HETEROLOGOUS EXPRESSION OF ALCELAPHINE HERPESVIRUS 1 STRUCTURAL PROTEINS AND THEIR USE IN THE DEVELOPMENT OF AN ELISA

BY

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Submitted in partial fulfilment of the requirements for the degree

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DECLARATION

I, Makgantjake Dominic Rachidi, do hereby declare that Dr Christine Vroon and Dr Charlotte Ellis assisted with the development of the ELISA. Except where acknowledgements indicate otherwise and for advice from my previous supervisor where I am employed, Dr Christine Vroon, this dissertation represents my own original work. Neither the full dissertation nor any part of it has been submitted for another degree at this or any other University.

This dissertation is presented in partial fulfilment of the requirements for the degree of Magister Scientiae (Veterinary Science) in the Department of Veterinary Tropical Diseases, University of Pretoria.

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Above all, the All mighty God, for the strength, courage and belief that carried me through this study.

Contents

LIST OF ABBREVIATIONS

Ab	antibody
Ag	antigen
AlHV-1	Alcelaphine herpesvirus 1
ALP	Alkaline phosphatase
BEK	bovine embryonic kidney
BHK	baby hamster kidney
BT	bovine turbinate
CMV	cytomegalovirus
DNA	deoxyribonucleic acid
ELISA	enzyme-linked immunosorbent assay
FCS	foetal calf sera
FITC	Fluorescein isothiocyanate
His	histidine
HRP	horse radish peroxidase
IFA	indirect immunofluorescence assay
IgG	immunoglobulin, G class
LB	Luria Bertani
LEW buffer	Lysis-elution-wash buffer
MCF	malignant catarrhal fever
MCS	multiple cloning site

MOI	multiplicity of infection
OIE	Office International des Epizooties
ORF	open reading frame
OvHV-2	Ovine herpesvirus 2
PBS	phosphate buffered saline
PCR	polymerase chain reaction
RES	restriction enzyme site
SDS	sodium dodecyl sulphate
TAE buffer	Tris-acetate-EDTA buffer
TMB	tetramethylbenzidine
VERO	African green monkey kidney

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SUMMARY

Heterologous expression of alcelaphine herpesvirus-1 proteins and their use in the development of an ELISA.

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Malignant catarrhal fever (MCF), a disease that is usually fatal in cattle, is caused by two distinct but related bovine herpesviruses which are members of the genus *Macavirus*. The wildebeest-associated alcelaphine herpesvirus-1 (AlHV-1) occurs mainly in East and southern Africa, whereas the sheep-associated ovine herpesvirus-1 (OvHV-2) has an almost worldwide distribution. The natural hosts or carriers of these two viruses are subclinically infected. The 130 kilobase pair (kbp) AlHV-1 double stranded DNA genome consists of 18 open reading frames (ORFs) coding for structural proteins and approximately 50 ORFs coding for non-structural proteins. The 18 structural ORFs encode for 4 capsid proteins, 5 tegument proteins, 8 glycoproteins and a minor capsid scaffold protein. ORF8 encoding for glycoprotein B, is the most conserved of the proteins amongst gammaherpesviruses, whereas the minor capsid protein encoded by ORF65, is amongst the most variable. Thus, the minor capsid protein is one of the antigens of choice for the development of an ELISA for detection of AlHV-1 reactive antibodies and glycoprotein B could be of importance in developing a cross-protective vaccine for gammaherpesviruses.

The naming and annotation of most of the AlHV-1 ORFs is based on comparison with related gammaherpesviruses and bioinformatics. Most of these ORFs are putative as there is no direct experimental evidence confirming that they code for any particular protein. In order to investigate whether the ORFs code for any proteins, two ORFs were targeted for *in vitro* heterologous expression.

AlHV-1, isolate C500, was grown in fetal bovine turbinate (BT) cell culture and viral genomic DNA extracted. ORF8, the putative glycoprotein B, was amplified with a PCR assay and inserted into a mammalian expression vector, pCI. VERO cells were transfected with the recombinant vector. Expression of ORF8 was confirmed by an indirect immunofluorescence assay (IFA) with AlHV-1 polyclonal sera and rabbit anti-bovine IgG (whole molecule) FITC conjugate. Truncated forms of ORF8 were further expressed as baculovirus recombinants using the Bac-to-Bac baculovirus expression system. Expression of the truncated ORF8 was confirmed by SDS-PAGE and Western blot.

AlHV-1 ORF65, the minor capsid protein gene, was amplified with a PCR assay from the viral genomic DNA and cloned in frame with a histidine tag in a bacterial expression vector, pCOLD I. Expression of the minor capsid protein was confirmed by SDS-PAGE and Western blot with the histidine tag monoclonal as well as AlHV-1 polyclonal sera. Orf65 was expressed in large quantities and column purified using the histidine tag. Orf65 was also expressed as a baculovirus recombinant using the Bac-to-Bac baculovirus expression system. Expression of the protein was confirmed by SDS-PAGE and Western blot with the histidine tag and AlHV-1 polyclonal sera. ORF65 expression in the baculovirus Bac-to-Bac expression system was up-scaled and the expressed protein column purified. Antibodies raised in chicken against the purified antigen were used successfully in an indirect immunoassay to detect AlHV-1 infected cells.

An indirect enzyme-linked immunosorbent assay (ELISA) to detect antibodies against AlHV-1 was developed. It is based on the use of the AlHV-1 minor capsid protein as the capture antigen for antibodies. The primary antibodies are detected by the addition of enzyme-labelled (horseradish peroxidase) protein G which detects bovid, ovid and wildebeest antibodies. Addition of a substrate of the enzyme, in this case, 3,3',5,5'-tetramethylbenzidine (TMB), results in a colour reaction which is measured using

spectrophotometric procedures. At a selected cut-off point of 18, the ELISA test has a sensitivity of 100% and a specificity of 100% and has been shown to detect AlHV-1 antibodies in cattle and wildebeest. The ELISA showed no cross-reactivity with sera raised in cattle against related viruses such as ovine herpesvirus 2, bovine herpesvirus 1, 2 and 4.

The two expressed proteins used in this study were found to be amongst the antigens expressed in cattle suffering from malignant catarrhal fever. The experimental AlHV-1 indirect ELISA needs further validation and this research may be extended to determine the performance of these antigens as candidate subunit vaccines.

CHAPTER 1: LITERATURE REVIEW

Introduction

Alcelaphine herpesvirus 1 (AlHV-1) is a gammaherpesvirus carried subclinically by African antelope of the subfamily Alcelaphinae; related viruses are carried worldwide by sheep and goats (Plowright et al. 1960). Infection of domestic and captive species of Artiodactyla with either AlHV-1 or ovineherpesvirus-2 (OvHV-2), carried by domestic sheep (*Ovis aries*), causes a disease called malignant catarrhal fever (MCF). When the causative agent of the disease is from wildebeest (*Connchaetes* spp), the disease is referred to as wildebeest-associated (WA) MCF. In the case of the sheep form, it is called the sheep-associated (SA) MCF. The disease was known in Europe as early as the 1700's (Werner *et al*. 2001). The Masaai in eastern Africa were aware of the disease for centuries and referred to it as wildebeest disease in recognition of the fact that their cattle got the disease from grazing in areas grazed by wildebeest during calving seasons (Heuschelle 1988). It was only in the early part of the 20th century that people in southern Africa became aware of the disease and called it "snotsiekte", Afrikaans for snotting sickness (Mettam, 1923). The disease was found later in the 1930's to be the same as the malignant catarrhal fever earlier observed in Europe as a result of work done by veterinary researchers.

1.1 The virus

AlHV-1 is classified as a member of the family *Herpesviridae*, subfamily *Gammaherpesvirinae* (Plowright *et al*. 1965). Originally grouped within the genus *Rhadinovirus*, it was recently classified in the new genus *Macavirus*. Members of this group have a double-stranded DNA genome and an icosahedral capsid, 100 – 110 nm in diameter. The viral tegument covering the nucleocapsid is covered by an envelope that contains viral glycoproteins (Roizman, 1996), (Figure 1).

The virus has a varied range of cell tropism. AlHV-1 was successfully cultured in primary or secondary and higher passaged cell cultures of a number of bovine cell types. Plowright (1960), cultivated the virus in bovine thyroid cells (BTh), calf testis (BT) (Plowright 1986), and calf (BK) or bovine embryonic kidney (BEK) cells (Plowright 1981; Plowright 1986). Kidney

cells of non-domestic ovine origin were also used with success for growth of AlHV-1. Fetal aoudad (*Ammotragus lervia)* (FAK) and fetal mouflon sheep (*Ovis musimon)* (FMSK) kidney cells optimally supported viral cultivation (Heuschelle and Fletcher 1984; Seal *et al*. 1989a,b).

The virulent form of the virus, isolated from clinically affected animals, produces characteristic large multinucleate foci in cell culture. The virus is cell-associated and co-cultivation of intact blood cells with the cell culture is necessary for successful viral isolation. Serial passage of AlHV-1 in culture attenuates the virus. The cytopathic effect induced by passaged virus is characterised by swelling and rounding of cells with rapid destruction of the monolayer and production of cell-free virus (Hart *et al*. 2007).

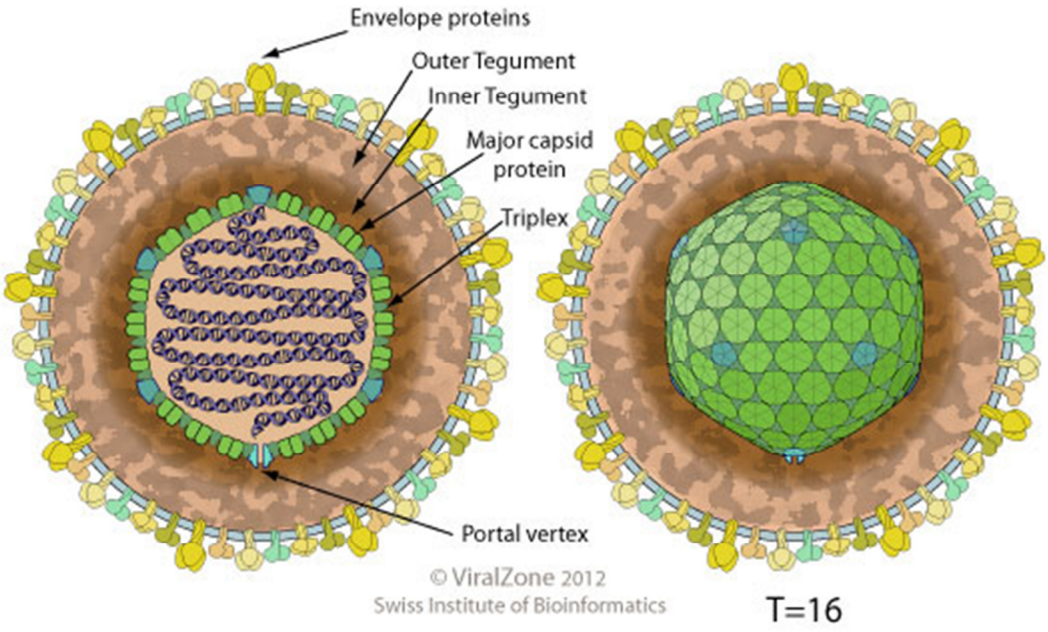


Figure 1: The general structure of gammaherpesviruses. The envelope proteins, which are glycoproteins are shown spanning the lipid envelope of the virus. (Image by Patrick Masson, Swiss Institute of Bioinformatics, Geneva, Switzerland, April 2012).

1.2 Pathogenicity

Malignant catarrhal fever is an infectious, sporadic and often fatal disease of ruminants caused by gammaherpesviruses, AlHV-1 and OvHV-2 (Reid and Buxton, 1984a; Loken *et al*., 1998; Schultheiss *et al*., 2000). The disease is distributed worldwide. In Africa, the disease in cattle is predominantly wildebeest-associated (WA) although the sheep-associated form does occur (Cleaveland *et al*., 2001; Bedelian *et al.,* 2007). Zoological gardens also experience problems with both the WA-MCF and SA-MCF. In the rest of the world, MCF is predominantly sheep-associated. The SA-MCF was reported in North and South America (Reid and Robinson, 1987; Berezowski *et al*., 2005; Rech *et al*., 2005), Europe (Collery and Foley, 1996; Frolich *et al*., 1998; Desmecht *et al*., 1999; Yus *et al*., 1999), the Middle East (Brenner *et al*., 2002; Abu Elzein *et al*., 2003), Asia (Dabak and Bulut, 2003), Africa (Rossiter, 1981) and New Zealand (Wilson, 2002).

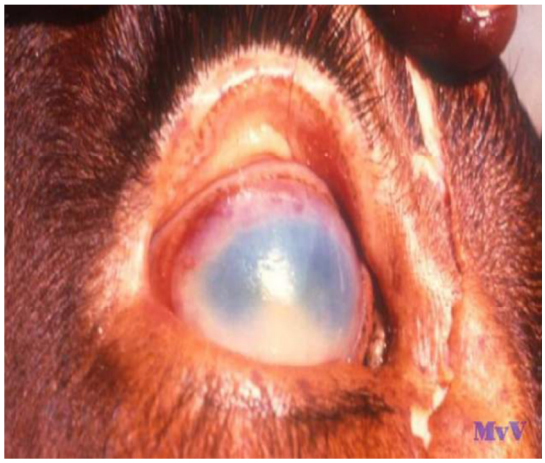
1.3 Diagnosis of MCF

Clinical signs for MCF vary between the most acutely to the chronic infected. But, in general common signs include high fever, mucopurulent discharge, corneal opacity, swollen limb joints, ulceration and necrosis of the muzzle and enlarged lymph nodes (Figure 2).

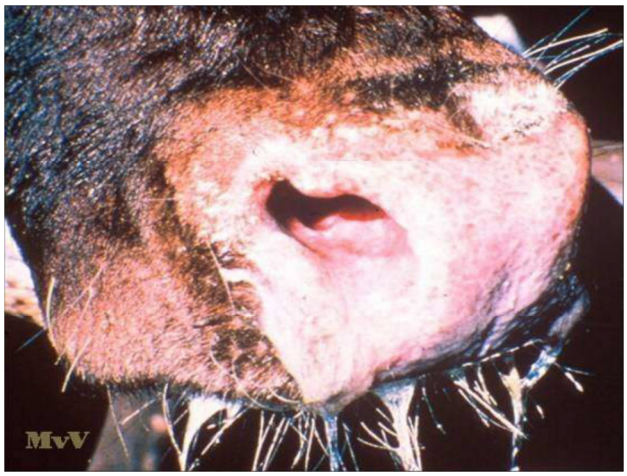
Animals showing some of these signs are bled and the blood sent for confirmatory laboratory tests for the presence of the MCF virus. Laboratories have developed a number of diagnostic tests such as the polymerase chain reaction (PCR) to detect MCF virus DNA, immunoperoxidase test, indirect immunofluorescence assay and ELISA to detect MCF specific antibodies, immunoblotting and virus neutralisation tests. PCR is the test normally performed to confirm the presence and classification of the virus. The World Organisation for Animal Health (OIE) regards histopathology as the definitive diagnostic tool for MCF in dead animals. But, PCR is still performed as a confirmatory tool for the MCF virus. With MCF, serological tests are performed as epidemiological tools rather than as diagnostic tests.

The virus neutralisation test is mostly used in determining gammaherpesvirus infections in wildlife (www.oie.int/fileadmin/Home/eng/Health_standards/thm/2.04.15_MCF.pdf). Since

clinically affected animals only develop virus neutralising antibodies in the terminal stage of the disease, the VN test cannot be used for the diagnosis of such cases. Haig et al. (2008) used the VN test in assessing the neutralising antibodies in cattle blood plasma and nasal secretions produced against a developmental stage AlHV-1 attenuated vaccine.



a.



b.

c.

Figure 2. Some of the clinical signs observed in cattle affected by malignant catarrhal fever. These include (a) severe ocular lesions, (b) ulceration and necrosis of the muzzle and (c) profuse mucopurulent nasal discharge. (MvV images by Prof. Moritz van Vuuren, Department of Veterinary Tropical Diseases, University of Pretoria; CBrown image by Dr. Corrie Brown, Department of Veterinary Pathology, University of Georgia).

1.4 Transmission of AlHV-1 and OvHV-2

Wildebeest calves below 3 months of age and lambs older than 6 months excrete the MCF virus via the ocular and nasal secretions (Mushi *et al*., 1981; Reid and Van Vuuren, 2004; Baxter *et al.* 1997; Li *et al*. 1998). Susceptible animals are infected by cell-free virus shed by neonatal wildebeest (Pretorius *et al.* 2008; Reid and Van Vuuren 2004). The cell-associated form of AlHV-1 propagated in vitro is pathogenic to animals. Following a limited number of passages in cell culture, a pathogenic cell-free AlHV-1 is generated. But, after multiple passages, the cell-free virus loses its pathogenicity owing to gene rearrangement. The mode of transmission is mostly by contact or aerosols from wildebeest calves and lambs. A proportion of wildebeest calves and lambs are infected in utero as observed in some gnotobiotic lambs with antibodies against MCF (Rossiter, 1981; Pretorius *et al*. 2008), with the majority being infected by the horizontal route. Transmission from wildebeest calves

occurs mostly at 1 to 2 months of age and with lambs, it is between 3 to 6 months of age. MCF – susceptible animals do not transmit virus to other animals.

1.5 Pathogenesis

The pathogenesis of MCF has not been fully elucidated. A number of hypotheses have been put forward regarding MCF pathogenesis. Work by Dewals and Vanderplasschen (2011) showed that in the terminal stages of MCF infection, 10% of the natural killer cells, $CD8^+$ T cells are latently infected with virus. The virus-infected cells become unregulated and proliferate uncontrollably producing IFN-γ. It is hypothesized that IFN-γ produced by these virus infected cells inhibits AlHV-1 reactivation from latency. Therefore, virus infection of the natural killer cells would lead to their uncontrolled proliferation and indirect evasion of the immune system through the IFN-γ-mediated inhibition of reactivation. Intense cell proliferation is not essential for a fatal outcome in MCF (Dewals and Vanderplasschen, 2011). This was shown in an experiment where OvHV-2 infected rabbits were treated with cyclosporine-A, but still developed SA-MCF even though cell infiltration was not observed (Buxton *et al*., 1984). Hyperacute MCF was observed to result in sudden death without the development of degenerative lesions (Plowright, 1990). Therefore, $CD8^+$ T-cell infiltration and the development of degenerative lesions appear not to be the cause of death in MCF infection.

Vascular lesions associated with MCF, are responsible for the observed clinical signs, (Reid and Van Vuuren, 2004). The fibrinoid necrosis of the walls blood vessels, which may be either focal or segmental, is accompanied by infiltration of lymphoblasts, lymphocytes and macrophages into the walls of the vessels and perivascular space. The lesions may occur throughout the whole body but are mostly abundant amongst other organs, the kidneys, lungs and meninges. The severity of the lesions correlates with the cellular infiltrations.

Palmeira et al. (2013), investigated the role played by ORF 73, a latency-associated nuclear antigen (LANA) homolog in MCF development. An ORF 73-deficient AlHV-1 mutant could not cause MCF when inoculated in susceptible animals. AlHV-1 ORF 73 is important for episomal maintenance of the virus. Whether ORF 73 plays any other pathogenic role during MCF

development, is a subject for investigation. AlHV-1 persistence *in vivo* is necessary for MCF induction in susceptible animals.

Cunha et al. (2012), demonstrated the presence of OvHV-2 ORF 25 transcripts, a lytic phase viral product, in peripheral blood leukocytes and tissues of cattle and bison with clinical and pre-clinical SA-MCF. It is argued that pre-clinical and clinical MCF is preceded by an increased OvHV-2 DNA replication, transcription and gene expression. The viral replication in cattle and bison is based in the cell cytoplasm as opposed to the nucleus in case of the natural host. An abortive OvHV-2 replication occurs in the cell cytoplasm, hence the lack of virus shedding and transmission from susceptible hosts. In support of this theory, Nelson et al. (2013) demonstrated the presence of ORF 25 transcripts in perivascular fibroblasts of bison with SA-MCF. The virus is thought to have reached the fibroblasts through localised lysis of OvHV-2 infected peripheral blood leukocytes. The OvHV-2 lytic products cause T-cell dysregulation with a concomitant vascular lymphocyte infiltration which in turn results in vascular lesions. Further support for this theory is that the severity of lesions in bison with clinical MCF correlated with the expression levels of ORF 25. Thus, the induction of SA- MCF is as a result of OvHV-2 replication, transcription and gene expression as opposed to latency.

1.6 Prevention and control

There is presently no commercially available vaccine against MCF. MCF susceptible animals should be separated from carrier species to prevent viral transmission. All carrier species, irrespective of their age, should be considered infective (Barnard, 1990). MCF susceptible animals are considered to be dead-end hosts and do not spread the disease to other animals. There has been concerted work done towards the development of a vaccine for MCF. Recent studies include those by Haig et al. (2008), Russell et al. (2012) and Palmeira et al. (2013). Haig et al. (2008) devised an immunisation strategy for the protection of cattle against AlHV-1 induced MCF. An attenuated C500 AlHV-1 strain was used together with an unlicensed Freund's adjuvant to vaccinate cattle intramuscularly (IM) in the neck. The cattle were then challenged 10 weeks after priming immunisation (6 weeks after booster immunisation) with virulent C500 AlHV-1 strain. Nine of ten cattle were protected. Russell et al. (2008), carried the work further by investigating the duration of immunity and the

antibody response involved. The group also used a licensed adjuvant in the form of oil-in water adjuvant, Emulsigen. Immunised cattle were challenged 3 or 6 months post immunisation. It was found that cattle that were immunised and protected from MCF had higher initial titres of anti-viral antibodies than animals that succumbed to the disease. Added to that, antibody titres in protected animals remained stable after challenge whilst antibody titres in animals that succumbed to the disease increased prior to the onset of clinical disease. From these studies it was concluded that a mucosal barrier of neutralising antibodies plays a role in protecting animals against infection and also that the initial antibody response determines the long-term protection of the animals.

The work by Palmeira et al. (2013) involved that investigation of the role of AlHV-1 ORF 73 in MCF pathogenesis. The group also looked at the possibility of using the ORF 73-deficient AlHV-1 as a vaccine candidate. Animals immunised with the ORF 73-deficient AlHV-1 were protected against lethal challenge with the wild-type AlHV-1. The recombinant AlHV-1, therefore, is a candidate vaccine. It is worth noting that the AlHV-1 specific antibodies produced prior to onset of MCF amongst immunised animals, are directed towards virus structural proteins including the major glycoprotein complex of the viral envelope (Herring et al. 1989)

1.6.1 AlHV-1 structural proteins

Herring et al. (1989), analysed the interaction of AlHV-1 strain WC11 structural proteins with sera from wildebeest, cattle with sheep-associated MCF and from sheep. Twenty five to thirty polypeptides were observed in immunoblots. Wildebeest sera reacted strongly with six polypeptides within immunoblots, whereas cattle and sheep sera reacted with a subset of the six polypetides. Adams and Hutt-Fletcher (1990), observed six glycoproteins and one non-glycosylated protein in their study to characterise AlHV-1 envelope proteins ranging in size from 48 kDa to 115 kDa. Five of the six glycoproteins form the gp115 complex, comprising of a 115, 110, 105, 78 and 48 kDa glycoproteins. The 115 kDa glycoprotein is cleaved to form a disulphide-linked heterodimer of 78 and 48 kDa. Antibodies produced against the gp115 complex neutralised virus infectivity as was also the case with the 130 kDa glycoprotein. Dry et al. (2008), found the AlHV-1 glycoprotein B to be a heterodimer comprised of 80 and 50 kDa glycoproteins in their proteomic analysis of AlHV-1. Six

glycoproteins, five capsid proteins, ten tegument and five nonstructural proteins were observed. The role of individual proteins in the pathogenesis of MCF can be investigated by using the AlHV-1 BAC clone (Dewals et al., 2006) or individually using heterologous expression systems.

1.7 Protein expression systems

Protein expression systems play a central and pivotal role in the production of recombinant proteins. The expression systems are driven by expression vectors, which are almost always plasmids. Recently, a number of viral vectors, for both mammalian and insect cells, have been developed for expression of recombinant proteins. The choice of the expression system is of paramount importance as it will ultimately determine the yields and quality of the recombinant protein. Some of the critical factors to consider when choosing an expression system include the mass of the protein, post-translational modifications of the expressed protein, number of disulphide bonds and the destination of the intended protein. The intended use of the expressed protein also plays a vital role in deciding which expression system to use. It is advisable to try multiple expression systems to be able to select an optimal one. Three common expression systems will be discussed, namely; bacterial, baculovirus (or insect cell), and the mammalian expression system.

1.7.1 Bacterial expression systems

Escherichia coli was the first bacterial host to be used for recombinant protein expression. It remains the expression system of choice for most researchers due to its simplicity, economic viability and its rapidity. The *E. coli* expression system affords the researcher the choice of directing expression of their proteins to the cytoplasm, periplasm and also secretion into the growth media. One can get recombinant protein yields of up to 30% of the biomass with cytoplasmic expression (Jana and Deb, 2005). The high yields of recombinant proteins often lead to the accumulation of insoluble aggregates of the protein called inclusion bodies. The formation of inclusion bodies with eukaryotic proteins

expressed in *E. coli* is thought to be a consequence of the 10-fold increased rate of translation and folding (Anderson *et al*. 1982; Goustin and Wilt, 1982). However, inclusion bodies can be an advantage at times: they are resistant to proteases, can be concentrated by centrifugation and refolded into active, soluble proteins.

The periplasmic expression of recombinant proteins offers some distinct advantages. The periplasm, unlike the cytoplasm with a reducing environment, has an oxidising environment that encourages disulphide bond formation. There are lesser amounts of proteins in the periplasm and thus a reduced probability of proteolysis. Toxic proteins can accumulate in the periplasm with less harm to the cell. However one disadvantage is that periplasmic protein expression results in very low yields.

1.7.1.1 The bacterial expression vector

The bacterial expression vector is a plasmid that can be maintained as an episome within specific bacterial cells through its origin of replication site. The vector contains a selectable marker, usually an antibiotic resistance gene that can be used to select cells containing the plasmid from those without it (Figure 3). Of importance for expression is the presence of a promoter in the vector that is active in bacterial cells, the Shine-Dalgarno sequence which is the ribosomal binding site, the multiple cloning site for insertion of the gene of interest and a transcription termination site. The gene of interest can be expressed as a fusion protein with either an affinity tag on the N-terminal or C-terminal site for ease of purification. Another fusion protein may be added to increase the solubility of the gene of interest. A protease cleavage site is normally included to cleave the fusion protein from the protein of interest.

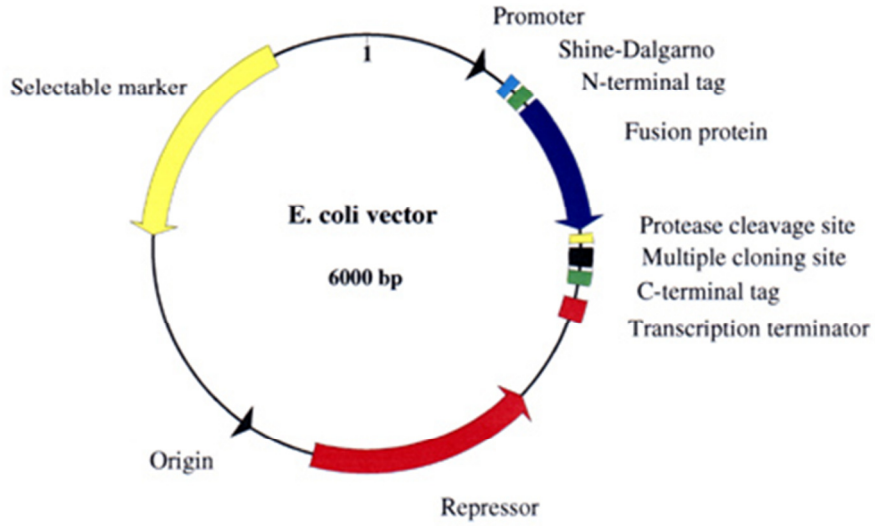


Figure 3: A typical bacterial expression vector showing the basic constituents necessary for heterologous expression in E. coli cells. (Image by Dr. Nikos Pinotsis, Center for Optimized Structural Studies, Max F Perutz Laboratories, University of Vienna).

1.7.1.2 Temperature and molecular chaperones

The temperature at which expression is performed and molecular chaperones are thought to be critical determining factors in the yield of soluble proteins. Sahdev *et al*. (2008), experimented with lowered temperatures between 15-30°C during the expression period. The reduced temperature slows the rate of transcription, translation and refolding, thus allowing for proper folding (Vera *et al*. 2006). Spies *et al*. (1999), showed lower temperature decreases heat shock protease activity. Protein solubility is promoted by co-expression with molecular chaperones (Young *et al*. 2004).

1.7.1.3 Genetically engineered *E. coli* strains

There are a number of genetically engineered *E. coli* strains that are commercially available. These genetically engineered strains were meant to overcome different constraints encountered when using bacterial expression systems for heterologous expression, Table 1.

Table 1: Some of the commonly used genetically engineered *E. coli* strains

Strain	Feature
C41 and C43(DE3), genotype- F^- *ompThsdS*$_B$($r_B^- m_B^-$)*gal dcm* (DE3)	For expression of toxic and membrane proteins (Miroux and Walker, 1996), (Lucigen, USA)
Origami or Origami (DE3), genotype- *Δ(ara-leu)7697 ΔlacX74 ΔphoA PvuII phoR araD139 ahpC gale galK rpsL F'[lac*$^+$*lacI*^{q}*pro](DE3) gor522:Tn10 trxB (Kan*^{R}*, Str*^{R}*, Tet*^{R}*)*	To enhance disulphide bond formation within the cytoplasm (Derman et al., 1993), (Novagen, Germany)
BL21 (DE3) CodonPlus-RIL, genotype- *E.coli B F*$^-$*ompT hsdS*($r_B^- m_B^-$)*dcm*$^+$*Tet*^{r} *gal λ(DE3)endA Hte [argU proL Cam*^{r}*] [argU ileY leuW Strep/Spec*^{r}*]*	For expression of rare *E. coli* codons. It co-expresses tRNAs for arginine (AGG/AGA), isoleucine (AUA) and leucine (CUA), (Stratagen, USA)
Rosetta or Rosetta (DE3), genotype- *F*$^-$*ompT hsdS*$_B$($r_B^- m_B^-$) *gal dcm* pRARE *(Cam*^{R}*)*	Co-expresses tRNAs for arginine (AGG/AGA/CGG), isoleucine (AUA) , Leucine (CUA), proline (CCC) and glycine (GGA), (Novagen, Germany)
Rosetta-gami, genotype – *Δ(ara-leu)7697 ΔlacX74 ΔphoA PvuII phoR araD139 ahpC galE galK rpsL F'[lac*$^+$*lacIqpro] gor522:Tn10 trxB pRARE (Cam*^{R}*, Kan*^{R}*, Str*^{R}*, Tet*^{R}*)*	Combines the enhanced disulfide bond formation of Origami cells with the enhanced expression of eukaryotic proteins that contain codons rarely used in *E. coli* (Novagen, Germany)

1.7.1.4 Inserting the sequence of interest into the vector

The sequence of interest may be inserted into the vector using either serial or parallel methods. Serial methods involve the use of restriction enzymes to generate complementary sites on both the vector and the insert sequence. The restriction digested vector and insert are ligated to each other by using a ligation enzyme. Parallel or universal systems offer the researcher an opportunity to transfer the target sequence to multiple vectors. The universal methods include ligation-independent cloning (LIC) (Aslanidis and de Jong, 1990; Haun *et al*.

1992), the Gateway (Esposito *et al*. 2009) and Infusion cloning (Zhu *et al*. 2007). With the LIC, target primer sequences include overhangs that are complementary to the vector ends that anneal strongly without ligation. The parallel systems are in general relatively less time consuming and cost effective.

1.7.1.5 Advantages and disadvantages of using bacterial expression systems

This is the cheapest of the expression systems, has the simplest of culture conditions and the expression levels are potentially very high. Culture growth is rapid, scaleable and many parameters can be altered to optimise expression levels. Although protein expression levels in bacteria are generally high, this often leads to inclusion body formation, which is disadvantageous. The post-translational modifications in bacteria are minimal and disulfide bond formation is inefficient, thus leading to misfolded proteins. Also, codon usage in bacteria is different to that in eukaryotes. *E. coli*, as a Gram-negative bacterium, poses a problem of the expressed proteins being contaminated with endotoxins.

1.7.2 Baculovirus/insect cell expression systems

Baculovirus/insect cell protein expression is based on inserting the gene of interest into a transfer vector, which is a plasmid, for integration into the baculovirus genome for expression in insect cell culture (Figure 4). The baculovirus genome may be linearized or maintained as a circular bacterial artificial chromosome (BAC) clone in *E. coli* cells. The basic constituents of this expression system are the insect cells and the recombinant baculovirus.

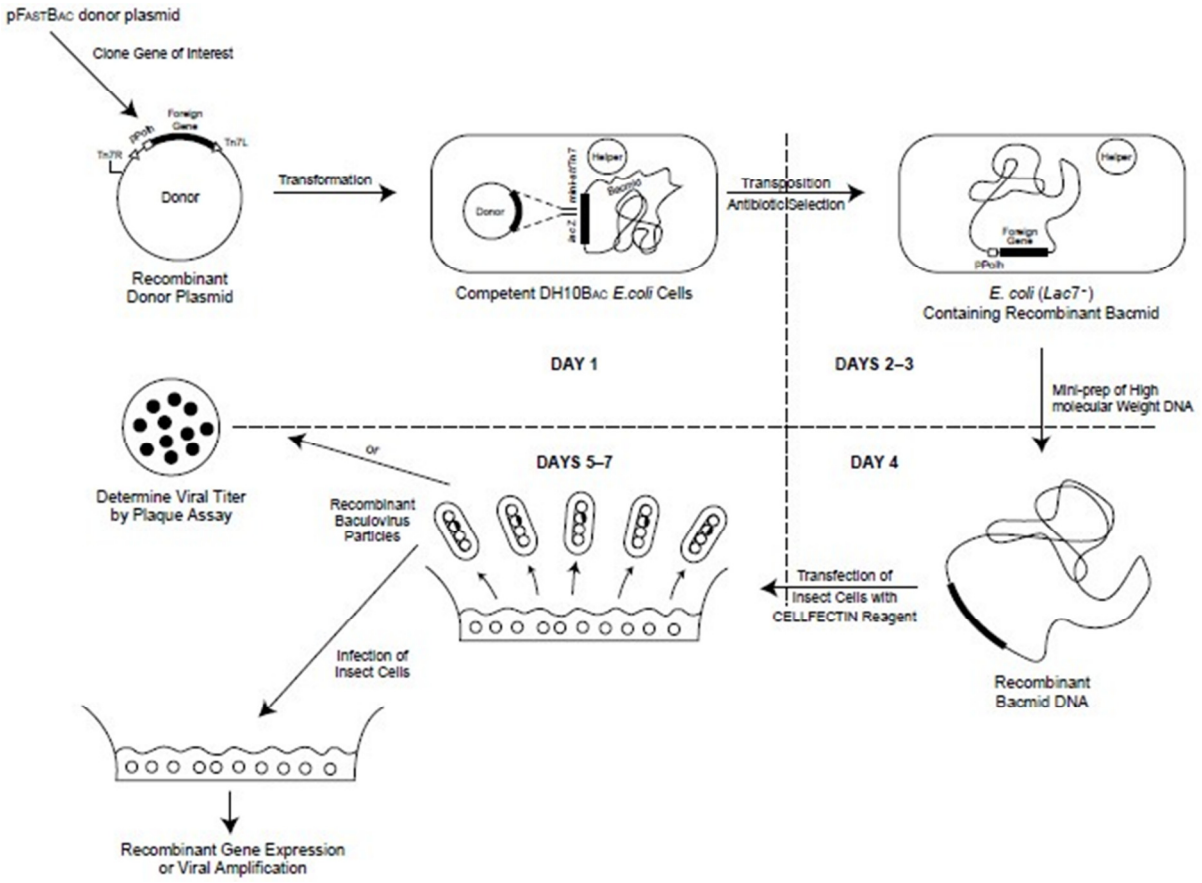


Figure 4 : Schematic representation of the steps involved in expressing the gene of interest in the baculovirus expression system. (Image from Invitrogen life technologies, Bac-to-BacR Baculovirus Expression System, Version D, 6 April 2004, cataloque number 10359).

1.7.2.1 The baculovirus vector

Baculoviruses belong to the family *Baculoviridae*, a group of viruses with large, double-stranded circular DNA genomes (Miller, 1988). The original baculovirus vectors were developed using the extensively studied *Autographa californica multicapsid nucleopolyhedrovirus* (AcMNPV) (Pennock et al. 1984; Smith *et al*. 1983). Baculoviruses have the ability to produce large amounts of polyhedrin towards the end of their infection cycle. Polyhedrin was found to form more than half of the total protein in the cells. The gene coding for expression of polyhedrin is non-essential for the survival of the virus in culture (Smith et al. 1983). This knowledge was exploited to form the first of the many available

baculovirus vectors. The polyhedrin gene within the recombinant baculovirus was replaced by a foreign gene under the polyhedrin promoter by homologous recombination between a transfer vector and the AcMNPV genome (Pennock *et al*. 1984; Smith *et al*. 1983). The foreign protein was expressed to high levels in insect cell culture, resulting in the birth of baculovirus/insect cell expression systems.

1.7.2.2 Protein tags

Heterologous expression of proteins may include fusion of the recombinant protein to a tag. The tag may serve the purpose of enhancing the solubility of the expressed protein or alternatively for affinity purification of the protein. Some of the commonly used tags for affinity purification are shown in Table 2 and Table 3 shows the most commonly used solubility tags. Solubility tags can be linked with affinity tags to enhance protein purification. These tags can either be included as N- or C-terminal fusion tags. Following expression and purification of the recombinant protein, the tag can be cleaved from the protein using the incorporated protease cleavage site (Table 4).

Table 2: Commonly used tags for protein purification (affinity tags)

Tag	**Affinity matrix**
His-tag , 6-10 His aa	Immobilized metal ions-Ni, Co, Cu, etc.
MBP (maltose-binding protein), 396 aa	Cross-linked amylose
GST (glutathione-S-transferase), 211 aa	Glutathione resin
Protein A (Staphylococcal Protein A), 280 aa	Immobilized IgG
FLAG-tag, 8 aa	Anti-FLAG mAb

Table 3: Commonly used tags to enhance protein solubility

Tag	Protein
SUMO, 100 aa	Small ubiquitin modifier
MBP, 396 aa	Maltose-binding protein
Trx, 109 aa	Thioredoxin
NusA, 495 aa	N-utilization substance
GB1, 56 aa	IgG domain B1 of streptococcus Protein G

Table 4: Proteases and their cleavage sites

Protease	Cleavage site
TEV (tobacco etch virus protease)	ENLYFQG
Enterokinase	DDDDK
Thrombin	LVPRGS
Factor Xa	IEGR
TVMV (tobacco vein mottling virus protease)	ETVRFQGS

1.7.2.3 Choice of tag

The type of tag to use depends on the characteristics of the protein under investigation. A researcher will have to try and test a number of tags before settling on one that best suits the protein under investigation. Some of the advantages and disadvantages of the affinity and solubility tags have been studied (Arnau *et al*. 2006; Waugh, 2005). As observed in Tables 2 and 3, the sizes of the affinity and solubility tags vary. A larger tag will impose a relatively higher metabolic load on the host cell than a smaller one. Larger tags interfere with the protein folding and hence the structure of the protein. Solubility tags such as NusA, GST and MBP, with their strong translational initiation signal, can be used when high expression levels of proteins are required (Cabrita *et al*. 2006; Kataeva *et al*. 2005).

1.7.2.4 Tagging N- or C-terminals

Different tags generally work more efficiently on either the N- or the C-terminal end of the expressed protein. The solubility enhancing tags, Trx and NusA, were found to be most efficient when positioned on the N-terminal (Sachdev and Chirgwin, 1998). The His-affinity tag, as well as the MBP solubility tag, works efficiently on either the N- or the C-terminal end of the protein (Dyson et al. 2004). With most proteases cleaving proteins towards the C-terminal of their recognition site, it is advantageous to place protein tags on the N-termini for their complete removal.

1.7.2.5 Tag removal from the expressed protein

Tags are removed by cleavage with the relevant endoprotease recognising the protease cleavage site (Table 4). As was previously mentioned, some tags interfere significantly with the structure and function of the recombinant proteins. For these proteins to be fully functional, most tags need to be removed from the expressed protein. However the His-tag was found to have no particular impact on the structure of some tagged proteins (Carson *et al*. 2007) and in some instances, was even found to be important for supporting the structure of the protein (Smits *et al*. 2008).

1.7.2.6 Transgenic insect cell lines

Although baculovirus/insect cell expression systems have eukaryotic protein processing capabilities, they are not identical to those of higher eukaryotes. This is evidenced by the differing glycosylation pattern observed in baculovirus-expressed proteins as compared to the mammalian-expressed proteins (Figure 5). A transgenic cell line, called MIMIC, commercialized by Invitrogen, was generated to overcome this deficiency. A glycosyltransferase cDNA was introduced into the insect cell line to enhance its ability to process glycosylation more in line with higher eukaryotes. A polydnavirus vankyrin gene was also introduced in some cell lines (Fath-Goodin *et al*. 2006). Co-expression of the polydnavirus vankyrin and the foreign protein resulted in a prolonged viability of the

baculovirus-infected cells and a concomitant increase in foreign protein expression. In another development, the baculovirus chitinase and cathepsin-like protease genes were deleted from the viral genome. Foreign protein expression from these baculovirus mutants resulted in reduced degradation of the expressed proteins as compared to baculovirus without the deletions (Kaba *et al*. 2004).

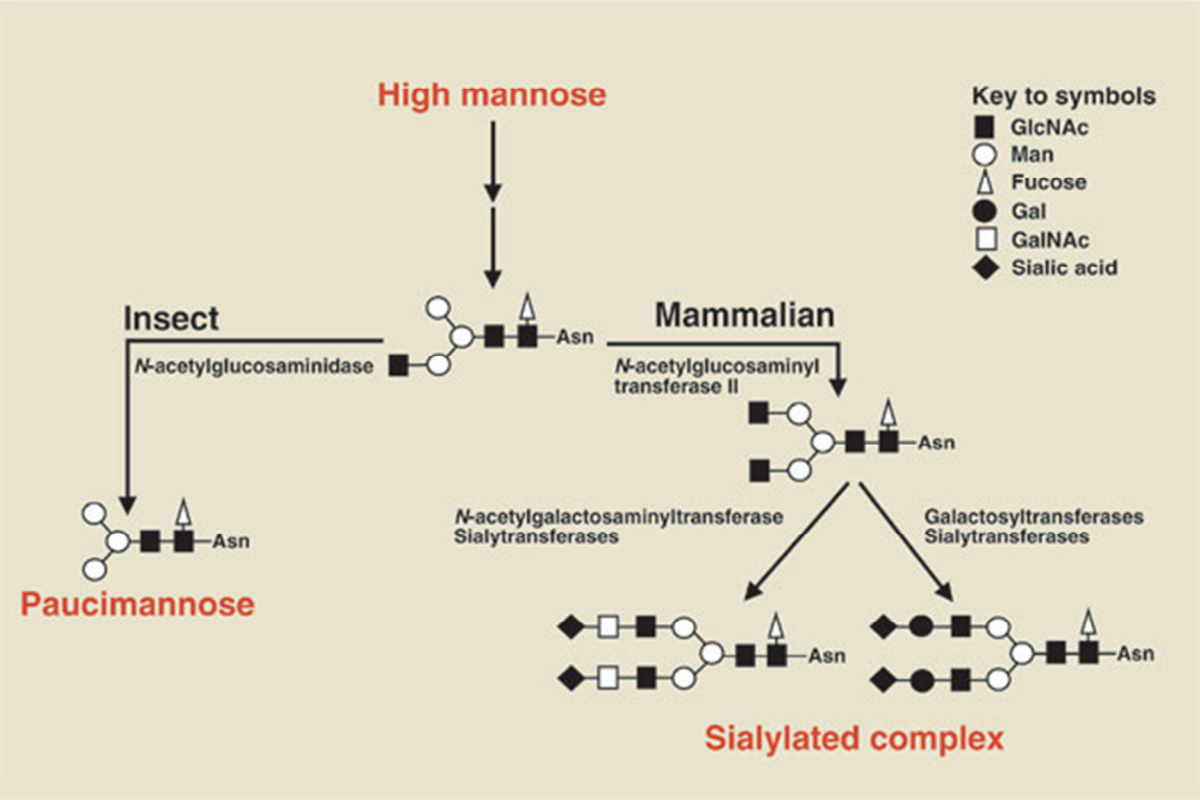


Figure 5: Glycosylation pattern observed with baculovirus/insect cell expressed proteins compared to mammalian expressed proteins. The N-glycans from the insect cells are relatively short structures with high mannose content and no terminal sialic acid residues (Marchal et al. 2001). The N-glycans in mammalian cells are extended by glycosyltransferases and terminate with a sialic acid residue. (Image from Kost et al. 2005)

1.7.2.7 Advantages and disadvantages of the baculovirus/insect cell expression system

Baculovirus/insect cell expression systems have the advantage of extensive posttranslational modifications of proteins. Although glycosylation is not identical to mammalian cells, it is functional. The expression levels are relatively good, especially for intracellular proteins, with efficient protein folding and products are free from endotoxins. Insect cells can be adapted to suspension cultures, thus facilitating scale-up processes. On the downside, baculovirus/insect cell expression systems utilize expensive culture media and need large volumes of virus for scale-up. And, baculovirus infection leads to cell lysis and potential degradation of expressed proteins.

1.7.3 The mammalian expression system

Mammalian expression systems have for years been considered the least efficient of the known expression systems. This was due to them being costly, time consuming and involved a number of problems in up-scaling the expression processes. Recent technological developments have significantly improved them, however. A mammalian expression vector, which either be a plasmid or a recombinant virus, and mammalian cells, are the basic tools required to express target genes.

1.7.3.1 The mammalian plasmid expression vector

The basic constituents of a mammalian plasmid expression vector are; the promoter, the multiple cloning sites (MCS), polyadenylation signal, a selection marker gene and an origin of replication compatible with bacterial cells. The promiscuous human cytomegalovirus (CMV) enhancer/promoter is usually used as the promoter of choice for mammalian expression. This allows the investigator the opportunity to use a number of different mammalian cell types to express target genes from one construct. The target gene is inserted into the MCS of the vector and must be directional with respect to the promoter for transcription of the target gene to occur. The polyadenylation signal stops continued RNA polymerase II transcription of the target gene. Transcription stoppage is immediately followed by addition of 200 – 250 adenosine residues to the 3'- end of the mRNA transcript

(Proudfoot, 1991). mRNA stability and translation were shown to be enhanced by polyadenylation (Bernstein and Ross, 1989; Jackson and Standart, 1990). The selection marker gene allows for selection of bacterial cells harbouring the plasmid vector after transformation. The bacterial origin of replication allows for maintenance and episomal replication of the plasmid vector.

1.7.3.2 Mammalian cell culture

A large number of mammalian cells have been grown in culture and successfully used for heterologous expression of proteins. Amongst the commonly used are the baby hamster kidney (BHK), African green monkey kidney (VERO), Chinese hamster ovary (CHO) and the human embryonic kidney (HEK293) cells. The cells may be grown as suspension or adherent cultures in the basic Dulbecco's modified eagle's medium (DMEM) supplemented with fetal calf serum. The cells may be transfected either transiently or stably depending on the investigator's need. Transgenic CHO and HEK293 cell lines have been engineered to improve their protein expression capabilities and scalability. These genetically engineered cell lines are available commercially.

1.7.3.3 Transgenic HEK293 and CHO cells

The HEK.EBNA cell line expresses the EBNA-1 protein from Epstein-Barr virus (EBV). The expression vector requires an EBV-derived origin of replication, *ori*P, to take advantage of this feature of the cell line. Interaction between the EBNA-1 and the *ori*P maintains the episomal existence of the expression vector within the nucleus. The expression vector is transferred to each daughter cell once upon cell division (Kishida et al. 2008; Lindner and Sugden, 2007). Viral elements from bovine papilloma virus, polyoma virus, and EBV have been added to some CHO transgenic cell lines. These elements were shown to have beneficial effects on protein expression (Kunaparaju, et al. 2005; Silla et al. 2005). Even with these added beneficial elements, CHO transgenic cells are less frequently used. Protein expression levels in CHO cells are usually lower than in HEK293 cells. Added to that, post-translational modifications are less appropriate leading to functionally non-relevant proteins (Dutta et al. 2012). A common drawback of stable transfected cell lines is the silencing of

transfected genes. This phenomenon may happen as a result of multiple transgene copies co-integarting at the same genomic locus (Martienssen, 2003). To circumvent this problem, matrix attachment regions are presently routinely used to shield transgenes from the silencing effects (Bell and Felsenfeld, 1999).

1.7.3.4 Advantages and disadvantages of the mammalian expression system

Mammalian expression systems offer post-translational protein modifications, endotoxin-free proteins and are good for obtaining secreted proteins. The disulphide bond formation and protein folding exhibited by mammalian cell expression is the most superior compared to the other expression systems. The glycosylation, formed from both the N-glycans and O-glycans, varies not only amongst proteins, but is also dependent on the cell type used (Jenkins, 1996). With stable transfections, one can archive protein-producing cell-lines, the need to purify large quantities of plasmid DNA is eliminated and thus a rapid route to protein expression is generated. The disadvantages of using mammalian expression systems includes amongst others, the expensive culture media required. For scale-up processes, large amounts of plasmid DNA, as well as expensive transfection reagents, are needed. Generating stably transfected cell lines can take months, especially if clonal selection is used, and there is a potential risk of expression loss with each passage.

1.7.4 Enzyme linked immunosorbent assays

The enzyme-linked immunosorbent assay (ELISA) was first described by Engvall and Perlmann (1971). It is a powerful method for the detection and quantification of a specific protein in a complex mixture. The advent of antigen-specific monoclonal antibodies by Kohler and Milstein (1975), and the development of methods to chemically link antibodies to biological enzymes by Avrameas (1969) and Pierce (1967) led to further advancement of ELISA techniques. These are commonly used in medical research laboratories has and have commercial applications, including the detection of disease markers and allergens in the food industries. They are also of immense importance for the veterinary industry, e.g. animals have to be tested, mostly by ELISA, for the presence or absence of antibodies against certain pathogens before they can be exported or imported.

1.7.4.1 The basic principle of the ELISA

The enzyme immunoassay is based on the fact that one of the test reagents is conjugated to an enzyme, and subsequent addition of the appropriate substrate and chromogen results in a color change that can be measured spectrophotometrically.The fact that proteins can be passively adsorbed to plastic led to the development of ELISAs. The result of an ELISA can either be a colour reaction that can be detected both visually and spectrophotometrically or that is measured by a fluorometer.

There are a number of variations of the basic ELISA format. The four common types of the ELISA techniques are; (i) direct, (ii) indirect, (iii) sandwich and (iv) competitive ELISA (Figure 6). The many variants of the ELISA all follow the same basic elements: (i) coating/capture (ii) plate blocking, (iii) probing/detection and (iv) signal measurement.

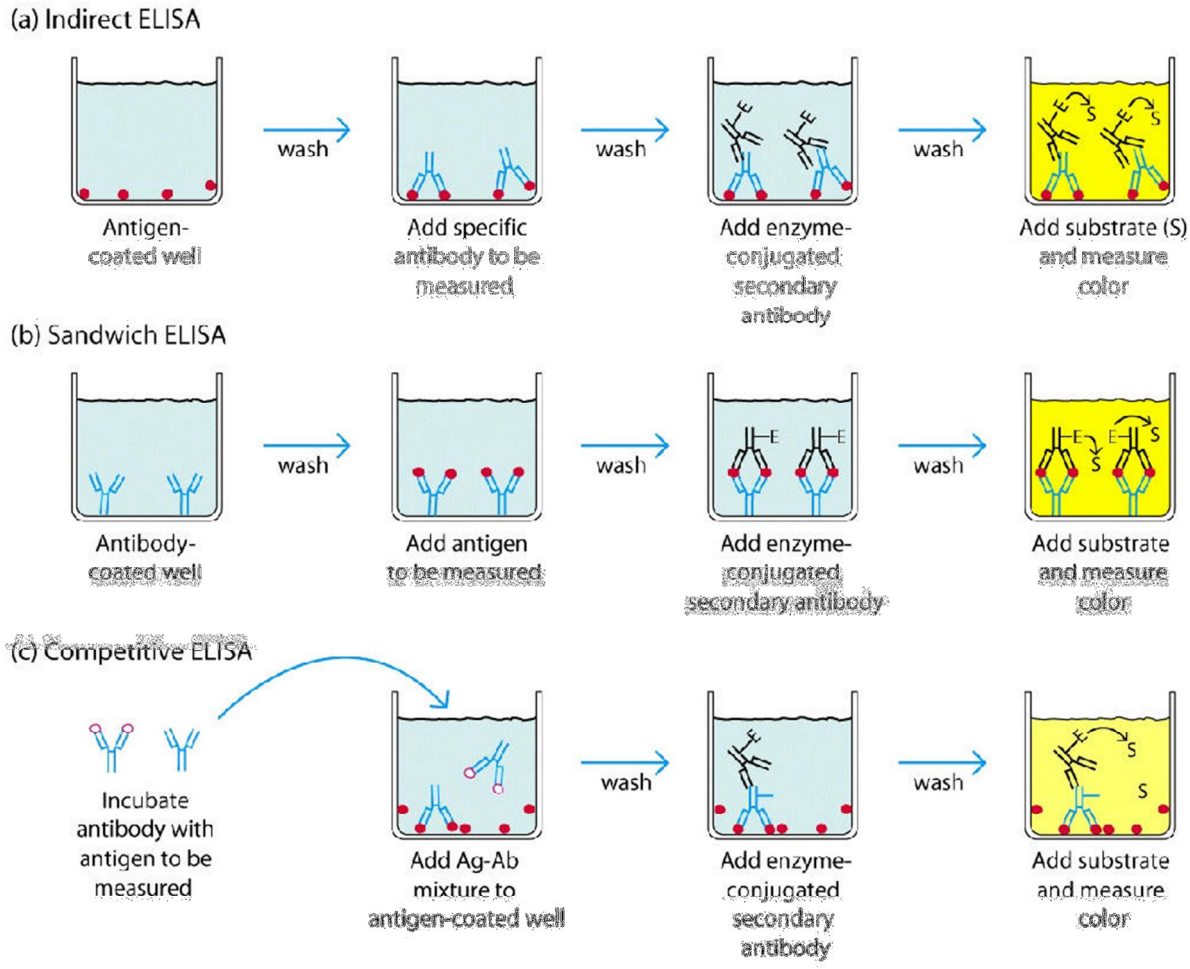


Figure 6: Variations in the ELISA technique. (a)An indirect ELISA method is used mostly for detection of serum antibodies. (b) Sandwich ELISA, usually used to detect and measure the amount of antigen, and (c) competitive ELISA, which is normally used for crude samples and still selectively binds any antigen that may be present.(Images by Dr. S. Sivakumar, Department of Biochemistry Sri Sankara Arts and Science College).

1.7.4.2 ELISA protocols

a) Plate coating

In the direct ELISA, an antigen dissolved in an alkaline buffer, such as carbonate buffer at pH≥9, is passively adsorbed to a 96-well plate. A sandwich or capture ELISA can be used in instances where the antigen does not adsorb adequately to the plate, or if the antigen is available in low amounts. With the capture ELISA, an antigen-specific antibody is adsorbed to the plate. The adsorbed antibody then captures the specific antigen when the sample is added. The capture/coating antibody and detection antibody must bind to different epitopes on the antigen for the ELISA to work efficiently.

b) Antigen detection

The direct ELISA approach uses an enzyme-tagged primary antibody that binds to the adsorbed antigen. A labelled secondary antibody is used to bind the antigen-bound primary antibody when performing an indirect detection in an ELISA. The primary aim of the secondary antibody is to amplify the signal for detection. In some cases, the enzyme-labelled secondary antibody is substituted by a biotinylated secondary antibody. A single secondary antibody can bind multiple biotin tags, the biotin in turn binds to multiple enzyme labelled avidin/streptavidin molecules. The signal in this instance is much stronger since the number of enzyme molecules for catalysis of the substrate is increased. Fluorescent assays using fluorophores may also be employed, although less sensitive than the colorimetric or chemiluminescent assays.

c) Enzymatic detection

Horseradish peroxidase (HRP) is preferred over alkaline phosphatase by most researchers. The smaller size (40 KDa) of HRP allows for more molecules of the enzyme to be conjugated to the antibody compared to the large (140 KDa) alkaline phosphatase enzyme. The higher number of antibody-conjugated HRP molecules amplifies the signal generated.

d) Substrates

Colorimetric and chemiluminescent substrates are commonly used in ELISAs for signal detection. A spectrophotometric plate reader, or a fluorometer, is used to measure the signal output from these reactions, respectively. Colorimetric substrates form coloured products with intensities proportional to the amount of enzyme present. The colour development is measured either directly or stopped with a stop solution to provide a fixed end point for the reaction. Chemiluminescent detection uses a fluorescent rather than a colorimetric product. Commercially available colorimetric substrates for HRP include 3.3',5,5'-tetramethylbenzidine (TMB), o-phenylenediamine dihydrochloride (OPD) and 2,2'-azino-bis(3-ethylbenzothiazoline -6-sulfonic acid (ABTS). For alkaline phosphtase, the substrate p-nitrophenyl phosphate (PNPP) is available.

Chemiluminescent detection is generally considered as being more sensitive compared to colorimetric detection.

1.7.4.3 Factors that affect ELISA development

Factors ranging from coating of the antigen, primary and secondary antibodies, blocking buffer to enzyme conjugate may affect ELISA performance. Traditionally, the coating antigen was attached to the plate by passive adsorption. Gamma irradiation of plates generates a positive charge that aids adsorption of the negatively charged protein. There are also pre-coated plates available commercially that enhance antigen adsorption. For example, nickel- or copper-coated plates may be used for immobilisation of histidine-tagged proteins. In instances where the interactions of the primary and secondary antibodies needs to be avoided, antibodies raised in different animal species can be used. Blocking buffer of non-mammalian origin may be used if cross-reactivity with the blocking buffer is observed. The addition of surfactants, like Tween 20, to blocking buffers helps in reducing the hydrophobic interactions between the blocking buffer proteins and the antigen or the antibodies. The washing of the ELISA plate in between the steps plays a very crucial role in eliminating non-specifically bound antigens or antibodies. Optimisation of the amount of enzyme conjugate to be used in the ELISA complex is also crucial. The amount of bound enzyme-conjugate

determines the level of signal generated. In general, optimisation of every element in the ELISA is crucial in developing a usable assay.

1.7.5 Objectives

The objectives of this study were to:

(a) Clone and express the AlHV-1 ORF65 (minor capsid protein) using the bacterial expression system, pCOLD I.

(b) Clone and express the full length AlHV-1 ORF8 (glycoprotein B) using the mammalian expression system, pCI.

(c) Clone and express the AlHV-1 ORF65 and the ORF8 truncated genes using the baculovirus/insect cells expression system.

(d) Develop an indirect ELISA based on the baculovirus/insect cell expressed AlHV-1 ORF65

CHAPTER 2

EXPRESSION OF ALHV-1 GLYCOPROTEIN B (ORF8) AND MINOR CAPSID PROTEIN (ORF65) IN HETEROLOGOUS EXPRESSION SYSTEMS

Abstract

The minor capsid protein (ORF65) was successfully expressed using both the bacterial and baculovirus/insect cell expression systems. The protein is highly soluble and expressed to relatively high levels. The full length AlHV-1 glycoprotein B was transiently expressed in the mammalian expression system, pCI. The full length glycoprotein B gene could not be expressed in the baculovirus/insect cell expression system. Two overlapping glycoprotein B gene fragments of approximately 800 base pairs (bp) each were successfully expressed using the baculovirus expression system. The expression levels of the two truncated genes were relatively low.

Introduction

Most of the open reading frames (ORFs) within the AlHV-1 genome were not previously confirmed experimentally to code for specific proteins. Their annotation, using bioinformatics, was mostly based on comparisons with closely related gammaherpesviruses (Ensser *et al*. 1997). Dry et al. (2008) analysed the AlHV-1 virus proteins. ORF 8, coding for the glycoprotein B (gpB) was found to be highly conserved amongst gammaherpesviruses. The protein products from ORF 8 are two domains of 80 kDa and 50 kDa. Since gpB is amongst the glycoproteins that are highly conserved within the gammaherpesviruses, it can serve as a good candidate antigen for an ELISA and also for a vaccine since it would be cross-reacting. In contrast, ORF65 has a low percentage sequence similarity amongst gammaherpesviruses (Figure 31). Therefore, ORF 65 can serve as a good candidate for the selective detection of specific gammaherpesvirus. *In vitro* expression of some of these ORFs will serve a confirmatory role that indeed the ORFs code for a particular protein.

2.1 Methods used in DNA manipulation

2.1.1 PCR amplification of gene of interest

Alcelaphine herpesvirus 1, C500 strain, was cultivated in baby hamster kidney (BHK) cells in Dulbecco's Modified Eagle's medium/F12 + GlutaMAX (DMEM/F12), (GIBCO,USA) supplemented with 8% fetal bovine serum(FBS) (Sigma-Aldrich, USA), 100U/ml penicillin, 100µg/ml streptomycin and 0,25µg/ml fungizone mixture (Lonza, Switzerland). Total genomic DNA was extracted by using the DNeasy blood and tissue kit (Qiagen, Germany) according to the manufacturer's instructions. Briefly, the cultured cells were pelleted by centrifugation, resuspended in phosphate buffered saline (PBS) and lysed by addition of proteinase K, lysis buffer and vortexing. The DNA bound to the silica membrane in the columns, was washed and eluted. The two full length genes ORF65 and ORF8, and the two ORF8 truncated sequences were amplified using the primers in Table 5. A touch-down PCR was employed in the amplification of the gene fragments.

Table 5: Primers used to amplify the full length glycoprotein B (gpB) gene, ORF65, and the truncated gpB gene.

Primer sequence	Product
5'-ACAC**CTCGAG**ACATGCAACTAGCCAACAATAGG-3'	Full length GpB forward primer Xho I RES in bold (for insertion in pCI)
5'-ACAT**CTCGAG**TAGTGAGTCATACACCAGGAAG-3'	Full length GpB reverse primer Xho I RES in bold (for insertion in pCI)
5'-ATTG**CATATG**GCGCACGCTAGGCCTAAACTTC-3'	Full length ORF65 forward primer Nde I RES in bold (for insertion in pCOLD I)
5'-AGTC**CTCGAG**TTATCGGCCTTTGCCTAGTTTTC-3'	Full length ORF65 reverse primer Xho I RES in bold (for insertion in pCOLD I)
5'-AATA**GAATTC**ATGGCGCACGCTAGGCCTAAAC-3'	Full length ORF65 forward primer EcoRI RES in bold (for insertion in pFastBAC HT_a)
5'-ACTACTCGAGTTATCGGCCTTTGCCTAGTTTTCC-3'	Full length ORF65 reverse primer EcoRI RES in bold (for insertion in pFastBAC HT_a)

Primer sequence	Product
5'-AGTC**GAATTC**GGCAACCACTGGTTCAACAG-3'	Truncated glycoprotein B 1 forward (gpBtr-1) primer. EcoRI RES in bold (for insertion in pFastBac HT_a)
5'-ATTA**CTCGAG**TTAGTTTTCCACCAGGGTCAG-3'	Truncated glycoprotein B 1 reverse (gpBtr-1 Rev) primer. Xho I RES in bold (for insertion in pFastBac HT_a)
5'- AGTC**GAATTC**CCTGACCCTGGTGGAAAAC-3'	Truncated glycoprotein B 2 forward (gpBtr-2) primer. EcoRI RES in bold (for insertion in pFastBac HT_a)
5'-ACAT**CTCGAG**TAGTGAGTCATACACCAGGAAG-3'	Truncated glycoprotein B 2 reverse (gpBtr-2) primer. Xho I RES in bold (for insertion in pFastBac HT_a)

2.1.2 Purification of DNA from agarose gels

DNA fragments were purified from gels using the QIAquick Gel Extraction kit (QIAGEN, Germany) following the manufacturer's instructions. Briefly, the DNA fragments were run on a 0.8% agarose gel (Lonza, Switzerland), at 7V/cm. The DNA fragments were then excised from the gels and weighed, so as to determine the amount of buffer to use. The relevant buffer was added to the gel slice in an eppendorf tube. The gel slice was dissolved at 50°C for ten minutes in the buffer provided. For optimal binding of the DNA to the membrane, a pH indicator was included in the buffer. With the recommended pH level attained, the DNA was bound to the membrane. The bound DNA fragment was washed with the relevant supplied buffer provided and eluted from the column matrix by using the supplied elution buffer.

2.1.3 DNA dephosphorylation

Calf intestinal alkaline phosphatase (CIAP), (Promega, USA), was used to dephosphorylate the 5'-termini of digested DNA fragments. The enzyme removes and

re-ligation phosphates from the 5'-ends of the DNA fragments, thus preventing re-circularization of the vector (Sambrook et al., 1989). The reaction mix is based on the fact that each picomole of DNA ends will require 0.01u of CIAP. The reaction mix was incubated at 37°C for 30 minutes. CIAP stop buffer was added to the reaction mix and the DNA fragment purified using the MinElute Reaction Cleaup (QIAGEN, Germany).

2.1.4 MinElute reaction Cleanup

The MinElute Reaction Cleanup kit (Qiagen, Germany), uses a buffer that catalyses the destruction of enzymes from a reaction mix. The buffer has a pH indicator since the binding of the DNA fragment to the column matrix membrane is pH dependant. Following destruction of the enzyme in the reaction mix by the buffer provide, the sample was applied to the column. The matrix-bound DNA fragment was washed with the washing buffer provided and subsequently eluted with the supplied elution buffer.

2.1.5 DNA ligation

The LigaFast Rapid DNA Ligation (Promega, USA) was used to ligate DNA fragments into the plasmid vector DNA. It is a rapid ligation system that requires 5 minutes for ligation of cohesive ends and up to 15 minutes for blunt-end ligation. The system also does not require any reaction clean-up prior to transformation of competent cells. A 1:2 molar ratio of vector-to-insert DNA is recommended as a starting point. Three units of enzyme are used for the ligation of a 100 ng, 3kb vector DNA to 33.3 ng of a 0.5kb insert DNA in a 1:2 vector:insert ratio. The reaction mix was incubated at room temperature.

2.1.6 Preparation of competent cells

For the preparation of solutions TFB1 (pH 5.8) and TFB2 (pH6.8), refer to Appendix 1. Glycerol stocks of DH5α and BL21 *E. coli* cells were stored in a -70°C freezer. Using an

inoculation loop, the *E. coli* culture is streaked on Luria Bertani (LB) agar plate without antibiotics and the plate incubated at 37°C overnight. Single colonies were picked using a toothpick and inoculated into 5ml LB broth without antibiotics. The culture was grown overnight at 37°C on a shaking platform at 200 rounds per minute (rpm) for optimal aeration.

100mL LB broth was inoculated with 1mL of the overnight culture and incubated with shaking at 200rpm at 37°C. The *E. coli* culture was grown to a density of 0.5 measured at an optical density of 600, OD_{600} = 0.5. The culture was chilled on ice for 15 minutes and pelleted by centrifugation at 4000 x g for 5 minutes, at 4°C. The pellet was re-suspended in 35 ml of ice cold solution TFB1 (Appendix 1)(Hanahan, 1983) and incubated on ice for 15 minutes. The bacterial culture was pelleted again by centrifugation as above. Eight millilitres of ice cold TFB2 (Appendix 1) was used to re-suspend the bacteria and incubation was continued on ice for 15 minutes. Two hundred microliter aliquots of the competent cells were frozen in liquid nitrogen and stored at -80°C. The prepared competent cells have a transformation efficiency of $1x10^8$ transformants/µg plasmid DNA.

2.1.7 Transformation of competent cells

Competent cells previously stored at -80°C were thawed on ice. Five microliters of the ligation mix was added to fifty microliters of competent cells. The reaction mix was mixed by gently tapping the tube and incubating on ice for 30 minutes. The reaction mix was heat-shocked at 42°C for 90 seconds and cooled on ice for 2 minutes. Approximately 500µl of LB medium (Appendix 1) was added to the reaction mix. This was followed by incubation of the reaction mix with shaking at 37°C for an hour. The reaction mix was spread in 100µl aliquots on agar plates containing the appropriate antibiotic. The plates were incubated overnight at 37°C.

2.1.8 Colony screening

A method called Fast-prep screening for recombinants (Beuken et al., 1998) was used to select recombinant clones. Briefly, the method involves picking and inoculating colonies in 5ml of LB broth containing the relevant antibiotics. The inoculated cultures were grown overnight at 37°C in a shaking incubator. Two hundred microliter (200µl) aliquots of the cultures were pelleted by centrifugation at 13 000rpm. The supernatant was discarded and 42µl of a sucrose solution was added to the pellet. Fourteen microliters of phenol/ chloroform solution was added to the mixture, and the pellet re- suspended by vortexing. The mixture was centrifuged at 13 000rpm for 3 minutes to separate the aqueous phase from the organic phase. Ten microliters of the upper aqueous phase was loaded on a 0.8% agarose gel. The agarose gel was run at 100V for 30 minutes with TAE buffer.

2.1.9 Minipreparation of plasmid DNA

A mini-preparation of plasmid DNA was performed using the QIAgen plasmid mini kit (QIAGEN, Netherlands). Following overnight growth of the bacterial culture, a 1mL aliquot of the culture was pelleted in a 1,5ml eppendorf tube. The pellet was resuspended in Solution I. The kit uses the alkaline method of cell lysis with Solution II for a maximum of 5 minutes. The cell lysis process was stopped by a neutralization buffer, Solution III. The cell debris and proteins were separated from the plasmid DNA by centrifugation at 13 000rpm. The supernatant was applied to the supplied provided and centrifuged to bind the plasmid DNA to the matrix. The bound plasmid DNA was washed and eluted with the supplied elution buffer.

2.1.10 Agarose gel electrophoresis

A 0.8% agarose gel was used in the electrophoresis of the nucleic acids throughout this work. SeaKem^{R} LE Agarose powder (Lonza, Switzerland), was dissolved in 1 x TAE buffer prepared from a 50 x TAE stock solution (Appendix 1). The agarose mixture was heated in a microwave oven to facilitate dissolving of the powder. After cooling the agarose,

ethidium bromide was added to a final concentration of 0.2µg/mL. The gels were run at 100V for 30 minutes.

2.1.11 Gel visualization

A gel documentation system, GeneSnap (SYNGENE, UK), was used to visualise and take pictures of the agarose gels. In instances where DNA bands had to be cut from the gel, a UV transilluminator (Wealtec Corporation, USA) was used and the DNA bands removed with a sterile razor blade.

2.2 Mammalian cell expression of AlHV-1 glycoprotein B

The AlHV-1 glycoprotein B gene was amplified with PCR using the C500 AlHV-1 strain genomic DNA as template. The 2 560 bp PCR product was run on an agarose gel, the DNA band excised and purified as previously described. The 4 006 bp mammalian expression vector, pCI (Figure 8) (Promega, USA), was used for the cloning and expression of glycoprotein B. The gel purified gpB gene and the mammalian expression plasmid vector were both digested with the restriction enzyme Xho I (Fermentas, Canada). Incubation of the restriction enzyme mix was performed at 37°C for 90 minutes. The digested gene fragment DNA and vector were run on a 0.8% agarose gel , the DNA bands were excised and purified as described earlier. The gpB gene and pCI vector were ligated and transformed into competent DH5α cells as described earlier. The transformed DH5α cells were grown for 1 hour at 37°C in a shaking incubator and 100µl aliquots spread on LB agar plates containing 60µg/ml carbenicillin. Colonies were picked and screened for pCIgpB recombinants using the Fast prep method, as described.

2.2.1 Transfection of VERO cells with the recombinant pCI-gpB

Adherent VERO cells were grown to confluency in 75cm^2 flasks in DMEM F12 medium supplemented with 8% fetal calf sera(GIBCO,USA) and antibiotics at 37°C and in an

atmosphere of 5% CO_2. A day before transfection, 2 x 10^5 cells were seeded on coverslips per well in a six well plate, and incubated at 37°C with 5% CO_2. A 0.4µg high quality recombinant pCI-gpB DNA, prepared a Qiagen Maxiprep kit, was used to transfect the seeded VERO cells using the Qiagen Effectene Transfection Reagent (Germany), according to the manufacturer's instructions. The transfected cells were incubated at 37°C with 5% CO_2 for 72 hours. For a positive control , VERO cells were infected with AlHV-1 and also incubated for 72 hours. For the negative control , VERO cells were transfected with pCI, the mammalian expression vector plasmid DNA without the gpB, and incubated for 72 hours. After 72 hours, the VERO cells were assayed for expression of the transfected gene using an indirect immunofluorescence assay (IFA).

2.2.2 Indirect immunofluorescence assay

Growth medium was aspirated from VERO cells growing on coverslips within the 6-well plates. The cells were washed once with 2 ml of PBS and fixed on the coverslips with 2 ml of ice cold acetone (100%) for five minutes at room temperature. PBS was again used to wash the cells and the coverslip was divided into two equal pieces using a diamond pencil whilst still in PBS. A 1:100 dilution of MCF-positive serum was prepared in 2% milk powder. One half of the coverslip was transferred to a dry well, the surface covered with 100µl of the prepared MCF serum and incubated at room temperature for 30 minutes. The coverslip was rinsed once in PBS and washed four times for five minutes each with PBS on a rocking platform. The rabbit anti-bovine IgG (whole molecule) FITC conjugate was diluted 1/4000 with 2% milk powder and Evan's blue (to a final concetration of 0.01%). Hundred microliters of the rabbit anti-bovine IgG (whole molecule) FITC conjugate mixture was used to detect the absorbed primary antibody on the coverslip. At this step, the coverslip was incubated at room temperature for 30 minutes in the dark. The coverslip was rinsed once with PBS and washed four times for five (5) minutes with PBS on a rocking platform. The final wash was with double distilled water (dH_2O) and the coverslip blow-dried in the dark. Mounting fluid was dropped on a slide and the coverslip mounted on the slide. The prepared slide was viewed under a fluorescence microscope with an attached camera and pictures taken of the cells.

2.3 Bacterial expression of the AlHV-1 ORF65

The ORF65 amplified by means of a PCR assay and the bacterial expression vector pCOLD I DNA (Figure 15)(TAKARA BIO. INC., USA) were double-digested with Nde I and Xho I (Fermentas, Canada) for 90 minutes at 37°C. A 0.8% agarose gel was used to analyse the linearised vector and ORF65 gene fragment . The gel-purified DNA fragments were ligated, DH5α cells transformed and the mixture spread on LB agar plates containing 60µg/ml carbenicillin. Colonies were picked from the overnight incubated plates, inoculated into LB broth with carbenicillin and grown overnight in a shaking incubator. The overnight cultures were used to select for recombinants using the Fast prep method as already described and also by restriction enzyme digestion. Minipreparation of the recombinant pCOLDORF65 DNA was performed using the Qiagen Miniprep kit as described previously. The recombinant DNA was used to transform competent BL21, plated on LB agar plates and incubated overnight at 37°C. To express ORF65, recombinant pCOLDORF65 colonies were picked from the overnight plate, inoculated into 5ml LB broth containing 50ug/ml of carbenicillin and cultured with shaking at 37°C. At a culture density of OD_{600}= 0,5, the culture was refrigerated at 15°C for 30 minutes. After the 30 minutes incubation period, IPTG at a final concentration of 1mM was added to the culture. The IPTG-induced culture was further incubated with shaking for 24 hours at 15°C. Following the 24 hour incubation, the cells were collected and expression of ORF65 was confirmed with SDS-PAGE and western blot analysis.

2.3.2 Large-scale preparation of the expressed ORF65 protein

A single colony was picked from the pCOLDIORF65 plate, using a toothpick, and inoculated into LB broth medium containing 60µg/ml carbenicillin. The culture was grown overnight at 37°C in a shaking incubator. On the following day, 1ml of the

overnight culture was transferred to 100ml of LB broth containing 60µg/ml carbenicillin. The 100ml culture was grown to mid-log phase (OD_{600}=0.5) and refrigerated at 15°C for 30 minutes. IPTG was then added to the culture at a final concentration of 1mM. The induced culture was incubated at 15°C in a shaking incubator for 24 hours.

Purification of the expressed protein was done using the Protino protein purification kit (Macherey-Nagel,Germany) with slight modifications to the manufaturer's instructions. The 100ml culture was pelleted by centrifugation at 6 000 rpm for 10 minutes at 4°C, the supernatant decanted and the pellet stored at -20°C.

2.3.3 ProtinoR Ni-TED Packed column ORF65 purification

The frozen pellet was resuspended in 4ml LEW buffer (Appendix 1) containing 2.5 kU rLysozyme™ solution and 50 units Benzonase® Nuclease. The resuspended cells were sonicated on ice for 15 minutes according to the manufacturer's instructions. The crude lysate was centrifuged for 30 minutes at 10 000 x g to remove cellular debris. The cleared supernatant was transferred to a clean tube and loaded on a pre-equilibrated Protino® Ni-TED 2000 packed column. The column was washed twice with 4ml of LEW buffer (Appendix 1) and the buffer allowed to drain under gravity. Three x 3 ml aliquots of Protino elution buffer (Appendix 1) were used to elute the bound protein from the column.

2.3.4 ORF65 antibodies raised in chickens

The column-purified ORF65 was used to raise antibodies in chicken. The antibodies were purified and checked for their authenticity by immunofluorescence. VERO cells were infected with AlHV-1 and viral protein expression assayed by IFA using the chicken raised antibodies as the primary antibody. The secondary antibody for this assay was the goat anti-chicken IgY (whole molecule) FITC, (Abcam,UK), used at a 1:250 dilution.

2.4 Baculovirus expression of ORF65 and truncated gpB

ORF65 and the truncated gpB were amplified by means of a PCR assay using the primer pairs in Table 5. The PCR products were gel-purified and double-digested together with the baculovirus transfer vector, pFastBac HT_a (Invitrogen, USA), with EcoRI and Xho I (Fermentas,Canada) for 1 hour and 30 minutes at 37°C. The digested DNA fragments were electrophoretically separated on a 0.8% agarose gel, excised from the gel and the inserts ligated to the vector using Ligafast Rapid DNA Ligation (Promega,USA), as described earlier. Competent DH5α cells were transformed with the ligation mix, grown with shaking at 37°C for 1 hour and plated on LB agar plates containing the antibiotic carbenicillin. The plates were incubated overnight at 37°C. Using toothpicks, single colonies were picked and inoculted into 5 ml LB broth in test tubes and incubated with shaking overnight. The Fast prep method for colony screening was used to screen for recombinants. The recombinants were further confirmed by restriction enzyme digestion.

2.4.2 Transformation of DH10Bac™ *E. coli*

Competent DH10Bac *E. coli* cells were transformed with the confirmed pFastBac HT_a recombinants, as described earlier. Briefly, the recombinant pFastBac HTa was mixed with 100µl DH10Bac competent cells and incubated on ice for 30 minutes. The cells were heat-shocked for 90 seconds at 42°C. The cells were incubated for 2 minutes on ice. 900µl of room temperature S.O.C medium was added to the cells and incubated with shaking at 37°C for 4 hours. A 10-fold serial dilution of the cells (10^{-1}, 10^{-2}, 10^{-3}) was prepared with S.O.C. medium. Hundred microliters of each dilution was spread on LB agar plates containing 50µg/ml kanamycin, 7µg/ml gentamycin, 10µg/ml tetracycline, 100µg/ml Bluo-gal (Invitrogen,USA), and 40µg/ml IPTG (Invitrogen,USA). Ten white colonies were picked from the plates, re-streaked on fresh plates and incubated overnight at 37°C. Colonies confirmed to have a white phenotype were picked, inoculated in LB medium containing 50µg/ml kanamycin, 7µg/ml gentamicin, 10µg/ml tetracycline and incubated with shaking at 37°C overnight for recombinant bacmid isolation.

2.4.3 Recombinant bacmid DNA isolation

The alkaline method of plasmid isolation was used to isolate the recombinant bacmids. 1.5 ml of the overnight culture was transferred to a 1.5 ml microcentrifuge tube and centrifuged at 13,000 x g for 3 minutes to pellet the cells. The supernatant was removed and the cells resuspended in 0.3 ml of Solution I (Appendix I) by vortexing. An amount of 0.3 ml of Solution II (Appendix I) was added, gently mixed with the cell suspension and incubated for 5 minutes at room temperature to lyse the cells. Then, 0.3 ml of Solution III (Appendix I) was added, mixed gently with the cell lysate and incubated on ice for 10 minutes. The mixture was centrifuged for 10 minutes at 13, 000 x g. The supernatant was then transferred to a microcentrifuge tube containing 0.8 ml of isopropanol. To mix, the tube was inverted a few times and placed on ice for 10 minutes. The sample was centrifuged for 15 minutes at room temperature at 13, 000 x g to pellet the DNA. The supernatant was removed and the pellet washed twice with 0.5 ml 70% ethanol. The pellet was then air dried for 10 minutes and re-suspended in 40µl of TE buffer.

2.4.4 Transfection of insect cells with the recombinant bacmid

The *Trans*IT^{R}-LT1 Transfection reagent (Mirus, USA) was used to transfect adherent Sf9 insect cells grown in Grace's complete insect medium (TNM-FH) (Lonza, Switzerland). A day before transfection, 5 x 10^{5} cells/well were seeded in 6-well plates. The *Trans*IT^{R}-LT1 Transfection Reagent was warmed to room temperature and vortexed gently before use. Two hundred and fifty microliters of serum-free Grace's Insect medium (TNM-FH) was placed in a sterile tube, 2.5µg of recombinant bacmid DNA added and mixed gently by tapping the sides of the tube. 7.5 µl of *Trans*IT^{R}-LT1 Transfection reagent was added to the DNA mixture, mixed gently as above and incubated at room temperature for 30 minutes. The *Trans*IT^{R}-LT1 Reagent:DNA complexes were added evenly drop-wise to the wells. The 6-well plate was rocked back-and-forth and from side-to-side to ensure even distribution of the *Trans*IT^{R}-LT1 Reagent:DNA complexes. The plates were incubated for 72 hours at 27°C. The medium containing the recombinant baculovirus was collected from the wells after 72 hours and transferred to

15 ml tubes. The tubes were centrifuged for 5 minutes at 500 x g to remove cells and debris. The clarified supernatant was transferred to a fresh 15 ml tube and stored at +4°C, with the tube covered in foil for protection from light.

2.4.5 Recombinant baculovirus plaque assay

Sf9 insect cells were seeded in 6-well plates and allowed to attach for 1 hour to 50% confluency. Two 6-well plates for each recombinant baculovirus stock were prepared. 8-log serial dilutions (10^{-1} to 10^{-8}) were prepared using the clarified baculovirus stocks in Grace's Insect medium (TNM-FH) (Lonza, Switzerland) without FBS. Working in a sterile hood, medium was removed from the 6-well plate and replaced with 1 ml of the appropriate recombinant baculovirus dilution (10^{-3} to 10^{-8}). The baculovirus infected cells were incubated for 1 hour at room temperature. After this period, the virus inoculum was removed and the cells overlayed with 2 ml of plaque-enhancing medium. After the agarose overlay had set (≈30 minutes), the plates were incubated at 27°C in a humidified incubator for 10 days. On the 10^{th} day, 0.5 ml of a 1 mg/ml Neutral Red solution (UNILAB, Philippines) was added to each well containing the plaque-enhancing overlay and the plates incubated for 2 hours at room temperature. Excess stain was removed using a pipette and the plaques counted.

2.4.6 Recombinant recombinant baculovirus stock amplification

On the day of infection, Sf9 cells were seeded at 2 x 10^6 cells/well in 2 ml of complete Grace's insect medium (TNM-FH) (Lonza, Switzerland) and incubated at room temperature for 1 hour to allow attachment. The cells were infected at a multiplicity of infection (MOI) of 0.1 and incubated for 48 hours at 27°C in a humidified incubator. The recombinant baculovirus-rich medium was collected from the cells, transferred to 15 ml tubes and clarified by centrifugation at 500 x g for 5 minutes. The clarified supernatant was transferred to fresh 15 ml tubes and stored at +4°C, protected from light by covering the tubes with foil.

2.4.7 Expression from the recombinant baculoviruses in S9 cells

Sf9 cells were infected with the three baculovirus recombinants at MOIs of 1, 2, 5, 7 and 10, in 6-well plates. The plates were incubated at 27°C in a humidified incubator. The Sf9 cells, infected at the different MOIs, were harvested over a time period of 24, 48 and 72 hours post-infection. The aim of this part of the work was to determine optimum conditions for protein expression from the inserted genes in the recombinant baculoviruses. The harvested cells were lysed in 1 x SDS-PAGE buffer and run on an SDS-PAGE to separate the expressed proteins.

2.4.8 Large-scale purification of AlHV-1 ORF65 and the truncated GpB in insect cells

Sf9 insect cells were seeded in 75 cm^2 flasks (Thermo Scientific, USA) at 2 x 10^6cells/flask in Grace's Insect medium (TNM-FH) (Lonza, Switzerland). The flasks were incubated at 27°C for 2 hours to allow for cell attachment. The Sf9 cells were infected with the relevant recombinant baculovirus at an MOI of 5 and incubated for 72 hours at 27°C in a humidified incubator. Seventy two hours post-infection, the cells were harvested and the expressed proteins purified using the Protino^R Ni-TED packed columns (Macherey-Nagel, Germany).

2.4.9 SDS/PAGE and Western blot analysis

Precise 12% Tris-Glycine precast gels (Thermo Scientific, USA), run in Tris-glycine buffer, as well as the Novex Bolt^{TM} mini precast gels (Life technologies, USA), run in MES buffer, were used for separating the proteins. The SEMI-PHOR semi-dry blotter (Hoefer Scientific Instruments, UK) was used for transferring the proteins to Immobilon^R-P transfer membranes (EMD Millipore, Germany). In brief, the transfer membrane was cut to the size of the gel. To prepare the membrane for the protein transfer, it was soaked in methanol for 5 minutes, followed by a 10 minute wash in distilled water and then soaked in the transfer buffer (Appendix 1) for 5 minutes. Six pieces of blotting

paper (GE Healthcare Life Sciences, UK), approximately 0.5 cm larger than the transfer membrane, were prepared for protein transfer by soaking them in transfer buffer for 5 minutes. A mylar mask was placed on the anode of the blotting instrument, then three transfer buffer-soaked pieces blotting paper were placed over the opening of the mask. The prepared transfer membrane, the gel on top of the membrane and the other three buffer-soaked blotting papers completed the sandwich. The lid, containing the cathode, was used to complete the circuit, and the transfer was performed for 45 minutes at 80 mA.

The transfer membrane was then removed and washed in PBS, blocked in blocking buffer and washed three times, 5 minutes each, on a rocking platform in PBS. The membrane was then incubated at room temperature soaked in primary antibody, namely AlHV-1 polyclonal sera, diluted in blocking buffer. Another washing step as above followed before the transfer membrane was soaked in a secondary antibody (rec-Protein G-peroxidase conjugate) [Invitrogen] for 1 hour, diluted in blocking buffer. The membrane was washed as above. Approximately 0.01g of 3,3'-diaminobenzidine tetrahydrochloride hydrate (Sigma-Adrich, USA) was dissolved in 10 ml PBS and 10µl hydrogen peroxide (BDH chemicals, UK) solution. The solution was used immediately as substrate for the peroxidase conjugated secondary antibody. Relevant proteins become visible as a colour reaction develops.

For the Coomassie staining, the gel was stained for 30 minutes in Brilliant Blue R concentrate (Sigma-Aldrich, USA) and then washed in 10% acetic acid solution.

2.5 RESULTS

2.5.2 Mammalian expression of the AlHV-1 full length gpB

2.5.2.1 Generation of the pCI-gpB recombinant

The AlHV-1 glycoprotein B was expressed in VERO cells using the mammalian expression vector pCI. The Xho I digested pCI vector and ORF8 gene are shown in figure 7. The vector size is approximately 4006 base pairs (bp) whilst the insert being 2 600 bp.

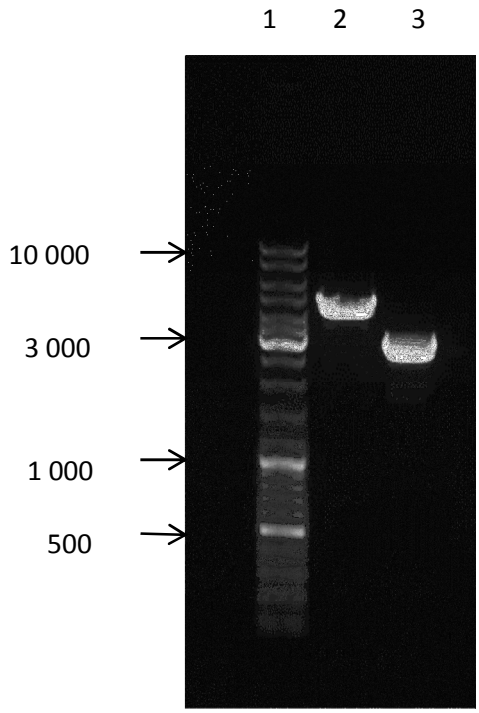


Figure 7: A 0.8% agarose gel showing the Xho I digested mammalian expression vector, pCI, and the full length glycoprotein B. Lane 1,Fermentas GeneRuler™ DNA ladder mix, Xho I linearised pCI in lane 2 and Xho I digested gpB (ORF8) in lane 3.

Figure 8 shows the diagrammatic construction of the recombinant pCI-gpB vector from the parental plasmid vector, pCI. The inserted gpB gene is shown in red as well as its orientation within the vector. The cytomegalovirus immediate early promoter driving the transcription of the insert is shown just upstream of the multiple cloning site (MCS). The SV40 poly adenylation is also shown downstream of the MCS. The selection marker, in this case ampicillin is shown in yellow.

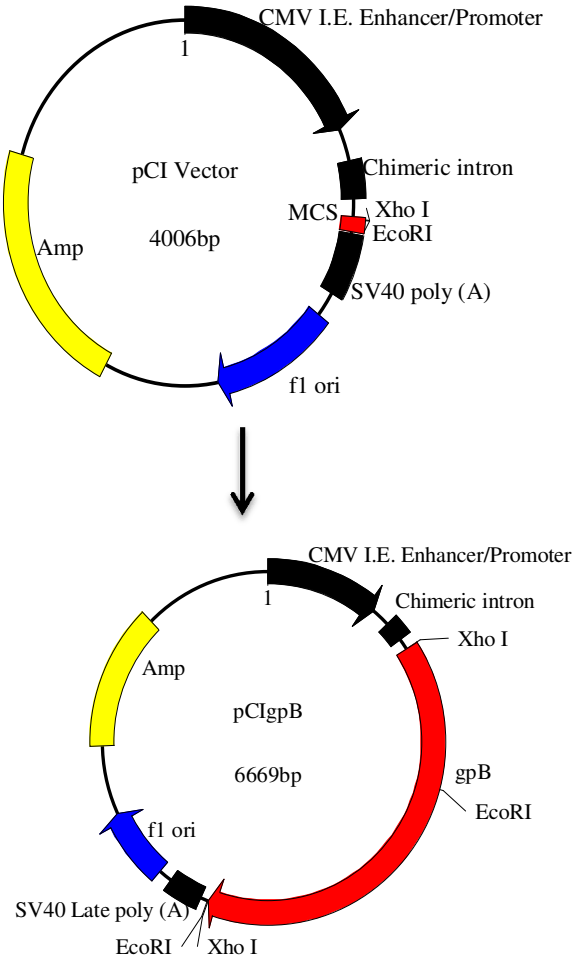


Figure 8: Schematic representation of the pCI mammalian expression vector and the resulting pCI-gpB recombinant vector (drawn by myself using the programme DNAMAN, plasmid sequence data from Promega, USA). The inserted gene is under the control of the upstream human cytomegalovirus immediate early (CMV I.E.) enhancer/promoter and the transcribed mRNA is stabilised by the SV40 poly (A) tail.

2.5.2.2 Fast prep screening of the pCIgpB recombinants

The ten pCI-gpB transformed DH5α *E. coli* colonies, picked and grown in LB broth yielded eight recombinant and only two non-recombinant plasmids, (Figure 9). The strong bands of approximately 1000 bp are due to RNA (Beuken *et al*. 1998)

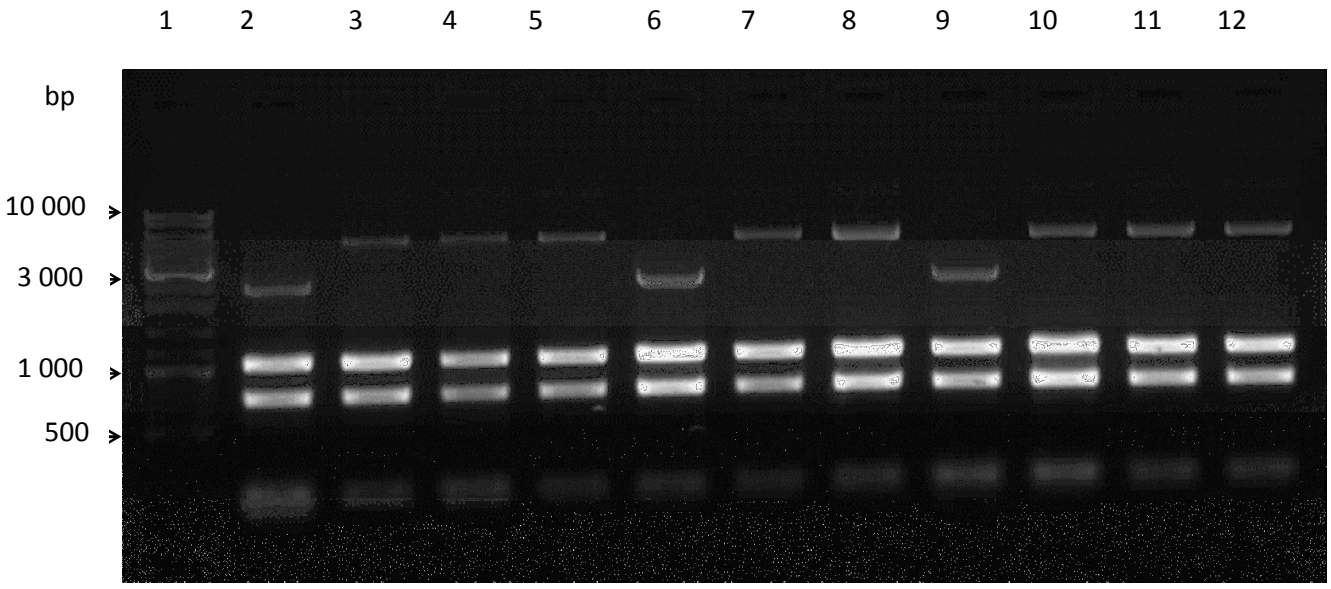


Figure 9: A 0.8 % gel showing pCIgpB recombinants prepared using the fastprep screening method. Lane 1, Fermentas GeneRuler™ DNA ladder mix, lane 2, control empty pCI vector DNA, lanes 6 and 9, non-recombinant empty pCI vector DNA, lanes 3, 4, 5, 7, 8, 9, 10, 11, and 12 recombinant pCIgpB plasmid DNAs, migrating slower than the pCI vector. Note: the bands of ~1000 bp and below are due to RNA (Beuken et al. 1998)

2.5.2.3 Confirmation of the orientation of gpB inserts within the pCI vector

The AlHV-1 gpB gene was cloned in the Xho I site of the mammalian expression vector pCI, upstream of the EcoRI within the multiple cloning site (figure 8). Digestion of the recombinant pCI-gpB with EcoRI resulted in an expected fragment of approximately 1 800 bp for the correct orientation of the insert within the vector (Figure 10).

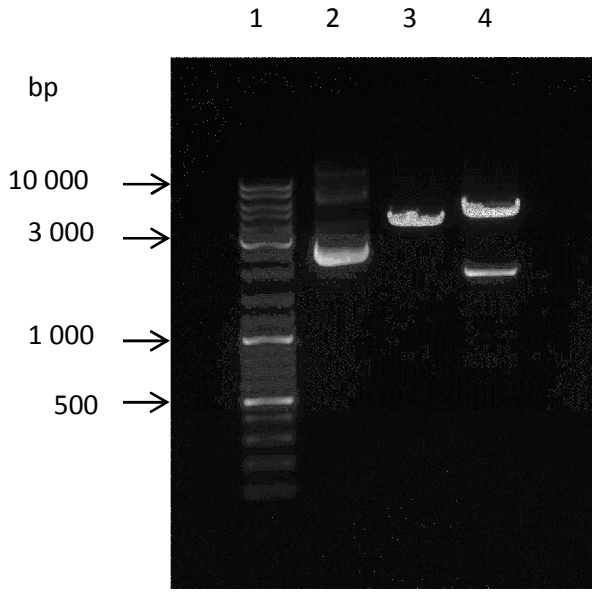


Figure 10: A 0.8% gel showing a digestion profile for the recombinant pCIgpB. Lane 1, Fermentas GeneRuler™ DNA ladder mix, lane 2, the undigested pCI vector, lane 3, EcoRI digested pCI vector and lane 4 is the EcoRI digested pCIgpB with th 1 800bp and the 4 900bp fragments clearly shown.

2.5.2.4 Expression of the full length AlHV-1 glycoprotein B gene in VERO cells

African green monkey kidney (VERO) cells were used for the expression of the full length AlHV-1 gpB as explained earlier. Fluorescence was not observed with the negative control slide of VERO cells transfected with an empty pCI vector DNA, when viewed under the fluorescent microscope (Figure 11).

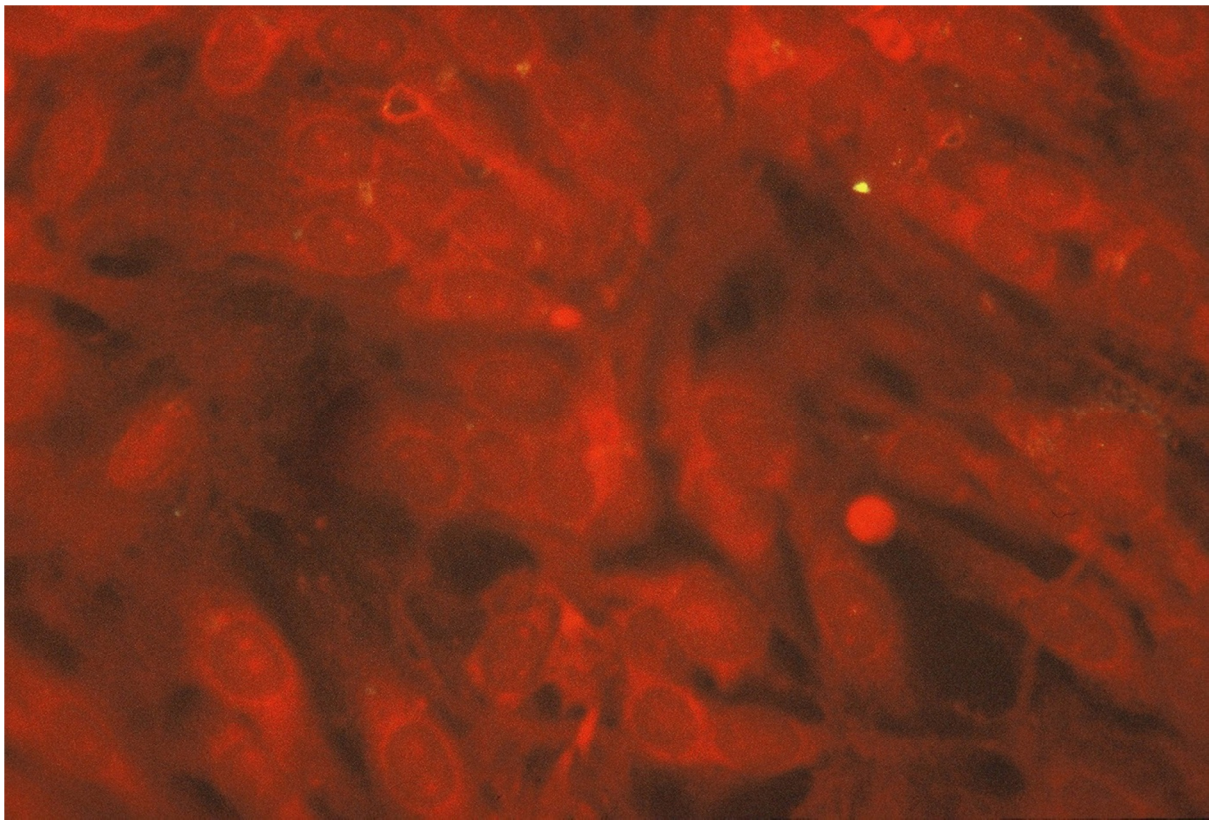


Figure 7: Indirect immunofluorescence assay for the negative control cells, transfected with an empty pCI plasmid. Fluorescence was not observed for these negative control cells, thus showing that the AlHV-1 positive sera only react specifically with proteins expressed in MCF infected animals or cells. Magnification, 100X.

Fluorescence of the VERO cells, interpreted as a positive assay result, was observed for the positive control, that is, VERO cells infected with AlHV-1 (Figure 12). The virus infected cells have aggregated and rounded up as seen in figure 12.Expression of glycoprotein B was also observed with VERO cells transfected with the construct pCI-gpB (Figure13).

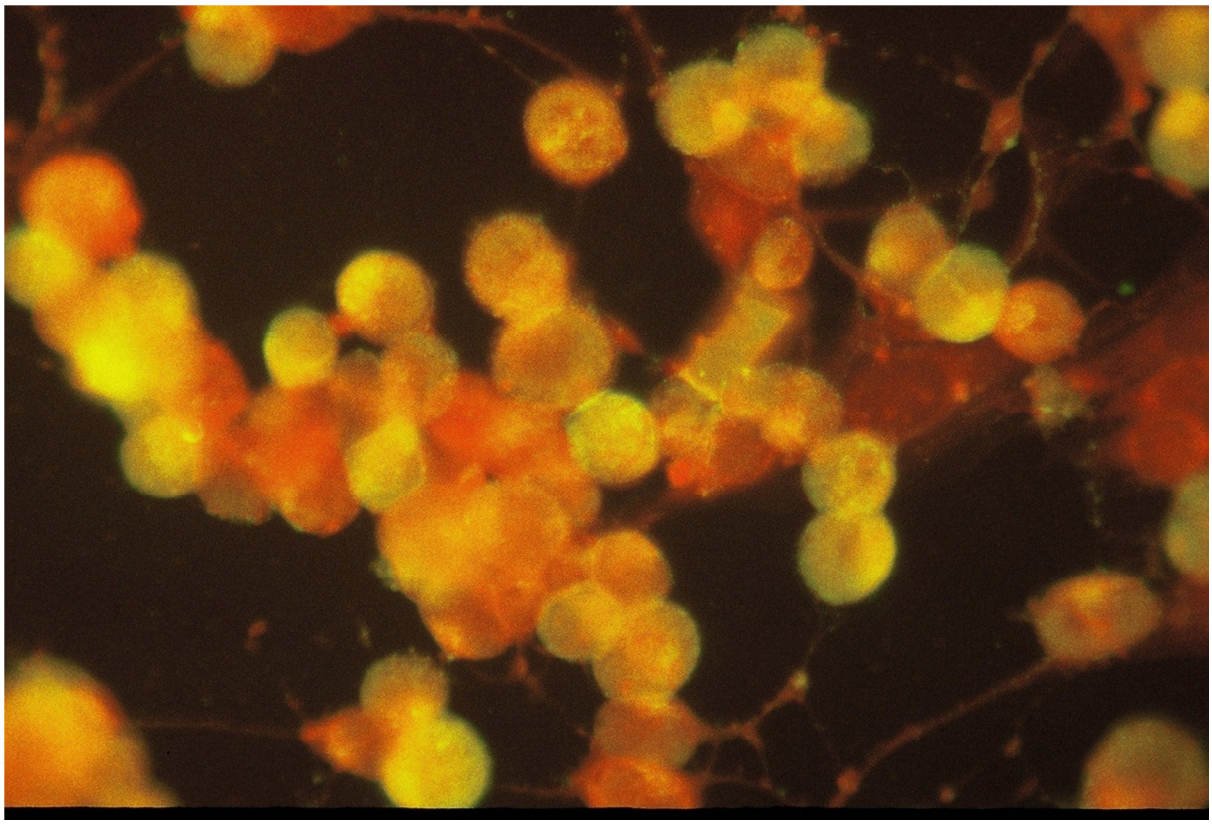


Figure 8: Indirect immunofluorescence assay for the positive control cells, VERO cells infected with AlHV-1, WC-11 strain. The VERO cells were stained three days post-infection when the cytopathic effect (CPE) could be observed. As it can be observed, AlHV-1 protein expression is shown by the fluorescent cells. Magnification, 100X.

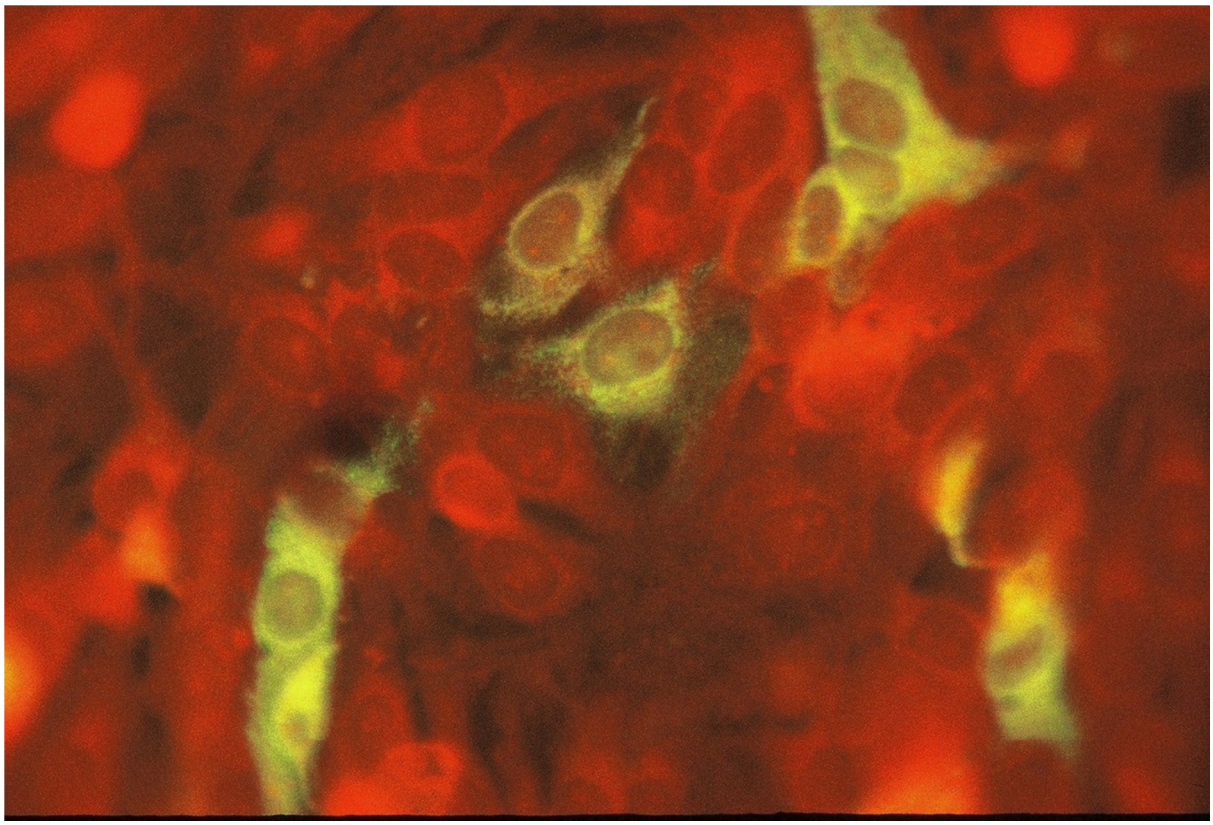


Figure 9: Indirect immunofluorescence assay for the pCI-gpB transfected cells. The AlHV-1 positive sera reacted specifically with the expressed AlHV-1 gpB protein. Expression levels were around 20-30%. Magnification, 100X.

2.5.3 Bacterial expression of AlHV-1 ORF65

2.5.3.1 Generation of the pCOLD I-ORF65 recombinant

The AlHV-1 ORF65 gene was expressed in BL21 *E. coli* cells using the bacterial expression vector pCOLD-I. The approximately 4407 bp Nde I and Xho I linearised pCOLD I vector DNA as well as the 800 bp ORF65 gene are shown in figure 14.

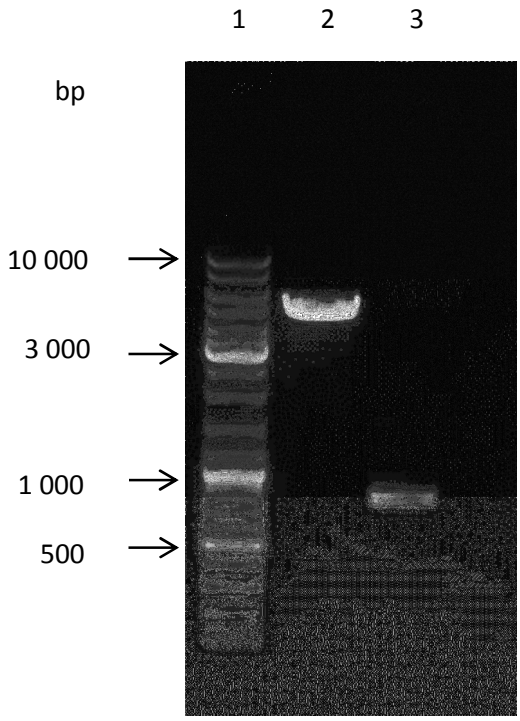


Figure 14: A 0.8% agarose gel showing the Nde I, Xho I digested pCOLD I in lane 2 and ORF65 in lane 3. Lane 1 is the Fermentas GeneRuler™ DNA ladder mix.

Figure 15 shows the diagrammatic construction of the pCOLDI-ORF65 recombinant from the parental plasmid vector pCOLD I. All the vector features are as shown in the figure and discussed later in the chapter. The insert was cloned directionally within the MCS using the Nde I and Xho I restriction enzymes.

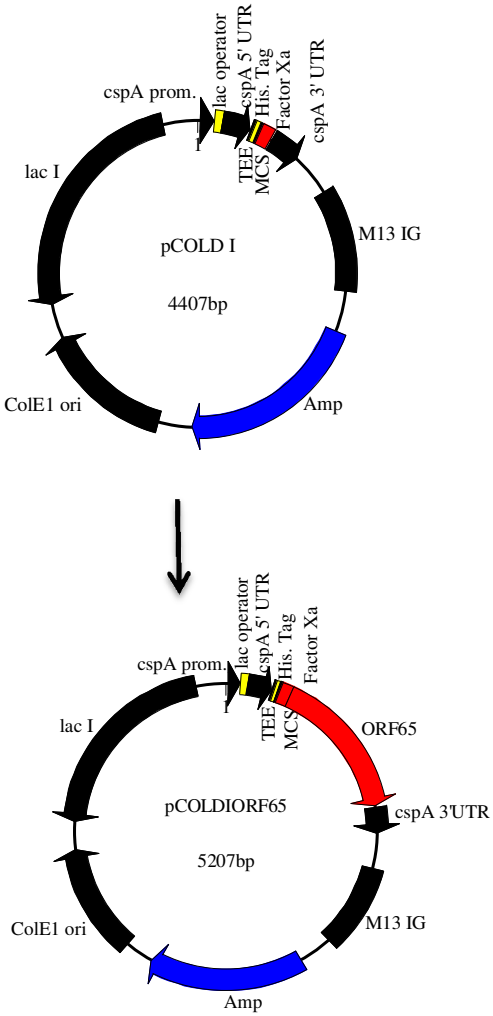


Figure 10: Schematic representations of pCOLD I-ORF65 recombinant generation from the pCOLD-I bacterial expression vector (drawn by myself using the programme DNAMAN, with data from the GenBank Accession number AB186388). The gene was inserted directionally using EcoRI and Xho I. The E. coli cold shock protein A promoter drives expression of genes inserted in the MCS of this vector.

2.5.3.2 Fast prep screening of the pCOLD I-ORF65 recombinants

The eleven pCOLDI-ORF65 transformed DH5α *E. coli* colonies, picked and grown in LB broth, yielded nine recombinant and only two non-recombinant plasmids (figure 16). The non-recombinant plasmids arose from incomplete digestion of the vector which has re-circularised.

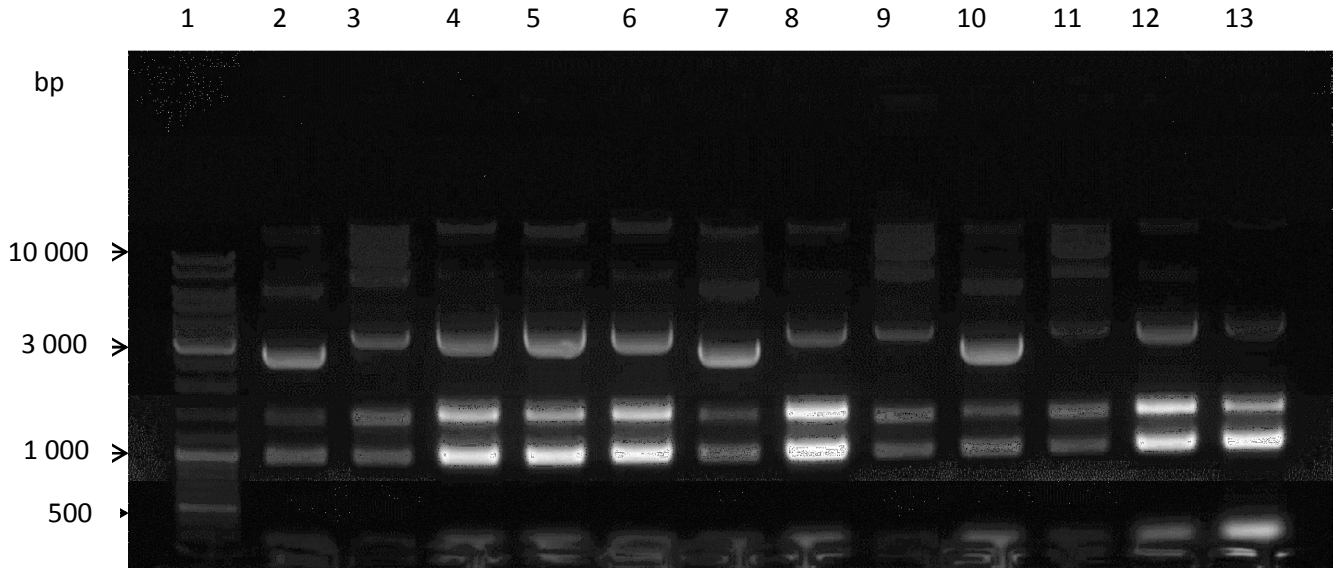


Figure 11: A 0.8% gel showing the pCOLD I-ORF65 recombinants prepared using the Fast prep method for screening. Lane 1, Fermentas GeneRuler™ DNA ladder mix, lane 2, empty control vector pCOLD I, lanes 3-6, 8-9 and 11 -13, the pCOLD I-ORF65 recombinant plasmids, and lanes 7 and 10, non-recombinant plasmid. Note: the bands of ~1000 bp and below are due to RNA (Beuken et al. 1998)

2.5.3.3 Confirmation of the orientation of ORF65 within the pCOLD I vector

A microgram of the recombinant pCOLD I-ORF65 plasmid prepared using a miniprep, as explained earlier, was digested in a final volume of 20µl. The digested pCOLD I-ORF65 recombinant plasmid is shown in figure 17. The Nde I/ Xho I digested pCOLD I-ORF65 gave a correct size insert of approximately 800 bp.

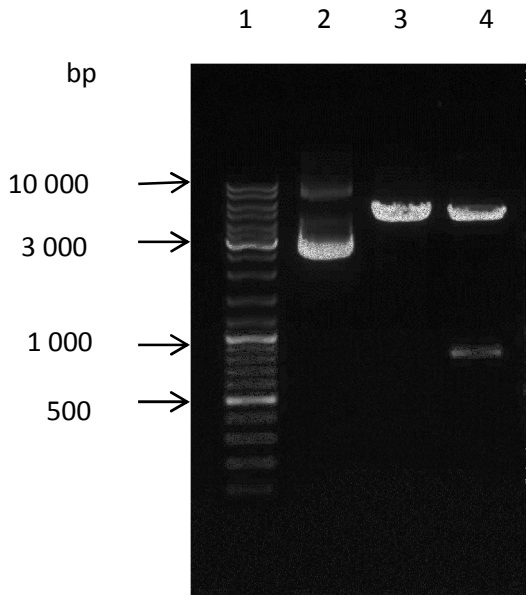


Figure 12: A 0.8% agarose gel showing the pCOLD I-ORF65 recombinant digestion. Lane 1, Fermentas GeneRuler™ DNA Ladder mix, lane 2, pCOLD I plasmid, lane 3, NdeI/Xho I digested pCOLD I and lane 4 is the Nde I/Xho I digested pCOLD I-ORF65 recombinant.

2.5.3.4 Expression and purification of ORF65 in E.coli BL21 cells

Chemically competent BL21 *E.coli* cells were transformed with the recombinant pCOLD I-ORF65 as previously explained to express the ORF65 protein. The observed protein size of 40 kDa, figure 18, is a bit larger than the expected size of 24,56 kDa (calculated using the Protein Molecular weight-Bioinformatics.Org programme from www.bioinformatics.org/sms/prot/_mw.html). There are also endogenous bacterial proteins of approximately 100 kDa that co-eluted with the expressed ORF65 protein, as observed in figure 18.

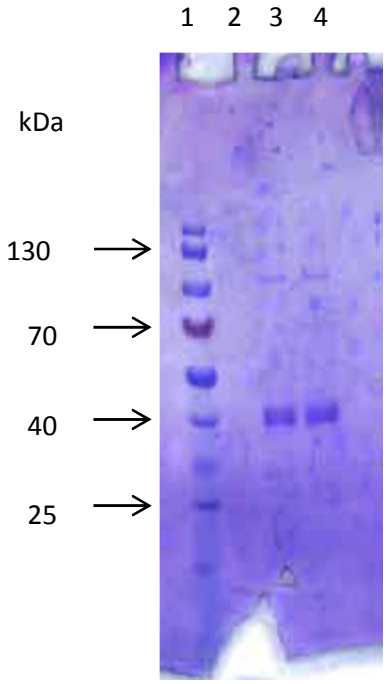


Figure 13: Coomassie stained SDS/PAGE gel of the BL21 expressed ORF65. The expressed proteins were purified using the Protino^{R} Ni-TED packed columns, run on 12% Precise Tris-Glycine precast gels, stained with Brilliant Blue R concentrate and destained with 10% acetic acid solution. In lane 1 is the PageRulerTM prestained protein ladder (Thermo Scientific), lane 2 is the flow through from the wash step, lanes 3 and 4 are aliquots from the two 3 ml protein elution. As seen in lanes 3 and 4, there is a 100 kDa protein that co-elutes with the purified ORF65 running at 40kDa.

Figure 19 shows a western blot of the bacterial expressed ORF65. As it can be seen the AlHV-1 reactive antibodies used to probe for the expressed ORF65 protein did not react with the 100 kDa endogenous bacterial protein observed in figure 18.

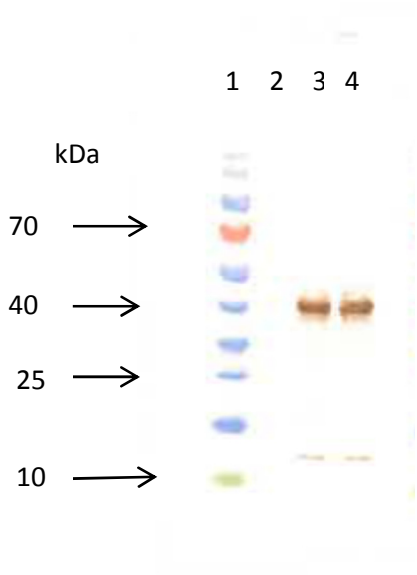


Figure 19: A western blot of the Protino[R] Ni-TED packed column purified ORF65. Lane 1, PageRuler[Tm] Prestained Protein ladder (Thermo Scientific), lane 2, flow through from the wash, lanes 3 and 4, aliquots from the two 3 ml protein elutions. As can be seen in lanes 3 and 4, the 100 kDa protein that co-elutes with the purified ORF65 does not react with the AlHV-1 polyclonal sera and is likely a bacterial protein.

2.5.3.5 Specificity of the chicken raised AlHV-1 ORF65 antibodies

As explained earlier the purified AlHV-1 ORF65 was used to raise antibodies in chicken. The purified antibodies were then used in an IFA to detect AlHV-1 proteins expressed in infected VERO cells. There was no fluorescence observed when observing the negative control VERO cells under a fluorescent microscope, (figure 20) prepared using IFA, thus indicating that the antibody did not react with cellular components.

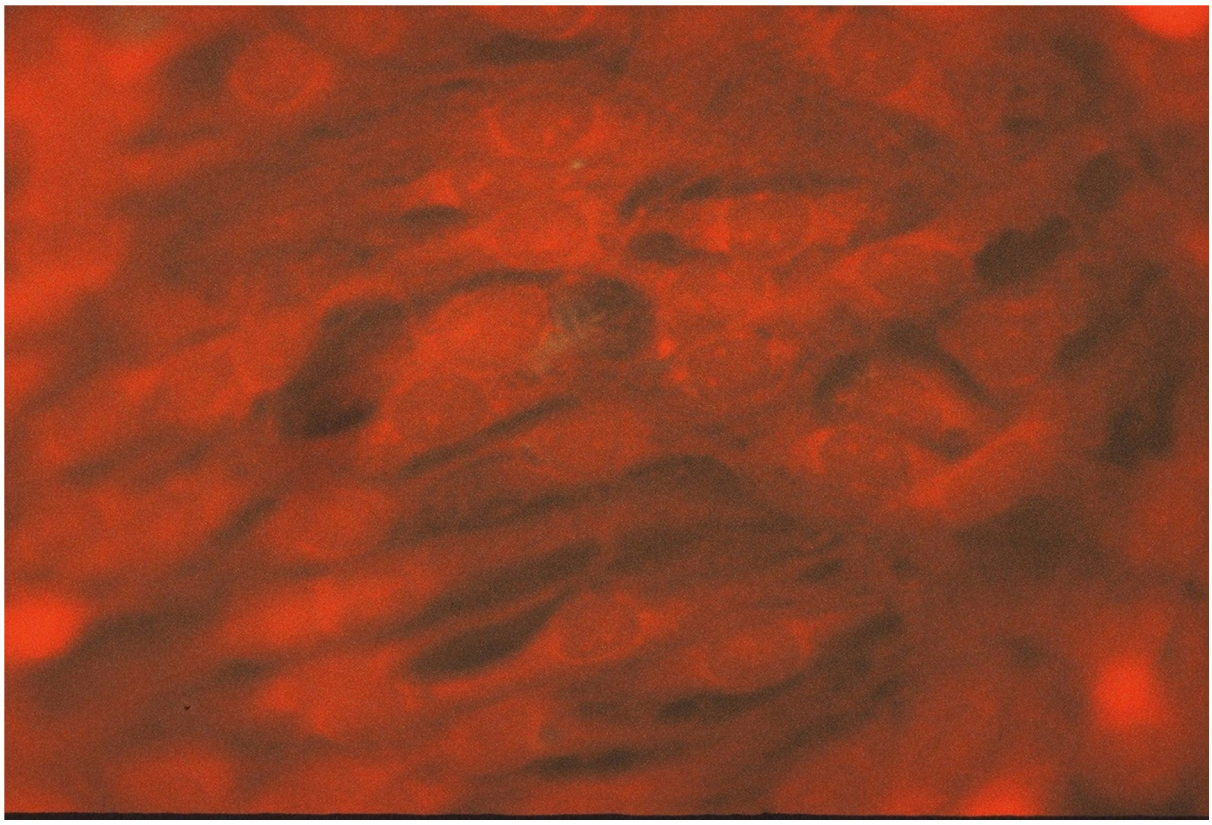


Figure 14: Uninfected negative control VERO cells, stained and probed by IFA for any interaction with antibodies raised in chicken against AlHV-1 ORF65. As can be seen from the lack of fluorescence, there was no interaction between the VERO cells and the ORF65 antibodies from chicken. Interaction between the two would be observed as fluorescence, coming from the bound FITC-conjugated secondary antibody when viewed under the fluorescent microscope. Magnification, 100X.

Figure 21 shows an IFA result for the AlHV-1 infected VERo cells probed with the chicken-raised antibodies. The AlHV-1 ORF65 chicken raised antibodies reacted with the AlHV-1 infected cells, thus indicating that they recognise the virus or proteins expressed by the virus.

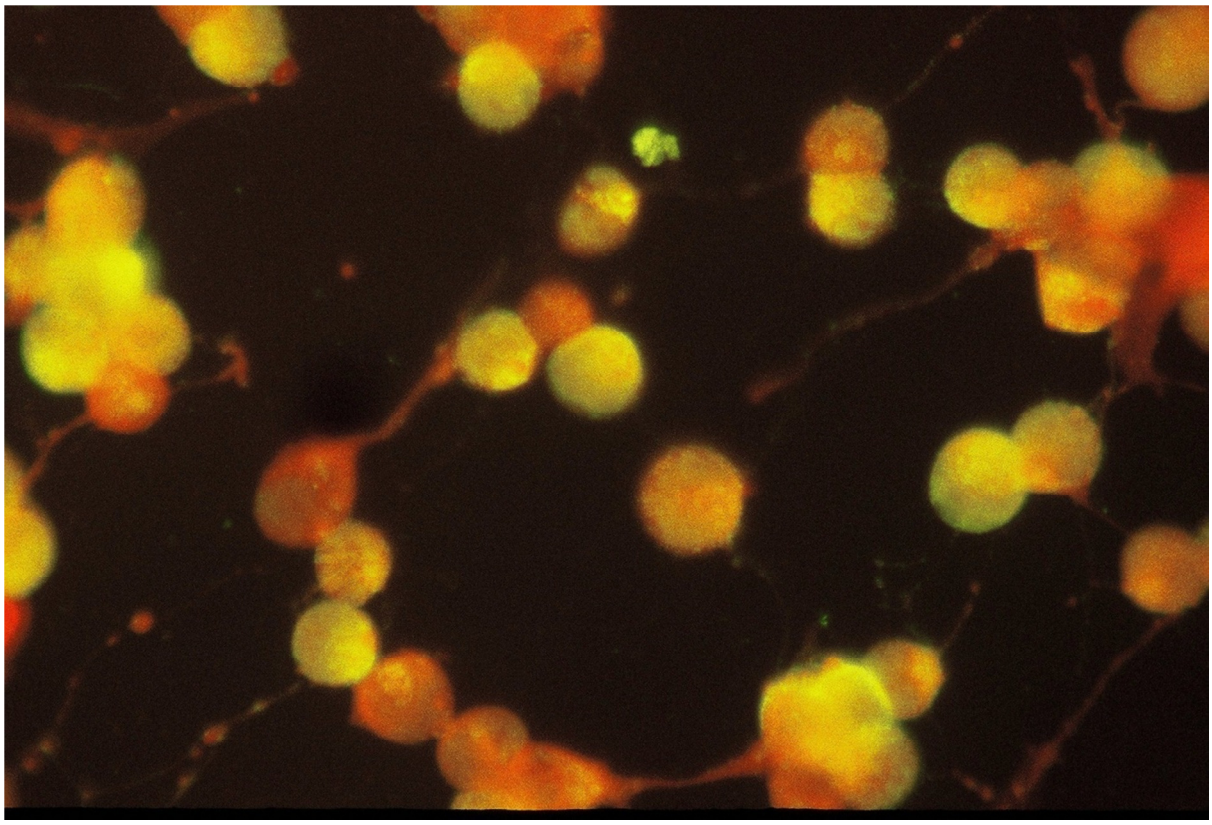


Figure 15: IFA-probed AlHV-1 infected VERO cells, 3 days post-infection with visible cytopathic effects. The infected cells were probed by IFA for interaction between the ORF65 antibodies raised in chicken and AlHV-1/AlHV-1 expressed proteins. The cells fluoresce strongly showing that there is recognition and binding between the antibodies and the AlHV-1/AlHV-1 expressed proteins. Magnification, 100X.

2.5.4 Baculovirus/Insect cell expression of truncated ORF65 and GpB gene

The full length ORF65 and the two truncated glycoprotein B genes were inserted in a continuous reading frame with the poly-histidine sequence of pFastBac HT_a. Figure 22 shows the EcoRI and Xho I linearized pFastBac HT_a plasmid DNA of approximately 4856 bp. The inserts, ORF65 and the two truncated gpB genes are also shown in figure 22.

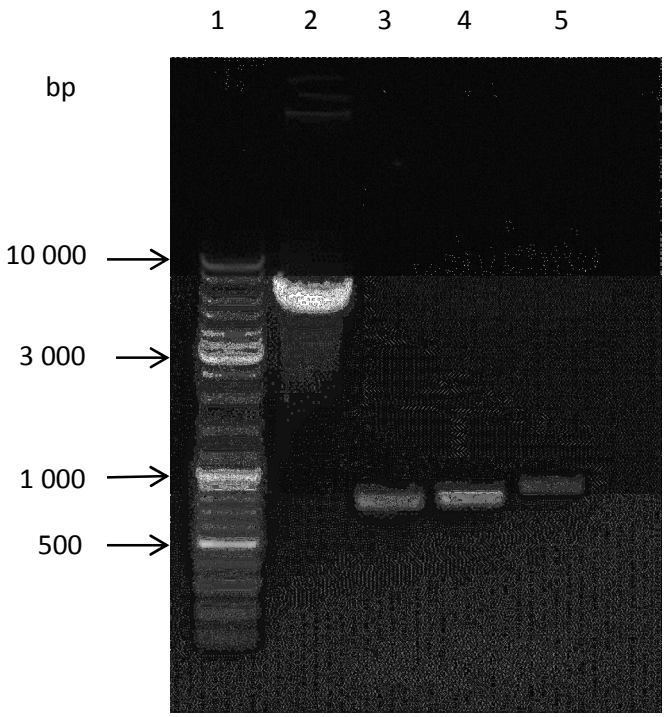


Figure 22: A 0.8% agarose gel showing the EcoRI and Xho I digested pFastBac HT$_a$ in lane 2, lane 3,ORF65, lane 4, truncatead gpB 1 and lane 5, truncated gpB 2. Lane 1, Fermentas GeneRuler^{TM} DNA ladder mix.

Figure 23 shows a diagrammatic representation for the construction of the pFastBacHT-ORF65 recombinant from the parental plasmid DNA, pFastBac HTa. The procedure was followed in generating the other two pFastBacHT recombinants with the two truncated gpB gene. From the diagram it can be seen that the foreign gene transcription, inserted in the MCS of the plasmid vector, will be driven by the polyhedron promoter. The transposon elements (Tn7L and Tn7R) for translocation of the gene of interest from the plasmid vector into the baculovirus genome are also shown.

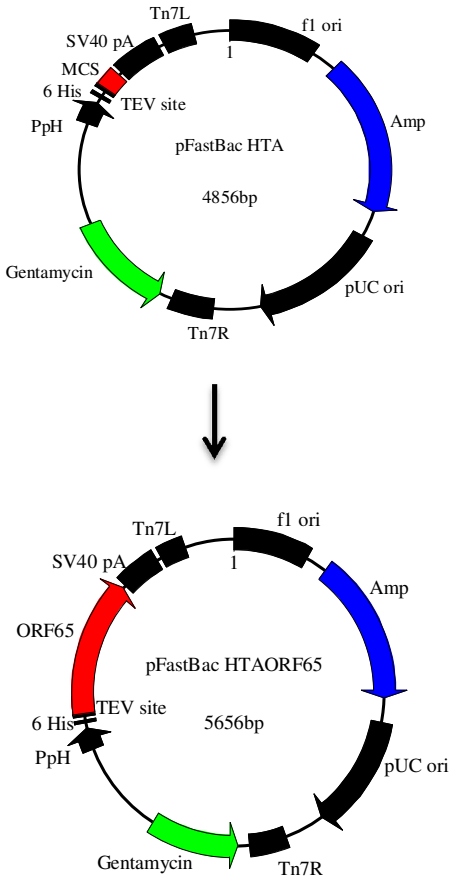


Figure 16: Schematic representation of the generation of the recombinant pFastBac HTa-ORF65 from pFastBac HT$_a$, the cloned gene is shown in red. The two glycoprotein B-pFastBac HT$_a$ truncated recombinants were generated in the same manner. The gene is transposed into the bacmid as part of a cassette, from Tn7R to Tn7L, thus imparting gentamycin resistance to the DH10Bac cells.

2.5.4.1 Fast prep screening of the pFastbac HTa recombinants

Eleven recombinant pFastBac HT$_a$+ORF65, ten recombinant pFastBac HTa+gpBtrunc-1 and pFastBac HTa+gpBtrunc-2 colonies were picked from the plates and grown overnight from transformation mixes. Analysis of the clones run on 0.8% agarose gels gave the following results: seven of eleven picked colonies from the pFastHT$_a$+65 plate were recombinants (Figure 24), six out of ten were recombinants for the pFastBac

HTa+gpBtrunc-1 (Figure 25) and seven out of ten for the pFastBac HTa+gpBtrunc-2 colonies (Figure 26).

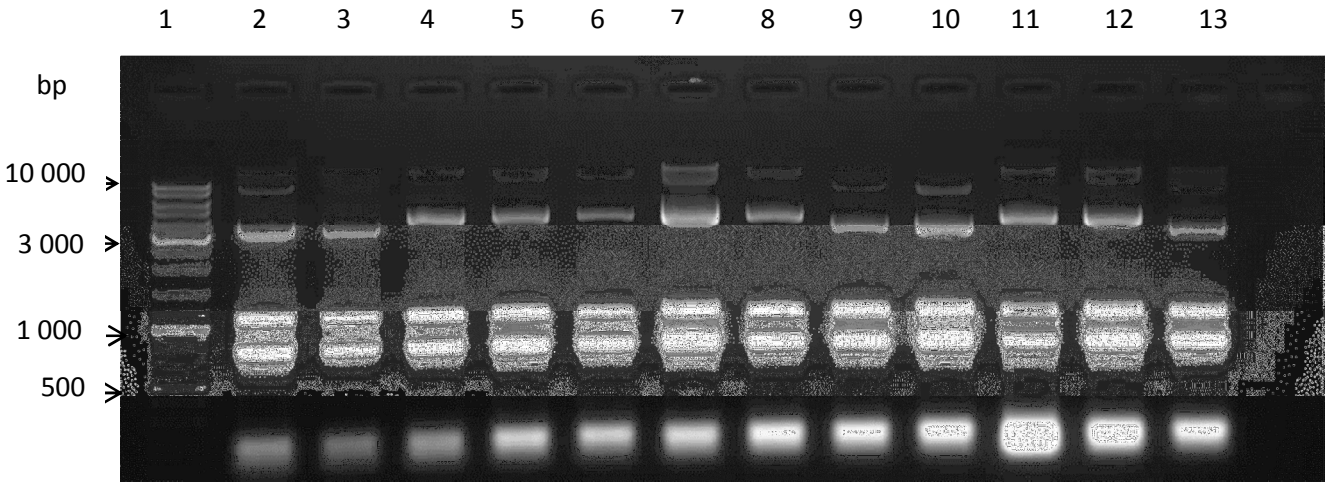


Figure 17: A 0.8% agarose gel showing the pFastBacHT_a+ORF65 recombinants. Lane 1, Fermentas GeneRuler™ DNA Ladder mix; lane 2, empty vector control; lanes 4-8 and 11 and 12, pFastBac HT_a+ORF65 recombinants; and lanes 3, 9, 10, and 13, non-recombinant, re-circularised plasmid. Note: the bands of ~1000bp and below are due to RNA (Beuken et al. 1998).

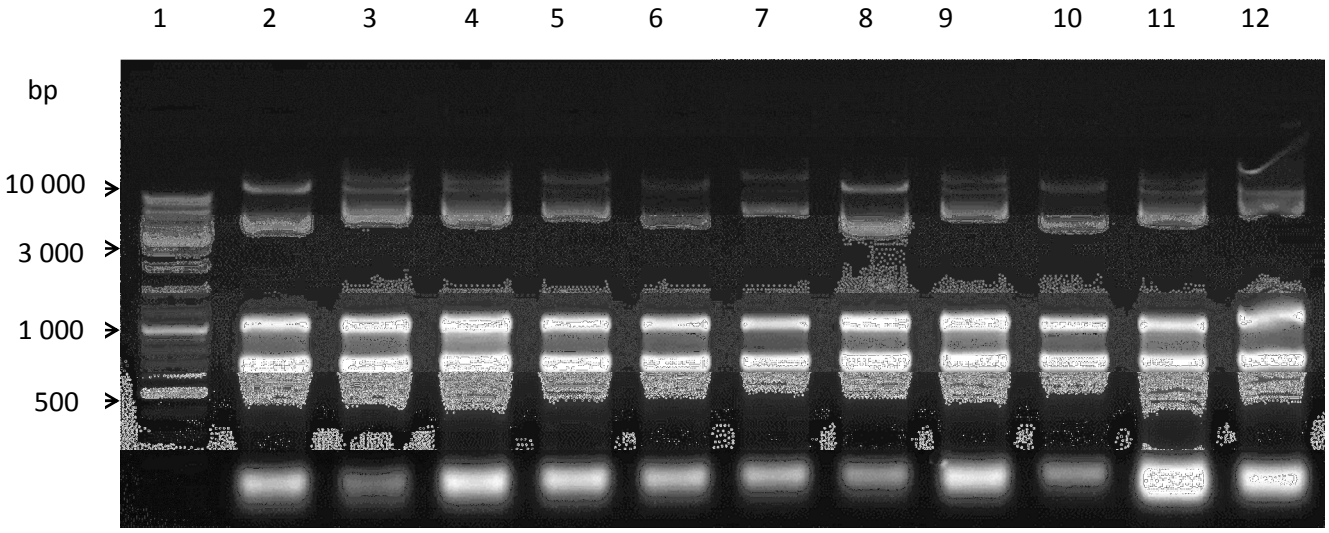


Figure 18: A 0.8% gel showing pFastBac HT_a+gpBtrunc-1 recombinants. Lane 1, Fermentas GeneRuler™ DNA ladder mix, lane 2, empty vector control; lanes 3-5, 7, 9 and 12, pFastBac Hta+gpBtrunc-1 recombinants; and lanes 6, 8, 10 and 11, non-recombinant re-circularised plasmid.

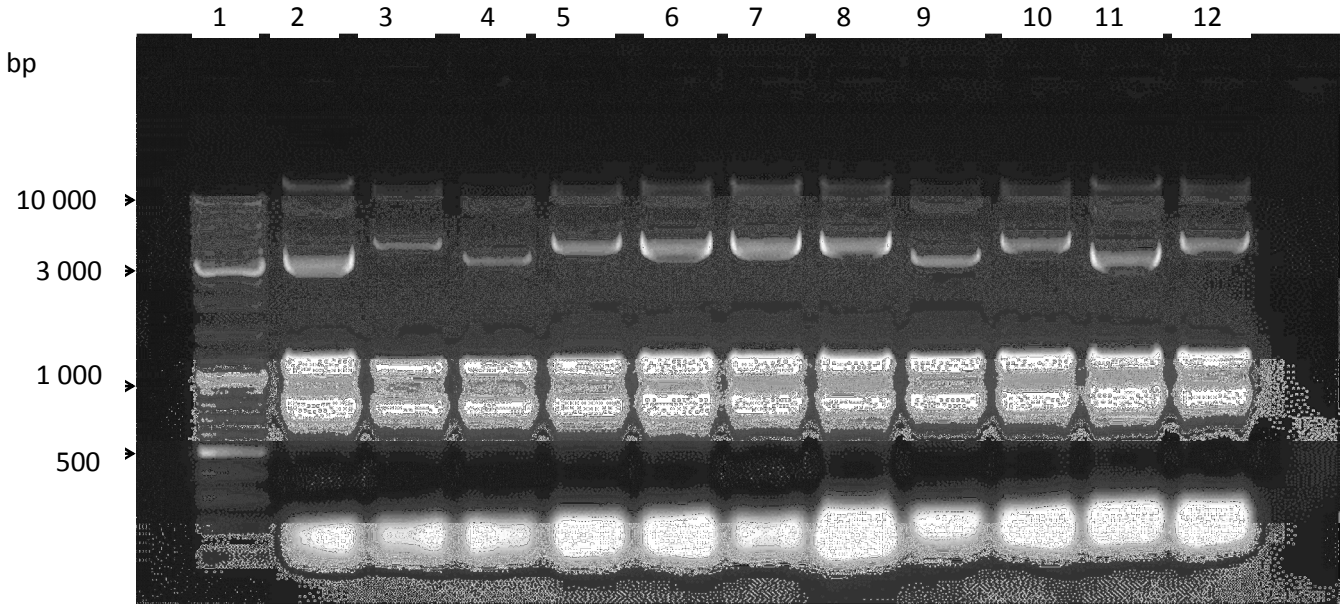


Figure 19: A 0.8% gel showing pFastBac HT$_a$+gpBtrunc-2 recombinants. Lanes 1, Fermentas GeneRuler^{TM} DNA Ladder mix; lane 2, is the empty plasmid control, Lnanes 3, 5-8, 10 and 12 are the pFastBac HT$_a$+gpBtrunc-2 recombinants; and lanes 4, 9 and 11, non-recombinant, re-circularised plasmid. Note:the bands of ~1000 bp and below are due to RNA (Beukes et al. 1998).

2.5.4.2 Confirmation of the presence of inserts within the recombinant pFastBac HTa

Minipreps of the recombinant pFastBac HTa plasmid DNA were performed as described earlier. A microgram of each of the recombinant plasmid DNA preps was digested with EcoRI and Xho I. The digestion profile is shown in Figure 27. The expected insert digestion fragments are as follows; approximately 800 bp for ORF65 and gpBtrunc-1 and 700bp for gptrunc-2. As seen in figure 27, the expected sizes were obtained.

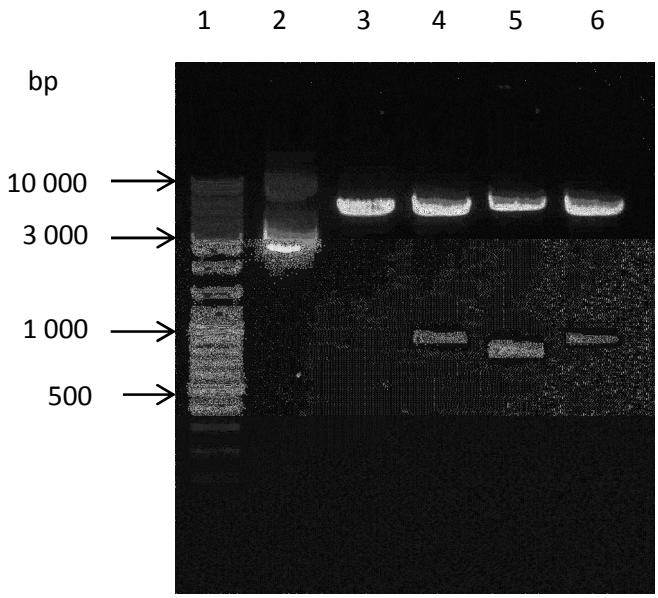


Figure 20: Digestion profiles for EcoRI and Xho I digestions of pFastBac HTa+65 (lane 4), pFastBac HTa+gpBtrunc-2 (lane 5) and pFastBac HTa+gpBtrunc-1 (lane 6). Lane 1, Fermentas GeneRuler™ DNA Ladder mix, lane 2 undigested pFastBac HTa vector DNA and lane 3, linearised vector.

2.5.4.3 Expression of the recombinant proteins from baculovirus infected insect cell cultures

The full length ORF65 gene was successfully expressed in insect cells from the relevant recombinant baculovirus (Figure 28). The two truncated gpB genes were also expressed in insect cells from the relevant recombinant baculoviruses (Figure 29 and 30). Protein expression levels for ORF65 and gpBtrunc-1 were relatively high as seen in figures 28 and 29 for the column purified products. The observed protein sizes of 40 kDa are larger than the calculated sizes of approximately 25 kDa. The expression levels for the gpBtrunc-2 was poor as seen in the western blot with the infected cell lyastes, figure 30. The observed protein size of approximately 35 kDa is also larger than the expected size of 25 kDa.

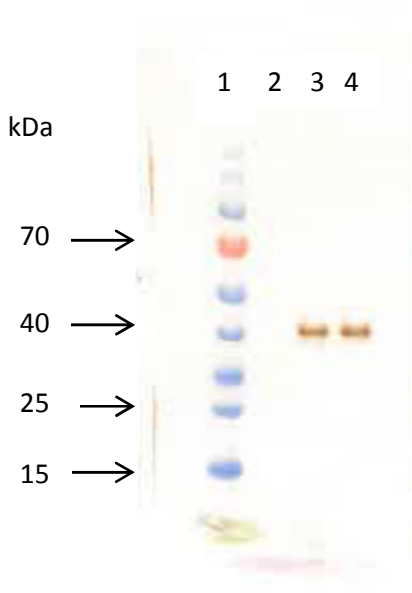


Figure 21: Western blot analysis of the baculovirus expressed AlHV-1 ORF 65 purified through Protino^{R} Ni-TED packed columns. Lane 1 is the PageRuler^{TM} Prestained Protein Ladder (Thermo Scientific), lane 2 is the flow through from the washing step. Lanes 3 and 4 are aliquots from the two 3 ml elutions.

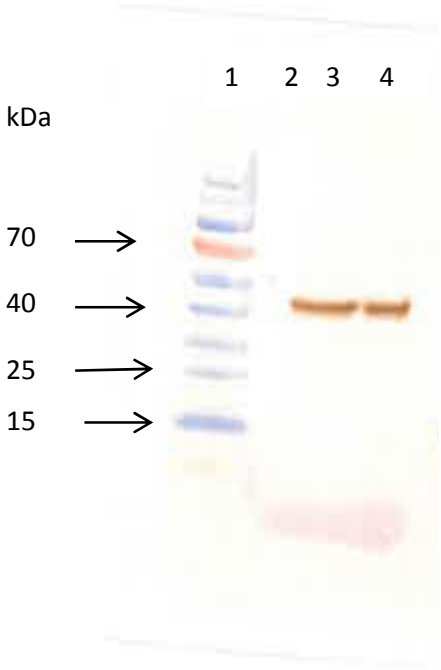


Figure 22: Western blot analysis of the baculovirus expressed AlHV-1 gpBtrunc-1 purified through ProtinoR Ni-TED packed columns. Lane 1 is the PageRuler^{TM} Prestained Protein Ladder (Thermo Scientific), lane 2 is the flow through from the wash step, lanes 3 and 4 are aliquots from the two 3 ml elutions.

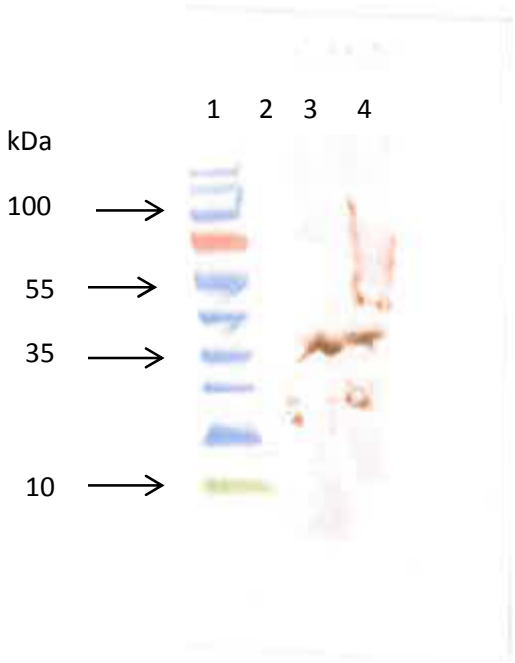


Figure 23: Western blot analysis of the baculovirus expressed AlHV-1 gpBtrunc-2 crude lysate. Lane 1 is the PageRuler™ Prestained Protein Ladder (Thermo Scientific), lane 2 is the insect cells crude lysate, lanes 3 and 4 are crude lysates from insect cells infected with recombinant baculovirus expressing the AlHV-1 gpBtrunc-2.

Discussion

Recombinant proteins play a pivotal role in the development of therapeutics, vaccines, diagnostics and in the study of biological systems. In this study, AlHV-1 ORF65, which is a capsid protein, was expressed in a bacterial expression system as well as within the insect cell/baculovirus expression system. The expressed ORF65 protein size was larger than the expected and calculated size of 25 kDa. The work by Rath et al., 2009, on the migration of membrane proteins through SDS-PAGE gels explains this phenomenon. It is explained as “gel shifting” which is caused by the loading or binding of SDS to the protein during sample preparation. Proteins vary in the ratios of detergent that they can load, normally varying from 3.4-10 g SDS/g protein. The full length AlHV-1 glycoprotein B was expressed in a mammalian expression system and two truncates of the protein were expressed in an insect cell expression system.

ORF65 expression was performed using the pCOLD bacterial expression system, which expresses heterologous proteins at low temperatures, to avoid protein aggregation and inclusion body formation. *E. coli* protein expression at low temperatures increases the chances of stability, solubility and correct folding of the expressed protein (Schirano et al. 1990) and suppresses the hydrophobic interactions necessary for inclusion body formation. The gene was positioned in a continuous reading frame with that of the pCOLD I vector, using the EcoRI and Xho I restriction sites, thus generating a fusion protein with the histidine tag (Figure 15). Transcription of the inserted gene is driven by the cold shock promoter, *csp* A. For regulation of gene transcription, the vector has constitutively expressed lac repressor, as well as a lac operator site. The 5’ and 3’ untranslated region of the cold shock protein is included in the vector for further stringent regulation of gene transcription. The pCOLD I vector uses the ColE1 *ori* for replication in bacterial cells. With this origin of replication, the plasmid is expected to maintain a copy number of 10-20 per cell, although this seems to vary based on the bacterial strain used. The only disadvantage of protein expression at low temperatures is that transcription and translation rates are reduced resulting in less protein being produced.

Expression was enhanced by using *E. coli* BL21 cells. These cells are deficient in *lon* and *Omp*T proteases, thus safe-guarding the stability of the expressed recombinant protein. BL21 cells have a *rec*A$^-$ genotype which has been shown to stabilize target plasmids thus creating an environment conducive for optimal protein expression. Although *E. coli* is not an ideal vector for expression of proteins that need post-translational modification, it was possible to express a biologically active capsid protein (ORF65) using the pCOLD bacterial expression system.

The mammalian expression system offers the best platform for posttranslational modifications of expressed proteins. AlHV-1 glycoprotein B was expressed in VERO cells driven by the promiscuous immediate-early human cytomegalovirus (CMV) promoter/enhancer (Figure 8). The CMV I.E enhancer/promoter is considered to be promiscuous as it is active in a number of cell lines (Schmidt, 1990). A chimeric intron placed immediately upstream of MCS in the expression vector, was found to increase transcription levels of genes (Evans and Scarpula, 1989; Huang and Gorman, 1990). An Sv40 late poly (A) tail terminates transcription of the inserted gpB gene and signals addition of 200 to 250 stabilising adenosine residues at the 3'-end of the transcribed mRNA (Proudfoot, 1991). The filamentous phage's origin of replication (f1 *ori*) can be used to synthesize and package single-stranded DNA from bacteria transfected with the pCI vector in the presence of an appropriate helper phage. Furthermore, the pCI vector has a T7 promoter sequence which can be used to synthesize RNA transcripts in vitro using the T7 RNA polymerase. This RNA can then be used for in vitro expression of the protein of interest in cell-free extracts. Recombinant DNA delivery into the mammalian cells plays a major role in determining the level of protein expression when using this system for transient expression. The cationic polymer, Effectene transfection reagent (Qiagen, USA), a liposome-based reagent and TransIT^R Transfection reagent, a broad spectrum reagent, were evaluated in an effort to optimally deliver the recombinant plasmid DNA to the adherent VERO cells. The Effectene transfection reagent performed better for delivery of the recombinant plasmid DNA without any evidence of toxicity to the cells. This transient protein expression is suitable only for analytical purposes.

Major challenges are encountered when up-scaling such processes. Firstly, transfection reagents used in delivering the recombinant plasmids are extremely expensive.

Secondly, transfection efficiency is usually very low, normally ranging from 20 to 30% of the total cells in culture. To circumvent these obstacles, stable transfections using either plasmid vectors or viral vectors may be employed. Although stable transfections using recombinant plasmids still require use of transfection reagents, transfected cells are selected, maintained and can be passaged a number of times with concomitant expression of the heterologous proteins. Viral vectors do not need transfection reagents for cell entry. Their natural ability to infect cells can be harnessed to express heterologous genes using the virus as a vector.

The insect cell/baculovirus expression system used in this study falls mid-way between the bacterial and mammalian system used-it has a posttranslational ability that is absent in bacterial cells but with less complexity compared to the mammalian system. The cost of protein expression in insect cells is more than that in bacterial expression system, but less than that in mammalian expression system. Recombinant bacmids for expression of the gene of interest in the “bac-to-bac” baculovirus expression system require the use of transfection reagents, like the one used for the recombinant plasmids in the mammalian expression system. The difference is that with insect cell/baculovirus expression system, following the initial transfection, a recombinant baculovirus is generated and subsequent expression of the gene of interest is achieved through infection of cell cultures with the recombinant baculovirus. Upscaling the reaction levels is also relatively cost-effective since previously amplified recombinant baculovirus is used for the infection.

The disadvantage of using the insect cell/baculovirus expression system is the limited number of passages one can use with the recombinant baculovirus. Performance of more than three consecutive passages of the recombinant baculovirus results in the virus losing the cloned gene of interest. Recombinant baculovirus serial passage introduces mutations and an accumulation of defective viral particles with no expression of the recombinant protein (Kool et al., 1991). Also, the glycosylation in insect cells is not identical to that in mammalian cells. They lack the N-terminal sialic acid and thus expose the glycoprotein to easy removal by carbohydrate-specific receptors in mammalian species (Szkudlinski et al., 1995).

Major advances had been made in improving the shortcomings of the insect cell/baculovirus expression system. For example, Vankyrin-enhanced ensect cell lines, with a much longer survival rate and delayed lysis after infection with baculovirus, have been generated. The improved survival of this cell line increases the period available for recombinant protein expression. Mimic^{TM}, an engineered cell line, commercialised by Invitrogen, expresses a mammalian-type glycosylation: a mammalian glycosyltransferase gene was introduced into these insect cells. More patents on improved baculovirus genomes and host insect cells are in the pipeline.

There is presently no universal expression system available for expression of all protein types. An investigator attempting to express a recombinant protein for the first time needs to assess multiple expression systems before an optimal one is identified. Background knowledge of the protein to be expressed assists in guiding the researcher towards choosing an optimal expression system. The intended use of the protein also plays a critical role. Fusion tags, whether to assist with solubility or purification, also need to be selected carefully. For example, the maltose-binding protein can help in improving the solubility of the fusion protein, but its large size may interfere with protein folding. On the other side, the relatively smaller histidine tag may not interfere with protein folding, but it is not one of the best purification tags. The tag's simple and short sequence increases the probability of it being similar to some of the host proteins, thus increasing the possibility of these contaminating the purified protein. As observed with the bacterial expressed ORF65 protein, some of the host proteins co-eluted and contaminated the eluent (Figure 18). Futhermore, the expressed ORF65 was used to raise active antibodies in chicken without cleaving the histidine tag from the protein. This is observed in Figure as the antibodies were able to bind AlHV-1 ifected VERO cells. It can be concluded from this observation that the hitidine tag did not interfere with ORF65 protein folding.

For removal of the fusion tag, promiscuous proteases like enterokinase and Factor Xa should be avoided if possible. They can cleave at secondary sites, often at other basic residues. Therefore, it is critical for the investigator to strike a balance between these factors for an optimal expression and purification of the protein of interest.

CHAPTER 3

THE DEVELOPMENT OF AN INDIRECT ELISA FOR THE DETECTION OF ANTIBODIES AGAINST ALCELAPHINE HERPESVIRUS 1

Abstract

We previously developed an AlHV-1 ORF65 antigen-based, indirect ELISA for the detection of antibodies against AlHV-1, based on a bacterial-expressed ORF65 antigen (unpublished). Some of the tested sera interacted with what we suspected to be *E. coli* endogenous proteins co-eluting with the ORF65 from Ni-TED columns. Therefore, a baculovirus-expressed AlHV-1 ORF65 antigen was column-purified and used to coat plates for the ELISA format in order to compare its performance to that of the bacterial-expressed protein. A cut off value of 17 was determined using the GraphPad Prism 6 programme and yielded equal test parameters (Sensitivity=Specificity=100%). The 44 AlHV-1 positive samples confirmed by polymerase chain reaction (PCR) and by the competitive inhibition ELISA (CI-ELISA), also tested positive with this indirect ELISA. There was no cross-reactivity between the antigen and sera against related viruses such as bovine herpesvirus 1, 2 or 4 as well as sera from cattle infected with ovine herpesvirus-2. The assay was highly repeatable, thus suggesting that the test can be used for routine detection of AlHV-1 antibodies in infected cattle.

Introduction

As discussed previously in the literature review, the OIE's preferred method for the detection of MCF is histopathology for dead animals, but most laboratories use PCR for detection of MCF virus presence in living animal blood.

Li et al. (1994), developed an indirect competitive inhibition enzyme-linked immunosorbent assay (CI-ELISA) for the detection of antibodies against both malignant catarrhal fever viruses. Li et al. (2001), improved on this indirect CI-ELISA by conjugating the monoclonal antibody, 15- A, with horseradish peroxidase in order to develop a

direct CI-ELISA. Both of these CI-ELISAs use the whole virus, AlHV 1, as the capture antigen, which has limitations as the AlHV-1 antigen and the monoclonal antibody are expensive and time-consuming to produce.

These MCF CI-ELISAs both detect antibodies from sera of animals infected with any of the MCF-causing viruses. The monoclonal antibody used in these CI-ELISA tests was raised against a highly conserved epitope in the viral glycoprotein complex (Li et al. 1994).

We previously developed an indirect ELISA using a bacterial expressed AlHV-1 minor capsid protein encoded by ORF65. The AlHV-1 capsid protein sequence is different to that of OvHV-2, as shown in Figure 31. The aim was therefore to develop an indirect ELISA that will detect antibodies raised against AlHV-1 only. The problem we encountered was that certain bacterial endogenous proteins co-eluted with the antigen during purification and cross-reacted with the cattle sera. This chapter describes the performance of a baculovirus-expressed ORF65 antigen in an attempt to improve the AlHV-1 indirect ELISA.

3.1 Materials and methods

3.1.1 Preparation of the ORF65 antigen for use in the ELISA

AlHV-1Alcelaphine herpesvirus 1 ORF65 protein was expressed in ten 175cm^2 monolayer flasks using insect cells grown in EXPRESS FIVE SFM medium (GIBCO, UK) infected with the recombinant baculovirus-ORF65, as described in Chapter 2. The purified ORF 65 antigen, stored at -20°C was used in this test.

```
CLUSTAL 2.0.12 multiple sequence alignment OvHV-2 and AlHV-1 ORF 65 proteins

OvHV-2 MSYARPRLPRIHVRLEQDYPHDPRVQQLQVQVLNNPNYANNVRAPYTYLVFLTAQQTYDA 60
AlHV-1 MAHARPKLPRINLRLDAEYPNDPRVQQLHLQILNNPNYANNVRAPLSYLVFLTAQEMYEV 60
       *::***:****::**: :**:********::*:************** :*********: *:.

OvHV-2 YVRQARGVNKKKLPPSNKPPPQLNNQLNNQQANNLPPVPPHPSGAGSGGG--PPNAPPLP 118
AlHV-1 FVRQARGVNKKRSGGPEKPILPANPGAGPQNAPGCGGGGSGSGGSGPPGGSAPQIQPPNN 120
       :**********:   .:**    *   . *:* .     . ..*:*. **  *   **

OvHV-2 DKPDPQQ------------GGGANNQS---QGSGGGNPLPPDPGC--------------- 148
AlHV-1 GVPSNQVSSNSGSAAGSGSGSGASSNSGAGSGAGAGSSACPSSGAGSGAGAGSSAGSNVQ 180
       . *. *              *.**..:*   .*:*.*..  *..*.

OvHV-2 --PPQPRDDRGGLGPGIPG-----LPPGLPKGLPLQLGLVGSD-GGGPPASP-VDSPPTK 199
AlHV-1 APPAPPPLPQVPMVPGVVNGQEMYLPLGPVNGLPQQLGTAGSDNSGGQASSPGSQNPPTK 240
         *. *   :  : **: .     ** *  :*** *** .*** .** .:**  :.****

OvHV-2 PIAPK-KGGKGAR  211
AlHV-1 PVAPRGKLGKGR  252
       *:**: * ***
```

Figure 24: A clustal 2.0 multiple sequence alignment for OvHV-2 and AlHV-1 minor capsid proteins (encoded by ORF65). The '.' depicts the weakly conserved residues within the group, the ':' depicts the highly conserved residues within the group and the '' depicts identical residues in both sequences. There is a 50.24% percentage identity between the protein sequences of OvHV-2 and AlHV-1 ORF 65.*

3.1.2 Antisera for use in the indirect ELISA

The sera used in this experiment were obtained from the diagnostic laboratory, ARC-Onderstepoort Veterinary Institute, Biotechnology division. Both the MCF negative (n=44) and positive (n=44) sera were from samples previously tested by PCR and CI-ELISA for AlHV-1 viral DNA and antibody presence, respectively. Bovine herpesvirus 1, 2 and 4 positive sera were obtained from Dr. Christine Vroon's collection. Sera from sheep infected with ovine pleuropneumonia (OPP), sera from merino sheep that tested positive for OvHV-2 using the commercial CI-ELISA, sera from cattle infected with OvHV-2 and tested positive with the commercial CI-ELISA kit and sera from wildebeest that tested positive with the commercial ELISA kit were also used in this study.

3.1.3 Checkerboard titration of the ORF 65 antigen and the AlHV-1 positive sera

NUNC U96 polysorp immunoplates (Thermoscientific, USA) were used as a solid phase for the assay. A volume of 100µl/well was used for all reagents throughout the test. Reagents were diluted in PBS [with 1% NONIDET P40 (NP40) (USB Corporation, USA), a non-ionic detergent, and added to wells].

Two-fold serial dilutions (1/100 to 1/51200) of the purified AlHV-1 ORF65 antigen in PBS, pH 7.4 were prepared in 10 duplicate wells across the columns of the ELISA plate, with the 11^{th} and 12^{th} columns receiving the diluent (i.e PBS+NP40) only. The plates were covered with their lids and incubated overnight at 4°C overnight. The following day, the antigen-coated plates were washed in an ELX 50 Autostrip washer (BIO-TEK Instruments, Inc., USA) programmed for a triple wash with 300µl PBS. MCF positive and negative sera (NVSL, Ames, USA) were added in replicate dilutions (1/50 to 1/3200) across the seven rows, the eighth row (row H) receiving the diluent only. The plates were then covered and incubated at 37°C in a THERMOstar (BMG LABTECH, Germany) orbital shaker for 1 hour. Unbound reagents were removed by washing with PBS in an ELX 50 Autostrip washer (BIO-TEK Instruments, Inc., USA) programmed for a triple 300µl wash.

The conjugate, rec-Protein G-Peroxidase (ZYMED Laboratories, USA), was added to the wells at a dilution of 1/8000, as recommended by the manufacturer and incubated at 37°C for 1 hour in the orbital shaker. Following the wash, this time with PBS+1%NP40, 50µl of 3,3',5,5'-Tetramethylbenzidine, a chromogenic substrate, was added to the wells and they were incubated at room temperature for 5 minutes. After the five minute incubation period, the reaction was stopped by the addition of 50µl of 0,2N H_2SO_4. The plates were read spectrophotometrically using the Wallac ELISA plate reader (PerkinElmer Life Sciences, USA).

3.1.3.1 Titration of the conjugate for use in the indirect ELISA

A two-fold serial dilution (1/100 to 1/51200) of the AlHV-1 ORF65 antigen was performed and used to coat polysorb plates as described (Section 3.1.3) and the plates were incubated overnight at 4°C. After one wash, optimally-diluted MCF positive and negative sera were added to the wells in duplicates, and the plates incubated in a THERMOstar orbital shaker for 1 hour at 37°C.

Following another washing, rec-Protein G-Peroxidase diluted in PBS+NP40 (1/2000 to 1/32000), was added to the different rows in replicates. The plates were incubated in an orbital shaker for an hour at 37°C, followed by another wash. A 100µl of the 3,3',5,5'-Tetramethylbenzidine chromogenic substrate was then added to the wells. Following a 5 minute incubation at room temperature, 100µl of the stop solution, 0.2N H_2SO_4 was added to the reactions. The plates were read spectrophotometrically at 450 nm in a Wallac ELISA plate reader.

3.1.4 Cross-reactivity with related ruminant herpesviruses

Two AlHV-1 positive sera and one negative serum (AMES), previously confirmed with the CI-ELISA, were used as controls in this experiment. A conjugate control well, lacking antigen, was also included in the assay to screen for background. Sera from cattle that had been infected with related bovine herpesviruses (BHV) 1, 2 and 4 (2 samples each); sera from sheep infected with ovine pleuropneumonia (OPP)(4 samples); sera from

merino sheep that tested positive for OvHV-2 using a commercial CI-ELISA kit; sera from cattle that had been infected with OvHV-2 and that had tested positive with the commercial ELISA kit(5 samples); and sera from five wildebeest that had tested positive with the commercial CI-ELISA kit were tested for their reactivity in the assay.

3.1.5 Repeatability of the assay

The plate to plate variation was determined by coating ten plates with quadruple wells AlHV-1 ORF 65 antigen, 1/100. A strong MCF positive serum was used in this experiment. The experiment was carried out over a ten-day period, that is; one plate per day.

3.1.6 Receiver Operating Characteristics (ROC) analysis

The ROC analysis was performed using the GraphPad Prism 6 programme. Fourty four AlHV-1 positive and 44 negative sera samples, previously confirmed by CI-ELISA and PCR, were used in this experiment. The ROC curve assists in the determination of the cut-off point between the AlHV-1 negative and positive samples.

3.2 Results

3.2.1 Checkerboard titration of the AlHV-1 ORF 65 antigen

The concentration and dilution of reagents used in this experiment were adapted from the previously determined bacterial-expressed AlHV-1 ORF65 antigen. The antigen concentration determined to be optimal for the assay was found to be 0,12µg with the antisera dilution of 1/100 in PBS. The conjugate, rec-Protein G-Peroxidase, was determined to work optimally for this assay at a dilution of 1/16000 in PBS+1%NP40. These pre-determined concentrations of the three reagents gave optical densities between 1.0 and 2.0 for the positives and produced minimal background and a clear differentiation between negatives and positives.

3.2.2 Cross-reactivity of the assay with related ruminant herpesviruses

The ELISA clearly differentiated between the AlHV-1 positive and negative samples (Figure 32). One of the wildebeest samples even produced an absorbance reading higher than the positive control samples. All the positive controls as well as the AlHV-1 positive wildebeest samples, gave an absorbance reading above 1.0. There was a significant difference in absorbance readings between the AlHV-1 positive samples and the negatives, with no overlap.

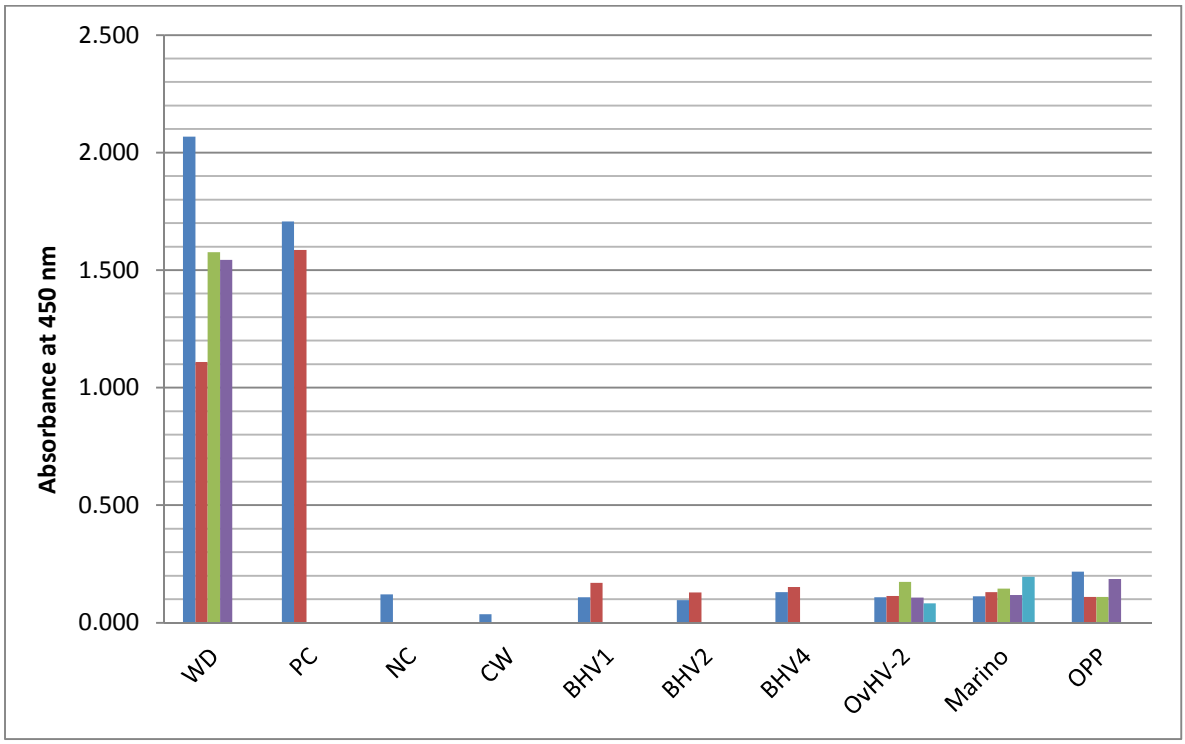


Figure 25: Results of the cross-reactivity test of the developed ELISA and related herpesviruses from ruminants. A 1/100 dilution of the samples in PBS was used. The positive (PC) and wildebeest (WD) samples are clearly comparable. The remaining samples compare well with the negative controls (NC). WD-wildebeest sera; PC-positive control; NC-negative control; BHV1, 2, 4-bovine herpesvirus 1, 2, 4; OvHV-2- sera from cattle infected with sheep-associated MCFV; Merino-sera from merino sheep that had tested positive for OvHV-2 using the commercial CI-ELISA kit; OPP-sera from sheep infected with ovine pleuropneumonia; CW-conjugate well, in which no antigen was added.

3.2.3 Repeatability of the assay

Ten quadruplets (on ten separate Nunc polysorp plates) of a strong positive AlHV-1 serum produced a mean absorbance value (± 1 standard deviation) of 1.76 ± 0.05 within

a single run of the indirect ELISA (Figure 33). The coefficient of variation for these quadruplets was 2.92%.

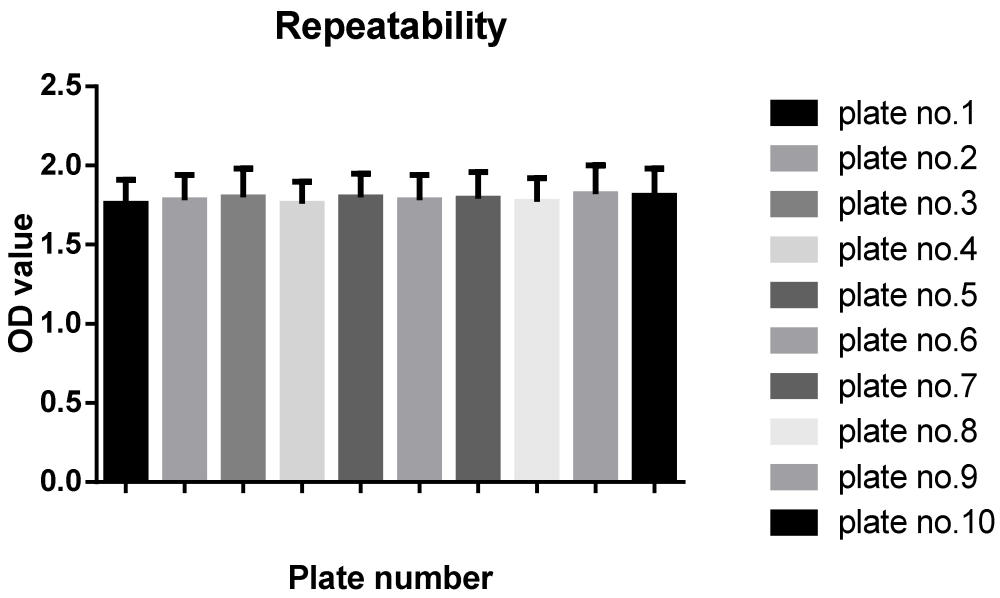


Figure 26: Comparison of the mean absorbance values for the ten quadruplet sera repeated in ten different plates. The error bars represent one standard deviation from the mean.

3.2.4 Receiver Operating Characteristics (ROC) analysis of the assay

3.2.4.1 Positive and negative samples

OD readings for the AlHV-1 positive and negative samples were normalised to calculate the percentage positive (PP) values using the following equation:

PP value= OD of test sample – OD of negative control sample/OD of reference positive serum x100

The distributions of the AlHV-1 positive sample PP values are shown in Figure 34. The PP values ranged from the a low of 24.8 to a high of 117.3

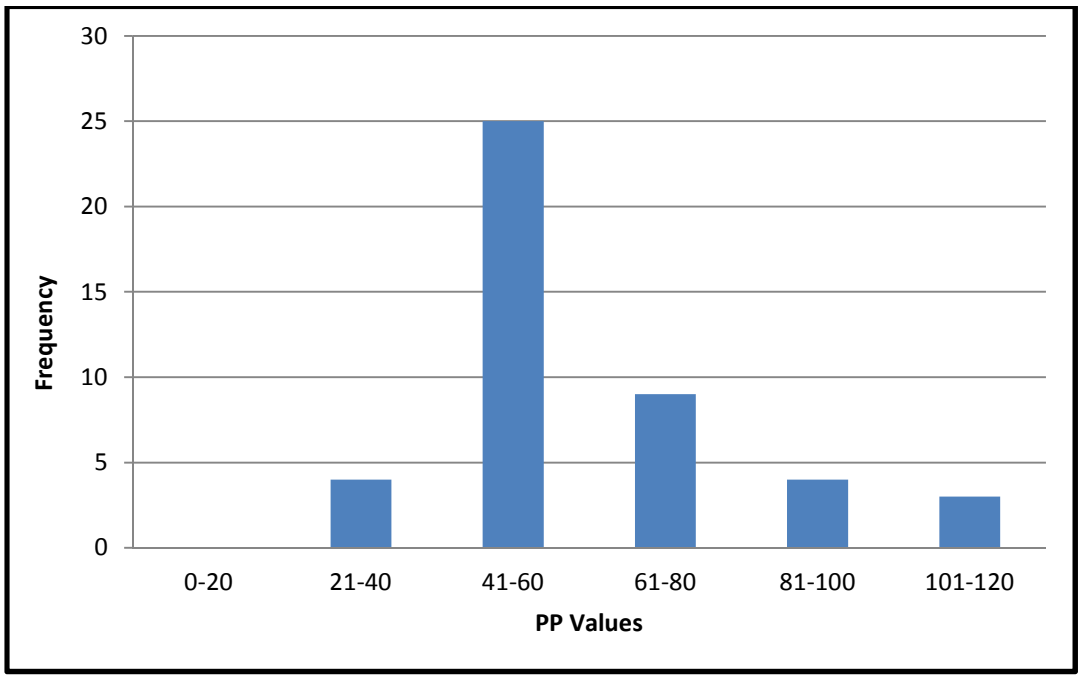


Figure 27: Normalised percentage positive values (PP values) for the 44 AlHV-1 positive cattle sera.

The distributions of the AlHV-1 negative sample PP values are shown in Figure 35. The highest PP value reading in the graph is the positive control. The normalised OD readings (PP values) for the AlHV-1 negative samples ranged from a low of -24 to a high of -4. The PP values for the AlHV-1 negative and positive samples were used with the GraphPad Prism 6 programme to draw a ROC curve.

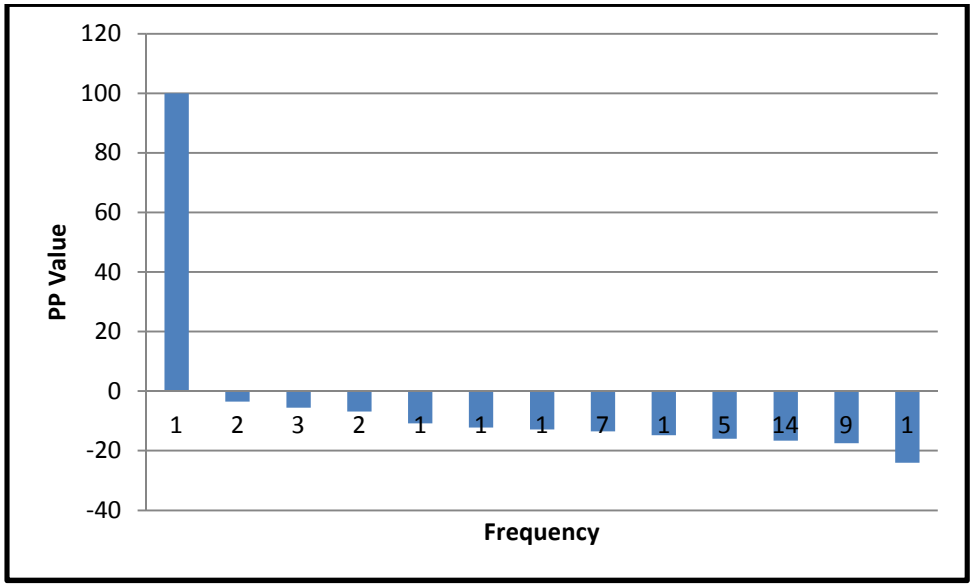


Figure 28: Normalised percentage positive values (PP values) for the 44 AlHV-1 negative cattle sera. The numbers on the bars depicts the frequency of appearance for each of the given PP values. The serum with a PP value of 100 is the positive control.

Area under the ROC curve

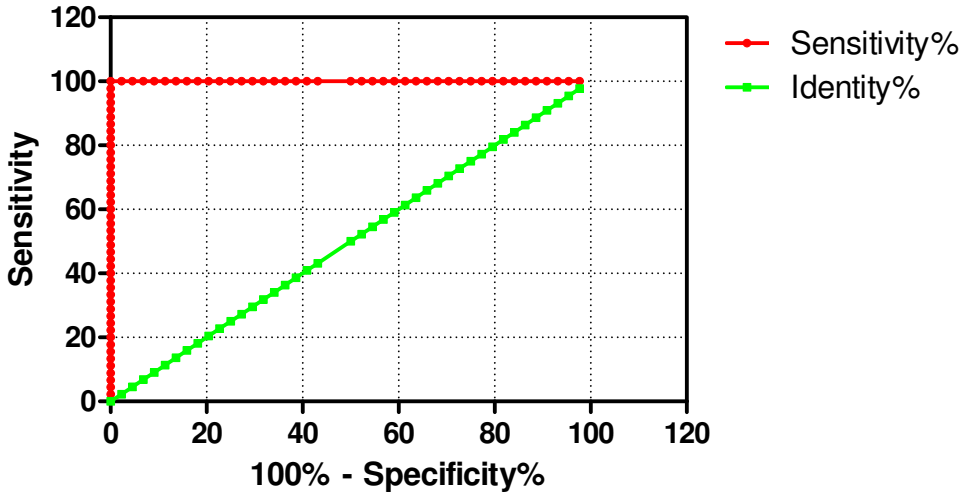


Figure 29: A ROC curve for AlHV-1 positive and negative samples.

Area under the ROC curve	
Area	1.000
Std. Error	0.0
95% confidence interval	1.000 to 1.000
P value	< 0.0001
Data	
Controls (Pos)	44
Patients (Neg)	45
Missing Patients	0
Missing Patients	0

The area under the plotted ROC curve equals 1.0. This depicts a perfect test with a specificity of 100% as well as sensitivity of 100%.

3.4.4.3 Cut-off determination

Eighty eight previously characterised samples (by both PCR and CI-ELISA) were used in determining the cut-off value. The GraphPad Prism tabulates and plots the sensitivity and specificity of the test at various cut-off values. A maximal combination of 100% specificity as well as sensitivity was achieved at a cut-off value of 17 PP.

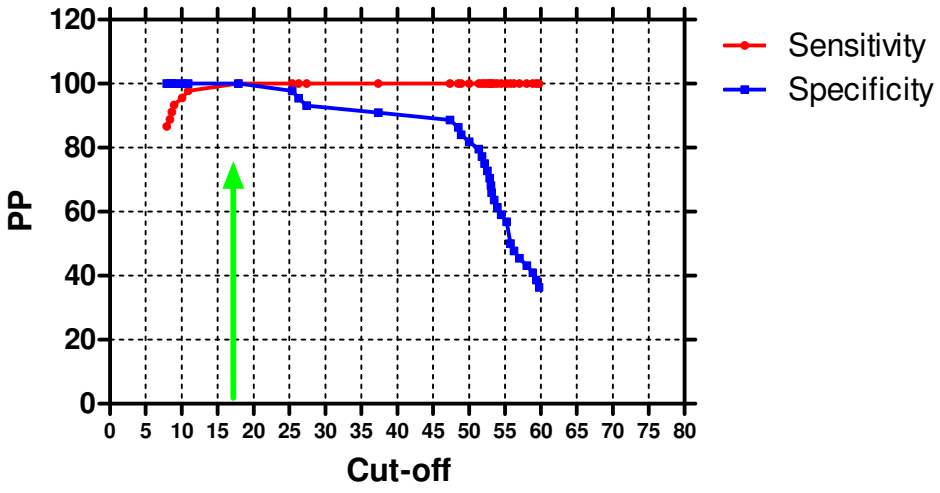


Figure 30: Determination of the cut-off value for the developed ELISA test. A cut-off value of 17 PP was obtained (green arrow) the intersection point of the two graphs.

Discussion

Three ELISAs were previously developed for the detection of MCF antibodies in animal sera, a direct and indirect CI-ELISA (Li et al. 1994; Li et al. 2001) and an indirect ELISA, WC11-ELISA (Fraser et al. 2006). The three tests all use the Minnesota isolate of malignant catarrhal fever virus (MCFV), WC11 strain, as the coating antigen. The MCF ELISA developed by Russell et al. (2012) uses the AlHV-1 C500 attenuted strain as a source of ELISA antigen. However, the tests detect the entire group of MCF viruses, including AlHV-1, OvHV-2 and AlHV-2. The problem encountered with these antibody based MCFV diagnostic tests is that they do not discriminate amongst the different MCF viruses. The objective of this study was to improve on a locally developed MCF indirect ELISA based on *in vitro* expressed recombinant AlHV-1 ORF65 antigen that selectively detects AlHV-1 antibodies.

The ORF 65 antigen is a highly variable protein amongst the gammaherpesviruses. As described earlier, using bioinformatics to compare the AlHV-1 and the OvHV-2 ORF65 antigen, it was found to have approximately a 50% amino acid difference. Whether this major difference in protein sequence between ORF65 antigens from the two viruses would translate into a major difference for detection by sera from animals infected by the two viruses, remains to be proved. The advantage of an assay based on a recombinant protein is that it is more specific when compared to a whole virus-based assay. Individual proteins, as observed with ORF65, tend to vary amongst related viruses. Therefore, antibodies produced against those specific proteins are expected to be very specific for them.

Generally, in developing an assay, quantities of reagents to be used need to be optimised. The checkerboard titration method was used in this study for the purpose of finding optimal working concentrations of each component. For this indirect ELISA, three components, namely, the antigen, the antibody and secondary antibody (conjugate) had to be optimised. With the checkerboard titration only two components can be optimised at any one time whilst keeping the third component's concentration constant. The ultimate aim of the checkerboard titration assay is to find optimal

conditions that will lead to a fully defined test with reagents used in a cost effective manner.

The assay showed no cross-reactivity with sera from animals infected with either bovine herpesvirus 1, 2 or 4; or ovine pleuropneumonia; or ovine herpesvirus 2. Antibodies in sera from animals infected with AlHV-1 were efficiently detected. With the optimum conditions now defined for the assay, 44 wildebeest-associated MCF positive sera as well as 44 negative serum samples were tested. The results for these samples were used in determining the cut-off value using the receiver operating characteristics (ROC) analysis. The ROC analysis also determines the sensitivity and specificity of the assay. For the ROC analysis, the data was normalised and raw test results were adjusted relative to values of controls included in each run of the assay. Normalisation of raw test results standardises ELISA results. Raw absorbance values are influenced by factors such as ambient temperature, photometric instrumentation and test parameters.

The GraphPad Prism ROC analysis used for analysis of the developed assay tabulates and plots the sensitivity and specificity of the test at various cut-off values (Appendix 2). The program calculates the specificity and sensitivity using each value in the data set and provides giving a cut-off value for each pair. In selecting a cut-off value, the operator is guided by the need to maximise both the sensitivity and specificity of the assay. A high cut-off value normally translates into an increased specificity of the test, but the sensitivity is lost in the process. A cut-off value of 17 percentage positive (PP) value with a sensitivity of 100% and a specificity of 100% was chosen for this test. The choice was simplified by the fact that there was a significant difference between the AlHV-1 negative and positive cattle sera values. Sera giving a PP value of anything between 16 and 18 should be treated as suspicious. There are instances where the distributions of test values from infected and uninfected animals overlap considerably. Such cases make it difficult to classify the disease or risk status of animals correctly. It is common in such instances for two cut-off values to be selected, one covering up to 99% of the infected animals(increased sensitivity), and one covering up to 99% of the uninfected animals(high specificity). Any values falling between the two chosen cut-offs would be classified as suspicious and would require a confirmatory assay.

The area under a ROC curve determines the ability of an assay to distinguish between positive and negative individuals. A perfect assay would have an area under the ROC curve of approximately 1, whereas an area under the curve of 0.5 depicts a useless test. Such a test would have no diagnostic value. The area under the ROC curve of the ELISA assay developed in this study is 1, thus suggesting an optimal ELISA. The test has a standard error of 0.00 and a small P value (<0.0001), thus one can confidently conclude that the test does discriminate between positive and negative sera samples for AlHV-1

In summary, a cost effective and efficient indirect ELISA that detects antibodies against wildebeest-associated MCF was developed. The assay data was highly repeatable thus suggesting a test that can be valuable for epidemiological studies of AlHV-1. Generally, a coefficient of variation of less than 20% for raw absorbance values indicates adequate repeatability (Jacobson, 1996). Epidemiological studies and sero-surveillance of this disease will be further simplified by the fact that there is presently no commercially available vaccine for the disease, therefore any antibody detected would be from natural infection. However, the ELISA developed during this research project must still be validated.

CHAPTER 4

GENERAL DISCUSSION AND CONCLUSION

Two of the Alcelaphine herpesvirus 1 structural proteins were expressed in three different expression systems. Glycoprotein B, encoded by ORF8, was expressed as a full length gene of approximately 2 620 bp in the mammalian expression vector, pCI. Two overlapping ORF8 truncated genes of approximately 700 bp each, were expressed in the baculovirus/insect cell expression system. The AlHV-1 minor capsid protein, encoded by ORF65, was expressed in the bacterial expression system, pCOLD I. The expressed ORF65 protein was purified through Ni-TED columns using the His_6 fusion tag. It was used to develop an indirect ELISA that detects antibodies to AlHV-1 from infected cattle sera. However, a number of false positives were recorded when using this assay. The minor capsid protein was also expressed in the baculovirus/insect cell expression system and the column purified protein was used to improve the AlHV-1 indirect ELISA. Using this baculovirus expressed ORF65 antigen in the ELISA, sensitivity and specificity of 100% was achieved at an optimally derived cut-off value of 17 percentage positive PP. There were no false negatives or false positives recorded with this test.

Glycoprotein B is a highly conserved protein amongst the gammaherpesviruses. The work by Dry et al. (2008) suggests the likelihood of gpB being part of the complex targeted by the 15-A monoclonal antibody used in the development of the CI-ELISA. The mature gpB protein is cleaved into two domains of 80 kDa and 50 kDa at a furin cleavage site very much similar to the 78 kDa and 48 kDa domains observed from the gp115 complex cleavage (Adams and Hutt-Fletcher, 1990). Previously, glycoprotein B from herpes simplex virus (HSV) was tested for use as a post infection treatment reagent for herpes simplex virus infections (Burke et al. 1997). The HSV glycoprotein B vaccine protected guinea pigs against the clinical manifestation of HSV-2 genital infection. Co-administration of adjuvant and the immunising dose dictated the extent of protection. With no commercially available vaccine or treatment for MCF, the highly conserved AlHV-1 glycoprotein B looks promising as a candidate for MCF vaccines.

Resulting from this study it should be possible to evaluate the transiently expressed full length glycoprotein B in pCI as a candidate DNA vaccine and the baculovirus expressed truncated gene assayed for candidacy as subunit vaccine. A prime-boost combination of the two could be assayed, using the candidate pCI-gpB DNA vaccine for priming followed by two consecutive booster vaccinations with the truncated genes. This combination may be further boosted by the addition of the full repertoire of the AlHV-1 glycoproteins, seven in total.

The improvedAlHV-1 indirect ELISA presents a cost effective, rapid and simplified assay for the detection of wildebeest-associated antibodies in cattle infected with AlHV-1. The assay is highly sensitive and specific and will prove invaluable in epidemiological studies on AlHV-1. The CI-ELISA was used as a “gold standard” in determining the performance of this AlHV-1 indirect ELISA. All the samples that were wildebeest-associated MCF PCR and CI-ELISA positive were positive with the improved indirect ELISA. The test also agreed with the PCR and CI-ELISA negative results. There is a high level of concordance between the AlHV-1 indirect ELISA and the CI-ELISA. Since there is a high level of agreement between the results from the WC11-ELISA and those from the CI-ELISA (Fraser et al., 2006), one can conclude that there will be a high level of concordance between the WC11-ELISA and the AlHV-1 indirect ELISA. The specificity of the indirect ELISA for AlHV-1 antibodies was demonstrated when tested against antigens of bovine herpesvirus 1, 2, 4; ovine herpesvirus 2 and ovine pleuropneumonia as the results were all negative.

The AlHV-1 and OvHV-2 isolate minor capsid proteins compared have an amino acid sequence identity of approximately 50%. As seen with the improved AlHV-1 indirect ELISA,this difference resulted in the assay detecting antibodies raised against the AlHV-1 only. Therefore, using the same reasoning, an OvHV-2- specific indirect ELISA could be developed using the ovine herpesvirus 2 minor capsid protein. Extending the idea further, a combination of the two, that is, AlHV-1 and OvHV-2 minor capsid proteins, together in a single ELISA assay should be able to detect antibodies to both viruses. Such tests would be very useful in terms of economics and ease of preparation of antigens. It is acknowledged however, that the ELISA assay is not the most sensitive amongst the tests for MCF detection. Muller-Doblies et al. (1998) compared the

performance of PCR, CI-ELISA and histopathology to detect MCF. It was concluded that CI-ELISA is less sensitive than either histopathology or PCR. The study concluded that a negative CI-ELISA test must be confirmed by PCR.

In conclusion, MCF is an economically important disease. The causative agent has an increasing host range, as recently documented with infections in African buffaloes (Pfitzer et al., 2013). The flourishing game farming industry, as well as zoological collections has brought reservoir hosts of the MCF virus into close proximity to susceptible host species. Therefore, there is a high demand for MCF control measures to minimise the infection rate of susceptible animals. Reliable, rapid and cost effective testing assays will play a major role in some of these control measures, as will an effective vaccine thus making the availability of both a high priority.

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APPENDIX 1

Buffers and Solutions

1. Luria Broth

   For 1 Liter of LB

Tryptone	10g
Yeast extract	5g
NaCl	10g

   Dissolve in 1 liter distilled water and autoclave for 20 inutes at at 15 psi and 120°C.

2. 50 X TAE Stock Solution

   242g Tris Base (MW=121.1)

   57.1 mL Glacial Acetic Acid

   100 mL 0.5 M EDTA

   Dissolve Tris in 600 mL of distilled water, add the EDTA and Acetic Acid and bring the final volume to 1 L with distilled water.

3. Rubidium chloride competent cells solutions

   (a)TFB 1

   For a 250 mL solution, dissolve the following in 100ml;

Potassium acetate	0.74g
Calcium chloride	1.25ml of 2M
Manganese chloride	2.47g
Rubidium chloride	3.023g
15% glycerol	37.5ml

   Bring the final volume to 250 ml with distilled water. Adjust the ph to 5.8 with 1M glacial acetic acid and filter sterilize (0.2µm)

   (b) TFB 2

   For a 100 ml solution, dissolve the following in 50ml;

MOPS/PIPES	0.335g
Calcium chloride	3.75ml of 2M
Rubidium chloride	0.12g

15% glycerol 15ml

Bring the final volume to 100ml with distilled water. Adjust pH to 6.5 with 1M KOH and filter sterilize (0.2µm).

4. Buffers for protein purification

(a) LEW buffer

50 mM NaH_2PO_4

300mM NaCl

Adjust pH to 8.0 with NaOH.

(b) Elution buffer

50 mM Na_2PO_4

300mM NaCl

250 mM imidazole

Adjust pH to 8.0 with NaOH.

5. Transfer buffer (Western blot)

For 1 liter of buffer

Tri base 3.03 g

Glycine 14.4 g

Dissolve in 600 ml of dH2O. Add 200 ml of methanol and add dH2O to a final volume of 1 liter.

6. Minipreparation of plasmid DNA

(a) Solution I

50 mM glucose, 25 mM Tris-Cl (pH 8.0) and 10 mM EDTA (pH 8.0)

(b) Solution II

0.2 N NaOH and 1% SDS

© Solution III

For 100ml solution

5M KoAC/Acetic Acid 60.0 ml

Glacial Acetic acid 11,5 ml

dH_2O 28.5 ml

APPENDIX 2

Data for ROC analysis

Cut-off	Sensitivity%	95% CI	Specificity%	95% CI
< -1.560	2.222	0.05625% to 11.77%	100	91.96% to 100.0%
< -1.515	4.444	0.5428% to 15.15%	100	91.96% to 100.0%
< -1.430	6.667	1.397% to 18.27%	100	91.96% to 100.0%
< -1.320	8.889	2.475% to 21.22%	100	91.96% to 100.0%
< -1.250	11.11	3.708% to 24.05%	100	91.96% to 100.0%
< -1.215	13.33	5.054% to 26.79%	100	91.96% to 100.0%
< -1.125	15.56	6.491% to 29.46%	100	91.96% to 100.0%
< -1.015	17.78	8.002% to 32.05%	100	91.96% to 100.0%
< -0.9600	20	9.576% to 34.60%	100	91.96% to 100.0%
< -0.9350	22.22	11.20% to 37.09%	100	91.96% to 100.0%
< -0.9100	24.44	12.88% to 39.54%	100	91.96% to 100.0%
< -0.8800	26.67	14.60% to 41.94%	100	91.96% to 100.0%
< -0.8100	28.89	16.37% to 44.31%	100	91.96% to 100.0%
< -0.7450	31.11	18.17% to 46.65%	100	91.96% to 100.0%
< -0.7200	33.33	20.00% to 48.95%	100	91.96% to 100.0%
< -0.6950	35.56	21.87% to 51.22%	100	91.96% to 100.0%
< -0.6300	37.78	23.77% to 53.46%	100	91.96% to 100.0%
< -0.5600	40	25.70% to 55.67%	100	91.96% to 100.0%
< -0.4750	42.22	27.66% to 57.85%	100	91.96% to 100.0%
< -0.4050	44.44	29.64% to 60.00%	100	91.96% to 100.0%
< -0.3400	46.67	31.66% to 62.13%	100	91.96% to 100.0%
< -0.1800	48.89	33.70% to 64.23%	100	91.96% to 100.0%
< -0.0550	51.11	35.77% to 66.30%	100	91.96% to 100.0%
< 0.0200	53.33	37.87% to 68.34%	100	91.96% to 100.0%
< 0.1100	55.56	40.00% to 70.36%	100	91.96% to 100.0%
< 0.2100	57.78	42.15% to 72.34%	100	91.96% to 100.0%
< 0.7750	60	44.33% to 74.30%	100	91.96% to 100.0%
< 1.425	62.22	46.54% to 76.23%	100	91.96% to 100.0%
< 1.660	64.44	48.78% to 78.13%	100	91.96% to 100.0%
< 1.955	66.67	51.05% to 80.00%	100	91.96% to 100.0%
< 2.225	68.89	53.35% to 81.83%	100	91.96% to 100.0%
< 2.300	71.11	55.69% to 83.63%	100	91.96% to 100.0%
< 2.330	73.33	58.06% to 85.40%	100	91.96% to 100.0%
< 2.360	75.56	60.46% to 87.12%	100	91.96% to 100.0%
< 2.675	77.78	62.91% to 88.80%	100	91.96% to 100.0%
< 3.240	80	65.40% to 90.42%	100	91.96% to 100.0%
< 4.090	82.22	67.95% to 92.00%	100	91.96% to 100.0%
< 6.210	84.44	70.54% to 93.51%	100	91.96% to 100.0%
< 7.925	86.67	73.21% to 94.95%	100	91.96% to 100.0%
< 8.360	88.89	75.95% to 96.29%	100	91.96% to 100.0%

Cut-off	Sensitivity%	95% CI	Specificity%	95% CI
< 8.640	91.11	78.78% to 97.52%	100	91.96% to 100.0%
< 8.965	93.33	81.73% to 98.60%	100	91.96% to 100.0%
< 10.06	95.56	84.85% to 99.46%	100	91.96% to 100.0%
< 10.91	97.78	88.23% to 99.94%	100	91.96% to 100.0%
< 17.88	100	92.13% to 100.0%	100	91.96% to 100.0%
< 25.40	100	92.13% to 100.0%	97.73	87.98% to 99.94%
< 26.30	100	92.13% to 100.0%	95.45	84.53% to 99.44%
< 27.40	100	92.13% to 100.0%	93.18	81.34% to 98.57%
< 37.35	100	92.13% to 100.0%	90.91	78.33% to 97.47%
< 47.35	100	92.13% to 100.0%	88.64	75.44% to 96.21%
< 48.50	100	92.13% to 100.0%	86.36	72.65% to 94.83%
< 48.90	100	92.13% to 100.0%	84.09	69.93% to 93.36%
< 50.00	100	92.13% to 100.0%	81.82	67.29% to 91.81%
< 51.35	100	92.13% to 100.0%	79.55	64.70% to 90.20%
< 51.80	100	92.13% to 100.0%	77.27	62.16% to 88.53%
< 52.15	100	92.13% to 100.0%	75	59.66% to 86.81%
< 52.55	100	92.13% to 100.0%	72.73	57.21% to 85.04%
< 52.85	100	92.13% to 100.0%	70.45	54.80% to 83.24%
< 53.05	100	92.13% to 100.0%	68.18	52.42% to 81.39%
< 53.15	100	92.13% to 100.0%	65.91	50.08% to 79.51%
< 53.50	100	92.13% to 100.0%	63.64	47.77% to 77.59%
< 53.95	100	92.13% to 100.0%	61.36	45.50% to 75.64%
< 54.45	100	92.13% to 100.0%	59.09	43.25% to 73.66%
< 55.20	100	92.13% to 100.0%	56.82	41.03% to 71.65%
< 55.75	100	92.13% to 100.0%	50	34.56% to 65.44%
< 56.25	100	92.13% to 100.0%	47.73	32.46% to 63.31%
< 57.00	100	92.13% to 100.0%	45.45	30.39% to 61.15%
< 58.00	100	92.13% to 100.0%	43.18	28.35% to 58.97%
< 58.85	100	92.13% to 100.0%	40.91	26.34% to 56.75%
< 59.35	100	92.13% to 100.0%	38.64	24.36% to 54.50%
< 59.75	100	92.13% to 100.0%	36.36	22.41% to 52.23%
< 60.60	100	92.13% to 100.0%	34.09	20.49% to 49.92%
< 61.55	100	92.13% to 100.0%	31.82	18.61% to 47.58%
< 63.15	100	92.13% to 100.0%	29.55	16.76% to 45.20%
< 64.55	100	92.13% to 100.0%	27.27	14.96% to 42.79%
< 64.95	100	92.13% to 100.0%	25	13.19% to 40.34%
< 65.75	100	92.13% to 100.0%	22.73	11.47% to 37.84%
< 66.75	100	92.13% to 100.0%	20.45	9.804% to 35.30%
< 70.60	100	92.13% to 100.0%	18.18	8.192% to 32.71%
< 74.65	100	92.13% to 100.0%	15.91	6.644% to 30.07%
< 78.35	100	92.13% to 100.0%	13.64	5.173% to 27.35%
< 82.35	100	92.13% to 100.0%	11.36	3.794% to 24.56%
< 86.95	100	92.13% to 100.0%	9.091	2.533% to 21.67%

Cut-off	Sensitivity%	95% CI	Specificity%	95% CI
< 98.60	100	92.13% to 100.0%	6.818	1.429% to 18.66%
< 108.4	100	92.13% to 100.0%	4.545	0.5553% to 15.47%
< 113.7	100	92.13% to 100.0%	2.273	0.05752% to 12.02%