubility, particle formation and immune display of trimers of major capsid protein 7 of African Horsesickness virus fused with enhanced green fluorescence protein. MSc Thesis.

Monastyrskaya K, Gould EA, Roy P (1995) Characterization and modification of the carboxy-terminal sequences of bluetongue virus type 10 NS1 protein in relation to tubule formation and location of antigenic epitope in the vicinity of the carboxy terminus of the protein. *Journal of Virology*, **69**, 2831-41.

Monastyrskaya K, Staeuber N, Sutton G, Roy P (1997) Effects of domain-switching and site-directed mutagenesis on the properties. *Virology*, **237**, 217-227.

Murby M, Sameulsson E, Nguyen TN, Mignard L, Power U, Binz H, Uhlen M, Stahl S (1995) Hydrophobicity engineering to increase solubility and stability of a recombinant protein from respiratory syncytial virus. *European Journal of Biochemistry*, **230**, 38-44.

Murphy A and Roy P (2008) Manipulation of the bluetongue virus tubules for immunogen delivery *Future medicine*, **3**, 351-359.

Netter HJ, Macnaughton TB, Woo W-P, Tindle R (2001) Antigenicity and immunogenicity of novel chimeric hepatitis B surface antigen particles with exposed hepatitis C. *Journal of Virology*, **75**, 2130 -2141.

Noad R and Roy P (2003) Virus-like particles as immunogens. *Trends in Microbiology*, **11**, 438-444.

Norton IT, Goodall DM, Anstren KRJ, Morris ER, Rees DA (1986) Dynamics of molecular organization in agarose sulphate. *Biopolymers*, **25**, 1009-1030.

Olive C, Toth I, Jackson D (2001) Technological advances in antigen delivery and synthetic peptide. *Reviews in Medicinal Chemistry*, **1**, 429-438.

Oldfield S, Adachi A, Urakawa T, Hirasawa T, Roy P (1990) Purification and characterization of the major group-specific core antigen VP7 of bluetongue virus synthesized by a recombinant baculovirus. *Journal of General Virology,* **71**, 2649-2656.

Ornstein L (1964) Disc electrophoresis. I. Background and theory. Annual New York Academy of *Science*, **121**, 321-349.

Owens RJ, Limn C, Roy P (2004) Role of an Abrovirus non-structural protein in cellular pathogenesis and virus release. *Journal of Virology*, **78**, 6649-6656.

Pengelley SC, Chapman DC, Abbott WM, Lin H-H, Huang W, Dalton K, Jones IM (2006) A suite of parallel vectors for baculovirus expression. *Protein expression and purification*, **48**, 173-181.

Perrie Y, Mohammed AR, Kirby DJ, McNeil SE, Bramwell VW (2008) Vaccine adjuvant systems: Enhancing the efficacy of sub-unit protein antigens. *International Journal of Pharmaceutics*, **364**, 272-280.

Philo JS and Arakawa T (2009) Mechanisms of Protein Aggregation. *Current Pharmaceutical Biotechnology*, **10**, 348-351.

Purcell AW, McCluskey J, Rossjohn J (2007) More than one reason to rethink the use of peptides in vaccine design. *Nature reviews*, **6**, 404-414.

Querec T, Bonnouna S, Alkan S, Laouar Y (2006) Yellow fever vaccine YF-17D activates multiple dendritic cell subsets via TLR2, 7, 8, and 9 to stimulate polyvalent immunity. *Journal of Experimental Medicine*, **203**, 2413-2424.

Ramshaw IA and Ramsay AJ (2000) Prime-boost strategy: exciting prospects for improved vaccinations. *Immunology Today*, **21**, 163-165.

Reddy RC, Lilie H, Rudolph R, Lange C (2005) L-Arginine increases the solubility of unfolded species of hen egg white lysozyme. *Protein Science*, **14**, 929-935.

Remond M, Da Costa B, Riffault S, Parida F, Breard E, Lebreton F, Zientara S, Delmas B (2009)Infectious bursal disease subviral particles displaying disease virus major antigenic site. *Vaccine*, **27**, 93-98.

Riley JE (2003) Construction of a new peptide insertion site in the top domain of major core protein VP7 of African horsesickness virus, MSc thesis

Rodriguez LL, Grubman MJ (2009) Foot and mouth disease virus vaccines. *Vaccine*, **27**, D90-D94.

Roy P, Urakawa T, Van Dijk AA, Erasmus BJ (1990) Recombinant virus vaccine for Bluetongue disease in sheep. *Journal of Virology*, **64**, 1998-2003.

Roy P, French T, Erasmus BJ (1992) Protective efficacy of virus-like particles for bluetongue disease. *Vaccine*, **10**, 28-32.

Roy P, Bishop DHL, Le Blois H, Erasmus BJ (1994) Long-lasting protection of sheep against bluetongue challenge after vaccination with virus-like particles: evidence for homologous and partial heterologous protection. *Vaccine*, **12**, 801-811.

Roy P (1996a) Orbivirus structure and assembly. *Virology*, **216**, 1-11.

Roy P, Bishop DHL, Howard S, Aitchison H, Erasmus B (1996b) Recombinant baculovirus-synthesized African horsesickness virus (AHSV) outer-capsid protein VP2 provides protection against virulent AHSV challenge. *Virology*, **77**, 2053-2057.

Roy P and Noad R (2006) Bluetongue virus assembly and morphogenesis. *Current topics in microbiology and immunology*, **309**, 87-116.

Roy P, Boyce M, Noad R (2009) Prospects for improved bluetongue vaccines. *Nature reviews*, **7**, 120-128.

Rutkowska D, Meyer QC, Maree F, Vosloo W, Fick W, Huismans H (2011) The use of soluble African horse sickness viral protein 7 as an antigen delivery and presentation system. *Virus research*, 1-2, 35-48.

Sambrook J, Russell DW (2001) **Molecular Cloning A Laboratory Manual**. Third edition, Cold Spring Harbor Laboratory Press, Cold Spring Harbor, New York.

Sette A, Fikes J (2003) Epitope-based vaccines: an update on epitope identification, vaccine design and delivery. *Current opinion in Immunology*, **4**, 461-470.

Shimomura O, Johnson FH, Saiga Y (1962) *Journal of Cellular Composition and Physiology*, **59**, 223-239.

Shin HS, Lim HJ, Cha HJ (2003) Quantitative Monitoring for Secreted Production of Human Interleukin-2 in Stable Insect Drosophila S2 Cells Using a Green Fluorescent Protein Fusion Partner. *Biotechnology Progress*, **19**, 152-157.

Slupetzky K, Gambhira R, Culp TD, Shafti-Keramat S, Schellnbacher C, Christensen ND, Roden RBS, Kirnbauer R (2007) A papillomavirus-like particle (VLP) vaccine displaying HPV16 L2 epitopes induces cross-neutralizing antibodies to HPV11. *Vaccine*, **25**, 2001-2010.

Smith G, Cherry C, Summers MD (1983) Product information sheet for CRL-1711, American Type culture collection.

Song H, Wang Z, Zheng D, Fang W, Li Y, Liu Y Niu Z, Qiu B (2005) A novel mucosal vaccine against foot-and-mouth disease virus induces protection in mice and swine. *Biotechnology Letters*, **27**, 1669-1674.

Sominskaya I, Skrastina D, Dislers A, Vasiljev D, Mihailova M, Ose V, Dreilina D, Pumpens P (2010) Construction and immunological evaluation of multivalent Hepatitis B Virus (HBV) Core virus-like particles. *Clinical and Vaccine Immunology*, **17**, 1027-1033.

Stone-Marschat MA, Moss SR, Burrage TG, Barber ML, Roy P (1996) Immunization with VP2 is sufficient for protection against lethal challenge with African Horsesickness Virus Type 4. *Virology*, **220**, 219-222.

Storni T, Kundig TM, Senti G, Johansen P (2005) Immunity in response to particulate antigen-delivery systems. *Advanced Drug Delivery reviews*, **57**, 333-355.

Summers, M.D. & Smith, G.E. (1987). A manual of methods for Baculovirus vectors and insect cell culture procedures. Texan Agricultural Experiment Station Bulletin No. 1555. Texas Agricultural Experiment Station, College Station, Tex.

Tan B-H, Nason E, Staeuber N, Jiang W, Monastryrskaya K, Roy P (2001) RGD Tripeptide of Bluetongue virus VP7 protein is responsible for core attachment to *Culicoides* cells. *Journal of Virology*, **75**, 3937-3947.

Tanaka S, Mikhailov M, Roy P (1995) Synthesis of Bluetongue virus chimeric VP3 molecules and their interactions with VP7 Protein to assemble into virus core-like particles. *Virology*, **214**, 593-601.

Tanaka Y, Tsumoto K, Umetsu M, Nakanishi Y, Yasutake Y, Sakai N et al. (2004) Structural evidence for guanidine-protein side chain interactions: crystal structure of CutA from Pyrococcus harikoshii in 3M guanidine hydrochloride. *Biochemical and Biophysical Research Communications*, **323**, 185-191.

Thanavala Y and Lugade AA (2010) Oral transgenic plant-based vaccine for hepatitis B. *Immunology Research*, **46,** 4-11.

Tian P, Hu Y, Schilling WP, Lindsay DA, Eiden J, Estes MK (1994) The nonstructural glycoprotein of Rotavirus affects intracellular calcium levels. *Journal of Virology*, **68**, 251-257.

Towbin H, Staehelin T, Gordon J (1979) Electrophoretic transfer of proteins from polyacrylamide gels to nitrocellulose sheets: Procedure and some applications. *Proceedings of the National Academy of Science*, **76**, 4350-4353.

Tsien RY (1998) The green fluorescent protein. *Annual review of Biochemistry*, **67**, 509-544.

Tsumoto K, Umetsu m, Kumagai D, Ejima T, Arakawa T (2003) Solubilization of active green fluorescent protein from insoluble particles by guanidine and arginine. *Biochemical and Biophysical Research Communications,* **312**, 1383-1386.

Urakawa T and Roy P (1988) Bluetongue virus tubules made in insect cells by recombinant baculoviruses: expression of the NS1 gene of bluetongue virus serotype 10. *Journal of Virology*, **62**, 3919-3927.

Valenzuala P, Medina A, Rutter WJ, Ammemer G, Hall BD (1982) Synthesis and assembly of hepatitis B virus surface antigen particles in yeast. *Nature*, **298**, 347-350.

Van Rensburg R (2003) Construction and structural evaluation of viral protein 7 of African horse sickness virus as a particulate, multiple peptide vaccine delivery system. MSc thesis.

Verwoerd DW and Huismans H (1969) On the relationship between bluetongue, Africanhorsesickness and reoviruses: hybridization studies. *Onderstepoort Journal of Veternary Research,* **36**, 175-179.

Verwoerd DW, Els HJ, De Villiers EM, Huismans H (1972) Structure of the bluetongue virus capsid. *Journal of Virology,* **10**, 783-94.

Verwoerd DW, Huismans H, Erasmus BJ (1979) Orbiviruses. In: Fraenkel-Conrat H & Wagner RR (eds). *Comprehensive Virology*, **14**, 285 -345.

Wade-Evans, Pullen L, Hamblin C, O'Hara R, Burroughs JN, Mertens PPC (1997) African horsesickness virus VP7 sub-unit vaccine protects mice against a lethal, heterologous serotype challenge. *Journal of General Virology*, **78,**1611-1616.

Wang W, Nema S, Teagarden D (2010) Protein aggregation—Pathways and influencing factors. *International Journal of Pharmaceutics*, **390**, 89-99.

Wilson CM (1983) Staining of proteins on gels: Comparison of dyes and procedures. *Methods in Enzymology*, **91**, 2336-247.

Wright PF, Webster RG, Knipe DM (eds) (2001) *Fields Virology*: Lippencott Williams and Wilkins.

Yang F, Moss LG, Phillips GN Jr. (1996) *Nature Biotechnology*, **14**, 1246-1251.

Zhang T, Xu Y, Qiao L, Wang Y, Wu X, Fan D, Peng Q, Xu X (2010) Trivalent Human Papillomavirus (HPV) VLP vaccine covering HPV type 58 can elicit high level of humoral immunity but also induce immune interference among component types. *Vaccine*, **28**, 3479-3487.

Appendix A

Sequencing data for:

VP7-169

VP7-169-FMDVEpi

VP7-169-eGFP

VP7-144-FMDVEpi

Complete BTV VP7-169 vector sequence

- from the cloning strategy of Wilma Fick & primers used for cloning

  note that a large part of the MCS of pFB1 was removed
- pFastbac 1 sequences in black
- Construct total length: 5766 bp
- BTV10 lab strain (Vida) VP7-169 sequences in red, ATG underlined

  insert length: 1068 bp 355 aa

  insert position: bases 4037-5105
- f1 origin: bases 2-457

  amp^R gene: bases 589-1449

  genta^R stop: bases 2802-3335

⌈f1 origin: bases 2-457

```
        10         20         30         40         50         60
GACGCGCCCT GTAGCGGCGC ATTAAGCGCG GCGGGTGTGG TGGTTACGCG CAGCGTGACC
CTGCGCGGGA CATCGCCGCG TAATTCGCGC CGCCCACACC ACCAATGCGC GTCGCACTGG

        70         80         90        100        110        120
GCTACACTTG CCAGCGCCCT AGCGCCCGCT CCTTTCGCTT TCTTCCCTTC CTTTCTCGCC
CGATGTGAAC GGTCGCGGGA TCGCGGGCGA GGAAAGCGAA AGAAGGGAAG GAAAGAGCGG

       130        140        150        160        170        180
ACGTTCGCCG GCTTTCCCCG TCAAGCTCTA AATCGGGGGC TCCCTTTAGG GTTCCGATTT
TGCAAGCGGC CGAAAGGGGC AGTTCGAGAT TTAGCCCCCG AGGGAAATCC CAAGGCTAAA

       190        200        210        220        230        240
AGTGCTTTAC GGCACCTCGA CCCCAAAAAA CTTGATTAGG GTGATGGTTC ACGTAGTGGG
TCACGAAATG CCGTGGAGCT GGGGTTTTTT GAACTAATCC CACTACCAAG TGCATCACCC

       250        260        270        280        290        300
CCATCGCCCT GATAGACGGT TTTTCGCCCT TTGACGTTGG AGTCCACGTT CTTTAATAGT
GGTAGCGGGA CTATCTGCCA AAAAGCGGGA AACTGCAACC TCAGGTGCAA GAAATTATCA

       310        320        330        340        350        360
GGACTCTTGT TCCAAACTGG AACAACACTC AACCCTATCT CGGTCTATTC TTTTGATTTA
CCTGAGAACA AGGTTTGACC TTGTTGTGAG TTGGGATAGA GCCAGATAAG AAAACTAAAT

       370        380        390        400        410        420
TAAGGGATTT TGCCGATTTC GGCCTATTGG TTAAAAAATG AGCTGATTTA ACAAAAATTT
ATTCCCTAAA ACGGCTAAAG CCGGATAACC AATTTTTTAC TCGACTAAAT TGTTTTTAAA
```

f1 origin end⌉

430 440 450 460 470 480
AACGCGAATT TTAACAAAAT ATTAACGTTT ACAATTTCAG GTGGCACTTT TCGGGGAAAT
TTGCGCTTAA AATTGTTTTA TAATTGCAAA TGTTAAAGTC CACCGTGAAA AGCCCCTTTA

490 500 510 520 530 540
GTGCGCGGAA CCCCTATTTG TTTATTTTTC TAAATACATT CAAATATGTA TCCGCTCATG
CACGCGCCTT GGGGATAAAC AAATAAAAAG ATTTATGTAA GTTTATACAT AGGCGAGTAC

⌈amp^R gene: bases 589-1449

550 560 570 580 590 600
AGACAATAAC CCTGATAAAT GCTTCAATAA TATTGAAAAA GGAAGAGT**AT** **G**AGTATTCAA
TCTGTTATTG GGACTATTTA CGAAGTTATT ATAACTTTTT CCTTCTCATA CTCATAAGTT

610 620 630 640 650 660
CATTTCCGTG TCGCCCTTAT TCCCTTTTTT GCGGCATTTT GCCTTCCTGT TTTTGCTCAC
GTAAAGGCAC AGCGGGAATA AGGGAAAAAA CGCCGTAAAA CGGAAGGACA AAAACGAGTG

670 680 690 700 710 720
CCAGAAACGC TGGTGAAAGT AAAAGATGCT GAAGATCAGT TGGGTGCACG AGTGGGTTAC
GGTCTTTGCG ACCACTTTCA TTTTCTACGA CTTCTAGTCA ACCCACGTGC TCACCCAATG

730 740 750 760 770 780
ATCGAACTGG ATCTCAACAG CGGTAAGATC CTTGAGAGTT TTCGCCCCGA AGAACGTTTT
TAGCTTGACC TAGAGTTGTC GCCATTCTAG GAACTCTCAA AAGCGGGGCT TCTTGCAAAA

790 800 810 820 830 840
CCAATGATGA GCACTTTTAA AGTTCTGCTA TGTGGCGCGG TATTATCCCG TATTGACGCC
GGTTACTACT CGTGAAAATT TCAAGACGAT ACACCGCGCC ATAATAGGGC ATAACTGCGG

850 860 870 880 890 900
GGGCAAGAGC AACTCGGTCG CCGCATACAC TATTCTCAGA ATGACTTGGT TGAGTACTCA
CCCGTTCTCG TTGAGCCAGC GGCGTATGTG ATAAGAGTCT TACTGAACCA ACTCATGAGT

910 920 930 940 950 960
CCAGTCACAG AAAAGCATCT TACGGATGGC ATGACAGTAA GAGAATTATG CAGTGCTGCC
GGTCAGTGTC TTTTCGTAGA ATGCCTACCG TACTGTCATT CTCTTAATAC GTCACGACGG

970 980 990 1000 1010 1020
ATAACCATGA GTGATAACAC TGCGGCCAAC TTACTTCTGA CAACGATCGG AGGACCGAAG
TATTGGTACT CACTATTGTG ACGCCGGTTG AATGAAGACT GTTGCTAGCC TCCTGGCTTC

1030 1040 1050 1060 1070 1080
GAGCTAACCG CTTTTTTGCA CAACATGGGG GATCATGTAA CTCGCCTTGA TCGTTGGGAA
CTCGATTGGC GAAAAAACGT GTTGTACCCC CTAGTACATT GAGCGGAACT AGCAACCCTT

```
      1090       1100       1110       1120       1130       1140
CCGGAGCTGA ATGAAGCCAT ACCAAACGAC GAGCGTGACA CCACGATGCC TGTAGCAATG
GGCCTCGACT TACTTCGGTA TGGTTTGCTG CTCGCACTGT GGTGCTACGG ACATCGTTAC

      1150       1160       1170       1180       1190       1200
GCAACAACGT TGCGCAAACT ATTAACTGGC GAACTACTTA CTCTAGCTTC CCGGCAACAA
CGTTGTTGCA ACGCGTTTGA TAATTGACCG CTTGATGAAT GAGATCGAAG GGCCGTTGTT

      1210       1220       1230       1240       1250       1260
TTAATAGACT GGATGGAGGC GGATAAAGTT GCAGGACCAC TTCTGCGCTC GGCCCTTCCG
AATTATCTGA CCTACCTCCG CCTATTTCAA CGTCCTGGTG AAGACGCGAG CCGGGAAGGC

      1270       1280       1290       1300       1310       1320
GCTGGCTGGT TTATTGCTGA TAAATCTGGA GCCGGTGAGC GTGGGTCTCG CGGTATCATT
CGACCGACCA AATAACGACT ATTTAGACCT CGGCCACTCG CACCCAGAGC GCCATAGTAA

      1330       1340       1350       1360       1370       1380
GCAGCACTGG GGCCAGATGG TAAGCCCTCC CGTATCGTAG TTATCTACAC GACGGGGAGT
CGTCGTGACC CCGGTCTACC ATTCGGGAGG GCATAGCATC AATAGATGTG CTGCCCCTCA

      1390       1400       1410       1420       1430       1440
CAGGCAACTA TGGATGAACG AAATAGACAG ATCGCTGAGA TAGGTGCCTC ACTGATTAAG
GTCCGTTGAT ACCTACTTGC TTTATCTGTC TAGCGACTCT ATCCACGGAG TGACTAATTC

 ampR stop ⌉
      1450       1460       1470       1480       1490       1500
CATTGGTAAC TGTCAGACCA AGTTTACTCA TATATACTTT AGATTGATTT AAAACTTCAT
GTAACCATTG ACAGTCTGGT TCAAATGAGT ATATATGAAA TCTAACTAAA TTTTGAAGTA

      1510       1520       1530       1540       1550       1560
TTTTAATTTA AAAGGATCTA GGTGAAGATC CTTTTTGATA ATCTCATGAC CAAAATCCCT
AAAATTAAAT TTTCCTAGAT CCACTTCTAG GAAAAACTAT TAGAGTACTG GTTTTAGGGA

      1570       1580       1590       1600       1610       1620
TAACGTGAGT TTTCGTTCCA CTGAGCGTCA GACCCCGTAG AAAAGATCAA AGGATCTTCT
ATTGCACTCA AAAGCAAGGT GACTCGCAGT CTGGGGCATC TTTTCTAGTT TCCTAGAAGA

      1630       1640       1650       1660       1670       1680
TGAGATCCTT TTTTTCTGCG CGTAATCTGC TGCTTGCAAA CAAAAAAACC ACCGCTACCA
ACTCTAGGAA AAAAAGACGC GCATTAGACG ACGAACGTTT GTTTTTTTGG TGGCGATGGT

      1690       1700       1710       1720       1730       1740
GCGGTGGTTT GTTTGCCGGA TCAAGAGCTA CCAACTCTTT TTCCGAAGGT AACTGGCTTC
CGCCACCAAA CAAACGGCCT AGTTCTCGAT GGTTGAGAAA AAGGCTTCCA TTGACCGAAG
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      1750       1760       1770       1780       1790       1800
AGCAGAGCGC AGATACCAAA TACTGTCCTT CTAGTGTAGC CGTAGTTAGG CCACCACTTC
TCGTCTCGCG TCTATGGTTT ATGACAGGAA GATCACATCG GCATCAATCC GGTGGTGAAG

      1810       1820       1830       1840       1850       1860
AAGAACTCTG TAGCACCGCC TACATACCTC GCTCTGCTAA TCCTGTTACC AGTGGCTGCT
TTCTTGAGAC ATCGTGGCGG ATGTATGGAG CGAGACGATT AGGACAATGG TCACCGACGA

      1870       1880       1890       1900       1910       1920
GCCAGTGGCG ATAAGTCGTG TCTTACCGGG TTGGACTCAA GACGATAGTT ACCGGATAAG
CGGTCACCGC TATTCAGCAC AGAATGGCCC AACCTGAGTT CTGCTATCAA TGGCCTATTC

      1930       1940       1950       1960       1970       1980
GCGCAGCGGT CGGGCTGAAC GGGGGGTTCG TGCACACAGC CCAGCTTGGA GCGAACGACC
CGCGTCGCCA GCCCGACTTG CCCCCCAAGC ACGTGTGTCG GGTCGAACCT CGCTTGCTGG

      1990       2000       2010       2020       2030       2040
TACACCGAAC TGAGATACCT ACAGCGTGAG CATTGAGAAA GCGCCACGCT TCCCGAAGGG
ATGTGGCTTG ACTCTATGGA TGTCGCACTC GTAACTCTTT CGCGGTGCGA AGGGCTTCCC

      2050       2060       2070       2080       2090       2100
AGAAAGGCGG ACAGGTATCC GGTAAGCGGC AGGGTCGGAA CAGGAGAGCG CACGAGGGAG
TCTTTCCGCC TGTCCATAGG CCATTCGCCG TCCCAGCCTT GTCCTCTCGC GTGCTCCCTC

      2110       2120       2130       2140       2150       2160
CTTCCAGGGG GAAACGCCTG GTATCTTTAT AGTCCTGTCG GGTTTCGCCA CCTCTGACTT
GAAGGTCCCC CTTTGCGGAC CATAGAAATA TCAGGACAGC CCAAAGCGGT GGAGACTGAA

      2170       2180       2190       2200       2210       2220
GAGCGTCGAT TTTTGTGATG CTCGTCAGGG GGGCGGAGCC TATGGAAAAA CGCCAGCAAC
CTCGCAGCTA AAAACACTAC GAGCAGTCCC CCCGCCTCGG ATACCTTTTT GCGGTCGTTG

      2230       2240       2250       2260       2270       2280
GCGGCCTTTT TACGGTTCCT GGCCTTTTGC TGGCCTTTTG CTCACATGTT CTTTCCTGCG
CGCCGGAAAA ATGCCAAGGA CCGGAAAACG ACCGGAAAAC GAGTGTACAA GAAAGGACGC

      2290       2300       2310       2320       2330       2340
TTATCCCCTG ATTCTGTGGA TAACCGTATT ACCGCCTTTG AGTGAGCTGA TACCGCTCGC
AATAGGGGAC TAAGACACCT ATTGGCATAA TGGCGGAAAC TCACTCGACT ATGGCGAGCG

      2350       2360       2370       2380       2390       2400
CGCAGCCGAA CGACCGAGCG CAGCGAGTCA GTGAGCGAGG AAGCGGAAGA GCGCCTGATG
```

GCGTCGGCTT GCTGGCTCGC GTCGCTCAGT CACTCGCTCC TTCGCCTTCT CGCGGACTAC

2410 2420 2430 2440 2450 2460
CGGTATTTTC TCCTTACGCA TCTGTGCGGT ATTTCACACC GCAGACCAGC CGCGTAACCT
GCCATAAAAG AGGAATGCGT AGACACGCCA TAAAGTGTGG CGTCTGGTCG GCGCATTGGA

2470 2480 2490 2500 2510 2520
GGCAAAATCG GTTACGGTTG AGTAATAAAT GGATGCCCTG CGTAAGCGGG TGTGGGCGGA
CCGTTTTAGC CAATGCCAAC TCATTATTTA CCTACGGGAC GCATTCGCCC ACACCCGCCT

2530 2540 2550 2560 2570 2580
CAATAAAGTC TTAAACTGAA CAAAATAGAT CTAAACTATG ACAATAAAGT CTTAAACTAG
GTTATTTCAG AATTTGACTT GTTTTATCTA GATTTGATAC TGTTATTTCA GAATTTGATC

2590 2600 2610 2620 2630 2640
ACAGAATAGT TGTAAACTGA AATCAGTCCA GTTATGCTGT GAAAAAGCAT ACTGGACTTT
TGTCTTATCA ACATTTGACT TTAGTCAGGT CAATACGACA CTTTTTCGTA TGACCTGAAA

2650 2660 2670 2680 2690 2700
TGTTATGGCT AAAGCAAACT CTTCATTTTC TGAAGTGCAA ATTGCCCGTC GTATTAAAGA
ACAATACCGA TTTCGTTTGA GAAGTAAAAG ACTTCACGTT TAACGGGCAG CATAATTTCT

2710 2720 2730 2740 2750 2760
GGGGCGTGGC CAAGGGCATG GTAAAGACTA TATTCGCGGC GTTGTGACAA TTTACCGAAC
CCCCGCACCG GTTCCCGTAC CATTTCTGAT ATAAGCGCCG CAACACTGTT AAATGGCTTG

⌈$genta^R$ stop: bases 2802-3335
2770 2780 2790 2800 2810 2820
AACTCCGCGG CCGGGAAGCC GATCTCGGCT TGAACGAATT GTTAGGTGGC GGTACTTGGG
TTGAGGCGCC GGCCCTTCGG CTAGAGCCGA ACTTGCTTAA C**AAT**CCACCG CCATGAACCC

2830 2840 2850 2860 2870 2880
TCGATATCAA AGTGCATCAC TTCTTCCCGT ATGCCCAACT TTGTATAGAG AGCCACTGCG
AGCTATAGTT TCACGTAGTG AAGAAGGGCA TACGGGTTGA AACATATCTC TCGGTGACGC

2890 2900 2910 2920 2930 2940
GGATCGTCAC CGTAATCTGC TTGCACGTAG ATCACATAAG CACCAAGCGC GTTGGCCTCA
CCTAGCAGTG GCATTAGACG AACGTGCATC TAGTGTATTC GTGGTTCGCG CAACCGGAGT

2950 2960 2970 2980 2990 3000
TGCTTGAGGA GATTGATGAG CGCGGTGGCA ATGCCCTGCC TCCGGTGCTC GCCGGAGACT
ACGAACTCCT CTAACTACTC GCGCCACCGT TACGGGACGG AGGCCACGAG CGGCCTCTGA

3010 3020 3030 3040 3050 3060

GCGAGATCAT AGATATAGAT CTCACTACGC GGCTGCTCAA ACCTGGGCAG AACGTAAGCC
CGCTCTAGTA TCTATATCTA GAGTGATGCG CCGACGAGTT TGGACCCGTC TTGCATTCGG

3070 3080 3090 3100 3110 3120
GCGAGAGCGC CAACAACCGC TTCTTGGTCG AAGGCAGCAA GCGCGATGAA TGTCTTACTA
CGCTCTCGCG GTTGTTGGCG AAGAACCAGC TTCCGTCGTT CGCGCTACTT ACAGAATGAT

3130 3140 3150 3160 3170 3180
CGGAGCAAGT TCCCGAGGTA ATCGGAGTCC GGCTGATGTT GGGAGTAGGT GGCTACGTCT
GCCTCGTTCA AGGGCTCCAT TAGCCTCAGG CCGACTACAA CCCTCATCCA CCGATGCAGA

3190 3200 3210 3220 3230 3240
CCGAACTCAC GACCGAAAAG ATCAAGAGCA GCCCGCATGG ATTTGACTTG GTCAGGGCCG
GGCTTGAGTG CTGGCTTTTC TAGTTCTCGT CGGGCGTACC TAAACTGAAC CAGTCCCGGC

3250 3260 3270 3280 3290 3300
AGCCTACATG TGCGAATGAT GCCCATACTT GAGCCACCTA ACTTTGTTTT AGGGCGACTG
TCGGATGTAC ACGCTTACTA CGGGTATGAA CTCGGTGGAT TGAAACAAAA TCCCGCTGAC

genta^R start⌉ (opposite strand)

3310 3320 3330 3340 3350 3360
CCCTGCTGCG TAACATCGTT GCTGCTGCGT AACATCGTTG CTGCTCCATA ACATCAAACA
GGGACGACGC ATTGTAGCAA CGACGACGCA TT**GTA**GCAAC GACGAGGTAT TGTAGTTTGT

3370 3380 3390 3400 3410 3420
TCGACCCACG GCGTAACGCG CTTGCTGCTT GGATGCCCGA GGCATAGACT GTACAAAAAA
AGCTGGGTGC CGCATTGCGC GAACGACGAA CCTACGGGCT CCGTATCTGA CATGTTTTTT

3430 3440 3450 3460 3470 3480
ACAGTCATAA CAAGCCATGA AAACCGCCAC TGCGCCGTTA CCACCGCTGC GTTCGGTCAA
TGTCAGTATT GTTCGGTACT TTTGGCGGTG ACGCGGCAAT GGTGGCGACG CAAGCCAGTT

3490 3500 3510 3520 3530 3540
GGTTCTGGAC CAGTTGCGTG AGCGCATACG CTACTTGCAT TACAGTTTAC GAACCGAACA
CCAAGACCTG GTCAACGCAC TCGCGTATGC GATGAACGTA ATGTCAAATG CTTGGCTTGT

3550 3560 3570 3580 3590 3600
GGCTTATGTC AACTGGGTTC GTGCCTTCAT CCGTTTCCAC GGTGTGCGTC ACCCGGCAAC
CCGAATACAG TTGACCCAAG CACGGAAGTA GGCAAAGGTG CCACACGCAG TGGGCCGTTG

3610 3620 3630 3640 3650 3660
CTTGGGCAGC AGCGAAGTCG AGGCATTTCT GTCCTGGCTG GCGAACGAGC GCAAGGTTTC
GAACCCGTCG TCGCTTCAGC TCCGTAAAGA CAGGACCGAC CGCTTGCTCG CGTTCCAAAG

```
      3670       3680       3690       3700       3710       3720
GGTCTCCACG CATCGTCAGG CATTGGCGGC CTTGCTGTTC TTCTACGGCA AGGTGCTGTG
CCAGAGGTGC GTAGCAGTCC GTAACCGCCG GAACGACAAG AAGATGCCGT TCCACGACAC

      3730       3740       3750       3760       3770       3780
CACGGATCTG CCCTGGCTTC AGGAGATCGG AAGACCTCGG CCGTCGCGGC GCTTGCCGGT
GTGCCTAGAC GGGACCGAAG TCCTCTAGCC TTCTGGAGCC GGCAGCGCCG CGAACGGCCA

      3790       3800       3810       3820       3830       3840
GGTGCTGACC CCGGATGAAG TGGTTCGCAT CCTCGGTTTT CTGGAAGGCG AGCATCGTTT
CCACGACTGG GGCCTACTTC ACCAAGCGTA GGAGCCAAAA GACCTTCCGC TCGTAGCAAA

      3850       3860       3870       3880       3890       3900
GTTCGCCCAG GACTCTAGCT ATAGTTCTAG TGGTTGGCTA CGTATACTCC GGAATATTAA
CAAGCGGGTC CTGAGATCGA TATCAAGATC ACCAACCGAT GCATATGAGG CCTTATAATT
```

transcriptional start site of polyhedrin promoter

polyhedrin promoter: bases 3904-4032

```
                                                  ↦+1
      3910       3920       3930       3940       3950       3960
TAGATCATGG AGATAATTAA AATGATAACC ATCTCGCAAA TAAATAAGTA TTTTACTGTT
ATCTAGTACC TCTATTAATT TTACTATTGG TAGAGCGTTT ATTTATTCAT AAAATGACAA
```

5' pFB1 POLH Fw primer → 3'

```
      3970       3980       3990       4000       4010       4020
TTCGTAACAG TTTTGTAATA AAAAAACCTA TAAATATTCC GGATTATTCA TACCGTCCCA
AAGCATTGTC AAAACATTAT TTTTTTGGAT ATTTATAAGG CCTAATAAGT ATGGCAGGGT
```

BamHI **VP7-169 start**

```
                   M  D  T  I  A  A  R  A  L  T  V  M  R  A  C
      4030       4040       4050       4060       4070       4080
CCATCGGGCG CGGATCCATG GACACTATCG CTGCAAGAGC ACTCACTGTG ATGCGAGCAT
GGTAGCCCGC GCCTAGGTAC CTGTGATAGC GACGTTCTCG TGAGTGACAC TACGCTCGTA

  A  T  L  Q  E  A  R  I  V  L  E  A  N  V  M  E  I  L  G  I
      4090       4100       4110       4120       4130       4140
GTGCTACGCT TCAAGAAGCA AGAATTGTAT TGGAAGCTAA TGTGATGGAA ATATTGGGGA
CACGATGCGA AGTTCTTCGT TCTTAACATA ACCTTCGATT ACACTACCTT TATAACCCCT

  A  I  N  R  Y  N  G  L  T  L  R  G  V  T  M  R  P  T  S  L
      4150       4160       4170       4180       4190       4200
TAGCAATCAA CAGATATAAT GGATTAACTT TACGAGGGGT GACGATGCGC CCAACCTCAT
ATCGTTAGTT GTCTATATTA CCTAATTGAA ATGCTCCCCA CTGCTACGCG GGTTGGAGTA

  A  Q  R  N  E  M  F  F  M  C  L  D  M  M  L  S  A  A  G  I
```

```
      4210       4220       4230       4240       4250       4260
TGGCGCAGAG AAATGAGATG TTTTTTATGT GTTTGGATAT GATGCTGTCT GCGGCTGGGA
ACCGCGTCTC TTTACTCTAC AAAAAATACA CAAACCTATA CTACGACAGA CGCCGACCCT

 N  I  G   P  I  S   P  D  Y  T   Q  H  M   A  T  I   G  V  L  A
      4270       4280       4290       4300       4310       4320
TAAATATTGG ACCGATATCC CCAGATTATA CCCAACATAT GGCTACAATC GGTGTACTAG
ATTTATAACC TGGCTATAGG GGTCTAATAT GGGTTGTATA CCGATGTTAG CCACATGATC

 T  P  E   I  P  F   T  T  E  A   A  N  E   I  A  R   V  T  G  E
      4330       4340       4350       4360       4370       4380
CGACGCCTGA GATACCTTTT ACGACAGAAG CAGCGAATGA AATAGCTCGC GTGACTGGGG
GCTGCGGACT CTATGGAAAA TGCTGTCTTC GTCGCTTACT TTATCGAGCG CACTGACCCC

 T  S  T   W  G  P   A  R  Q  P   Y  G  F   F  L  E   T  E  E  T
      4390       4400       4410       4420       4430       4440
AGACTTCAAC GTGGGGACCA GCGCGTCAGC CCTATGGTTT TTTCCTTGAG ACGGAGGAGA
TCTGAAGTTG CACCCCTGGT CGCGCAGTCG GGATACCAAA AAAGGAACTC TGCCTCCTCT

 F  Q  P   G  R  W   F  M  R  A   A  Q  A   V  T  A   V  V  C  G
      4450       4460       4470       4480       4490       4500
CCTTCCAACC TGGGAGATGG TTTATGCGTG CCGCTCAAGC TGTGACTGCG GTAGTATGCG
GGAAGGTTGG ACCCTCTACC AAATACGCAC GGCGAGTTCG ACACTGACGC CATCATACGC

 P  D  M   I  Q  V   S  L  N  A   G  A  R   K  L  A   R  V  D  G
                                            HindIII  BssHII  Sal
      4510       4520       4530       4540       4550       4560
GCCCGGATAT GATTCAAGTG TCACTGAATG CTGGGGCAAG AAAGCTTGCG CGCGTCGACG
CGGGCCTATA CTAAGTTCAC AGTGACTTAC GACCCCGTTC TTTCGAACGC GCGCAGCTGC

 D  V  Q   Q  I  F   Q  G  R  N   D  P  M   M  I  Y   L  V  W  R
      4570       4580       4590       4600       4610       4620
GGGATGTGCA GCAGATATTT CAAGGCCGTA ATGATCCTAT GATGATATAT CTAGTGTGGA
CCCTACACGT CGTCTATAAA GTTCCGGCAT TACTAGGATA CTACTATATA GATCACACCT

 R  I  E   N  F  A   M  A  Q  G   N  S  Q   Q  T  Q   A  G  V  T
      4630       4640       4650       4660       4670       4680
GAAGAATTGA AAACTTTGCG ATGGCGCAAG GTAATTCACA GCAAACTCAA GCAGGTGTGA
CTTCTTAACT TTTGAAACGC TACCGCGTTC CATTAAGTGT CGTTTGAGTT CGTCCACACT

 V  S  V   G  G  V   D  M  R  A   G  R  I   I  A  W   D  G  Q  A
      4690       4700       4710       4720       4730       4740
CTGTTAGCGT TGGCGGAGTA GACATGCGGG CGGGACGTAT TATAGCGTGG GATGGACAAG
GACAATCGCA ACCGCCTCAT CTGTACGCCC GCCCTGCATA ATATCGCACC CTACCTGTTC
```

```
  A  L  H    V  H  N    P  T  Q  Q    N  A  M    V  Q  I    Q  V  V  F
        4750       4760        4770        4780        4790        4800
CCGCTCTACA  TGTGCATAAC  CCCACACAAC  AGAATGCAAT  GGTTCAAATA  CAAGTCGTGT
GGCGAGATGT  ACACGTATTG  GGGTGTGTTG  TCTTACGTTA  CCAAGTTTAT  GTTCAGCACA

  Y  I  S    M  D  K    T  L  N  Q    Y  P  A    L  T  A    E  I  F  N
        4810       4820        4830        4840        4850        4860
TTTACATTTC  CATGGATAAG  ACTTTAAACC  AATATCCCGC  TTTGACTGCT  GAAATCTTTA
AAATGTAAAG  GTACCTATTC  TGAAATTTGG  TTATAGGGCG  AAACTGACGA  CTTTAGAAAT

  V  Y  S    L  R  D    H  T  W  H    G  L  R    T  A  I    L  N  R  T
        4870       4880        4890        4900        4910        4920
ACGTTTATAG  CCTCAGAGAT  CATACATGGC  ACGGATTGAG  GACGGCCATA  CTAAACAGAA
TGCAAATATC  GGAGTCTCTA  GTATGTACCG  TGCCTAACTC  CTGCCGGTAT  GATTTGTCTT

  T  L  P    N  M  L    P  P  I  F    P  P  N    D  R  D    S  I  L  T
        4930       4940        4950        4960        4970        4980
CTACACTGCC  AAACATGCTG  CCACCTATCT  TCCCACCAAA  TGATCGAGAT  AGTATCCTGA
GATGTGACGG  TTTGTACGAC  GGTGGATAGA  AGGGTGGTTT  ACTAGCTCTA  TCATAGGACT

  L  L  L    L  S  T    L  A  D  V    Y  T  V    L  R  P    E  F  A  I
        4990       5000        5010        5020        5030        5040
CTCTTTTGCT  TTTGTCTACG  CTTGCTGATG  TTTATACCGT  TTTGAGACCA  GAGTTCGCGA
GAGAAAACGA  AAACAGATGC  GAACGACTAC  AAATATGGCA  AAACTCTGGT  CTCAAGCGCT

  H  G  V    N  P  M    P  G  P  L    T  R  A    I  A  R    A  A  Y  V
        5050       5060        5070        5080        5090        5100
TTCATGGCGT  AAACCCAATG  CCTGGGCCGC  TCACACGTGC  TATTGCACGC  GCCGCCTATG
AAGTACCGCA  TTTGGGTTAC  GGACCCGGCG  AGTGTGCACG  ATAACGTGCG  CGGCGGATAC

  *
VP7-169 stop codon ⏋
      EcoRI                                                 pFb stop codons
        5110       5120        5130        5140        5150        5160
TGTAGGAATT  CGGAATTCAA  AGGCCTACGT  CGAAGCTAGC  TTGTCGAGAA  GTACTAGAGG
ACATCCTTAA  GCCTTAAGTT  TCCGGATGCA  GCTTCGATCG  AACAGCTCTT  CATGATCTCC

        5170       5180        5190        5200        5210        5220
ATCATAATCA  GCCATACCAC  ATTTGTAGAG  GTTTTACTTG  CTTTAAAAAA  CCTCCCACAC
TAGTATTAGT  CGGTATGGTG  TAAACATCTC  CAAAATGAAC  GAAATTTTTT  GGAGGGTGTG
     3' ← pFB1 RV primer 5'

        5230       5240        5250        5260        5270        5280
CTCCCCCTGA  ACCTGAAACA  TAAAATGAAT  GCAATTGTTG  TTGTTAACTT  GTTTATTGCA
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GAGGGGGACT  TGGACTTTGT  ATTTTACTTA  CGTTAACAAC  AACAATTGAA  CAAATAACGT

      5290        5300        5310        5320        5330        5340
GCTTATAATG  GTTACAAATA  AAGCAATAGC  ATCACAAATT  TCACAAATAA  AGCATTTTTT
CGAATATTAC  CAATGTTTAT  TTCGTTATCG  TAGTGTTTAA  AGTGTTTATT  TCGTAAAAAA

      5350        5360        5370        5380        5390        5400
TCACTGCATT  CTAGTTGTGG  TTTGTCCAAA  CTCATCAATG  TATCTTATCA  TGTCTGGATC
AGTGACGTAA  GATCAACACC  AAACAGGTTT  GAGTAGTTAC  ATAGAATAGT  ACAGACCTAG

      5410        5420        5430        5440        5450        5460
TGATCACTGC  TTGAGCCTAG  GAGATCCGAA  CCAGATAAGT  GAAATCTAGT  TCCAAACTAT
ACTAGTGACG  AACTCGGATC  CTCTAGGCTT  GGTCTATTCA  CTTTAGATCA  AGGTTTGATA

      5470        5480        5490        5500        5510        5520
TTTGTCATTT  TTAATTTTCG  TATTAGCTTA  CGACGCTACA  CCCAGTTCCC  ATCTATTTTG
AAACAGTAAA  AATTAAAAGC  ATAATCGAAT  GCTGCGATGT  GGGTCAAGGG  TAGATAAAAC

      5530        5540        5550        5560        5570        5580
TCACTCTTCC  CTAAATAATC  CTTAAAAACT  CCATTTCCAC  CCCTCCCAGT  TCCCAACTAT
AGTGAGAAGG  GATTTATTAG  GAATTTTTGA  GGTAAAGGTG  GGGAGGGTCA  AGGGTTGATA

      5590        5600        5610        5620        5630        5640
TTTGTCCGCC  CACAGCGGGG  CATTTTTCTT  CCTGTTATGT  TTTTAATCAA  ACATCCTGCC
AAACAGGCGG  GTGTCGCCCC  GTAAAAAGAA  GGACAATACA  AAAATTAGTT  TGTAGGACGG

      5650        5660        5670        5680        5690        5700
AACTCCATGT  GACAAACCGT  CATCTTCGGC  TACTTTTTCT  CTGTCACAGA  ATGAAAATTT
TTGAGGTACA  CTGTTTGGCA  GTAGAAGCCG  ATGAAAAAGA  GACAGTGTCT  TACTTTTAAA

      5710        5720        5730        5740        5750        5760
TTCTGTCATC  TCTTCGTTAT  TAATGTTTGT  AATTGACTGA  ATATCAACGC  TTATTTGCAG
AAGACAGTAG  AGAAGCAATA  ATTACAAACA  TTAACTGACT  TATAGTTGCG  AATAAACGTC

      5770    5776
CCTGAATGGC  GAATGG
GGACTTACCG  CTTACC
```

Sequence of pFb.VP7-169- FMDV Epi

- Showing pFbmod2 regions surrounding BTV10 VP7-169 + insert
- Cloned by Debbie Bolton using primers VP7for307 and VP7rev652
- Construct total length: pFbmod2(4701bp)+VP7(1062bp)+insert(108bp) = 5871 bp
- Chimeric protein size: VP7(355aa=39 kDa) + Epi (36 aa=4 kDa) = 43 kDa
- BTV10 VP7-169 sequences in black, insert in green

polyhedrin promoter

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GATCATGGAG  ATAATTAAAA  TGATAACCAT  CTCGCAAATA  AATAAGTATT  TTACTGTTTT  CGTAACAGTT  TTGTAATAAA
CTAGTACCTC  TATTAATTTT  ACTATTGGTA  GAGCGTTTAT  TTATTCATAA  AATGACAAAA  GCATTGTCAA  AACATTATTT

                                                                   VP7-169 start
                                                            BamHI  1           5
                 5' pFB1 POLH Fw primer → 3'                        M  D   T  I  A   A  R  A  L
        90         100         110         120         130         140         150         160
AAAACCTATA  AATATTCCGG  ATTATTCATA  CCGTCCCACC  ATCGGGCGCG  GATCCATGGA  CACTATCGCT  GCAAGAGCAC
TTTTGGATAT  TTATAAGGCC  TAATAAGTAT  GGCAGGGTGG  TAGCCCGCGC  CTAGGTACCT  GTGATAGCGA  CGTTCTCGTG

  10           15           20           25           30          35
 T  V  M   R  A  C   A  T  L  Q   E  A  R   I  V  L   E  A  N  V   M  E  I   L  G  I
       170         180         190         200         210         220         230         240
TCACTGTGAT  GCGAGCATGT  GCTACGCTTC  AAGAAGCAAG  AATTGTATTG  GAAGCTAATG  TGATGGAAAT  ATTGGGGATA
AGTGACACTA  CGCTCGTACA  CGATGCGAAG  TTCTTCGTTC  TTAACATAAC  CTTCGATTAC  ACTACCTTTA  TAACCCCTAT

              40           45           50           55           60
A  I  N  R   Y  N  G   L  T  L   R  G  V  T   M  R  P   T  S  L   A  Q  R  N   E  M  F
       250         260         270         280         290         300         310         320
GCAATCAACA  GATATAATGG  ATTAACTTTA  CGAGGGGTGA  CGATGCGCCC  AACCTCATTG  GCGCAGAGAA  ATGAGATGTT
CGTTAGTTGT  CTATATTACC  TAATTGAAAT  GCTCCCCACT  GCTACGCGGG  TTGGAGTAAC  CGCGTCTCTT  TACTCTACAA

       65           70           75           80           85
 F  M  C   L  D  M  M   L  S  A   A  G  I   N  I  G  P   I  S  P   D  Y  T   Q  H  M  A
       330         340         350         360         370         380         390         400
TTTTATGTGT  TTGGATATGA  TGCTGTCTGC  GGCTGGGATA  AATATTGGAC  CGATATCCCC  AGATTATACC  CAACATATGG
AAAATACACA  AACCTATACT  ACGACAGACG  CCGACCCTAT  TTATAACCTG  GCTATAGGGG  TCTAATATGG  GTTGTATACC

  90           95           100          105           110          115
 T  I  G   V  L  A   T  P  E  I   P  F  T   T  E  A   A  N  E  I   A  R  V   T  G  E
       410         420         430         440         450         460         470         480
CTACAATCGG  TGTACTAGCG  ACGCCTGAGA  TACCTTTTAC  GACAGAAGCA  GCGAATGAAA  TAGCTCGCGT  GACTGGGGAG
GATGTTAGCC  ACATGATCGC  TGCGGACTCT  ATGGAAAATG  CTGTCTTCGT  CGCTTACTTT  ATCGAGCGCA  CTGACCCCTC

              120          125          130          135          140
T  S  T  W   G  P  A   R  Q  P   Y  G  F  F   L  E  T   E  E  T   F  Q  P  G   R  W  F
       490         500         510         520         530         540         550         560
ACTTCAACGT  GGGGACCAGC  GCGTCAGCCC  TATGGTTTTT  TCCTTGAGAC  GGAGGAGACC  TTCCAACCTG  GGAGATGGTT
TGAAGTTGCA  CCCCTGGTCG  CGCAGTCGGG  ATACCAAAAA  AGGAACTCTG  CCTCCTCTGG  AAGGTTGGAC  CCTCTACCAA

      145          150          155          160          165
 M  R  A   A  Q  A  V   T  A  V   V  C  G   P  D  M  I   Q  V  S   L  N  A   G  A  R  K
       570         580         590         600         610         620         630         640
TATGCGTGCC  GCTCAAGCTG  TGACTGCGGT  AGTATGCGGC  CCGGATATGA  TTCAAGTGTC  ACTGAATGCT  GGGGCAAGAA
ATACGCACGG  CGAGTTCGAC  ACTGACGCCA  TCATACGCCG  GGCCTATACT  AAGTTCACAG  TGACTTACGA  CCCCGTTCTT
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HindIII
170          175          180          185          190          195
 L  R  Y    N  G  E    C  K  Y  T    Q  Q  S    T  A  I    R  G  D  R    A  V  L    A  A  K
       650         660         670         680         690         700         710         720
AGCTTAGATA  CAACGGTGAG  TGCAAGTACA  CGCAGCAGTC  CACTGCCATT  CGCGGTGACC  GTGCCGTCTT  GGCCGCAAAG
TCGAATCTAT  GTTGCCACTC  ACGTTCATGT  GCGTCGTCAG  GTGACGGTAA  GCGCCACTGG  CACGGCAGAA  CCGGCGTTTC

             200          205        SalI      210          215                      220
Y  A  N  T    K  H  K    L  P  S    T  V  D  G    D  V  Q    Q  I  F    Q  G  R  N    D  P  M
       730         740         750         760         770         780         790         800
TACGCCAACA  CCAAACACAA  ACTCCCGTCT  ACCGTCGACG  GGGATGTGCA  GCAGATATTT  CAAGGCCGTA  ATGATCCTAT
ATGCGGTTGT  GGTTTGTGTT  TGAGGGCAGA  TGGCAGCTGC  CCCTACACGT  CGTCTATAAA  GTTCCGGCAT  TACTAGGATA

      255          260          265                     270          275
 M  I  Y    L  V  W  R    R  I  E    N  F  A    M  A  Q  G    N  S  Q    Q  T  Q    A  G  V  T
       810         820         830         840         850         860         870         880
GATGATATAT  CTAGTGTGGA  GAAGAATTGA  AAACTTTGCG  ATGGCGCAAG  GTAATTCACA  GCAAACTCAA  GCAGGTGTGA
CTACTATATA  GATCACACCT  CTTCTTAACT  TTTGAAACGC  TACCGCGTTC  CATTAAGTGT  CGTTTGAGTT  CGTCCACACT

280          285                      290          295                      300          305
 V  S  V    G  G  V    D  M  R  A    G  R  I    I  A  W    D  G  Q  A    A  L  H    V  H  N
       890         900         910         920         930         940         950         960
CTGTTAGCGT  TGGCGGAGTA  GACATGCGGG  CGGGACGTAT  TATAGCGTGG  GATGGACAAG  CCGCTCTACA  TGTGCATAAC
GACAATCGCA  ACCGCCTCAT  CTGTACGCCC  GCCCTGCATA  ATATCGCACC  CTACCTGTTC  GGCGAGATGT  ACACGTATTG

             310          315                      320          325                      330
P  T  Q  Q    N  A  M    V  Q  I    Q  V  V  F    Y  I  S    M  D  K    T  L  N  Q    Y  P  A
       970         980         990        1000        1010        1020        1030        1040
CCCACACAAC  AGAATGCAAT  GGTTCAAATA  CAAGTCGTGT  TTTACATTTC  CATGGATAAG  ACTTTAAACC  AATATCCCGC
GGGTGTGTTG  TCTTACGTTA  CCAAGTTTAT  GTTCAGCACA  AAATGTAAAG  GTACCTATTC  TGAAATTTGG  TTATAGGGCG

      335                      340          345                      350          355
 L  T  A    E  I  F  N    V  Y  S    F  R  D    H  T  W  H    G  L  R    T  A  I    L  N  R  T
      1050        1060        1070        1080        1090        1100        1110        1120
TTTGACTGCT  GAAATCTTTA  ACGTTTATAG  CTTCAGAGAT  CATACATGGC  ACGGATTGAG  GACGGCCATA  CTAAACAGAA
AAACTGACGA  CTTTAGAAAT  TGCAAATATC  GAAGTCTCTA  GTATGTACCG  TGCCTAACTC  CTGCCGGTAT  GATTTGTCTT

  360          365                      370          375                      380          385
 T  L  P    N  M  L    P  P  I  F    P  P  N    D  R  D    S  I  L  T    L  L  L    L  S  T
      1130        1140        1150        1160        1170        1180        1190        1200
CTACACTGCC  AAACATGCTG  CCACCTATCT  TCCCACCAAA  TGATCGAGAT  AGTATCCTGA  CTCTTTTGCT  TTTGTCTACG
GATGTGACGG  TTTGTACGAC  GGTGGATAGA  AGGGTGGTTT  ACTAGCTCTA  TCATAGGACT  GAGAAAACGA  AAACAGATGC

              390          395                      400          405                      410
L  A  D  V    Y  T  V    L  R  P    E  F  A  I    H  G  V    N  P  M    P  G  P  L    T  R  A
      1210        1220        1230        1240        1250        1260        1270        1280
CTTGCTGATG  TTTATACCGT  TTTGAGACCA  GAGTTCGCGA  TTCATGGCGT  AAACCCAATG  CCTGGGCCGC  TCACACGTGC
GAACGACTAC  AAATATGGCA  AAACTCTGGT  CTCAAGCGCT  AAGTACCGCA  TTTGGGTTAC  GGACCCGGCG  AGTGTGCACG

      VP7-169 stop codon ⌉
      415          419
 I  A  R    A  A  Y  V    *    EcoRI                                                pFb stop codons
      1290        1300        1310        1320        1330        1340        1350        1360
TATTGCACGC  GCCGCCTATG  TGTAGGAATT  CAAAGGCCTA  CGTCGAAGCT  AGCTTGTCGA  GAAGTACTAG  AGGATCATAA
ATAACGTGCG  CGGCGGATAC  ACATCCTTAA  GTTTCCGGAT  GCAGCTTCGA  TCGAACAGCT  CTTCATGATC  TCCTAGTATT

      1370        1377
TCAGCCATAC  CACATTT
AGTCGGTATG  GTGTAAA
3' ← pFB1 RV primer 5'
```

Sequence of pFb.VP7-169-eGFP

- Showing pFbmod2 regions surrounding BTV10 VP7-169 + insert
- Cloned by Debbie Bolton using primers eGFPHindF5'GCAAGCTTATGGTGAGCAAGGGCGAG
  and eGFPSalR 5'GCGTCGACCTTGTACAGCTCGTCCAT
- Designed for amplifying complete eGFP gene (717nt encoding 239aa).
- Forward primer has *Hind*III site, reverse primer has *Sal*I site on the end, for cloning directionally into the *Hind*III/*Sal*I site at position 169 of VP7-169 vector.
- Construct total length: pFb(4701 bp)+VP7(1062 bp)+insert(717bp) = 6633 bp
- Chimeric protein size: VP7(353aa=39 kDa) + eGFP(239 aa=27 kDa) = 66 kDa
- BTV10 VP7-169 sequences in black, insert in green
- NOTE: nt C at $eGFP_{192}$ Δ to A $\Rightarrow$ no aa Δ

polyhedrin promoter

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GATCATGGAG ATAATTAAAA TGATAACCAT CTCGCAAATA AATAAGTATT TTACTGTTTT CGTAACAGTT TTGTAATAAA
CTAGTACCTC TATTAATTTT ACTATTGGTA GAGCGTTTAT TTATTCATAA AATGACAAAA GCATTGTCAA AACATTATTT
```

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                                                                   VP7-169 start
                                                         BamHI 1           5
                    5' pFB1 POLH Fw primer → 3'                    M  D   T  I  A   A  R  A  L
        90        100        110        120        130        140        150        160
AAAACCTATA AATATTCCGG ATTATTCATA CCGTCCCACC ATCGGGCGCG GATCCATGGA CACTATCGCT GCAAGAGCAC
TTTTGGATAT TTATAAGGCC TAATAAGTAT GGCAGGGTGG TAGCCCGCGC CTAGGTACCT GTGATAGCGA CGTTCTCGTG
```

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 10          15          20          25          30          35
 T  V  M   R  A  C   A  T  L  Q   E  A  R   I  V  L   E  A  N  V   M  E  I   L  G  I
       170        180        190        200        210        220        230        240
TCACTGTGAT GCGAGCATGT GCTACGCTTC AAGAAGCAAG AATTGTATTG GAAGCTAATG TGATGGAAAT ATTGGGGATA
AGTGACACTA CGCTCGTACA CGATGCGAAG TTCTTCGTTC TTAACATAAC CTTCGATTAC ACTACCTTTA TAACCCCTAT
```

```
            40          45          50          55          60
A  I  N  R   Y  N  G   L  T  L   R  G  V  T   M  R  P   T  S  L   A  Q  R  N   E  M  F
       250        260        270        280        290        300        310        320
GCAATCAACA GATATAATGG ATTAACTTTA CGAGGGGTGA CGATGCGCCC AACCTCATTG GCGCAGAGAA ATGAGATGTT
CGTTAGTTGT CTATATTACC TAATTGAAAT GCTCCCCACT GCTACGCGGG TTGGAGTAAC CGCGTCTCTT TACTCTACAA
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        65          70          75          80          85
 F  M  C   L  D  M  M   L  S  A   A  G  I   N  I  G  P   I  S  P   D  Y  T   Q  H  M  A
       330        340        350        360        370        380        390        400
TTTTATGTGT TTGGATATGA TGCTGTCTGC GGCTGGGATA AATATTGGAC CGATATCCCC AGATTATACC CAACATATGG
AAAATACACA AACCTATACT ACGACAGACG CCGACCCTAT TTATAACCTG GCTATAGGGG TCTAATATGG GTTGTATACC
```

```
 90          95          100         105         110         115
 T  I  G   V  L  A   T  P  E  I   P  F  T   T  E  A   A  N  E  I   A  R  V   T  G  E
       410        420        430        440        450        460        470        480
CTACAATCGG TGTACTAGCG ACGCCTGAGA TACCTTTTAC GACAGAAGCA GCGAATGAAA TAGCTCGCGT GACTGGGGAG
GATGTTAGCC ACATGATCGC TGCGGACTCT ATGGAAAATG CTGTCTTCGT CGCTTACTTT ATCGAGCGCA CTGACCCCTC
```

```
            120         125         130         135         140
T  S  T  W   G  P  A   R  Q  P   Y  G  F  F   L  E  T   E  E  T   F  Q  P  G   R  W  F
       490        500        510        520        530        540        550        560
ACTTCAACGT GGGGACCAGC GCGTCAGCCC TATGGTTTTT TCCTTGAGAC GGAGGAGACC TTCCAACCTG GGAGATGGTT
TGAAGTTGCA CCCCTGGTCG CGCAGTCGGG ATACCAAAAA AGGAACTCTG CCTCCTCTGG AAGGTTGGAC CCTCTACCAA
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```
      145            150            155           160           165
 M  R  A    A  Q  A  V    T  A  V    V  C  G    P  D  M  I    Q  V  S    L  N  A    G  A  R  K
        570        580        590        600        610        620        630        640
TATGCGTGCC GCTCAAGCTG TGACTGCGGT AGTATGCGGC CCGGATATGA TTCAAGTGTC ACTGAATGCT GGGGCAAGAA
ATACGCACGG CGAGTTCGAC ACTGACGCCA TCATACGCCG GGCCTATACT AAGTTCACAG TGACTTACGA CCCCGTTCTT

HindIII
170              175             180            185              190            195
 L  M  V    S  K  G    E  E  L  F    T  G  V    V  P  I    L  V  E  L    D  G  D    V  N  G
        650        660        670        680        690        700        710        720
AGCTTATGGT GAGCAAGGGC GAGGAGCTGT TCACCGGGGT GGTGCCCATC CTGGTCGAGC TGGACGGCGA CGTAAACGGC
TCGAATACCA CTCGTTCCCG CTCCTCGACA AGTGGCCCCA CCACGGGTAG GACCAGCTCG ACCTGCCGCT GCATTTGCCG

                 200            205             210              215              220
H  K  F  S   V  S  G    E  G  E    G  D  A  T    Y  G  K    L  T  L    K  F  I  C    T  T  G
        730        740        750        760        770        780        790        800
CACAAGTTCA GCGTGTCCGG CGAGGGCGAG GGCGATGCCA CCTACGGCAA GCTGACCCTG AAGTTCATCT GCACCACCGG
GTGTTCAAGT CGCACAGGCC GCTCCCGCTC CCGCTACGGT GGATGCCGTT CGACTGGGAC TTCAAGTAGA CGTGGTGGCC

      225              230            235             240              245
 K  L  P    V  P  W  P    T  L  V    T  T  L    T  Y  G  V    Q  C  F    S  R  Y    P  D  H  M
        810        820        830        840        850        860        870        880
CAAGCTGCCC GTGCCCTGGC CCACCCTCGT GACCACACTG ACCTACGGCG TGCAGTGCTT CAGCCGCTAC CCCGACCACA
GTTCGACGGG CACGGGACCG GGTGGGAGCA CTGGTGTGAC TGGATGCCGC ACGTCACGAA GTCGGCGATG GGGCTGGTGT

250              255              260             265              270              275
 K  Q  H    D  F  F    K  S  A  M    P  E  G    Y  V  Q    E  R  T  I    F  F  K    D  D  G
        890        900        910        920        930        940        950        960
TGAAGCAGCA CGACTTCTTC AAGTCCGCCA TGCCCGAAGG CTACGTCCAG GAGCGCACCA TCTTCTTCAA GGACGACGGC
ACTTCGTCGT GCTGAAGAAG TTCAGGCGGT ACGGGCTTCC GATGCAGGTC CTCGCGTGGT AGAAGAAGTT CCTGCTGCCG

                 280              285             290              295              300
N  Y  K  T    R  A  E    V  K  F    E  G  D  T    L  V  N    R  I  E    L  K  G  I    D  F  K
        970        980        990       1000       1010       1020       1030       1040
AACTACAAGA CCCGCGCCGA GGTGAAGTTC GAGGGCGACA CCCTGGTGAA CCGCATCGAG CTGAAGGGCA TCGACTTCAA
TTGATGTTCT GGGCGCGGCT CCACTTCAAG CTCCCGCTGT GGGACCACTT GGCGTAGCTC GACTTCCCGT AGCTGAAGTT

       305             310              315              320              325
 E  D  G    N  I  L  G    H  K  L    E  Y  N    Y  N  S  H    N  V  Y    I  M  A    D  K  Q  K
       1050       1060       1070       1080       1090       1100       1110       1120
GGAGGACGGC AACATCCTGG GGCACAAGCT GGAGTACAAC TACAACAGCC ACAACGTCTA TATCATGGCC GACAAGCAGA
CCTCCTGCCG TTGTAGGACC CCGTGTTCGA CCTCATGTTG ATGTTGTCGG TGTTGCAGAT ATAGTACCGG CTGTTCGTCT

   330              335             340              345              350              355
 N  G  I    K  V  N    F  K  I  R    H  N  I    E  D  G    S  V  Q  L    A  D  H    Y  Q  Q
       1130       1140       1150       1160       1170       1180       1190       1200
AGAACGGCAT CAAGGTGAAC TTCAAGATCC GCCACAACAT CGAGGACGGC AGCGTGCAGC TCGCCGACCA CTACCAGCAG
TCTTGCCGTA GTTCCACTTG AAGTTCTAGG CGGTGTTGTA GCTCCTGCCG TCGCACGTCG AGCGGCTGGT GATGGTCGTC

                 360              365             370              375              380
N  T  P  I    G  D  G    P  V  L    L  P  D  N    H  Y  L    S  T  Q    S  A  L  S    K  D  P
       1210       1220       1230       1240       1250       1260       1270       1280
AACACCCCCA TCGGCGACGG CCCCGTGCTG CTGCCCGACA ACCACTACCT GAGCACCCAG TCCGCCCTGA GCAAAGACCC
TTGTGGGGGT AGCCGCTGCC GGGGCACGAC GACGGGCTGT TGGTGATGGA CTCGTGGGTC AGGCGGGACT CGTTTCTGGG

      385              390             395              400              405
 N  E  K    R  D  H  M    V  L  L    E  F  V    T  A  A  G    I  T  L    G  M  D    E  L  Y  K
       1290       1300       1310       1320       1330       1340       1350       1360
CAACGAGAAG CGCGATCACA TGGTCCTGCT GGAGTTCGTG ACCGCCGCCG GGATCACTCT CGGCATGGAC GAGCTGTACA
GTTGCTCTTC GCGCTAGTGT ACCAGGACGA CCTCAAGCAC TGGCGGCGGC CCTAGTGAGA GCCGTACCTG CTCGACATGT

410 SalI         415              420             425              430              435
 V  D  G    D  V  Q    Q  I  F  Q    G  R  N    D  P  M    M  I  Y  L    V  W  R    R  I  E
       1370       1380       1390       1400       1410       1420       1430       1440
AGGTCGACGG GGATGTGCAG CAGATATTTC AAGGCCGTAA TGATCCTATG ATGATATATC TAGTGTGGAG AAGAATTGAA
TCCAGCTGCC CCTACACGTC GTCTATAAAG TTCCGGCATT ACTAGGATAC TACTATATAG ATCACACCTC TTCTTAACTT
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            440               445                450             455              460
N  F  A  M     A  Q  G     N  S  Q     Q  T  Q  A    G  V  T     V  S  V     G  G  V  D    M  R  A
      1450       1460       1470       1480       1490       1500       1510       1520
AACTTTGCGA TGGCGCAAGG TAATTCACAG CAAACTCAAG CAGGTGTGAC TGTTAGCGTT GGCGGAGTAG ACATGCGGGC
TTGAAACGCT ACCGCGTTCC ATTAAGTGTC GTTTGAGTTC GTCCACACTG ACAATCGCAA CCGCCTCATC TGTACGCCCG


      465             470              475             480              485
 G  R  I     I  A  W  D    G  Q  A     A  L  H     V  H  N  P     T  Q  Q     N  A  M     V  Q  I  Q
      1530       1540       1550       1560       1570       1580       1590       1600
GGGACGTATT ATAGCGTGGG ATGGACAAGC CGCTCTACAT GTGCATAACC CCACACAACA GAATGCAATG GTTCAAATAC
CCCTGCATAA TATCGCACCC TACCTGTTCG GCGAGATGTA CACGTATTGG GGTGTGTTGT CTTACGTTAC CAAGTTTATG

 490              495              500              505              510              515
 V  V  F     Y  I  S     M  D  K  T     L  N  Q     Y  P  A     L  T  A  E     I  F  N     V  Y  S
      1610       1620       1630       1640       1650       1660       1670       1680
AAGTCGTGTT TTACATTTCC ATGGATAAGA CTTTAAACCA ATATCCCGCT TTGACTGCTG AAATCTTTAA CGTTTATAGC
TTCAGCACAA AATGTAAAGG TACCTATTCT GAAATTTGGT TATAGGGCGA AACTGACGAC TTTAGAAATT GCAAATATCG

            520               525               530              535              540
L  R  D  H     T  W  H     G  L  R     T  A  I  L     N  R  T     T  L  P     N  M  L  P     P  I  F
      1690       1700       1710       1720       1730       1740       1750       1760
CTCAGAGATC ATACATGGCA CGGATTGAGG ACGGCCATAC TAAACAGAAC TACACTGCCA AACATGCTGC CACCTATCTT
GAGTCTCTAG TATGTACCGT GCCTAACTCC TGCCGGTATG ATTTGTCTTG ATGTGACGGT TTGTACGACG GTGGATAGAA

      545              550              555              560              565
 P  P  N     D  R  D  S     I  L  T     L  L  L     L  S  T  L     A  D  V     Y  T  V     L  R  P  E
      1770       1780       1790       1800       1810       1820       1830       1840
CCCACCAAAT GATCGAGATA GTATCCTGAC TCTTTTGCTT TTGTCTACGC TTGCTGATGT TTATACCGTT TTGAGACCAG
GGGTGGTTTA CTAGCTCTAT CATAGGACTG AGAAAACGAA AACAGATGCG AACGACTACA AATATGGCAA AACTCTGGTC

                                                                 VP7-169 stop codon
570             575                 580               585              590    592      EcoRI
 F  A  I     H  G  V     N  P  M  P     G  P  L     T  R  A     I  A  R  A     A  Y  V      *
      1850       1860       1870       1880       1890       1900       1910       1920
AGTTCGCGAT TCATGGCGTA AACCCAATGC CTGGGCCGCT CACACGTGCT ATTGCACGCG CCGCCTATGT GTAGGAATTC
TCAAGCGCTA AGTACCGCAT TTGGGTTACG GACCCGGCGA GTGTGCACGA TAACGTGCGC GGCGGATACA CATCCTTAAG

                                            pFb stop codons
      1930       1940       1950       1960       1970       1980    1986
AAAGGCCTAC GTCGAAGCTA GCTTGTCGAG AAGTACTAGA GGATCATAAT CAGCCATACC ACATTT
TTTCCGGATG CAGCTTCGAT CGAACAGCTC TTCATGATCT CCTAGTATTA GTCGGTATGG TGTAAA
                                                   3' ← pFB1 RV primer 5'
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Sequence of pFb.VP7-144-FMDVEpi

- Showing VP7-144 ORF + insert
- Cloned by Debbie Bolton using primers jv2f 5' CGGAATTCGATCCAAATCATGATACATGG 3' and jv2r 5' GCCTCGAGATTCGCGCTCTGTTCTTTCTG 3'

  Designed for amplifying an 36 aa epitope region on FMDV VP1.
- Forward primer has *Eco*RI site, reverse primer has *Xho*I site on the end, for cloning directionally into the *Eco*RI/*Xho*I site at position 144 of VP7-144 vector.
- Construct total length: pFb (4700bp)+ VP7 (1172bp)+ insert (108bp) = 5980 bp
- Chimeric protein size: VP7 (353 aa) + Epitope B (36 aa) = 389 aa

  VP7 (39 kDa) + Epitope B (3 kDa) = 42 kDa
- AHSV9 VP7-144 sequences in black, insert in green

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25
M  D  A  I    A  A  R    A  L  S    V  V  R  A    C  V  T    V  T  D    A  R  V  S    L  D  P
         10          20          30          40          50          60          70          80
ATGGACGCGA  TAGCAGCAAG  AGCCTTGTCC  GTTGTACGGG  CATGTGTCAC  AGTGACAGAT  GCGAGAGTTA  GTTTGGATCC
TACCAGCGCT  ATCGACGTTC  TCGGAAGAGG  CAACATGCCC  GTACACAGTG  TCACTGTCTA  CGCTCTCAAT  CAAACCTAGG

 G  V  M    E  T  L  G    I  A  I    N  R  Y    N  G  L  T    N  H  S    V  S  M    R  P  Q  T
         90         100         110         120         130         140         150         160
AGGAGTGATG  GAGACGTTAG  GGATTGCAAT  CAATAGGTAT  AATGGTTTAA  CAAATCATTC  GGTATCGATG  AGGCCACAAA
TCCTCACTAC  CTCTGCAATC  CCTAACGTTA  GTTATCCATA  TTACCAAATT  GTTTAGTAAG  CCATAGCTAC  TCCGGTGTTT

  Q  A  E    R  N  E    M  F  F  M    C  T  D    M  V  L    A  A  L  N    V  Q  I    G  N  I
        170         180         190         200         210         220         230         240
CCCAAGCAGA  ACGAAATGAA  ATGTTTTTTA  TGTGTACTGA  TATGGTTTTA  GCGGCGCTGA  ACGTCCAAAT  TGGGAATATT
GGGTTCGTCT  TGCTTTACTT  TACAAAAAAT  ACACATGACT  ATACCAAAAT  CGCCGCGACA  TGCAGGTTTA  ACCCTTATAA

S  P  D  Y    D  Q  A    L  A  T    V  G  A  L    A  T  T    E  I  P    Y  N  V  Q    A  M  N
        250         260         270         280         290         300         310         320
TCACCAGATT  ATGATCAAGC  GTTGGCAACT  GTGGGAGCTC  TCGCAACGAC  TGAAATTCCA  TATAATGTTC  AGGCCATGAA
AGTGGTCTAA  TACTAGTTCG  CAACCGTTGA  CACCCTCGAG  AGCGTTGCTG  ACTTTAAGGT  ATATTACAAG  TCCGGTACTT

 D  I  V    R  I  T  G    Q  M  Q    T  F  G    P  S  K  V    Q  T  G    P  Y  A    G  A  V  E
        330         340         350         360         370         380         390         400
TGACATCGTT  AGAATAACGG  GTCAGATGCA  AACATTCGGA  CCAAGCAAAG  TGCAAACGGG  GCCTTATGCA  GGAGCGGTTG
ACTGTAGCAA  TCTTATTGCC  CAGTCTACGT  TTGTAAGCCT  GGTTCGTTTC  ACGTTTGCCC  CGGAATACGT  CCTCGCCAAC
                                          EcoRI
 V  Q  Q    S  G  R    Y  Y  V  P    E  F  R    Y  N  G    E  C  K  Y    T  Q  Q    S  T  A
        410         420         430         440         450         460         470         480
AGGTGCAACA  ATCTGGCAGA  TATTACGTAC  CGGAATTCAG  ATACAACGGT  GAGTGCAAGT  ACACGCAGCA  GTCCACTGCC
TCCACGTTGT  TAGACCGTCT  ATAATGCATG  GCCTTAAGTC  TATGTTGCCA  CTCAGCTTCA  TGTGCGTCGT  CAGGTGACGG
                                                                                  XhoI
 I  R  G  D    R  A  V    L  A  A    K  Y  A  N    T  K  H    K  L  P    S  T  L  E    Q
        490         500         510         520         530         540         550
 ATTCGCGGTG  ACCGTGCCGT CTTGGCCGCA  AAGTACGCCA  ACACCAAACA  CAAACTCCCG  TCTACCCTCGA GC
 TAAGCGCCAC  TGGCACGGCA GAACCGGCGT  TTCATGCGGT  TGTGGTTTGT  GTTTGAGGGC  AGATGGGAGCT CG


 G  R  T    R  G  G    Y  I  N  S    N  I  A    E  V  C    M  D  A  G    A  A  G    Q  V  N
       560         570         580         590         600         610         620         630
AAGGTCGAAC  GCGTGGTGGG  TACATCAATT  CAAATATTGC AGAAGTGTGT  ATGGATGCAG  GTGCTGCGGG  ACAGGTCAAT
TTCCAGCTTG  CGCACCACCC  ATGTAGTTAA  GTTTATAACG TCTTCACACA  TACCTACGTC  CACGACGCCC  TGTCCAGTTA
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A  L  L  A    P  R  R    G  D  A    V  M  I  Y   F  V  W    R  P  L    R  I  F  C    D  P  Q
     640        650        660        670        680        690        700        710
GCGCTGCTAG CCCCAAGGAG GGGGGACGCA GTCATGATCT ATTTCGTTTG GAGACCGTTG CGTATATTTT GTGATCCTCA
CGCGACGATC GGGGTTCCTC CCCCCTGCGT CAGTACTAGA TAAAGCAAAC CTCTGGCAAC GCATATAAAA CACTAGGAGT

 G  A  S    L  E  S  A    P  G  T    F  V  T
     720        730        740        750
AGGTGCGTCA CTTGAGAGCG CTCCAGGAAC TTTTGTCACC
TCCACGCAGT GAACTCTCGC GAGGTCCTTG AAAACAGTGG

V  D  G  V    N  V  A    A  G  D    V  V  A  W    N  T  I    A  P  V    N  V  G  N    P  G  A
     760        770        780        790        800        810        820        830
GTTGATGGAG TAAATGTTGC AGCTGGAGAT GTCGTCGCAT GGAATACTAT TGCACCAGTG AATGTTGGAA ATCCTGGGGC
CAACTACCTC ATTTACAACG TCGACCTCTA CAGCAGCGTA CCTTATGATA ACGTGGTCAC TTACAACCTT TAGGACCCCG

 R  R  S    I  L  Q  F    E  V  L    W  Y  T    S  L  D  R    S  L  D    T  V  P    E  L  A  P
     840        850        860        870        880        890        900        910
ACGCAGATCA ATTTTACAGT TTGAAGTGTT ATGGTATACG TCCTTGGATA GATCGCTAGA CACGGTTCCG GAATTGGCTC
TGCGTCTAGT TAAAATGTCA AACTTCACAA TACCATATGC AGGAACCTAT CTAGCGATCT GTGCCAAGGC CTTAACCGAG

  T  L  T    R  C  Y    A  Y  V  S    P  T  W    H  A  L    R  A  V  I    F  Q  Q    M  N  M
     920        930        940        950        960        970        980        990
CAACGCTCAC AAGATGTTAT GCGTATGTCT CTCCCACTTG GCACGCATTA CGCGCTGTCA TTTTTCAGCA GATGAATATG
GTTGCGAGTG TTCTACAATA CGCATACAGA GAGGGTCAAC CGTGCGTAAT GCGCGACAGT AAAAAGTCGT CTACTTATAC

Q  P  I  N    P  P  I    F  P  P    T  E  R  N    E  I  V    A  Y  L    L  V  A  S    L  A  D
    1000       1010       1020       1030       1040       1050       1060       1070
CAGCCTATTA ATCCGCCGAT TTTTCCACCG ACTGAAAGGA ATGAAATTGT TGCGTATCTA TTAGTAGCTT CTTTAGCTGA
GTCGGATAAT TAGGCGGCTA AAAAGGTGGC TGACTTTCCT TACTTTAACA ACGCATAGAT AATCATCGAA GAAATCGACT

 V  Y  A    A  L  R  P    D  F  R    M  N  G    V  V  A  P    V  G  Q    I  N  R    A  L  V  L
    1080       1090       1100       1110       1120       1130       1140       1150
TGTGTATGCG GCTTTGAGAC CAGATTTCAG AATGAATGGT GTTGTCGCGC CAGTAGGCCA GATTAACAGA GCTCTTGTGC
ACACATACGC CGAAACTCTG GTCTAAAGTC TTACTTACCA CTTCAGCGCG GTCATCCGGT CTAATTGTCT CGAGAACACG

 A  A  Y    H  *
    1160    1168
TAGCAGCCTA CCACTAG
ATCGTCGGAT GGTGATC
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